TEXTE
54/2017

Comparison of dung and soil fauna from pastures treated with and without ivermectin as an example of the effects of a veterinary pharmaceutical

Final Report

For our Environment

Umwelt Bundesamt

TEXTE 54/2017

Environmental Research of the
Federal Ministry for the
Environment, Nature Conservation,
Building and Nuclear Safety

Project No. (FKZ) 3710 63 412
Report No. (UBA-FB) 002155/E

Comparison of dung and soil fauna from pastures treated with and without ivermectin as an example of the effects of a veterinary pharmaceutical

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On behalf of the German Environment Agency

Imprint

Publisher:
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Study completed in:
December 2013

Edited by:
Section IV 2.2 Pharmaceuticals, Washing and Cleaning Agents
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Publication as pdf:
http://www.umweltbundesamt.de/publikationen
ISSN 1862-4804

Dessau-Roßlau, June 2017

The project underlying this report was financed by the Federal Ministry for the Environment, Nature Conservation, Building and Nuclear safety under project number FKZ 3710 63 412. The responsibility for the content of this publication lies with the author(s).

Kurzbeschreibung

Die Anwendung von Anthelmintika (z.B. Ivermectin) bei Nutztieren kann Dungorganismen beeinträchtigen und in einigen Fällen auch den Dungabbau hemmen. Während der Registrierung müssen Antiparasitika in “higher-tier”-Studien untersucht werden, sofern in Labor-Einzelarttests negative Effekte auf Dungorganismen beobachtet wurden. Da es bisher für solche komplexeren Studien keine Richtlinien wurde ein internationales Projekt durchgeführt, um (1) die Belastbarkeit von Freilandstudien, die von 4 Instituten in verschiedenen Regionen mit unterschiedlichen Dung- und Bodenorganismengemeinschaften sowie Umweltbedingungen durchgeführt wurden, zu untersuchen und um (2) die Auswirkungen dieser Unterschiede auf die Interpretation der Testergebnisse zu studieren. Die Versuche liefen in Lethbridge (Kanada), Montpellier (Frankreich), Zurich (Schweiz) und Wageningen (Holland). Als Testsubstanz wurde Ivermectin eingesetzt. Es zeigte sich, dass es, wie zu erwarten, große Unterschiede in der Zusammensetzung wichtiger Gruppen der Dunginsekten (Familienebene) an den 4 Standorten gab. Die Ergebnisse belegen zudem, dass Ivermectin negative Auswirkungen auf mehrere Gruppen der Dungfliegen bzw. Dungkäfer an allen Standorten hatte. Allerdings konnte kein Einfluss der Ivermektinbehandlung auf die Abbaurate des Rinder-dungs in gemäßigten Breiten festgestellt werden. Zudem wurden an einem Standort (Wageningen) negative Auswirkungen auf die unter den Dunghaufen lebende Bodenfauna (Collembolen, nicht aber Regenwürmer) gefunden. Das Studiendesign erwies sich als gut geeignet für die Untersuchung der Wirkungen von Antiparasitika auf Dunginsekten und die Bodenfauna, wie es für eine “higher-tier”-Risikobeurteilung erforderlich ist. Extreme Wetterereignisse während einer solchen Studie können aber die Abundanz einiger Dunginsektengruppen beeinflussen. Diese Ergebnisse wurden in Hinsicht auf die Eignung von Risikominderungsmaßnahmen diskutiert.

Abstract

The application of anthelmintics (e.g. ivermectin) to domestic animals can affect populations of dung-dwelling organisms and in some cases retard dung degradation. During their registration process, such parasiticides need to be tested at higher tier levels when adverse effects on dung organisms are observed in single species toxicity tests. Since no guidance on higher-tier testing was available, an international project was set up in order (1) to assess the robustness of field tests when conducted by 4 research groups at different geographic sites, varying in dung and soil faunas, in environmental conditions, and (2) to study the effects of these variable conditions on the interpretation of test results. The experiments were conducted in Lethbridge (Canada), Montpellier (France), Zurich (Switzerland), and Wageningen (The Netherlands). Ivermectin was used as test compound. The study demonstrated that there are considerable differences in the composition of the principal groups of dung insect fauna (family level) between different experimental sites in the study, as could be expected according to biogeography. The results indicate that ivermectin does negatively affect various groups of dung flies and also dung beetles at all study sites. However, ivermectin treatments do not seem to have an effect on the degradation rate of dung in temperate climate regions. Effects on soil fauna (Collembola, not earthworms) living below dung pats did occur only in Wageningen. The study design is suitable to evaluate the effects of parasiticides on dung insects and soil fauna under field conditions such as required in higher-tier testing for risk assessment. Extreme weather conditions during the experiments, however, may interfere with the abundance of certain important groups of dung insects. The results are discussed in the context of measures mitigating the risk of ivermectin.

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List of Abbreviations

AIC	Akaike Information Criterion
ANCOVA	Analysis of Co-VarianceAnalysis of Variance
ANOVA	Analysis of Variance
BEF	Biodiversity-Ecosystem Functioning
DPA	Days Post-Administration
DT	Dissipation Time of a chemical, here in dung or soil (usually given in days)
EC	European Community
ECx	Effect Concentration x% (x = 10 or 50)
EEA	European Environmental Agency
EFSA	European Food Safety Authority
EMA	European Medicines Agency
ERA	Environmental Risk Assessment
ESS	Ecosystem Services
EU	European Union
GLM	General Linear Model
GMP	Genetically Modified Plants
HPLC	High Performance Liquid Chromatography
HPLC-FLD	High Performance Liquid Chromatography with Fluorescence Detection
ISO	International Organisation for Standardisation (Genf)
IVM	Ivermectin
LCx	Lethal concentration x% (x = 50)
LOD	Limit of detection
LOQ	Limit of Quantification
LOEC	Lowest Observed Effect Concentration
LUFA	Agricultural Investigation and Research Institute (Landwirtschaftliche Untersuchungs- und Forschungsanstalt) (Speyer, Germany)
MEA	Millennium Ecosystem Assessment
NOEC	No Observed Effect Concentration
OECD	Organisation for Economic Co-operation and Development (Paris)
PRC	Principal Response Curve
RMM	Risk Mitigation Measures
RSD	Relative Standard Deviation
SOP	Standard Operation Procedure
SPE	Solid Phase Extraction

SSD	Species Sensitivity Distribution
TEEB	The Economics of Ecosystems and Biodiversity
UBA	Federal Environment Agency (Umweltbundesamt) (Dessau-Roßlau)
UK	United Kingdom
VICH	International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products
VMP	Veterinary Medicinal Products
WP	Work Package

Zusammenfassung

Ziele des Vorhabens

Dieses Vorhaben hatte primär zwei Ziele:

a) Beschreibung des aktuellen Wissenstandes zu den Wirkungen von Ivermectin auf die Diversität der Dungfauna, der Bodeninvertebraten und von Pflanzen;
b) Einbau der Projektinformationen in vorhandene Prozesse der Umweltrisikobeurteilung bzw. des Risikomanagements von Ivermectin und anderen Antiparasitika.

Grundlage der Arbeiten dieses Vorhabens war die Überzeugung, dass die Umweltrisiko-beurteilung für Antiparasitika zwei Schutzziele abzudecken hat: die Funktion sowie die Struktur (Biodiversität) des Dungökosystems. Letztere wird hier wie folgt definiert: "Biodiversity is the variability among living organisms from all sources, including, 'inter alia', terrestrial, marine, and other aquatic ecosystems, and the ecological complexes of which they are part: this includes diversity within species, between species and of ecosystems" (UNCED 1992). Ausgehend von diesem gemeinsamen Verständnis wurden die Ziele des Vorhabens wie folgt konkretisiert:

1. Hat der Einsatz von Ivermectin langfristige Folgen für die Biodiversität der Dung- und Bodenorganismen?
2. Gibt es einen Unterschied zwischen der Sensitivität der im Labor untersuchten Arten und denjenigen Arten, die in den vier Freilandstudien gefunden wurden? Sind die in den Standardtests verwendeten Arten repräsentativ für die Freiland-Gemeinschaften?
3. Gibt es Belege für die Erholung der Dungorganismengemeinschaften? Dafür ist die Zusammensetzung der jeweiligen Dung- und Bodenorganismengemeinschaften wichtig.

Die Umsetzung dieser Ziele wird zuerst anhand der praktischen Arbeiten an vier Stand-orten mit deutlich unterschiedlichen ökologischen Eigenschaften zusammengefasst. Darauf aufbauend war es möglich, allgemeine Regeln zur Durchführung von Freilandstudien im Rahmen der Umweltrisikobeurteilung von Antiparasitika in Form von Standardarbeitsanleitungen zu formulieren.

In dieser Zusammenfassung werden primär die verwendeten Methoden sowie die erzielten Ergebnisse der vier Freilandstudien zusammengefasst und diskutiert. Dabei war die Hauptfrage, inwieweit diese Studien eine relevante Ergänzung bestehender Vorgaben zur Umweltrisikobeurteilung von Antiparasitika darstellen können (VICH 2004; siehe auch Jochmann et al. (2011) sowie Adler et al. (2013)). Ausgehend von diesen Informationen werden am Ende dieses Dokuments Empfehlungen für die Testung von Antiparasitika vorgestellt. Dabei wird auch diskutiert, inwieweit die eigenen Ergebnisse aus der Literatur genutzt werden können, um die folgenden, allgemeineren Fragen zu beantworten:

- Gibt es Risikominimierungsmaßnahmen (RMM), mittels derer die Diversität der Dungorganismen geschützt werden kann?
- Ist ein nachhaltiges Weidenmanagement beim Einsatz von Antiparasitika möglich?

Durch die Kombination des Wissens zu den Wirkungen von Ivermectin auf die Struktur und Funktion von Dung- und Bodenorganismen mit Vorschlägen für RMM ist einschätzbar, welche Rolle RMM bei der Umweltrisikobeurteilung von Antiparasitika spielen können.

Kurze Beschreibung des methodischen Ansatzes

Alle vier Studien wurden anhand einer vorab von den Partnern festgelegten Standardarbeitsanweisung durchgeführt. Abweichungen davon mussten begründet werden.

Die wichtigsten Eigenschaften der vier Standorte sind in Tabelle A zusammengestellt. Die klimatischen Bedingungen unterscheiden sich deutlich hinsichtlich der durchschnittlichen Lufttemperatur und der

jährlichen Niederschlagsmenge. Die Bodeneigenschaften sind dagegen relativ ähnlich (Ausnahme: niedriger pH und sandige Textur in Wageningen).

Tabelle A: Überblick zu den wichtigsten Standort- und Bodeneigenschaften an den vier Versuchsstandorten

Standort- bzw. Bodeneigenschaften	Montpellier	Zurich	Wageningen	Lethbridge
Staat	Frankreich	Schweiz	Niederlande	Kanada
Koordinaten	43°79’33.40 N; 3°73’18.75 O	47°23’44.87 N; 8°33’02.62 O	51°59’32.16 N; 5°39’39.82 O	49°41’25.46 N; 112°46'26.15 W
Landnutzung	Grasstreifen am Ackerrand	Wiesenrand	Wiese (seit 2006)	Wiese (Rinderhaltung)
Niederschlag (mm/J)	700	1123	846	365
Mittl. Temperatur(°C)	13.0	7.9	10.5	5.8
Bodeneigenschaften				
pH (CaCl2)	7.6	7.4	5.2	7.3
Organ. Gehalt (%)	3.1	4.6	2.9	6.2
Lagerungsdichte (g/L)	1149	1254	1449	987
Max. Wasserhaltekap.	48.0	47.6	34.2	60.7
Kohlenstoff (g/kg)	16.75	24.28	12.55	27.35
Stickstoff (g/kg)	1.646	3.018	1,009	2.747
C/N-Verhältnis	10.18	8.05	12.44	9.96
Bodenart (Textur)	Schluff. Lehm	Toniger Lehm	Sand	Toniger Lehm

An jedem Standort wurde ein Strukturexperiment (Dauer: eine Woche; Messparameter: Diversität und Abundanz der Dungorganismen) und ein Funktionsexperiment (Dauer: ein Jahr; Messparameter: Dungabbau sowie Diversität und Abundanz der Bodenorganismen). In beiden Experimenten wurden künstliche Dunghaufen verwendet. Der Dung stammte von unbehandelten bzw. mit Ivermectin (IVM) behandelten Rindern aus Montpellier (Quelle für die drei europäischen Standorte) oder Lethbridge (Quelle für den kanadischen Standort). Im Frühjahr 2011 wurden Rinder mit einer topikalen Formulierung (Ivomec® pour-on) in empfohlener Dosierung (500 µg IVM/kg Körpergewicht) behandelt. Drei, 7, 14 und 28 Tage (in Lethbridge auch nach 56 Tagen) nach der Applikation wurde der Dung der behandelten Rinder (<3 Stunden alt) gesammelt. Aus diesem Material wurden Dunghaufen mit einem Gewicht von jeweils 500 g (Montpellier: 800 g) hergestellt und im Freiland ausgebracht.

Im Strukturexperiment wurden die künstlichen Dunghaufen in Wageningen, Zurich und Lethbridge für eine Woche im Freiland auf einer Platte in 10 Replikaten für jede der sechs Behandlungsstufen exponiert (Tag 0, 3, 7, 14, 28 (56) = 50 (60) Dunghaufen)). Nach einer Woche im Freiland wurden die Dunghaufen in Emergenzfallen im Labor überführt, in denen alle im Dung schlüpfenden Insekten in Ethanol während eines Zeitraums von 3 Monaten gefangen wurden. In Montpellier wurden Container (Größe: 7 L, Höhe: 25 cm, Ø 15 cm) im Freiland aufgestellt, in deren Mitte ein künstlicher Dunghaufen gelegt wurde. Danach blieb deren Öffnung für ein bis drei Wochen geöffnet, bevor diese mit Emergenz-fallen verschlossen wurde. Alle Emergenzfallen wurden solange geleert, bis keine Insekten mehr schlüpften. Dungkäfer wurden bis zur Art bestimmt, Staphyliniden bis zur Gattung sowie Fliegen und Wespen (Montpellier, Lethbridge) bis zur Familie. Barberfallen (gefüllt mit 4% Formaldehydlösung) mit Dung als Köder wurden in Wageningen, Zurich und Lethbridge verwendet, um diejenigen Dungorganismen zu identifizieren, die vor, während und nach dem Fangzeitraum der Emergenzfallen aktiv waren.

Im Funktionsexperiment wurden 25 Dunghaufen pro Behandlungsstufe (Tag 0, 3, 7, 14, 28 (56) = 125 / 150 Dunghaufen (Europa bzw. Kanada) verwendet. Jeder Dunghaufen wurde auf der Bodenoberfläche exponiert. Fünf "Funktionsdunghaufen" wurden zu unterschiedlichen Zeitpunkten (Dauer: bis zu 1 Jahr nach Ausbringung) ins Labor verbracht, zerkleinert, gewogen, und getrocknet (min. 48h bei 100°C). Wichtigster Parameter war dabei das aschefreie Trockengewicht (d.h. der organische Gehalt) der einzelnen Dungprobe.

Parallel zur Beprobung der "Funktions-Dunghaufen" wurden Bodenorganismen gesammelt. Dazu wurde mittels eines Bodenstechers je eine Probe für Mikroarthropoden bzw. die Rückstandsanalytik genommen. Danach wurde der Boden innerhalb eines Quadrats von 25 * 25 cm (Tiefe 10 cm) um den Liegeplatz des Dunghaufens abgegraben und nach per Hand nach Regenwürmern durchsucht (nicht in Lethbridge, da dort keine Regenwürmer vorkommen). Mikroarthropoden wurden aus den Bodenkernen mittels Hitzeextraktion ausgetrieben. Nur Collembolen und Regenwürmer wurden bis zur Art bzw. ökologischen Gruppe bestimmt, der Rest nach Großgruppen (z.B. Oribatida, Gamasida usw.) aufgeteilt.

In der Rückstandsanalytik wurde die Extraktionsmethode von Litskas et al. (2010) verwendet. Homogenisierte und gesiebte Boden- bzw. Dungproben wurden mit Acetonitril extrahiert (interner Standard Doramectin) und aufgereinigt. Die Derivatisierung erfolgte nach Berendsen et al. (2007). Die mittlere Wiederfindungsrate des internen Standards Doramectin bei den verschiedenen Boden- und Dungproben lag zwischen 97,7 % und 101,3 %. Ergebnisse mit unzureichender Wiederfindung des internen Standards Doramectin (<80 % und >120 %) wurden von der weiteren Bearbeitung ausgeschlossen. Insgesamt betraf dies 5.9 % (Anzahl gemessener Proben: 613).

Für das Strukturexperiment wurden die Anzahl der geschlüpften Taxa und Individuen sowie der daraus resultierende Shannon Diversitätsindex bestimmt. Diese Daten wurden in Abhängigkeit der absoluten Ivermectinkonzentration separat für jeden Standort analysiert (kontinuierlicher Regressionsansatz). Dieser Ansatz galt analog auch für die Auswertung der Regenwürmer und Collembolen. Für den Dungabbau (Trockengewicht) über die Zeit (Monate) im Funktionsexperiment wurde zuerst die beste beschreibende Funktion ermittelt (= linear) (Burnham & Anderson 1992). Danach wurden die Gewichtsdaten (log-transformiert oder nicht) in Abhängigkeit von der (kontinuierlichen) Zeit (Monat) zum Gesamtvergleich einer Varianzanalyse unterzogen mit Standort und Ivermectinkonzentration als zusätzlichen (diskreten) Faktoren. Unterschiedliche Abbaugeschwindigkeiten zwischen den Standorten bzw. Konzentrationen erscheinen in einer solchen Analyse als signifikante Interaktionen.

Ergebnisse der vier Freilandstudien

Aufgrund der komplexen Struktur dieses Vorhabens (vier Standorte, zwei Kompartimente (Dung, Boden), strukturelle und funktionale Endpunkte, insbesondere aber die Vielzahl der untersuchten Organismengruppen) ist es unmöglich, alle Ergebnisse in dieser Zusammenfassung wiederzugeben. Stattdessen werden zuerst für jeden Standort charakteristische Daten präsentiert, bevor die wichtigsten Ergebnisse überblicksartig vorgestellt werden.

Methodologische Vorbemerkung

Aufgrund unvorhersehbarer Faktoren gab es wenige Abweichungen vom ursprünglich vorgesehenen Design. Generell beeinflussten klimatische Bedingungen die Studien stark: Aufgrund einer langen Dürre in Montpellier war die Anzahl der Regenwürmer und Collembolen zu gering, um ausgewertet werden zu können. Eine Beprobung der Regenwürmer in Lethbridge entfiel, da in dieser Prärieregion aus natürlichen Gründen nur sehr wenige große Bodentiere vorkommen. Die Beprobung von Bodenorganismen unterhalb der Dunghaufen war kein Problem, da die entsprechenden Richtlinien (ISO 2006a, b) mit kleinen Modifikationen anwendbar waren. Die Bestimmung der Dung- und Bodenorganismen

verlief unproblematisch, da in diesem Vorhaben Spezialisten eingebunden waren. Bei einer zukünftigen Routineanwendung sollten eher genetische Methoden („Barcoding“) zur Bestimmung der Dung- und Bodenorganismendiversität angewendet werden sollten.

Rückstandsanalytik

Bei der Bestimmung von IVM in den Dung- und Bodenproben konnten niedrige Nachweis- und Bestimmungsgrenzen (NWG bzw. BG) erreicht werden. Für die Dungproben lag die NWG bei 5,1 µg IVM/kg TG und die BG bei 12,4 µg IVM/kg TG. Die entsprechenden Werte für die Bodenproben lagen bei 0,9 µg IVM/kg TG (NWG) bzw. 2,3 µg IVM/kg TG (BG). In den drei europäischen Studien wurde Dung aus Montpellier verwendet. Die Konzentration von IVM in den vier Behandlungsstufen (Tag 0, 3, 7, 14, 28) lag im Mittel bei 2,845, 2,480, 0,692 und 0,049 mg IVM/kg Dung TG (Abb. A). In den in Lethbridge verwendeten Dungproben lag die Anfangskonzentration von IVM bei 5,029 (Tag 3), 7,675 (Tag 7), 0,341 (Tag 14), 0,065 (Tag 28) und 0,015 mg IVM/kg Dung TG (Tag 56). Der Abbau von IVM folgte in allen Behandlungsstufen einer logarithmischen Kurve. Einem schnellen Abbau in den ersten Monaten der Exposition folgte eine deutlich langsamere Phase, wobei der DT50 im Dung in den beiden höchsten Behandlungsstufen bei ca. 2 – 3 Monaten lag. Außer in den Proben von Tag 28 war IVM auch noch 13 Monate nach Studienbeginn nachweisbar

Die IVM-Konzentrationen im Dung behandelter Rinder waren in den europäischen und kanadischen Proben vergleichbar. Die IVM-Ausscheidung mittels Dung folgte einem typischen Muster: Ein Maximum wird oft 2-3 Tage nach topikaler Applikation von IVM beobachtet, gefolgt von einem scharfen Rückgang, der in eine Phase deutlich geringerer Ausscheidung übergeht, die sich über einen Zeitraum von 4 bis 6 Wochen hinziehen kann (Herd et al. 1996). Generell entsprechen die hier gefunden IVM-Konzentrationen in ihrer Höhe und ihrem Muster den Ergebnissen aus der Literatur (z.B. Edwards et al. 2001; Boxall et al. 2004; Fernández et al. 2009).

Bodenproben wurden an den vier Standorten zu verschiedenen Zeiten in einem Zeitraum von zwei bis 13 Monaten nach Applikation genommen. Mit wenigen Ausnahmen wurde IVM nur in den Proben der Behandlungsstufen Tag 3 und 7 gefunden (Abb. B). In Lethbridge und bei späteren Probenahmen an den anderen Standorten wurden Konzentrationen zwischen 0,002 und 0,006 mg IVM/kg Boden TG gemessen. In der Literatur gibt es nur wenige Angaben zu IVM-Konzentrationen aus Bodenproben im Freiland (z.B. (Römbke et al. 2010b). Vergleichbare Konzentrationen, aber fast nur im obersten Zentimeter, wurden in einer Wiese nahe York (England) gemessen (Pope, 2010). Die in unserer Studie gefundenen Konzentrationen liegen in dem Bereich, der auch bei englischen Weiden, auf denen mit Ivermectin behandelte Rindern standen, gefunden wurde (Boxall et al. 2006).

Abbildung A: Ivermectin-Konzentration im Dung während der Studie (Einzelwerte). X-Achse: Expositionszeit der Dunghaufen im Freiland; Y-Achse: IVM-Konzentration in mg/kg Dung TG)

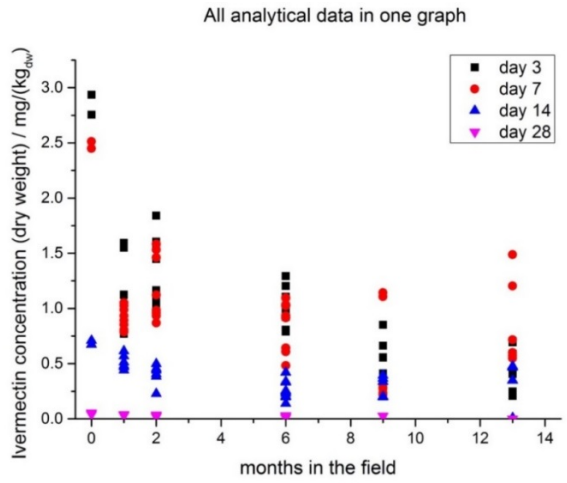
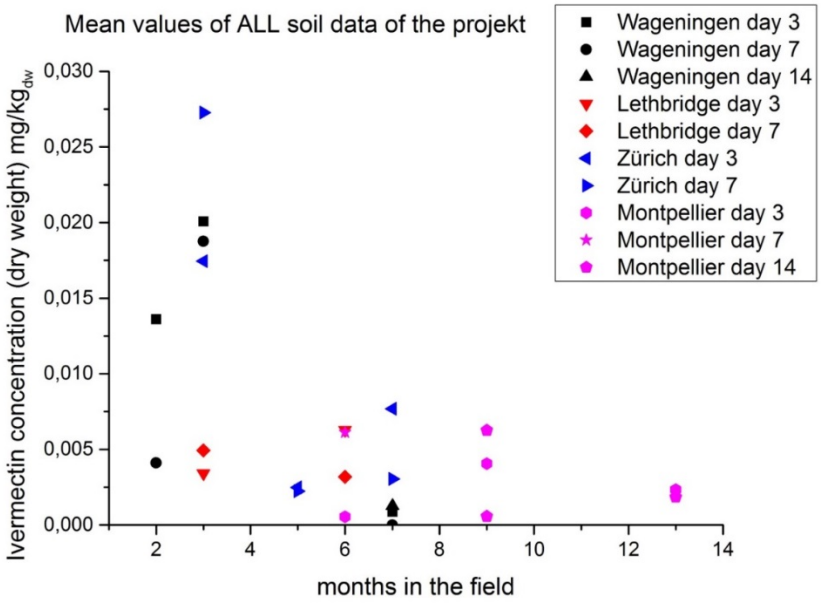


Abbildung B: Ivermectin-Konzentration im Boden (Einzelwerte). X-Achse: Expositionszeit der Dunghaufen im Freiland; Y-Achse: IVM-Konzentration in mg/kg Dung TG)



Genereller Überblick über die Ergebnisse der Freilandstudien

Im Folgenden werden die vier oben gestellten Ziele bzw. Fragen der Studie auf der Grundlage der eigenen Ergebnisse diskutiert (vgl. Liebig et al. 2010; Lumaret et al. 2012).

1. **Hat der Einsatz von Ivermectin langfristige Folgen für die Biodiversität der Dungorganismen? Speziell: Wie lange hält die "toxische" Wirkung im Dung an?**

Die Wirkungen von Ivermectin auf die Dung- und Bodenorganismen sind in Tabelle B zusammengestellt. In dieser einfachen Übersicht werden die jeweiligen Wirkungen (oder deren Fehlen) auf die verschiedenen biologischen Endpunkte in drei Klassen eingeteilt: ROT: Signifikante Wirkungen bei der angegebenen Konzentration auf Taxa aus der jeweiligen Organismengruppe. GRÜN: Keine signifikanten Wirkungen auf die verschiedenen biologischen Endpunkte aus der jeweiligen Organismengruppe.

GELB: Alle zwischen ROT und GRÜN liegenden Fälle. In einigen Fällen war eine entsprechende Klassifikation nicht möglich, da die jeweiligen Organismen nicht (oder nur in sehr geringer Anzahl) an dem jeweiligen Versuchsstandort vorkamen. Aufgrund dieses Überblicks lautet die Antwort auf Frage 1: Ja, der Einsatz von Ivermectin hat eine langanhaltende Wirkung auf verschiedene Gruppen von Dungorganismen, speziell Fliegen (meist Sphaeroceridae und Sepsidae). Selbst 28 Tage nach Applikation von IVM auf Rinder ist der von ihnen ausgeschiedene Dung, der niedrige IVM-Konzentrationen im Bereich von 0,01 – 0,05 mg/kg Dung TG enthält, hochtoxisch für verschiedene Fliegengruppen an allen Versuchsstandorten. Aufgrund der nur in Lethbridge durchgeführten Verwendung einer weiteren Dungcharge (56 Tage nach Versuchsbeginn), wurde nachgewiesen, dass diese Wirkung mindestens so lange anhält.

Ivermectin hat zudem negative Auswirkungen auf den Schlupf von Dungkäfern aus dem Dung behandelter Rinder, doch ist dieser Effekt auf die ersten zwei Wochen nach der Applikation begrenzt. Dabei ist nicht nur die Familie Aphodiidae (d.h. "typische" Dungkäfer) betroffen, sondern es wurden auch die Familien Hydrophilidae und, etwas weniger, Ptilidae (die beide oft übersehen werden) beeinträchtigt. Kurzflügelkäfer (Staphylinidae) und parasitische Wespen zeigen dagegen eine mittlere Sensitivität: Geringer als die vielen Fliegen aber höher als die der meisten Dungkäfer.

An allen Standorten (aber in unterschiedlicher Intensität) wurden signifikante Wirkungen auf Regenwürmer und vor allem Collembolen an verschiedenen Zeitpunkten und Behandlungsstufen beobachtet. Obwohl es nicht auszuschließen ist, dass Ivermectin für diese Unterschiede zwischen Kontrollen und einzelnen Behandlungsstufen verantwortlich war, fehlte oftmals eine klare Dosis-Wirkungs-Beziehung. Das heißt, dass eine mögliche Wirkung von IVM auf Bodenorganismen sicher schwächer als die auf Dungorganismen ist.

Trotz der eindeutigen Wirkungen auf Dunginsekten wurde keine signifikante Beeinträchtigung des Dungabbaus durch Ivermectin an allen Versuchsstandorten gefunden. Die Gründe dafür sind nicht klar, doch es könnte sein, dass der Dungabbau, zumindest in den späteren Stadien, mehr durch die Aktivität der Regenwürmer (die durch IVM kaum betroffen sind) sowie durch physikalische Prozesse erfolgt und nicht in erster Linie durch die Aktivität koprophiler Insekten. Offensichtlich haben auch (extreme) Wetterbedingungen das Versuchsergebnis an einigen Standorten beeinflusst, z.B. in dem sie sich negativ auf die Abundanz wichtiger Dungorganismengruppen auswirkten (z.B. in Montpellier, wo Dungkäfer viel seltener als in früheren Jahren auftraten).

In diesem Zusammenhang sollte nicht vergessen werden, dass im Freiland auch Arten beeinträchtigt waren (z.B. Wespen oder Kurzflügel-käfer), für die es bisher keine standardisierten Testverfahren gibt. Es sollte untersucht werden, ob es einen Bedarf für neue Testverfahren mit diesen Organismen gibt.

2. Gibt es einen Unterschied zwischen der Sensitivität der im Labor untersuchten Arten und denjenigen Arten, die in den vier Freilandstudien gefunden wurden? Mit anderen Worten: Sind die in den Standardtests verwendeten Arten repräsentativ für die Gemeinschaften im Freiland?

Die in diesem Vorhaben zusammen gestellten Ergebnisse aus Standardlabortests mit Dung- und Bodenorganismen (d.h. die Fliegen *Scathophaga stercoraria* und *Musca autumnalis*, die Käfer *Aphodius constans* und *Onthophagus taurus* (Kompartiment Dung) als auch die Regenwurmart *Eisenia fetida/andrei* sowie der Collembole *Folsomia candida* (Kompartiment Boden) lassen sich mit den Ergebnissen aus den hier beschriebenen Freilandstudien vergleichen, in dem jeweils die Testdaten aus einem Standardtest (Tab. C) den Testdaten aus dem Freiland für die gleiche Organismengruppe gegenübergestellt werden (Tab. B).

Demnach ähneln die Ergebnisse aus dem Labor mit Fliegen denjenigen aus dem Freiland, wobei Sepsidae meistens die sensitivste Gruppe sind, gefolgt von den Sphaeroceridae. Daher könnte eine Konse-

quenz aus dieser Studie sein, einen Standardtests mit Sepsiden zu entwickeln, da diese Familie in Hinsicht auf ihre Diversität, Sensitivität, weite Verbreitung und Praktikabilität für diesen Zweck gut geeignet ist (Blanckenhorn et al. 2013a, b).

Im Fall der Dungkäfer ist die Situation komplexer, da an den vier Standorten unterschiedliche Käfergruppen am sensitivsten reagierten. Speziell bei den Aphodiidae gab es sowohl sehr empfindliche (z.B. in Wageningen) als auch völlig unempfindliche (z.B. in Lethbridge) Arten. Die Hydrophilidae waren ebenfalls recht empfindlich, kamen aber nicht an allen Standorten vor. Es scheint, dass die Labortestergebnisse mit Dungkäfern eher am unteren Ende der in den Freilandstudien gefundenen Empfindlichkeitsskala anzusiedeln sind. Dies trifft besonders dann zu, wenn man auch die Ergebnisse aus verlängerten Labortests mit dem Dungkäfer *Aphodius constans* in den Vergleich mit einbezieht (Römbke et al. 2012). Allerdings ist Sensitivität nicht das einzige Entscheidungskriterium für die Auswahl der Testspezies. Die Abdeckung verschiedener ökologischer Rollen (und damit Expositions-szenarien) sowie der biogeographischen Verbreitung sollten auch berücksichtigt werden.

Im Fall der Bodenorganismen sieht es so aus, als ob die Ergebnisse aus den Standardlabor-tests protektiv für Regenwürmer sind; d.h. dass die Wirkwerte im Freiland höher sind als in den Labortests. Die Wirkungen auf die Collembolen im Freiland sind schwer zu interpretieren, teils, weil es keine eindeutigen Dosis-Wirkungs-Beziehungen gab, teils, weil nur in Wageningen ein signifikanter Effekt festgestellt wurde. Daher ist anzunehmen, dass die Ergebnisse der Labortests protektiv für diese wichtigen Organismen sind. Allerdings wäre es, um auf der sicheren Seite zu sein, hilfreich, Multi-Spezies Verfahren mit Collembolen zu entwickeln, da die Tiere in diesen Tests sehr empfindlich reagierten (e.g. Jensen & Scott-Fordsmand 2012).

Tabelle B: Überblick über die Wirkung von Ivermectin auf verschiedene biologische Parameter. ROT (e. = Effekt): Signifikante Wirkung bei der angegebenen Konzentration auf die jeweilige Organismengruppe. GRÜN (n.e. = kein Effekt): Keine signifikante Wirkung auf die jeweilige Organismengruppe. GELB (n.c. = nicht angebbar): Alle Fälle zwischen den beiden GRÜN und ROT. NA = nicht anwendbar: Die jeweilige Organismengruppe kam nicht vor, wurde nicht untersucht oder beurteilt. Die vollständigen Namen der aufgeführten Organismengruppen sind unterhalb von Tabelle C aufgeführt.

Org. Gruppe	**Montpellier**					**Zurich**					**Wageningen**					**Lethbridge**					**Alle zusammen**			
IVM Konz.[1]	**0.05**	**0.69**	**2.48**	**2.84**		**0.05**	**0.69**	**2.48**	**2.84**		**0.05**	**0.69**	**2.48**	**2.84**		**0.02**	**0.06**	**0.35**	**7.68**	**5.03**	**Niedrig ➔ Hoch**			
Käfer	n.e.	n.e.	n.e.	e.		n.c.	n.c.	n.c.	e.		n.c.	e.	e.	e.		n.c.	n.c.	n.c.	e.	e.	n.c.	n.c.	e.	e.
Hydro.	NA	NA	NA	NA		n.e.	n.e.	e.	e.		n.e.	e.	e.	e.		e.	e.	e.	e.	e.	n.e.	n.c.	e.	e.
Ptil.	NA	NA	NA	NA		n.e.	n.e.	n.e.	n.e.		NA	NA	NA	NA		n.e.	n.e.	n.e.	e.	e.	n.e.	n.e.	n.c.	n.c.
Apho.	n.e.	n.e.	n.e.	e.		n.e.	n.e.	n.e.	n.e.		e.	e.	e.	e.		n.e.	n.e.	n.e.	n.e.	n.e.	n.c.	n.c.	n.c.	n.c.
Staph.	n.e.	n.c.	n.c.	e.		e.	e.	n.c.	n.c.		n.c.	n.c.	n.c.	n.c.		n.c.	n.e.	n.e.	e.	e.	n.e.	n.c.	e.	e.
Fliegen	n.c.	e.	e.	e.		n.e.	n.e.	n.c.	n.c.		n.c.	n.c.	n.c.	n.c.		n.c.	e.	e.	e.	e.	n.c.	n.c.	e.	e.
Cecy.	NA	NA	NA	NA		n.e.	n.e.	n.e.	e.		n.e.	n.e.	n.e.	n.e.		NA	NA	NA	NA	NA	n.e.	n.e.	n.e.	n.c.
Chiro.	NA	NA	NA	NA		n.e.	n.e.	e.	e.		n.e.	n.e.	n.e.	n.e.		NA	NA	NA	NA	NA	n.e.	n.e.	n.c.	n.c.
Sepsi.	e.	e.	e.	e.		e.	e.	e.	e.		e.	e.	e.	e.		e.	e.	e.	e.	e.	e.	e.	e.	e.
Sphae.	n.e.	e.	e.	e.		NA	NA	NA	NA		e.	e.	e.	e.		n.e.	e.	e.	e.	e.	n.c.	e.	e.	e.
Wespen	n.e.	n.e.	e.	n.e.		e.	e.	e.	e.		NA	NA	NA	NA		NA	NA	NA	NA	NA	n.c.	n.c.	e.	n.c.
Earth.	NA	NA	NA	NA		e.	e.	e.	n.e.		n.e.	n.e.	e.	e.		NA	NA	NA	NA	NA	n.c.	n.c.	e.	n.c.
Coll.	n.e.	n.e.	n.e.	n.e.		e.	n.e.	e.	e.		e.	n.e.	n.e.	e.		n.e.	n.e.	n.e.	n.e.	e.	n.c.	n.e.	n.c.	n.c.
Degra.	n.e.					n.e.					n.e.					n.e.					n.e.			

[1]: IVM Konzentration im Dung in mg/kg Trockengewicht

Tabelle C: Überblick über die Wirkungen von Ivermectin auf verschiedene Organismengruppen und Endpunkte. Linke Spalte: Ergebnisse aus standardisierten Labortests mit dem EC50-Wert als Wirkungs-Endpunkt. Andere vier Spalten: Niedrigste Ivermectin-Konzentration mit einem signifikanten Effekt auf das jeweilige Taxon (meist auf Familienebene) an den vier Versuchsstandorten zu verschiedenen Zeitpunkten. Alle Ivermectinkonzentrationen sind in mg/kg Dung oder Boden TG. Zu den Details siehe WP I, Kapitel 6 (Ergebnisse der Labortests) oder WP II, Kapitel 11 – 15. NA = nicht anwendbar: Die jeweilige Organismengruppe kam nicht vor, wurde nicht untersucht oder beurteilt.

Labor (mg/kg TG)		**Montpellier**		**Zurich**		**Wageningen**		**Lethbridge**	
Spezies	EC50	Taxon	Signif. Effekt	Taxon	Signif. Effekt	Taxon	Signif. Effekt	Taxon	Signif. Effekt
M. autumnalis	0,035	Sepsidae	≤0,05	Sepsidae	≤0,05	Sepsidae	≤0,05	Sepsidae	≤0,02
S. stercoraria	0,150	Sphaerocer.	0,69	Chironomidae	2,48	Sphaerocer.	≤0,05	Sphaeroceridae	0,06
A. constans	0,880	Aphodiidae	2,84	Aphodiidae	>2,84	Aphodiidae	≤0,05	Aphodiidae	>7,68
				Hydrophilidae	2,48	Hydrophilidae	0,69	Hydrophilidae	≤0,02
O. taurus	0,220			Ptilidae	>2,84			Ptilidae	5,03
E. fetida/andrei	5,30	Nicht anwendbar		Lumbricidae	0,05?	Lumbricidae	2,48	Nicht anwendbar	
F. candida	1,70	Collembola	>2,84	Collembola	≤0,05?	Collembola	≤0,05	Collembola	5,03

? Aus verschiedenen Gründen (siehe Text), sind diese Werte nicht belastbar.

Käfer: Hydrophilidae, Ptilidae, Aphodiidae, Staphylinidae

Fliegen: Cecydomyiidae, Chironomidae, Sepsidae, Sphaeroceridae

Andere: Wespen, Lumbricidae, Collembola

3. Gibt es Belege für die Erholung der Dungorganismengemeinschaften – und kann man dabei die interne (d.h. intrinsische) Erholung von einer Erholung mittels Einwanderung ("Rekolonisierung") unterscheiden?

Hinsichtlich der Wirkungen von Ivermectin auf verschiedene Organismengruppen (Tab. B) in Dung vom Tag 28 wurde festgestellt, dass es trotz niedriger IVM-Konzentrationen im Versuchsverlauf keine Erholung bei Dungfliegen gab, speziell den Sphaeroceridae und Sepsidae. Diese Aussage trifft wahrscheinlich auch auf Dung zu, der 56 Tage nach Applikation ausgeschieden wurde, aber dies wurde nur am Standort Lethbridge untersucht. Zumindest einige Gruppen von Wespen und Kurzflügelkäfern reagieren ebenfalls stark auf Dung, der 28 Tage nach Applikation von IVM ausgeschieden wurde. Im Gegensatz dazu zeigten Dungkäfer keine Beeinträchtigung durch den Dung vom Tag 28. Aus der Literatur, ist bekannt, dass die wichtigsten Dungorganismengruppen unterschiedlich auf Ivermectin reagieren und zudem, in Abhängigkeit von ihren jeweiligen ökologischen Eigenschaften, über unterschiedliche Wiedererholungsstrategien verfügen. Diese Art von ökologischer Information auf der Artebene ist sehr hilfreich, wenn es um die Definition von Risikominimierungsstrategien (RMM). Leider sind diese Informationen entweder nicht vorhanden oder weit in der Literatur verstreut.

Dungorganismen sind häufig sehr mobil: So erscheint die gelbe Dungfliege *Scathophaga stercoraria* nur wenige Minuten nach deren Ausbringung an frischen Dunghaufen – obwohl die nächsten Plätze, an denen sie gewartet haben könnten, mindestens 50 m entfernt liegen. Die Entscheidung in dieser Studie, die vorhandenen Ressourcen für eine genaue Untersuchung der Wirkungen und nicht die der Wiedererholung zu nutzen hing nicht zuletzt vom großen Aufwand für letzteres ab. Spätestens wenn die Effekte von Ivermectin auf Dungorganismen modelliert werden sollen, ist es wichtig, Angaben zu den Bedingungen zu haben, unter denen eine Wiedererholung dieser Arten möglich ist (Brühl et al. 2012)).

Für die Bodenorganismen konnte eine Wiedererholung innerhalb der Projektlaufzeit nicht bestimmt werden, was u.a. daran lag, dass die Ergebnisse schwer zu interpretieren waren. Während der Versuchsdauer (bis zu 14 Monate nach der Applikation von IVM) war es schwierig, die Wirkungen von Ivermectin im Dung und den verschiedenen gleichzeitig auf die Regenwürmer und Collembolen wirkenden Umweltfaktoren zu differenzieren. Da der größte Teil des Dungs innerhalb weniger Monate abgebaut wurde, waren die IVM-Konzentrationen im Boden niedrig. Zudem war die durch Ivermectin beeinflusste Fläche im Vergleich zur Gesamtfläche des jeweiligen Versuchsstandorts klein, so dass schon aus diesen Gründen ein langanhaltender Effekt auf die Bodenorganismen unwahrscheinlich war.

4. Gibt es Risikominimierungsmaßnahmen (RMM), mittels derer die Diversität der Dungorganismen geschützt werden kann?

Wahrscheinlich nicht. Ausgehend von den Arbeiten von Liebig et al. (2011; 2014) wurden die im Einklang mit der gegenwärtigen europäischen und deutschen Rechtsprechung vorgeschlagenen RMM mit Bezug zum Schutz der Dungorganismen kurz vorgestellt. Allerdings ist darauf hinzuweisen, dass gegenwärtig eine detaillierte Beschreibung möglicher RMM und deren Anwendung unter Nutzung eines festen Kriteriensets (z.B. Praktikabilität) durch das Umweltbundesamt erarbeitet wird (Adler, pers. Mitteilung).

Empfehlungen und Ausblick

Die folgende Liste basiert auf den Erfahrungen aus diesem Vorhaben, d.h. primär zu den Auswirkungen einer einzelnen Applikation von Ivermectin auf Rinder für die Struktur und Funktion von Dung- und Bodenorganismengemeinschaften an vier verschiedenen Standorten. Zusätzlich wurde auf Erfahrungen aus der Literatur zurückgegriffen.

- Zukünftig durchzuführende Freilandstudien mit Ivermectin oder anderen Antiparasitika in Rinderdung sollten eine Probenahme nach 56 (oder mehr) Tagen nach Applikation einplanen.
- Es wird empfohlen, dass, in Abhängigkeit von den Ergebnissen aus Labortests, Bodenorganismen in solchen Freilandstudien mit abgedeckt werden.

- Das verwendete Testdesign ist dazu geeignet, Auswirkungen von Antiparasitika auf die Struktur der Dungorganismengemeinschaft sowie den Dungabbau im Freiland als Teil eines „higher-tier testing for risk assessment" zu erfassen.
- Da die vorhandenen Informationen zur Ökologie und Biogeographie von Dungorganismen nicht ausreichen, sind Forschungsanstrengungen zur Schließung dieser Datenlücken, differenziert für wichtige ökologische Regionen Europas, notwendig.
- Zusätzlich sollten diese Informationen in einer zentralen Datenbank erfasst werden (als Beispiel könnte die Datenbank zur Bodenbiologie in Deutschland dienen: EDAPHObase (Burkhardt et al. 2014)).
- Falls Freilandstudien Teil einer zukünftigen "higher-tier assessment strategy" werden, sollte die Durchführung solcher Studien in einem OECD "Guidance Document" beschrieben werden. Dieses Dokument könnte auf der in diesem Projekt erarbeiteten SOP sowie Literaturangaben basieren (speziell Jochmann et al. 2011).
- Der Schutz der Struktur (= Biodiversität) und Funktion (= Leistungen) von Dung- und Bodenorganismen sollte in die bestehenden VICH/EMA Dokumente aufgenommen werden – wie es auch schon für Pestizide erfolgt ist (EC 2009).
- Ausgehend von einem aktuellen Übersichtsartikel (Liebig et al. 2011; 2014) wurden bisher vorgeschlagene Risikominimierungsmaßnahmen (RMM) für Antiparasitika kritisch beurteilt. Demnach sind diese nicht ausreichend für den Schutz von Dung- und Bodenorganismengemeinschaften.

Summary

Aims of the project

This project had two main aims:

a) Description of the current knowledge about the effects of ivermectin on the diversity of dung fauna, soil invertebrates and plants;
b) Implementation of the new information into existing risk assessment and risk management schemes for ivermectin and other parasiticides.

Basic consideration when planning the practical work of this project was the common understanding that any risk assessment is performed in order to address two protection goals: both the function and the structure of the (dung) ecosystem have to be protected. The latter is defined as follows: "biodiversity" is "the variability among living organisms from all sources, including, 'inter alia', terrestrial, marine, and other aquatic ecosystems, and the ecological complexes of which they are part: this includes diversity within species, between species and of ecosystems" (UNCED 1992).

Based on this common understanding about the protection goals the aims of the project could be specified as follows:

1. Does the use of ivermectin cause any long-term effect on dung fauna and soil organism biodiversity?
2. Is there a difference between the sensitivity of the species tested in standard laboratory tests and those species found in the four field studies? Are the model species used in the laboratory representative for the communities in the field?
3. Is there evidence of recovery of dung organism populations? In this context, the composition of the respective dung and soil organism communities are important.

The implementation of these aims will firstly be described by summarizing the practical work at four sites with clearly different ecological conditions. Based on this information, general guidance could be given in terms how to perform field studies in the context of the environmental risk assessment of parasiticides. In fact, this guidance was laid down in the format of Standard Operation Procedures (SOPs).

In this summary chapter, the methods used and the results gained in the four field tests are compiled and discussed. The main question is whether field studies could be a relevant extension part of the current test strategy for the ERA of VMPs (VICH 2004; see also Jochmann et al. (2011) and Adler et al. (2013). Based on this information, recommendations for the testing of parasiticides will be presented at the end of this document. Finally, the following questions will be addressed:

- Do risk mitigation measures exist that can protect the biodiversity of dung organisms?
- Is there a possibility for a sustainable pasture management by using parasiticides?

Thus, by combining our knowledge on the effects of IVM on the structure and function of dung and soil communities with recommendations for risk mitigation measures (RMM) it will be possible to understand which role RMM can play in future ERA of parasiticides.

Short description of the methodological approach

The tests at all four sites followed the same SOP which was developed as a joint effort by all partners at the beginning of the project. Deviations had to be justified.

In Table A, the main characteristics of the four study sites are presented. Their climatic conditions clearly differ in terms of mean air temperature and the annual precipitation. However, soil properties do not differ that much (exceptions: low pH and sandy texture at Wageningen).

Table A: Overview on the main site and soil properties of the four study sites

Site / Soil Parameters	Montpellier	Zurich	Wageningen	Lethbridge
Country	France	Switzerland	Netherland	Canada
Coordinates	43°79'33.40 N; 3°73'18.75 O	47°23'44.87 N; 8°33'02.62 O	51°59'32.16 N; 5°39'39.82 O	49°41'25.46 N; 112°46'26.15 W
Land use	Grass strip near crop site	Borderline of a meadow	Meadow (since 2006)	Meadow, used for cattle
Ann. precipit. (mm/y)	700	1123	846	365
Mean ann. temp. (°C)	13.0	7.9	10.5	5.8
pH ($CaCl_2$ - method)	7.6	7.4	5.2	7.3
Organic matter (%)	3.1	4.6	2.9	6.2
Bulk density (g/L)	1149	1254	1449	987
WHCmax	48.0	47.6	34.2	60.7
Carbon	16.75	24.28	12.55	27.35
Nitrogen	1.646	3.018	1,009	2.747
C/N ratio	10.18	8.05	12.44	9.96
Soil texture	Silty loam	Clay loam	Pure sand	Weakly clay loam

Each study included a structural experiment (duration: one week; main endpoint: dung organism diversity) and a functional experiment (duration: up to a year; main endpoint: dung degradation; plus diversity and abundance of soil organisms). In both experiments, artificial dung pats prepared from the dung of cattle treated with ivermectin (IVM) were exposed in the field. Dung was collected from untreated cattle (Day 0) in Montpellier (used for the three European studies) and in Lethbridge (used for the Canadian study). In early spring 2011, cattle were treated with a topical formulation of ivermectin (Ivomec® pour-on) at the recommended dose (500 µg IVM/kg body weight). Fresh dung (<3 h old) was collected from treated animals 3, 7, 14, and 28 days post-application (in Lethbridge, dung was collected after 56 days too). Dung from each treatment was mixed, and pats of 500 g (Montpellier: 800 g) were prepared and placed in the field.

In the structural part of the experiment, the artificial dung pats (ten replicates for each of the five (six) treatments: Days 0, 3, 7, 14, 28 (56) = 50 (60) pats)) were exposed on plates in the field. After one week, the pats were collected and transported to the laboratory. Each dung pat was placed in a specially designed emergence trap that captured any flying and crawling insects emerging from the dung in ethanol. The pats were kept for more than three months in the traps. In Montpellier, a different approach was used. Containers (capacity 7L, 25 cm high, Ø 15 cm) were buried to their rim in the soil and one dung pat was deposited in each container. Dung pats were left free to be colonized by insects for one, two and three weeks, then emergence traps were set up to collect insects as they emerge. In all emergence traps, emergent insects were collected at regular intervals, preserved in 70% or 95% ethanol, and later identified and enumerated. Dung beetles were identified at species level, staphylinid beetles at the genus level and separated in two size classes (small and large). Flies and wasps (the latter only sampled in Montpellier and Lethbridge) were identified at the family level. Pitfall traps with dung as bait and filled with 4% formaldehyde solution were operated at all study sites (except Montpellier)

to determine the activity of insects at the study site before, during and after the time that pats were exposed in the field.

In the functional part of the experiment, 25 replicated pats were used per treatment (Days 0, 3, 7, 14, 28, (56) = 125 and 150 pats in Europe and Canada, respectively) and placed outdoors in a randomized grid. Each pat was exposed directly on the soil surface. Five 'function' pats per treatment were removed from the field at differing dates at the four sites up to twelve months after exposure. They were ground, weighed and sub-samples were oven-dried for at least 48h at 100°C. Approximately 50 g were heated in a muffle furnace at 500°C for 12 h to determine the ash content. Main measurement endpoint was dung mass loss, determined as ash-free dry weight (i.e. organic matter).

At the same dates of the functional experiments, soil organisms were sampled. First two soil cores were taken directly below the pat (one for micro-arthropods, one for residue analysis). Afterwards, a hole was dug into the soil (25 * 25 cm, depth 10 cm) and the taken soil was sorted for earthworms by hand directly in the field (not at Lethbridge because there no earthworms do occur). Micro-arthropods were extracted from the soil cores by heat extraction. Only earthworms and Collembola were determined on the species level and were also divided into three ecological groups. The rest was separated into larger taxonomic groups (e.g. Oribatida, Gamasida etc.).

For residue analysis, the extraction procedure according to Litskas et al. (2010) was used. Homogenized soil and dung samples were extracted with acetonitrile (internal standard: doramectin) and cleaned-up. Derivatization was performed according to Berendsen et al. (2007). For all soil samples a limit of detection (LOD) of 0.9 µg/kg d.w. and a limit of quantification (LOQ) of 2.3 µg/kg d.w. was determined. The LOD for dung samples was 5.1 µg/kg d.w. and the LOQ 12.4 µg/kg d.w. The mean recovery of the internal standard doramectin in the different soil and dung samples differed between 97.7 % and 101.3 %.

In the structural experiment the number of taxa emerged were analyzed using ANCOVA or, alternatively, the Shannon diversity index (when comparing individual sites) as a function of the absolute ivermectin concentration (continuous regression). In the functional experiment, for dung degradation (dry weight) over time (months) as a function of ivermectin concentration firstly the best-fit function (linear) to the data was identified (Burnham & Anderson 2001). For significance testing across ivermectin treatments and/or sites, ANCOVA of the pat dry weights (log10-transformed or not) against month was used, including ivermectin treatment (and site) as fixed factors. Retarded dung decomposition at higher ivermectin concentrations would in this analysis show as a time (i.e. month) by ivermectin concentration interaction.

Results of the four field studies

Due to the complexity of the study design (four sites, two compartments (dung, soil)), structural and functional endpoints, and in particular the high number of organism groups and species, it is impossible to present all results. Thus, for each study site, characteristic data are presented. Finally, the most relevant results are given in an overview

Methodological considerations

Only few deviations from the original design did occur. In general, climatic conditions did influence the studies strongly. For example, due to long periods of drought in Montpellier the number of earthworms and springtails was too low to be interpreted. Earthworm sampling was not performed in Lethbridge, since large soil organisms are rare or absent at prairie pastures for natural reasons. Technically, the sampling of earthworms and spring-tails below the dung pats was no problem following the respective guidelines (ISO 2006a, b) Regarding species determination, no problems occurred since in this study taxonomists were included. However, in order to decrease the considerable effort needed

for species determination for almost all groups of soil organisms, it is recommended to use genetic methods („Barcoding“) in order to facilitate addressing the species level in the future.

Residue analysis

For all soil samples a limit of detection (LOD) of 0.9 µg/kg d.w. and a limit of quantification (LOQ) of 2.3 µg/kg d.w. was determined. The LOD for dung samples was 5.1 µg/kg d.w. and the LOQ 12.4 µg/kg d.w. In the three European studies dung samples prepared in Montpellier were used. The mean concentrations of IVM in the four treatment groups collected 3, 7, 14 and 28 days after treatment were 2.845, 2.480, 0.692 and 0.049 mg IVM/kg dung d.w., respectively (see Fig. A). In dung from treated cattle in Lethbridge, detected levels of ivermectin residues at the time of excretion were 5.029 (Day 3), 7.675 (Day 7), 0.341 (Day 14), 0.065 (Day 28) and 0.015 mg IVM/kg dung d.w. (Day 56). The degradation of ivermectin in dung pats deposited in the field followed a logarithmic curve for all the treatments. A quick decrease of ivermectin concentration in dung was observed in the first months, with a DT50 for the two highest initial concentrations (3 and 7 days post-administration (DPA)) obtained after 2-3 months post deposit, followed by a slower decrease. For all treatments except 28 DPA, ivermectin was still detectable in dung pats after 13 months.

Residue levels of ivermectin in dung of treated cattle were generally comparable between European and Canadian studies. Peak excretion of residues following topical application of ivermectin occurs 2-3 days post-treatment, followed by a sharp decline to form a long tail that may persist for more than 4 to 6 weeks (e.g., Herd et al. 1996). Results of chemical analyses documented the presence of ivermectin residues in dung of treated cattle, declining in a pattern consistent with previous studies. (e.g. Edwards et al. 2001; Boxall et al. 2004; Fernández et al. 2009).

Soil samples were taken at different times at the four study sites over the period between two and 13 months after starting the study. With few exceptions, the concentration of ivermectin was only detectable in the D3 and D7 treatments (Fig. B). In Lethbridge and at later dates at the other sites, almost similar mean values between 0.002 and 0.006 mg IVM/kg soil d.w. were found. The concentration of ivermectin in soil has rarely been measured in field studies (e.g. Römbke et al. 2010b). Comparable concentrations, but almost only in the uppermost centimeter of soil, have been found in a field study performed near York, England (Pope, 2010). Concentrations found in soil in this study are well within the range determined at farm sites in England where ivermectin has regularly been applied to livestock (Boxall et al. 2006).

Figure A: Ivermectin concentration in dung over time (individual values). Months in the field post-deposit are on the abscissa and ivermectin concentration (mg/kg Dung dw) on the ordinate.

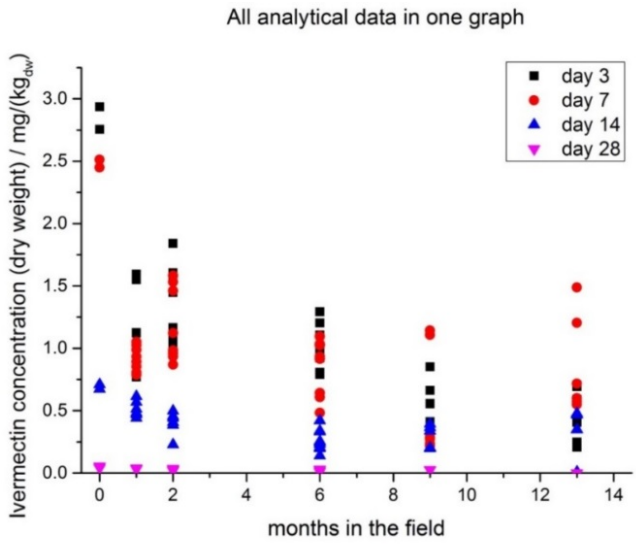
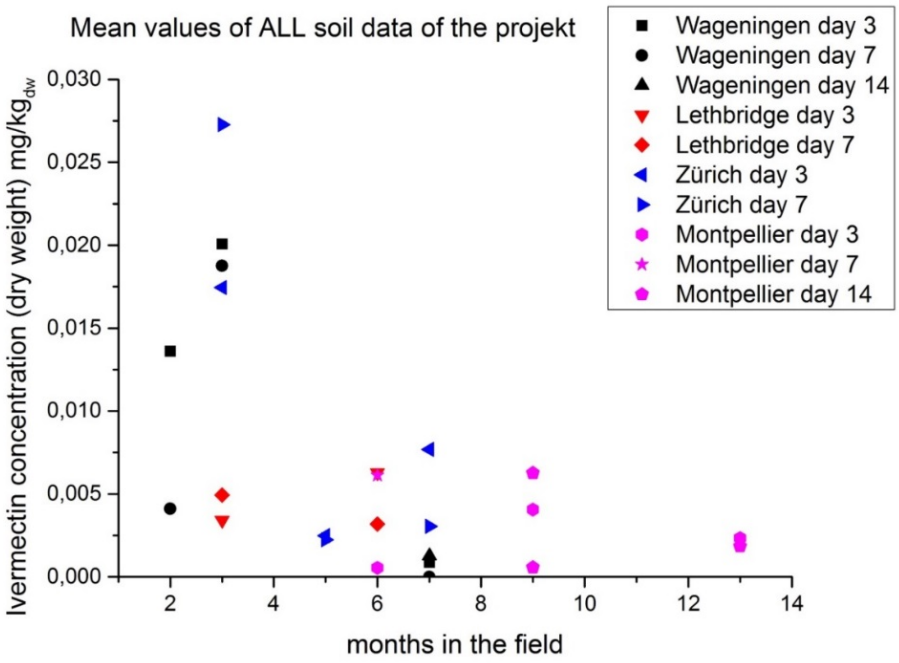


Figure B: Ivermectin concentration in soil over time (individual values). Months in the field post-deposit are on the abscissa and ivermectin concentration (mg/kg soil dw) on the ordinate.



Overall summary of the results of the field studies

In the following, the four main aims (or questions) listed in the Introduction are reconsidered in light of our overall results (vgl. Liebig et al. 2010; Lumaret et al. 2012).

1. Does the use of ivermectin cause any long-term effect on dung fauna biodiversity? In particular, how long stays the "toxic" impact of the dung?

The effects of ivermectin on the dung and soil organism communities is summarized in Table B. In this simple approach, the effect (or the lack of it) of ivermectin on various biological endpoints is classified in one of three classes: RED: Significant effects at the respective concentration on the taxa belonging to the respective group; GREEN: No significant effects at the respective concentration on the taxa belonging to the respective group; YELLOW: all cases in-between RED and GREEN. In some cases, one endpoint could not be evaluated, because these organisms were not (or only in very few numbers) present at the specific site.

According to this overview the answer to Question 1 is: Yes, the use of ivermectin has a long-term effect on various groups of dung organisms, in particular flies - most notably the Sphaeroceridae and Sepsidae. Even dung excreted 28 days after application of this VMP, containing ivermectin at concentrations as low as 0.01 - 0.05 mg/kg d.w., is highly toxic for various fly groups at all test sites. Actually, due to the study of an extra treatment only performed at the Lethbridge site it is highly likely that this effect does last at least 56 days after application of ivermectin, if not longer.

Ivermectin also negatively impacts the emergence of dung beetles from treated cattle dung, but in this case the effect is mainly limited to the first two weeks after the application of ivermectin. In addition, the impact is not restricted to species of the family Aphodiidae (i.e. "typical" dung beetles), but species of the families Hydrophilidae and, to a lesser extent, Ptilidae (both often overlooked) were also impacted. Staphylinid beetles and parasitic wasps show an intermediate sensitivity: less than various flies but more than many dung beetles. This result was not expected from the relatively few studies with rove beetles found in the literature.

At all sites (but in different intensities) significant reductions of the number of earthworms and springtails could be found at several ivermectin concentrations and sampling dates. However, despite an indication that ivermectin was responsible for these differences, a clear concentration-effect relationship could not be identified, so overall the effect of ivermectin on these two soil organism groups was certainly weaker than that on the dung decomposing groups.

Despite these noticeable detrimental effects on arthropods, ivermectin overall did not significantly hamper dung degradation at all test sites. As discussed in the respective section above, this indicates that dung pat degradation, at least at the later stages, is more a function of the activity of earthworms, which were only rarely affected by ivermectin, and physical deterioration, rather than biological degradation by coprophilic insects. Obviously, (extreme) weather conditions may have influenced the outcome of these studies at some sites, e.g. via affecting the abundance of certain important groups of dung insects (such as in Montpellier, where dung beetles were much rarer than in previous years).

Last but not least it should not be forgotten that in the field regularly other organism groups were affected for which so far no standard tests exist, such as wasps or staphylinid beetles. The need for further tests with such organisms should be assessed.

2. Is there a difference between the sensitivity of the species tested in standard laboratory tests and those species found in the four field studies? In other words, are the model species used in the laboratory representative for the communities in the field?

The results of laboratory tests with dung and soil organisms can roughly be compared quantitatively with the effects determined in the field. The flies *Scathophaga stercoraria* and *Musca autumnalis*, the beetles *Aphodius species* and *Onthophagus taurus* (dung compartment) as well as the earthworm *Eisenia fetida/andrei* and the springtail *Folsomia candida* (soil compartment) have been used as standard laboratory test species (Table C).

Referring to this compilation the results of standard laboratory tests with flies are at the same order of magnitude as the effects found at most field sites (Table B). Sepsidae are always the most sensitive group, followed by the Sphaeroceridae. Therefore, one consequence of these results could be to standardise a test with sepsid flies, since they would be in terms of diversity, sensitivity, biogeographic, and practicability most suitable (Blanckenhorn et al. 2013a, b).

In the case of dung beetles the situation is more complex since their sensitivity appears to differ at the four sites. Especially Aphodiid species could be very sensitive (e.g. in Wageningen) or not at all (e.g. in Lethbridge). Hydrophilidae are also quite sensitive but do not occur at all sites. In general, the laboratory test results seem to be at the lower end of the sensitivity spectrum found in the field. This is especially true when considering the results of extended laboratory tests with the dung beetle *Aphodius constans* (not shown here), where EC50 values of 0.16 mg/kg IVM / kg dung d.w. were found (Römbke et al. 2012). However, sensitivity is not the only criterion when selecting test species. Different ecological roles (and thus exposure scenarios) and/or biogeographical ranges have also to be considered.

In case of soil organisms, it seems that the results of standard laboratory tests are protective (i.e. the effect values are higher in the field) for earthworms, despite some results from Zurich that need further evaluation. The effects on Collembola found in the field are difficult to interpret, partly because of a lack of dose-response relationships, partly because of the fact that only in Wageningen a clear significant effect was detected. Therefore, for the time being it is assumed that the laboratory tests are protective for these important non-targets. However, in order to be on the safe side, it would be very helpful to standardize multi-species laboratory or semi-field tests with Collembola since they are known to react very sensitively to Ivermectin in such complex situations (e.g. Jensen & Scott-Fordsmand 2012).

Table B: Overview on the effects of ivermectin on various biological endpoints: RED (e. = effect): Significant effects at the respective concentration on the respective group. GREEN (n.e. = no effect): No significant effects of the respective concentration on the respective group. YELLOW (n.c. = not clear): all cases in-between RED and GREEN. NA = Not applicable: Organism group not available, studied or evaluated. Full names of organism groups written-out below Table C.

Org. Group	Montpellier				Zurich				Wageningen				Lethbridge					All together			
IVM conc.[1]	0.05	0.69	2.48	2.84	0.05	0.69	2.48	2.84	0.05	0.69	2.48	2.84	0.02	0.06	0.35	7.68	5.03	Low → High			
Beetles	n.e.	n.e.	n.e.	e.	n.c.	n.c.	n.c.	e.	n.c.	e.	e.	e.	n.c.	n.c.	n.c.	e.	e.	n.c.	n.c.	e.	e.
Hydro.	NA	NA	NA	NA	n.e.	n.e.	e.	e.	n.e.	e.	e.	e.	e.	e.	e.	e.	e.	n.e.	n.c.	e.	e.
Ptil.	NA	NA	NA	NA	n.e.	n.e.	n.e.	n.e.	NA	NA	NA	NA	n.e.	n.e.	n.e.	e.	e.	n.e.	n.e.	n.c.	n.c.
Apho.	n.e.	n.e.	n.e.	e.	n.e.	n.e.	n.e.	n.e.	e.	e.	e.	e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.c.	n.c.	n.c.	n.c.
Staph.	n.e.	n.c.	n.c.	e.	e.	e.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.e.	n.e.	e.	e.	n.e.	n.c.	e.	e.
Flies	n.c.	e.	e.	e.	n.e.	n.e.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	e.	e.	e.	e.	n.c.	n.c.	e.	e.
Cecy.	NA	NA	NA	NA	n.e.	n.e.	n.e.	e.	n.e.	n.e.	n.e.	n.e.	NA	NA	NA	NA	NA	n.e.	n.e.	n.e.	n.c.
Chiro.	NA	NA	NA	NA	n.e.	n.e.	e.	e.	n.e.	n.e.	n.e.	n.e.	NA	NA	NA	NA	NA	n.e.	n.e.	n.c.	n.c.
Sepsi.	e.	e.	e.	e.	e.	e.	e.	e.	e.	e.	e.	e.	e.	e.	e.	e.	e.	e.	e.	e.	e.
Sphae.	n.e.	e.	e.	e.	NA	NA	NA	NA	e.	e.	e.	e.	n.e.	e.	e.	e.	e.	n.c.	e.	e.	e.
Wasps	n.e.	n.e.	e.	n.e.	e.	e.	e.	e.	NA	NA	NA	NA	NA	NA	NA	NA	NA	n.c.	n.c.	e.	n.c.
Earth.	NA	NA	NA	NA	e.	e.	e.	n.e.	n.e.	n.e.	e.	e.	NA	NA	NA	NA	NA	n.c.	n.c.	e.	n.c.
Coll.	n.e.	n.e.	n.e.	n.e.	e.	n.e.	e.	e.	e.	n.e.	n.e.	e.	n.e.	n.e.	n.e.	n.e.	e.	n.c.	n.e.	n.c.	n.c.
Degra.	n.e.				n.e.				n.e.				n.e.					n.e.			

[1]: IVM concentration indung in mg/kg dry weight

Table C: Overview on the effects of ivermectin on various organisms and endpoints. Left column: results of standard laboratory tests using the EC50 as effect value. Other four columns: lowest ivermectin concentration with a significant effect on taxa (usually family level) at the four study sites at different points in time. All ivermectin concentrations given in mg/kg dung or soil dry weight. NA: Not applicable: Organism group not available, studied or evaluated.

Labor. (mg/kg d.w.)		Montpellier		Zurich		Wageningen		Lethbridge	
Species	EC50	Taxon	Signif. effect	Taxon	Signif. effect	Taxon	Signif. effect	Taxon	Signif. effect
M. autumnalis	0.035	Sepsidae	≤0.05	Sepsidae	≤0.05	Sepsidae	≤0.05	Sepsidae	≤0.02
S. stercoraria	0.150	Sphaerocer.	0.69	Chironomidae	2.48	Sphaerocer.	≤0.05	Sphaeroceridae	0.06
A. constans	0.880	Aphodiidae	2.84	Aphodiidae	>2.84	Aphodiidae	≤0.05	Aphodiidae	>7.68
				Hydrophilidae	2.48	Hydrophilidae	0.69	Hydrophilidae	≤0.02
O. taurus	0.220			Ptilidae	>2.84			Ptilidae	5.03
E. fetida/andrei	5.30	Not applicable		Earthworms	0.05?	Earthworms	2.48	Not applicable	
F. candida	1.70	Collembola	>2.84	Collembola	≤0.05?	Collembola	≤0.05	Collembola	5.03

? For different reasons, these values are not robust.
Beetles: Hydrophilidae, Ptilidae, Aphodiidae, Staphylinidae
Flies: Cecydomyiidae, Chironomidae, Sepsidae, Sphaeroceridae
Others: Wasps, Earthworms, Collembola

3. Is there evidence of recovery of dung organism populations (if possible, can internal (intrinsic) recovery be distinguished from immigration ("re-colonization"))?

Referring to the effects visualized in Table B, there is no recovery of several dung fly families, especially the Sphaeroceridae and Sepsidae, in dung excreted 28 days after application of IVM. As already stated, this statement is probably also true for dung excreted after 56 days, but this was tested only at one site (Lethbridge). At least several groups of wasps and staphylinid beetles did also not recover in dung excreted 28 days after application of IVM. In contrast, the majority of dung beetles did recover at Day 28. Surely, as known from literature in general and from the results of laboratory tests in particular, the major organism groups do react differently towards IVM – and also do have different recovery strategies, depending on their ecology. This kind of ecological information on the species level could be helpful when defining risk mitigation measures (RMM), since they would be different depending on, for example, how long it may take for a population to recover after being affected by ivermectin the RMM. Unfortunately, this information is either not existing or so widely scattered, that it is not easily available.

Most of the dung organisms are very mobile: for example, according to own observations dung flies such as *Scathophaga stercoraria* occur at fresh dung pats within minutes after they have been deposited. The next place where they might have been waited was about 50 m away. The decision to focus our work on the potential effects was made in the light of the high efforts to study recovery in detail versus the limited resources available. However, especially when trying to model the effects of IVM on dung organisms it is extremely important to clarify under which conditions recovery will occur (cf. similar experiences with non-target arthropods (Brühl et al. 2012)).

In the case of soil organisms, full recovery could not be proven till the end of the experiment, but the results are difficult to interpret. During the study time (up to 14 months after application of IVM) the interaction between the effect of IVM in dung (probably one of the major food sources of the organisms studied) and the many environmental factors also affecting earthworms and springtails are difficult to separate. Since most of the dung was degraded within a few months, IVM concentrations in soil were relatively low, and the area impacted by IVM is relatively small in comparison to the overall area of the field a long-lasting effect of IVM on soil invertebrates is unlikely. The field tests were not designed in a way that recovery of dung or soil organisms could have been studied in detail. Especially the distinction between immigration and intrinsic recovery is difficult to make.

4. Do any risk mitigation measures (RMM) exist that can guarantee dung fauna biodiversity?

Probably not. However, some RMM might be helpful. Starting with the work of Liebig et al. (2011; 2014) those referring to the protection of dung organisms and which are in accord with European and German law were briefly summarized. It should be noted that the German Environmental Agency (UBA) is currently reviewing existing RMM and their usage on the basis of a fixed set of criteria (e.g. practicability) (Adler, pers. communication).

Recommendations and outlook

This list is based on the experiences made in this project, i.e. mainly regarding the effects of a single application of ivermectin to cattle on the structure and function of dung and soil organism communities at four different sites. In addition, information from literature has been taken into account.

- Future higher tier field experiments with ivermectin (or comparable VMP) and dung from cattle should include dung samples taken 56 days after treatment or even later.
- It is recommended to include the study of soil organism in field studies assessing side-effects of veterinary pharmaceuticals, depending on the results of laboratory tests.
- The study design is suitable to evaluate the effects of VMP on dung fauna structure and dung degradation under field conditions in higher-tier testing for risk assessment.

- Since the available information on dung organisms' biogeography and ecology is not existing further research is needed in order to improve our knowledge on the diversity, biogeography and ecology of dung organism communities in the different European ecological zones.
- In addition, a central database should be set for this kind of information (e.g. following the central German database on soil organisms: EDAPHObase (Burkhardt et al. 2014)).
- In case field studies will be part of a higher-tier assessment strategy, the performance of such studies has to be described in an OECD Guidance Document, which could be based on the SOP prepared in this project; see also Jochmann et al. 2011).
- The protection of the structure (= biodiversity) and function (= services) of dung and soil organism communities should be incorporated in the current VICH/EMA guidance documents as it has already been done for pesticides (EC 2009).
- Using a recent review (Liebig et al. 2011; 2014), existing risk mitigation measures (RMM) for VMP were critically evaluated. Those proposed so far are not sufficiently helpful for the protection of dung and soil organism communities.

1 Introduction

1.1 Aims of the project

According to the terms of this project it had two main aims:

a) Collection of knowledge about the effects of ivermectin on the diversity of dung fauna, soil invertebrates and plants (including the identification of knowledge gaps)
(Note that plants were not studied since the project focuses on dung fauna and associated soil fauna)
b) Implementation of the new information into existing risk assessment and risk management schemes for ivermectin and other parasiticides

Based on discussions with the sponsor these aims could be specified as follows:

1. Does the use of ivermectin cause any long-term effect on dung fauna biodiversity?
In particular, how long stays the “toxic” impact of the dung?
2. Is there a difference between the sensitivity of the species tested in standard laboratory tests and those species found in the four field studies? In other words, are the model species used in the laboratory representative for the communities in the field?
3. Is there evidence of recovery of dung organism populations (if possible, can internal (intrinsic) recovery be distinguished from immigration (“re-colonization”))?
In this context, the ecological properties of the dung and soil organisms have to be taken into account (e.g. how quickly they can recover)
4. Is it possible to use own results and literature data to address more general questions:
Do any risk mitigation measures exist that can guarantee dung fauna biodiversity?
Is there a possibility for a sustainable pasture management by using parasiticides?

Basic consideration when planning the practical work of this project, was the common understanding that any risk assessment is performed in order to address two protection goals: both the function and the structure of the (dung) ecosystem have to be protected. When talking about structure in fact the biodiversity of the dung organism community is meant as defined by the 1992 United Nations Earth Summit in Rio de Janeiro: "biodiversity" is "the variability among living organisms from all sources, including, 'inter alia', terrestrial, marine, and other aquatic ecosystems, and the ecological complexes of which they are part: this includes diversity within species, between species and of ecosystems" (UNCED 1992).

1.2 Legal background including risk assessment and protection goals

This short overview on the legal context is mainly based on a recent paper addressing this subject (Adler et al. 2013). To address the potential risk of Veterinary Medicinal Products (VMP), and in particular, parasiticides in an authorization process, guidelines have been published by the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH), a trilateral program to harmonize technical requirements for these drugs in Europe, Japan, and the United States. The Environmental Risk Assessment (ERA) allows a tiered approach: whereas in Phase I (VICH 2000) general aspects regarding use and exposure are handled, ecotoxicological test requirements are specified in Phase II (VICH 2004). An ERA of VMP for dung fauna is required if the substance acts as a parasiticide for the treatment of pasture animals.

As part of the ERA in the authorization process of parasiticides for animals reared on pasture, the VICH guideline on the Tier A of Phase II requires a clarification whether or what kind of non-target effects fecal-excreted parasiticides have on dung beetles and flies. Both dung beetle and dung fly data are required to assess the effects of parasiticides on dung fauna. In case a risk is identified at the end of

Phase II Tier A, a refinement regarding the effects of the product on the representative non-target organisms is required (Tier B). Nevertheless, the VICH guideline (VICH 2004) recommends that "For certain VMP, it may be necessary to go beyond Tier B because more complex studies, specific to issues being addressed or to a particular region, are necessary to complete the risk assessment." However, further information on Tier B studies (and beyond) for dung organisms is missing, although numerous studies have assessed the effects of VMP on dung organisms, both in the laboratory and in the field using different methods (Lumaret et al. 2012) within the last 25 years. So far, however, an ERA was conducted for only a few parasiticides, therefore the performance of higher-tier studies with dung and soil organisms as described in this report publication is strongly needed by the European Medicines Agency (EMA) as well as national authorities, industry, and consultants. In fact, the only advice given on how to proceed beyond Tier A is a statement in the EMA VICH guideline: "Regulatory guidance should be sought on appropriate studies." In the "Revised Guideline on Environmental Impact Assessment for Veterinary Medicinal Products" (EMA 2008) no further information on higher tier-testing of dung organisms is given. The work described in this report addresses specifically the performance of field studies as part of higher-tier work (see also Jochmann et al. 2012).

1.3 Structure of the report

For reasons of practicability, the project was divided into two work packages (WP). In any case, the results of WP I was considered when planning the work of WP II.

In the following, the individual chapters of each work package are listed (numbers indicate the chapter numbering used in later parts of this report).

WP I: Theoretical work and background considerations:

1. Literature review and database
2. Definition of dung organism communities
3. Characterization of exposed habitats in Europe (partly also North America) and their dung communities including the zoning of relevant regions in Europe
4. Description of different routes of exposure of parasiticides (consideration of new developments and strategies of parasiticides
5. Identification of the most relevant and most sensitive species of dung communities, which might be affected by antiparasiticides
6. Verification of existing models for the risk assessment of parasiticides related to dung fauna communities
7. Verification of existing risk assessment approaches (analysis of deficits) for VMP and formulation of new approaches, in particular regarding evaluation criteria and methods
8. Verification of existing risk management and risk mitigation measures (analysis of deficits) for VMP and formulation of new approaches
9. References (Part I)

It should be noted that much of the content of WP I is based on previous work performed by some (or all) of the partners of this project, usually in close cooperation with the sponsor. In several cases, this work has already been published (or submitted) elsewhere, meaning that it will not be repeated here in detail.
In addition to these subchapters, several issues specifically important to the situation in Germany were addressed in this work package. They were written in German and can be found in the Annex of this report.

WP II: Performance of four field studies in Europe (three) and Canada (one):

This WP is divided into six subchapters (10 – 15), each of them describing the field experiments with ivermectin at one of the four study sites.

10. Description of the field study with dung organisms in Montpellier, France
11. Description of the field study with dung organisms in Zurich, Switzerland
12. Description of the field study with dung organisms in Wageningen, Netherlands
13. Description of the field study with dung organisms in Lethbridge, Canada
14. Description of the field studies with soil organisms at all four sites
15. Summary of the work performed including an overall assessment of the risk of ivermectin for dung and soil organisms at different field sites.

The first four chapters have the same structure, since they follow the same study design and address the same structural (biodiversity) and functional (dung decomposition) endpoints. In addition, the concentration of ivermectin in the compartments dung and soil was measured at all study sites. In varying depth, they also address two specific ecological questions, based on additional work not originally planned when setting up the project:

- Description of population dynamics of dung organisms during one season, using data from pitfall traps
- Evaluation of the occurrence of secondary poisoning, focusing on the role of predatory staphylinid beetles

In the fifth subchapter the investigation of soil organisms' (earthworms, springtails) at all four sites is described. Due to site properties and the history of the respective sites no earthworm sampling was performed in Canada (no such animals are present at the study site). The whole practical work was based on Standard Operation Procedures (SOPs) which were developed as part of this project. Finally, all results are summarized and evaluated in a final subchapter, containing also recommendations how to improve the risk assessment of veterinary pharmaceuticals.

The outcome of this project will be presented to the responsible working group of the European Medicine Agency (EMA; London) as part of the discussion on how to improve the assessment and management of veterinary medicinal products.

2 WP I: Literature review and database

2.1 Introduction

In the context of this report a detailed literature review was performed. It focused on the biogeography and ecology of dung organisms in Europe. In addition, ecotoxicological effects of VMP, in particular ivermectin, were also compiled. Most of the data found were generated in studies with cattle (in rare cases also with horses or sheep), usually performed on grassland. In addition, the biogeography of selected soil organisms, in particular earthworms (Lumbricidae) and springtails (Collembola), was also studied. However, due to limited resources, this work could only be done exemplary (in particular looking at the situation in Germany). In parallel to the work on dung and soil invertebrates it was investigated whether plants could be affected by VMP. Own experience gained in EU FP6 project ERA-Pharm as well as results from other literature reviews (e.g. Edwards et al. 2001; Boxall et al. 2004; Liebig et al. 2010) show that plants do not react sensitively to ivermectin. Thus, these organisms are not considered anymore in this report.

Originally, it was planned to set-up two databases with similar structures, one for each compartment (dung and soil). In detail, the following information was asked for:

- Study site, e.g. coordinates, description
- Compartment dung: e.g. origin (i.e. from which farm animal), pH, or structure
- Methods: determination of abiotic parameters as well as sampling of the organisms
- Taxonomic information: species, genus, family etc.
- Bibliographical details regarding the publications
- Evaluation of the quality of the individual paper or data sets

Right now, the data base of dung organisms consists of taxonomic details (species, genus, family) and geographical data about the location of the respective study sites. The description of the locations varies in quality and is accurate for only approximately 40% of all individuals. The remaining proportion of dung organisms could only be located on a regional level. Additional information concerning sampling methods, the dung compartment, or other ecological data was missing in most of the relevant literature.

In the case of soil organisms, it was possible to refer to the database Bo-Info, which was developed in another UBA project (FKZ 3708 72 201; Römbke et al. 2012). In Bo-Info, both the taxonomic as well as the ecological information is compiled – but just for Germany.

2.2 Dung organisms:

Right now, the database contains 19,366 data sets from 76 publications. In total, 25 European countries and parts of western Russia and Turkey with about 406 regions as well as 985 clearly defined sites are presented. In addition, information from a small number of North American publications was also included.

Unfortunately, the outcome of this exercise was disappointing, due to the following reasons:

- Most paper do not contain any ecological information
- Rarely data on the sampling methods is provided
- Usually, there are many individual sampling spots in each region
- The distribution of sampling sites is very heterogeneous, both on a European scale as well as for individual regions
- In fact, the result of this review is primarily a list of species (or higher taxa) found at individual sites which are rarely characterized (in the best case, a site name and land use are given)

The information compiled in the data set can be presented in different ways; for example:

- According to the distribution patterns of the dung organisms at a family level (Figure 1):
- About half of the sampled dung organisms in Europe belong to the Coleoptera family Aphodiidae (54%), followed by Scarabaeidae (19%), Geotrupidae (7%), Hydrophilidae, (5%), the Diptera family Sepsidae (5%) and the rove beetles Staphylinidae 3%. The dung beetle families Trogidae, Cetoniidae and Melolonthidae account together 5% of all sampled animals. The other dung organism counts together 3% within the whole data set.
- By the geographical distribution of the sample data at a country level (Figure 2): About 37% of the sampled data are from France, while Spain has a rate of 13%, Germany 11%, the Netherlands 9%, Italy 8% and Switzerland 6%. England (3%), Portugal (2%), the Czech Republic (2%) and Sweden (2%) are underrepresented. The other counties used in this study count together 4% of the sampled data.
- The dung source of the studied organisms (Figure 3): 81 % of the paper showed a lack of information concerning animal dung sources as well as ecological material. Only 15.2 % were sampled using the pitfall method. The other methods were highly underrepresented, except for water extraction with 4%.
- The trapping methods used in the studies (Figure 4): In the overwhelming majority of studies (74%) no data concerning the trapping method were available.

Figure 1: Family based distribution pattern of dung organism abundance

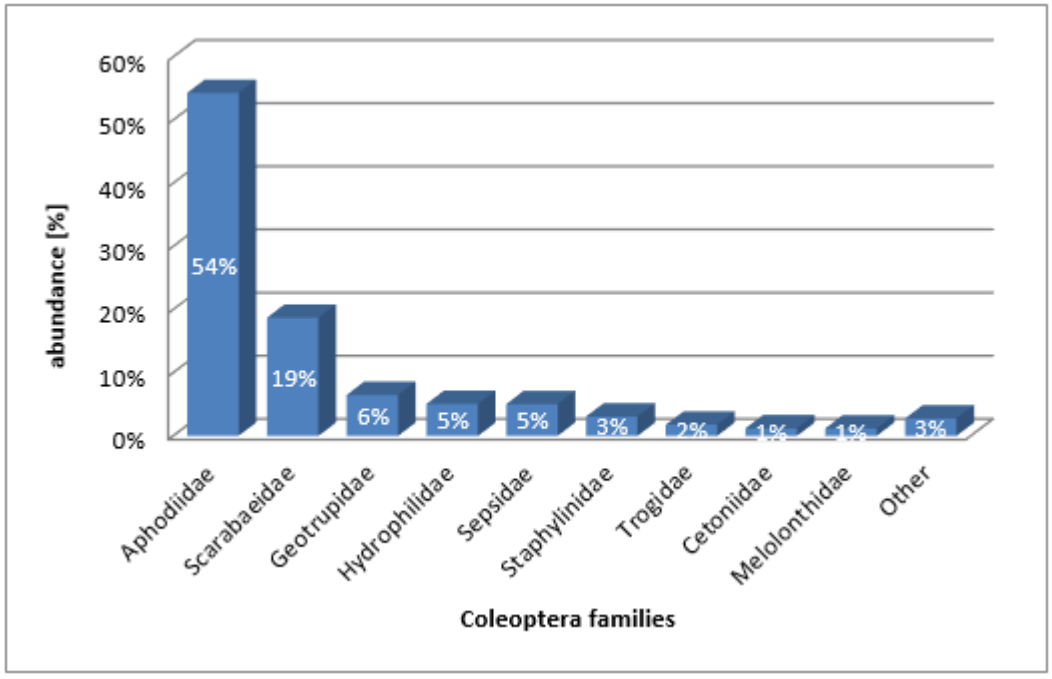


Figure 2: Geographical distribution of the sample data according at country level

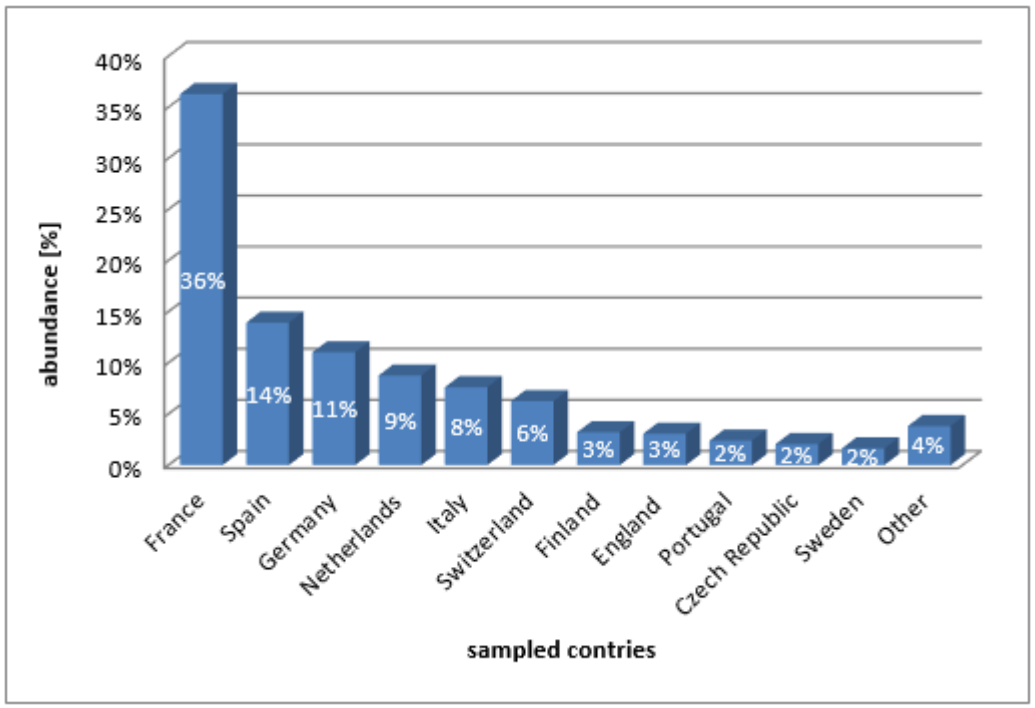


Figure 3: Distribution of animal dung sources

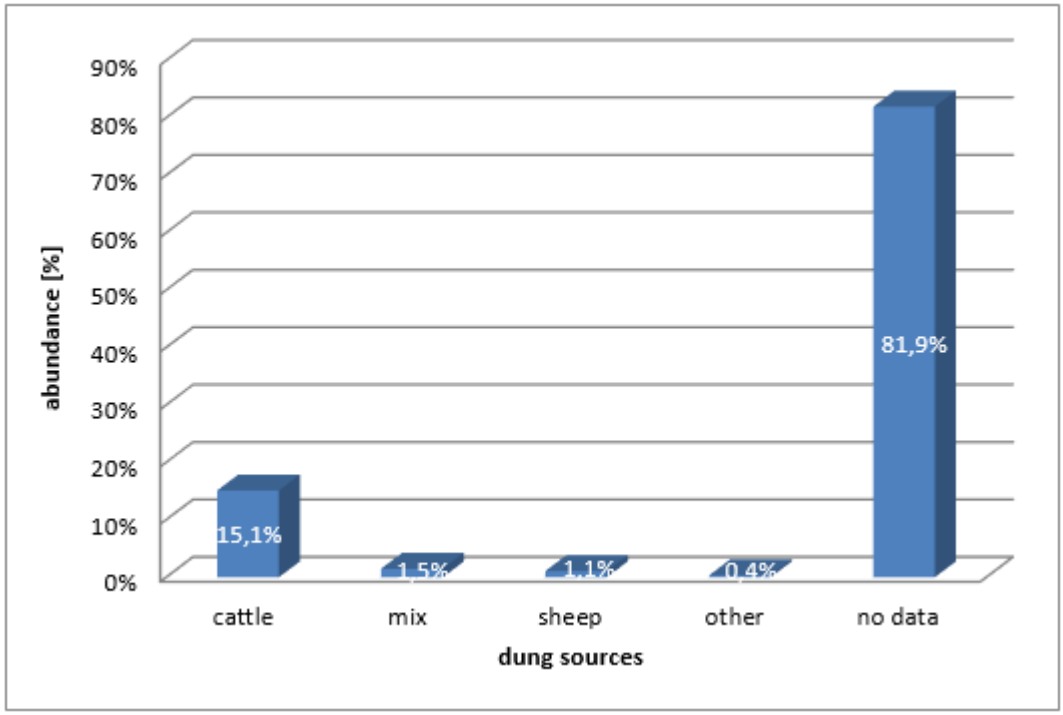
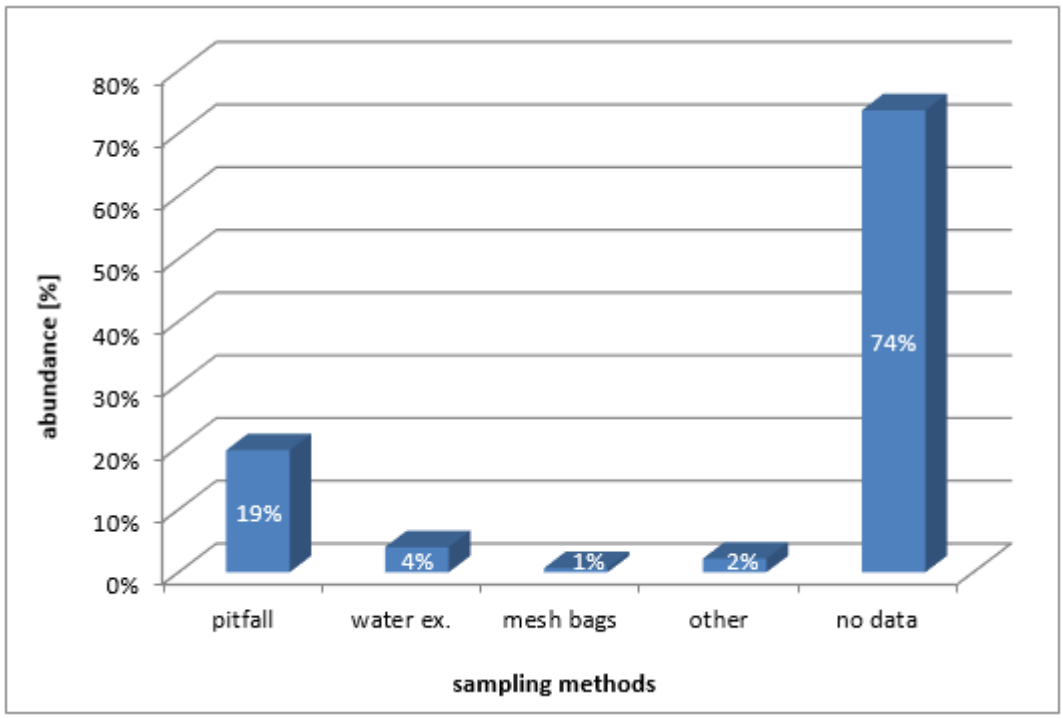


Figure 4: Distribution of trapping method



The literature evaluated can be classified roughly in two groups:

- Ecotoxicological field studies (see also 5), in particular data from the control plots (McCracken & Foster 1993, Errouissi et al. 2004, Lumaret 2010, Förster et al. 2011, Droevendaal, pers. Comm., Jochmann, pers. Comm.)
- Biogeographical or ecological studies unrelated to any use of VMP (e.g. Allenspach 1970, Lumaret 1990, Skidmore 1991, Köhler & Klausnitzer 1998, Pont & Meier 2002, Dellacase & Dellacasa 2006, Jurena & Bezdek 2008, Rößner 2012)

The evaluation of the literature shows that very rarely all relevant information as listed above is presented in few papers. Data on the characterization of the sampling site, the specific exposure conditions or details of the sampling methodology is usually not provided. This is especially true in older work (in particular the lack of site characteristics), but the situation is not much better even in recent publications. Exceptions are the – few – ecotoxicological studies performed in the context of VMP registration, since they have to fulfill minimum requirements of quality assurance. In addition, such studies are often performed in a comparable way as field studies focusing on the effects of pesticides on soil organisms, especially earthworms. For about 20 years, the latter have to be performed according to international guidelines (e.g. ISO 1999), which contain detailed requirements regarding background information such as site characteristics. In contrast, biogeographical or ecological studies usually provide only the name of the species as well as the name of the sampling site. This deficit is not going to change when increasing efforts of such a literature review.

2.3 Soil organisms

In the context of the literature search on dung organisms also the few papers were identified in which also soil organisms were sampled – but these examples are very rare (e.g. Svendsen et al. 2003; Römbke et al. 2010b). Therefore, within a project aiming to improve the preconditions for the protection of the habitat function of soils in Germany, the database 'Bo-Info' was established. In this database soil biological data from permanent soil monitoring sites of several German states as well as from the

literature were compiled. Soil biological data on abundance and dominance for Lumbricidae (earthworms), Enchytraeidae (potworms), Collembola (springtails) and moss mites (Oribatida) were analyzed with respect to their distribution, site characteristics (habitat type, land use) and soil properties (pH, texture, organic matter) (Römbke et al. 2012). This work was performed by four partner institutions, i.e. ECT Oekotoxikologie GmbH (Flörsheim), the Institute for Environmental Research of the University of Aachen in co-operation with the Research Institute for Ecosystem Analysis (Aachen), the Senckenberg- Museum Görlitz, and the Federal Museum for Natural History Karlsruhe. The information extracted from literature was compiled in an Access data base with a similar structure as the one used here for dung organisms. One aim of this project was the identification of the „normal" occurrence of soil organism communities in different soils and land use types in Germany. Despite its focus on Germany the data compiled in Bo-Info do allow to get an idea which soil organisms may primarily be affected by the usage of VMP, assuming that the structure of the grassland communities is comparable in wide parts of Europe.

Currently, the Bo-Info does contain about 45,000 data sets from all over Germany (Figure 5). One data set is defined as the combination of the name of a species with the name of a site (plus its abiotic characteristics), often together with information on the abundance at this site. The data are, however, very unequally distributed within the studied organism groups: about half of them belong to the Annelida (earthworms and enchytraeids), while the rest is distributed between Collembola and Oribatida in a ratio of 2 to 1. The number of nematode data sets is still very low. The aim of this project was the improvement of the preconditions for the protection of soils' habitat function as described in § 2 of the German Federal Soil Protection Act (1998), in particular by, first, identifying suitable biological indicators (i.e. organism groups) for the assessment of soil quality and, second, establishing reference values useful for selected habitat types to be used for evaluating whether a soil fulfils the habitat function or not (BBodSchG 1998; EU 2006).

Figure 5: Sites in Germany where soil invertebrates have been sampled. Black dots: German permanent soil monitoring sites. Brown dots: Sampling sites used in various research projects (Römbke et al. 2012)

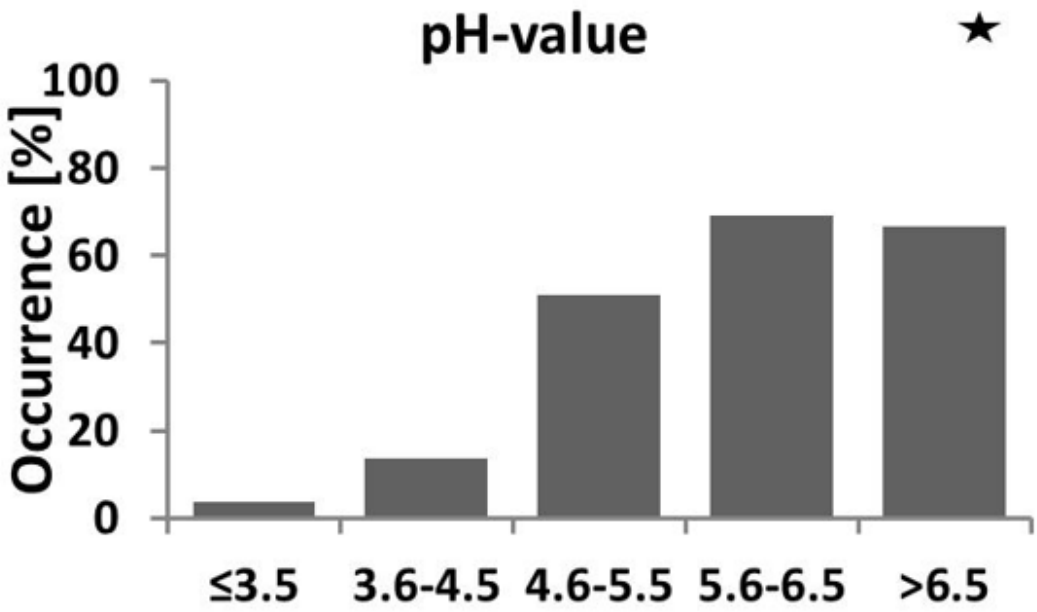
In the following, the characterization of one earthworm species is used as an example for the evaluation of ecological and biogeographical data. *Lumbricus terrestris* (Lumbricidae, Oligochaeta) is a very important inhabitant of crop and grassland sites, because it is an anecic species: They construct deep (up to several meters) vertical burrows but feed at the soil surface, meaning that the strongly influence the water regime of the soil, its physical structure and also nutrient cycles. For these reasons, they are considered to be ecological engineers, i.e. due to their activity they provide ecological niches for other species (Lavelle et al. 1997). *L. terrestris* has been found at 363 sites all over Germany, i.e. it is one of the most common earthworm species (Figure 6). However, it is still too early to assess at this map in detail, since it is strongly influenced by the sampling activity and the availability of data. For example, earthworms have been regularly sampled at permanent soil monitoring sites in the far north of Germany for the last ten years, meaning that this region is well covered. The lack of observations in south-eastern Germany (mainly Bavaria) does not indicate that this species is not occurring there. In fact, it is also abundant in that region, but the sampling data have only very recently been transferred to our database – and their implementation in the maps has not been finished yet. The lack of findings sites in Northwest Germany, however, indicates truly the lack of sampling over there.

Figure 6: Sampling sites in Germany where *Lumbricus terrestris* has been found (Römbke et al. 2012)

More importantly, data on the abiotic (mainly soil) properties of the sites where this species has been found can be used to identify the ecological requirements of individual species, meaning that their occurrence (or that of whole communities) can be predicted for other sites as long as their abiotic properties are known. In other words, this information can be used as a reference system (or yardstick) for the classification and assessment of the biological quality of soils (Breure et al. 2005). A difference between predicted and occurring species would be an indication for concern regarding the function of

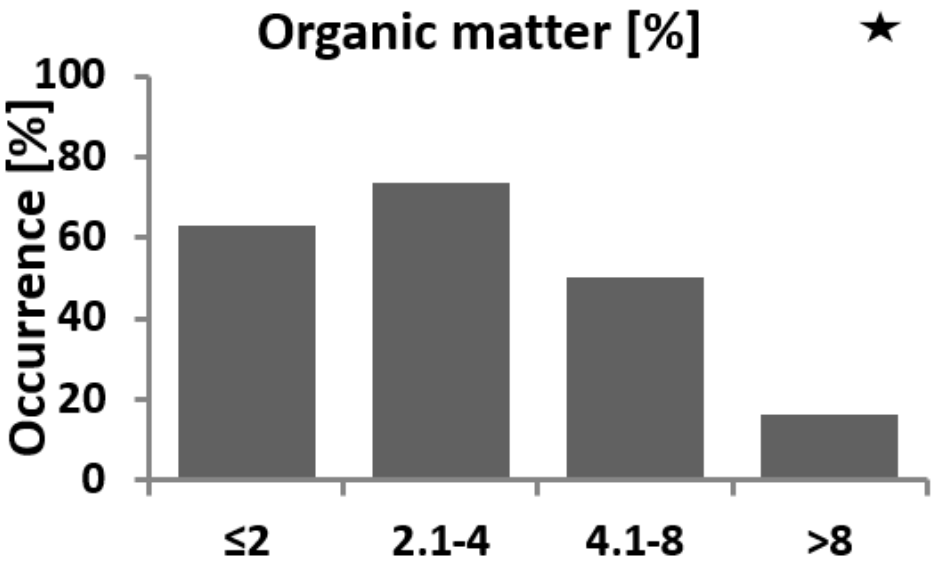
that soil as a habitat for soil organisms. In a further step the reason for this difference has to be identified, e.g. whether VMP could be responsible for the changed community structure (e.g. the lack of species). In the following, the ecological requirements of *L. terrestris*, based on the German database are briefly presented (Figure 7-Figure 10). According to these results, this species prefer significantly soils with a neutral pH, medium organic matter content, loamy-clayey texture and grasslands. Similar information is available for 13 other lumbricid species, common in wide parts of Europe (Römbke et al. 2012).

Figure 7: Relative frequency of *L. terrestris* depending on the pH-value of the soil (Römbke et al. 2012)



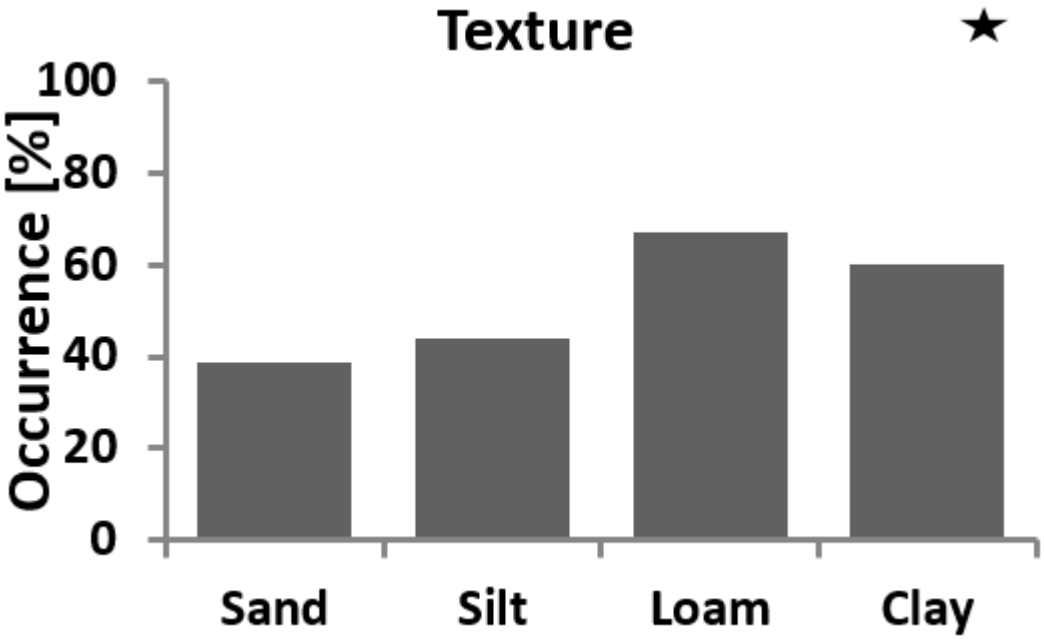
Data basis: number of sites at which this species was found (n = 363). Asterisks indicate statistically significant differences (Chi^2-Test): * $p \leq 0.05$.

Figure 8: Relative frequency of *L. terrestris* depending from the organic matter content of the soil (Römbke et al. 2012)



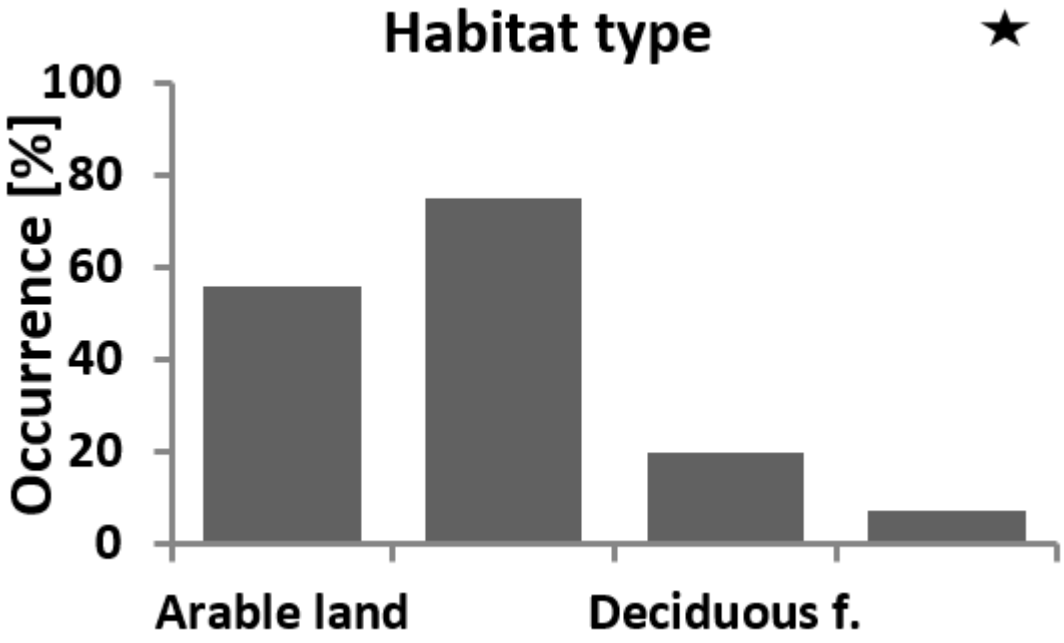
Data basis: number of sites at which this species was found (n = 12). Asterisks indicate statistically significant differences (Chi2-Test): * p ≤ 0.05

Figure 9: Relative frequency of *L. terrestris* depending from the texture of the soil (Römbke et al. 2012)



Data basis: number of sites at which this species was found (n = 363). Asterisks indicate statistically significant differences (Chi2-Test): * p ≤ 0.05

Figure 10: Relative frequency of *L. terrestris* depending from the habitat type of the soil (Römbke et al. 2012)



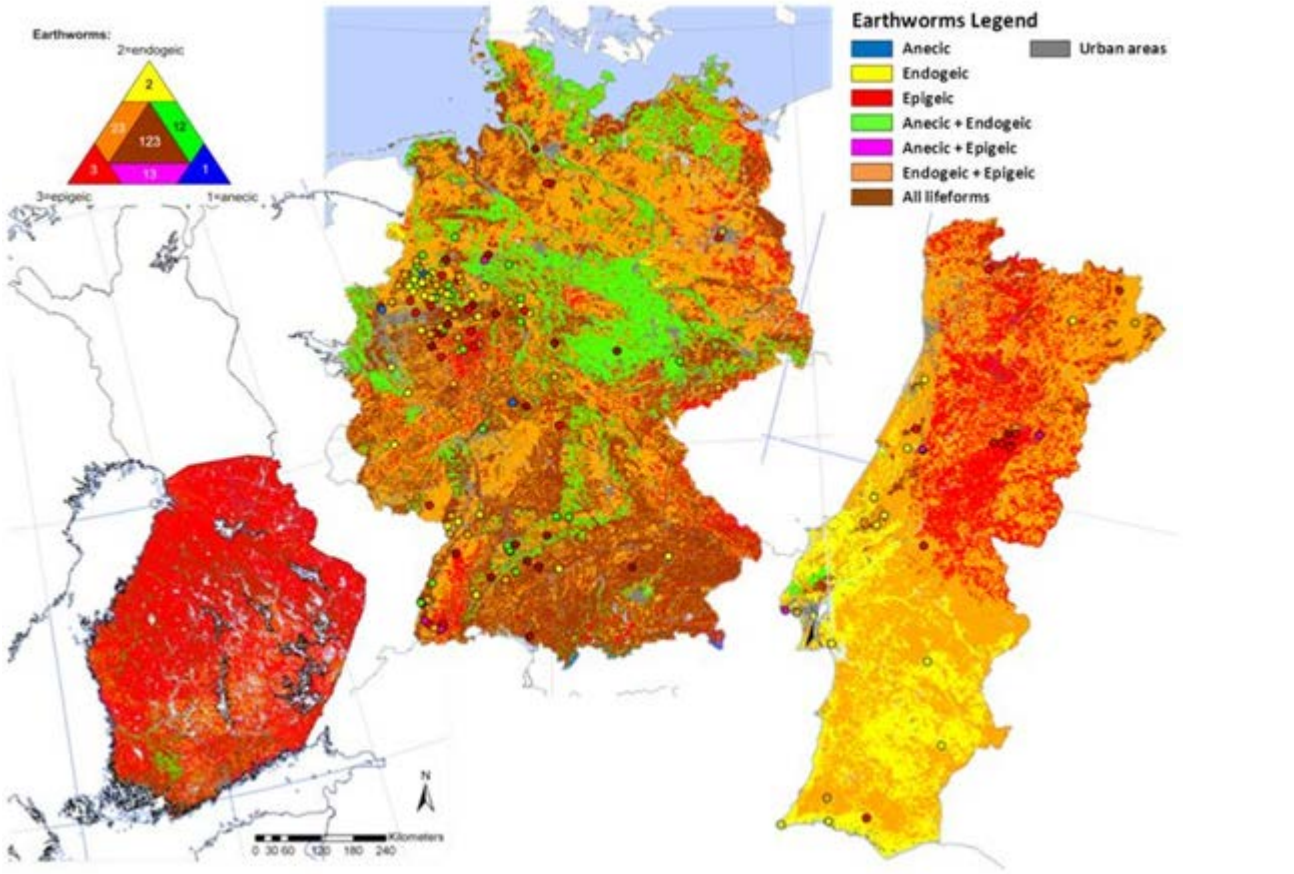
Data basis: number of sites at which this species was found (n = 363). Asterisks indicate statistically significant differences (Chi2-Test): * p ≤ 0.05

In parallel to the work in Germany, a modeling approach for defining soil ecoregions within Europe was developed to improve the realism of exposure scenarios for plant protection products (EFSA 2009). Biological information on four soil animal groups (earthworms, enchytraeids, collembolans and isopods) was used to assign each species to different life forms, representing depth horizons in which they occur. Based on information from three countries covering a North-South gradient (Finland, Germany, Portugal), species presence-absence data were modeled using pedological and climatological information (provided by the Joint Research Centre (JRC, Ispra, Italy) of the European Union). Ecoregion maps were produced for the four organisms' groups but worked best for earthworms for most of the countries and revealed marked differences between the countries. Maps are not predictive on a local scale, but give a probability of the soil biota community to be found on a regional scale. The main results obtained are:

- Maps based on modeled information are in line with ecological and biogeographical information for the organism groups considered.
- Factors determining the distribution of the organisms could be identified, in particular for earthworms (Bouché 1977; Henneberg 2007).
- Differences could be observed between the three countries in community composition based on life form groups of earthworms (Figure 11).

This approach, originally developed for pesticides, should be evaluated for VMP.

Figure 11: Extrapolated occurrence of the three ecological groups of earthworms in three countries of the European Union, Portugal, Germany and Finland



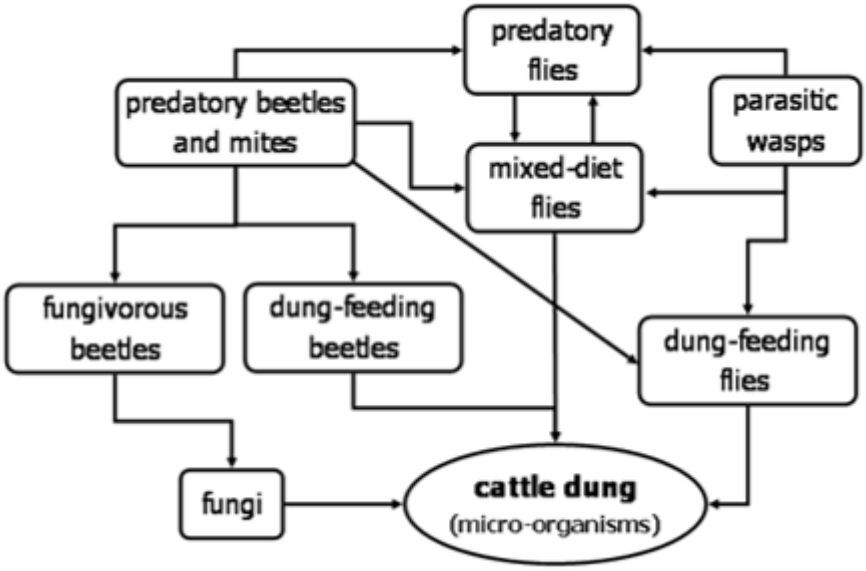
Explanation of colors see upper right corner; Source: EFSA (2010b)

3 WP I: Definition of dung organism communities

The information compiled here is based on OECD (2010) and Jochmann et al. (2010).

So far, there is no definition of a dung organism community which is generally accepted. However, it could be said that all species belong to this community which spend a "relevant part" of their life cycle in dung pats or close to them – or which depend on dung as their main food source. The term „relevant" can be understood regarding time (i.e. the main part of their life time is spent in or close to dung) or in a biological sense (e.g. dung is necessary as the place where eggs are deposited). However, there is a "grey zone" of species: for examples, those feeding on dung, especially at later stages of dung pat decomposition, but do not depend on this food source. Many saprophagous species (e.g. earthworms, Collembola or some Nematoda) belong to this group, but also many predators such as staphylinid beetles, which visit dung pats because of the high density of prey organisms. All these organisms together form a highly complex, temporally and spatially very variable community which can be considered as an ecosystem on its own (Figure 12). In the following, the text will focus on dung organisms in temperate grasslands, i.e. species from other regions (such as the tropics) or land use forms (e.g. forest) will not be covered.

Figure 12: Various groups of the dung organism community, shown in a simplified food web



Nematoda as well as soil organisms, usually only relevant at later stages of dung pat decomposition, or sporadic visitors are not shown (Boxall at el. 2004)

In the following some examples dung organism communities are listed (Jochmann et al. 2010). Often these communities are very rich in species and individuals. From time of deposition to total degradation, a dung pat may contain several dozen species of coprophilous arthropods (insects and mites) exceeding 1000 individuals (Laurence 1954; Mohr 1943). For Britain, Skidmore (1991) listed 275, 213, and 110 species of insects in dung of cattle, horses and sheep, respectively. For North America, Blume (1985) listed 450+ species of insects associated with cattle dung. The vast majority of dung-associated taxa are either innocuous or desired either as natural enemies of pest flies or to accelerate dung degradation. Worldwide, only a few of these taxa are considered pest species; e.g., horn fly (*Haematobia irritans irritans*), buffalo fly (*H. i. exigua*), face fly (*Musca autumnalis*), or stable fly (*Stomoxys calcitrans* (L.)).

Dung pat communities are comprised of arthropod guilds that are characterized by differences in diet (Figure 12). The larvae of dung-feeding flies, which include most species of coprophilous flies, feed on

microorganisms. Early-instar larvae of mixed diet flies feed on microorganisms and then switch, usually in the last instar, to feed on insects. Larvae of predatory flies feed only on insects. Dung-feeding beetles (mainly Scarabaeidae) feed solely or primarily on dung. Adults within this guild are filter-feeders (Holter 2000) and probably feed mostly on the microorganisms present in the fluid component of fresh dung (Aschenborn et al. 1989). In contrast, larvae of dung-feeding beetles ingest undigested plant fiber from which nutrients are extracted through the action of symbiotic cellulose-digesting bacteria housed in the larval hindgut (Terra 1990). Predatory beetles (mainly Staphylinidae) feed on other insects, particularly the eggs and larvae of flies. Fungivorous beetles colonize pats at later stages of decomposition and feed on fungal hyphae and spores. Wasps associated with dung are mainly parasitoids of dung-breeding flies. In addition, arthropods that arrive to colonize fresh dung may carry mites, nematodes, bacterial and fungal spores that quickly increase in number once introduced to the pat. Predatory mites feed on nematodes or immature insects. The growth of bacteria and fungi accelerate dung degradation.

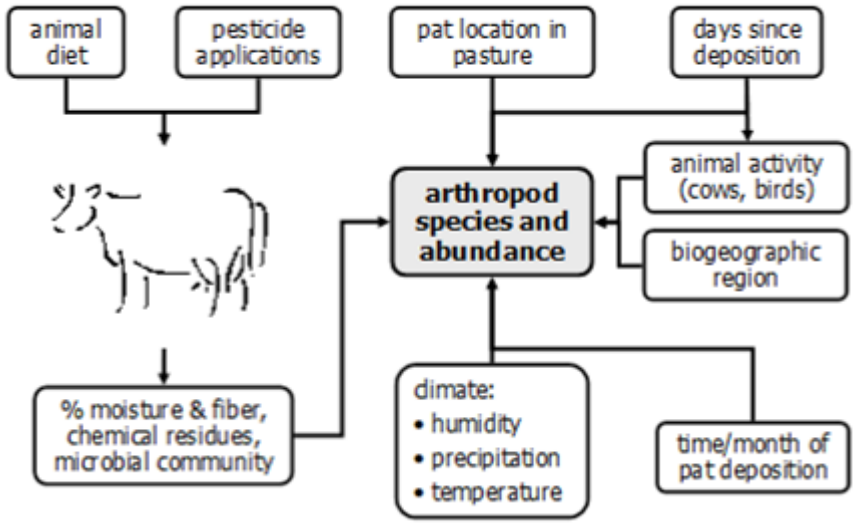
Colonization of fresh dung usually starts with flies and winged beetles, some of which arrive immediately after deposition, and which feed, mate, and lay eggs that produce a new generation in about 2 to 3 weeks. Fly numbers rapidly decline after a few hours, by which time crust formation on the pat has reduced the release of volatile attractants. Most dung-feeding beetles arrive shortly thereafter to feed and oviposit, with colonization peaking usually within the first week after deposition. Dung-feeding beetles form three general groups termed ‘dwellers’, ‘tunnelers’, and ‘rollers’ (Hanski & Cambefort 1991). Dwellers complete egg-to-adult development within the pat or at the interface between the pat and soil surface, and are the dominant group in temperate climates. Adult tunnelers remove from the fresh pat, dung that is buried in more or less vertical tunnels that may extend 10 cm or more into the soil. This dung provides food for larvae that hatch from eggs laid in the buried dung. Rollers have the same nesting behavior as tunnelers, but dung removed from the pat is first formed into balls that are rolled some distance from the pat prior to burial. Egg-to-adult development time of dung-feeding beetles may take weeks to months (Merritt & Anderson 1977). Parasitic wasps, mites and predaceous beetles arrive concurrently with the flies and dung-feeding beetles and may either oviposit or feed on immature insects developing in the dung pat. There is very little additional colonization of dung by coprophilous insects 2 to 3 weeks after deposition, but adult beetles of some species may remain within the dung for more than 2 weeks after arrival.

The final colonization phase occurs with the breakdown of the interface between the dung and the soil surface. This process provides soil-dwelling organisms (e.g., earthworms, enchytraeids, bacteria) access to complete the breakdown of the dung (Swift et al. 1979). Depending on geographic region and season, earthworms may play a greater role in dung degradation than dung-dwelling insects (Holter 1979; Lumaret & Errouissi 2002). During this latter phase, decomposing pats may be visited by taxa searching for food, shelter, or which are attracted to rich organic soils and rotting vegetation. Such taxa may include centipedes (Chilopoda), woodlice (Isopoda), millipedes (Diplopoda), harvestmen (Opiliones), spiders (Araneae), earwigs (Dermaptera), springtails (Collembola), termites (Isoptera), ants (Formicidae), click beetles (Elateridae), ground beetles (Carabidae), and bugs (Hemiptera). These incidental species are not normally considered to be part of the dung pat community, because they do not rely on dung as a breeding substrate.

Because insect activity accelerates dung degradation, rapid removal of dung from the pasture surface often is used – incorrectly – as an indicator of the ‘health’ of the dung insect community. Degradation reflects the interaction of a complex of biotic and abiotic factors (Figure 13). Livestock stocking rates affect the likelihood of pats being disrupted by trampling. Birds foraging for insects or seeds can quickly fragment pats. Shade reduces the rate of pat desiccation, which makes the pat attractive to insect colonists for a longer period of time. Heavy rainfall quickly causes the dissolution of fresh pats. Warm and/or wet conditions usually initiate peak insect activity, which generally is lowest when con-

ditions are cold and/or dry. The moisture and fibre content of the animal's diet affects the compactness of the dung and its resistance to degradation. North temperate regions are often characterized by small species (dwellers), which do not bury dung but only slowly degrade the pat during a period of weeks through the feeding activity of their larvae (Cambefort and Hanski 1991). Depending upon this complex of factors (potentially, including the impact of VMP), complete incorporation of a 'healthy' dung pat into the soil may vary from weeks to years (Merritt and Anderson 1977).

Figure 13: Biotic and abiotic factors influencing the degradation of dung pats (Floate, pers. comm.)



Based on the information available in literature, Jochmann et al. (2010) prepared a list of organism groups (mainly families) which can be considered as typical for dung organism communities in temperate grasslands (Table). Depending on the regional and local conditions the species composition and ecological importance of individual groups can differ (in particular regarding sporadic visitors).

Table 1: List of important dung organisms (family level) occurring in the dung of farm animals

Taxon (common name)	Taxon (common name)
Coleoptera (beetles)	Diptera (flies)
Clambidae (fringe-winged beetles)	Brachycera
Cryptophagidae (silken fungus beetles)	Anthomyiidae (anthomyiid flies)
Lathridiidae (minute brown scavenger beetles)	Calliphoridae (blow flies)
Pselaphidae (short-winged mold beetles)	Dolichopodidae (long-legged flies)
Ptiliidae (feather-winged beetles)	Empididae
Histeridae (hister beetles)	Muscidae (muscid flies)
Hydrophilidae (water scavenger beetles)	Phoridae (scuttle flies)
Scarabaeidae (scarab beetles)	Sarcophagidae (flesh flies)
► Aphodiinae (aphodian dung beetles) ► Geotrupinae (earth-boring dung beetles) ► Scarabaeinae (dung beetles, tumble bugs)	Scathophagidae (dung flies) Sepsidae (black scavenger flies) Sphaeroceridae (small dung flies)
Staphylinidae (rove beetles)	Stratiomyidae (soldier flies)

Taxon (common name)	Taxon (common name)
	Syrphidae (hover flies)
Hymenoptera (wasps)	Nematocera
Braconidae	Anisopodidae (window gnats)
Diapriidae	Cecidomyiidae (gall midges)
Eucoilidae	Ceratopogonidae (biting midges, punkies, or no-see-ums)
Figitidae	Chironomidae (midges)
Ichneumonidae	Mycetophilidae (fungus gnats)
Mymaridae (fairyflies)	Psychodidae (moth flies)
Proctotrupidae	Scatopsidae (minute black scavenger flies
Pteromalidae	Sciaridae (dark-winged fungus gnats)
Scelionidae	Tipulidae (crane flies)
Tiphiidae	
Acari (mites)	Nematoda (roundworms)
Eviphididae	Bunonematidae
Halolaelapidae	Diplogastridae
Macrochelidae	Panagrolaimidae
Parasitidae	Rhabditidae
Uropodidae	Tylopharyngidae
Annelida	Collembola (springtails)
Enchytraeidae (potworms)	
Lumbricidae (earthworms)	

The species distribution differs in the different ecological regions (Jochmann et al. 2010)

4 WP I: Characterization of exposed habitats of European Communities and their dung organisms - Differentiated protection target and protected property descriptions on zonings within relevant regions of Europe

4.1 Introduction

Within a natural spatially heterogeneous region like Europe (or even the EU-27) there are significant differences between the local dung organism communities, mainly on the basis of climatic and site-specific (e.g. land use, soil properties, vegetation etc.) factors. So far, the composition of dung organism communities has not yet been used to define specific regions, though this purpose at the level of individual organism groups has already been approached (e.g. dung beetles: Hanski & Cambefort 1991). In fact, there is a division of Europe into a northern temperate and southern Mediterranean region, with no exact specified boundaries. However, the regulatory requirements for the environmental risk assessment of veterinary pharmaceuticals do not address this differentiation. In contrast, as part of the registration process of pesticides, Europe has been divided into three zones: a northern, central and a southern zone. Their borders follow existing national borders (EC 2009), which clearly indicates that this zoning is not based on scientific criteria but rather on administrative grounds. In contrast, there are suggestions for a regionalization concept of soil organism communities in Europe, based on experiences made in a few countries (specifically: Germany, Finland, Portugal) (EFSA 2010b). In this case, the concept is based on the species distribution of few important groups of soil organisms (mainly earthworms (Lumbricidae) and springtails (Collembola).

Prerequisite for such a regionalization is the availability of sufficient knowledge on the occurrence of dung organisms. Not only the taxonomic-and biogeographical information but in particular ecological data must be available for individual species. What kind of information is needed, depends on the organism groups: while for dung organisms land use, climate and vegetation are probably most important, species distribution of soil organisms depends more on soil properties such as organic matter content or pH (Römbke et al. 2012).

4.2 Biogeography of selected dung organism groups and species

The following maps represent the current state of knowledge regarding the occurrence of selected dung organism groups (family to species level). They are based on the current stage-of-the-art of data points collected in our database. Firstly, all sampling points are compiled in one map (Figure 14). It should be noted that one dot could mean one site or, more often in countries such as France or Italy, a region (e.g. a French department, or an Italian province). This presentation was selected since the number of individual sampling points in these countries is far too high to be handled in this project. In addition, it must be reminded that in almost all cases the available information consisted of the name of a taxon (usually a species), the name of a sampling site (but often without coordinates) and the date of sampling; i.e. any ecological information regarding the site characteristics is missing (by the way, rarely the sampling method is described in detail).

Figure 14: Distribution of the sampled dung organisms according to the project database

Most sample points present geographically not specified locations but rather widespread regions such as federal states

In the following, firstly the occurrence of selected beetle families (Figure 15-Figure 17) and of the Dipteran family Sepsidae (Figure 18) is presented. Afterwards, the distribution of the dung beetle species used or proposed for standard dung organism laboratory testing is shown (Figure 19Figure 21). Due to the small number of available data for other groups of dung beetles and dung flies (including soil organisms found in studies with dung pats (nematodes, earthworms)), no maps have been prepared for those organisms. On the European level, maps showing the distribution of soil organisms are not available (Jeffrey et al. 2010). On the national level, map availability increases but is still very low (e.g. Rutgers & Dirven-Van Breemen 2012, Römbke et al. 2012).

As can be seen from Figure 14, the documented sampling of dung organisms is unevenly distributed within Europe. The focus is clearly on the western part, while other regions, do not yet seem to have been sampled, such as large parts of Eastern Europe. However, this map provides a partially distorted picture the distribution of dung organisms for the following reasons:

- Individual sample points are available for several sampling programs, but often they cannot be identified geographically. For example, dung organisms were caught at 250 locations in Slovakia and the Czech Republic, but the individual locations are not well documented. Therefore, for both countries just a single data point per species has been included in the map (Jurena et al. 2008). A similar situation has been found in Germany (Köhler & Klausnitzer 1998) and France (Lumaret 1990), where only states or departments were presented instead of specific sample points, respectively.
- To make sure that no work was left out, specialists from conservation organizations or regional museum were contacted; e.g. Dr. D. Mann (Museum of Natural History, Oxford, UK) and Prof. R. Wall (University of Nottingham, UK). Their sometimes very long reading lists were helpful to sum up the data contained in this report. The respective colleagues agreed that the number of usable (and especially meaningful) literature sources is low in general.
- In this context, it must be stated that much of the collected information has not been properly published. For example, according to our map almost nothing is known about the dung organism communities of the British Isles. However, in a workshop held at the University of Oxford Dr. Mann gave a talk entitled “British Coprophagic Scarabaeoidea: a synopsis”, in which dozens of sampling sites were shown (usually nested around the place where Dr. Mann worked during his career). This data set is not publicly available.

In the following, the distribution of the three most important families of dung beetles plus one Dipteran family are shown. The species-rich Aphodiidae (256 species in our database) is widespread throughout Europe (Figure 15) The Scarabaeidae (91 species) show a similar distribution pattern, with a lower number of individual evidences in England and Italy (Figure 16). Even the distribution pattern of the third typical dung beetle family, the Geotrupidae (30 species), does not significantly differ from the former two. Striking is only their substantial absence in Italy, but this is probably due to a lack of appropriate publications (Figure 17). The dung fly family Sepsidae is nearly solely situated in the north of Europe, mainly because of lack of publications for the rest of Europe (Figure 18). From what has been said it must be concluded that the family level for biogeographic statements is of limited value. Probably the distribution pattern visible in these four maps is mainly indicating the respective sampling effort in the individual countries – and/or their publication efficiency.

Figure 15: Locations of the existing collection points of species from the family Aphodiidae according to the project database (red dots)

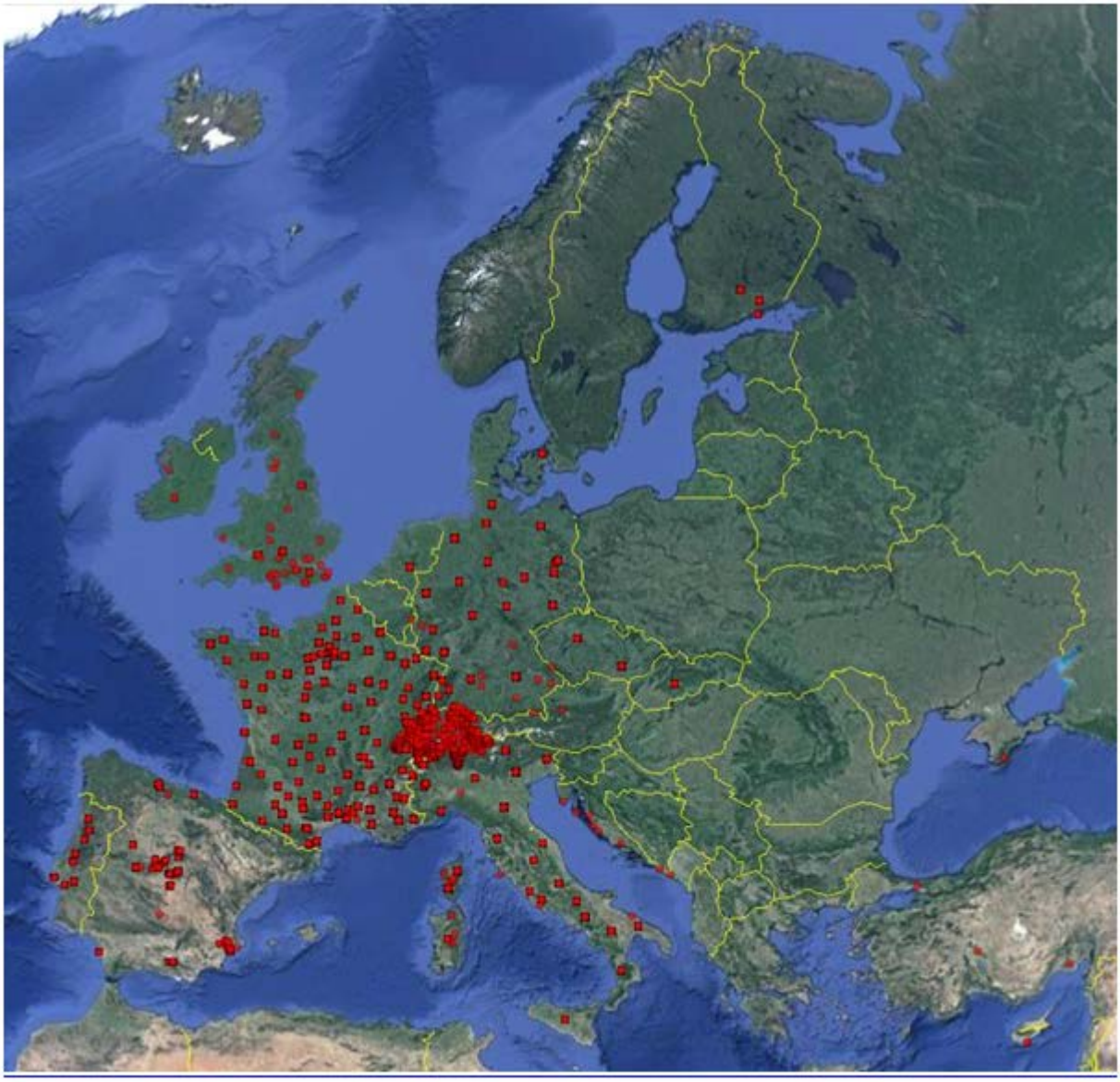


Figure 16: Locations of the previously existing collection points of species from the Coleoptera family Scarabaeidae according to the project database (yellow dots)

The high density of individual sampling points in Switzerland is caused by the fact, that geographic coordinates are given for many sampling sites (Allensbach 1970). Together with the information provided by Prof. W. Blanckenhorn and co-workers, this country is probably the one with the highest density of sampling points in our database. However, assuming that all sampling points in East Germany would be shown individually, this region would look like Switzerland, especially after the impressive publication by Rößner (2012). In fact, it can be assumed that the information on the distribution of dung beetles is almost similar in Western and central Europe, including Britain. Less sampling efforts but probably mainly lower species numbers and densities are responsible for the lack of data sets from Scandinavia. Regarding the Mediterranean countries, it seems that sampling efforts and taxonomic experience are only available in the Western part of that region. Not much can be said about Eastern Europe, but, extrapolating from the situation for soil organisms, there is probably much information hidden in regional publications, written in local languages.

Figure 17: Locations of the previously existing collection points of species from the Coleoptera family Geotrupidae according to the project database (pink dots)

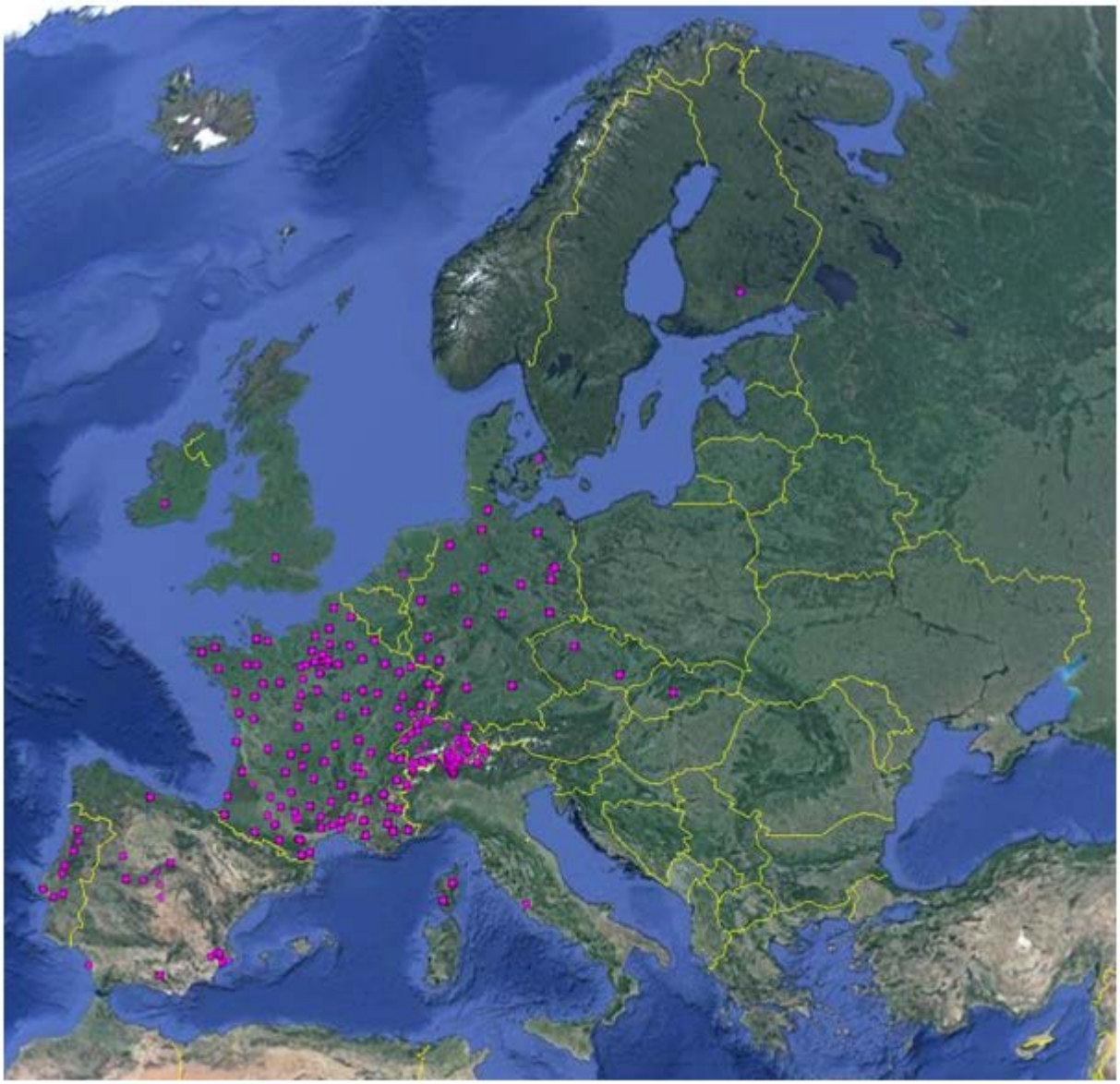


Figure 18: Locations of the previously existing collection points of species from the Diptera family Sepsidae according to the project database (light blue dots)

Despite the fact that dung flies are extremely important in dung degradation, especially in fresh dung pats, the number of Dipteran data sets is much lower than that of dung beetles. One reason might be that dung flies (like most other Dipterans) are more difficult to determine than beetles (partly, because the flies are usually much smaller). In addition, the number of "freelance" taxonomists interested in these organisms is very low. Therefore, the map showing the distribution of sepsid flies is an artifact (Figure 18), caused by one publication (Pont & Mexer 2002). This is a pity since some species of this family have recently been identified as extremely sensitive towards ivermectin and related compounds (e.g. Blanckenhorn et al. 2013a, b). Still, it is strange that in the literature search only few records for the well-known species *Scathophaga stercoraria* (the yellow dung fly) have been found.

Because of the high species richness (795 species) but a poor sample amount of 19,366 individuals, a specific distribution overview on the recorded dung organisms on a species level is highly improperly. Therefore, we focused on two species, used already in standard laboratory tests (*Aphodius constans, Onthophagus taurus*) and *Onthophagus vacca*, a potential new test dung beetle proposed by J-P. Lumaret (University of Montpellier).

Figure 19: Locations of the previously existing collection points of the species A. constans from the Coleoptera family Aphodidae according to the project database (orange dots)

O. taurus has been selected as an ecotoxicological standard species since it can be well kept in the laboratory (however, long term culturing is difficult). *Aphodius constans* is a typical representative of the fauna of southern France, which at certain times may reach high abundances and dominance especially in the Pyrenees foothills, around the city of Montpellier and also on the coast of Portugal (Figure 19). Its distribution (and avoiding misidentifications, e.g. from Germany (Rößner 2010)) focuses on Southwestern Europe, with some "outliers" as north as England.

Figure 20: Locations of the previously existing collection points of the species Onthophagus taurus from the Coleoptera family Scarabaeidae according to the project database (green dots)

The second test species whose potential is currently under review in a ring test is *Onthophagus taurus* (Figure 20). It has been frequently detected in Switzerland, Germany, France, Portugal, the Czech Republic, Slovakia and in Central Spain. *O. taurus* is also widely distributed on a global scale, i.e. it has been introduced in North America and Australia (Blume 1985, Edwards 2007). This is an additional argument for its choice as a standard test species. The distribution pattern of the potential new test species *Onthophagus vacca* indicates a wide distribution in Germany, France, Portugal, South-east Switzerland and central Spain (Figure 21). Its distribution pattern in Europe is more or less similar than that of *O. taurus*, but on a global scale it seems to be rarer. Right now, it is too early to decide which of these two species is more suitable as a standard test organism.

Figure 21: Locations of the previously existing collection points of the species Onthophagus vacca from the Coleoptera family Scarabaeidae according to the project database (rosa dots)

Other groups of organisms considered to be typical dung organisms are largely missing in the database, partly because they are late visitors to the dung pats (e.g. the rove beetles (Staphylinidae)), partly because they seem to be generally rare (e.g. Histeridae).

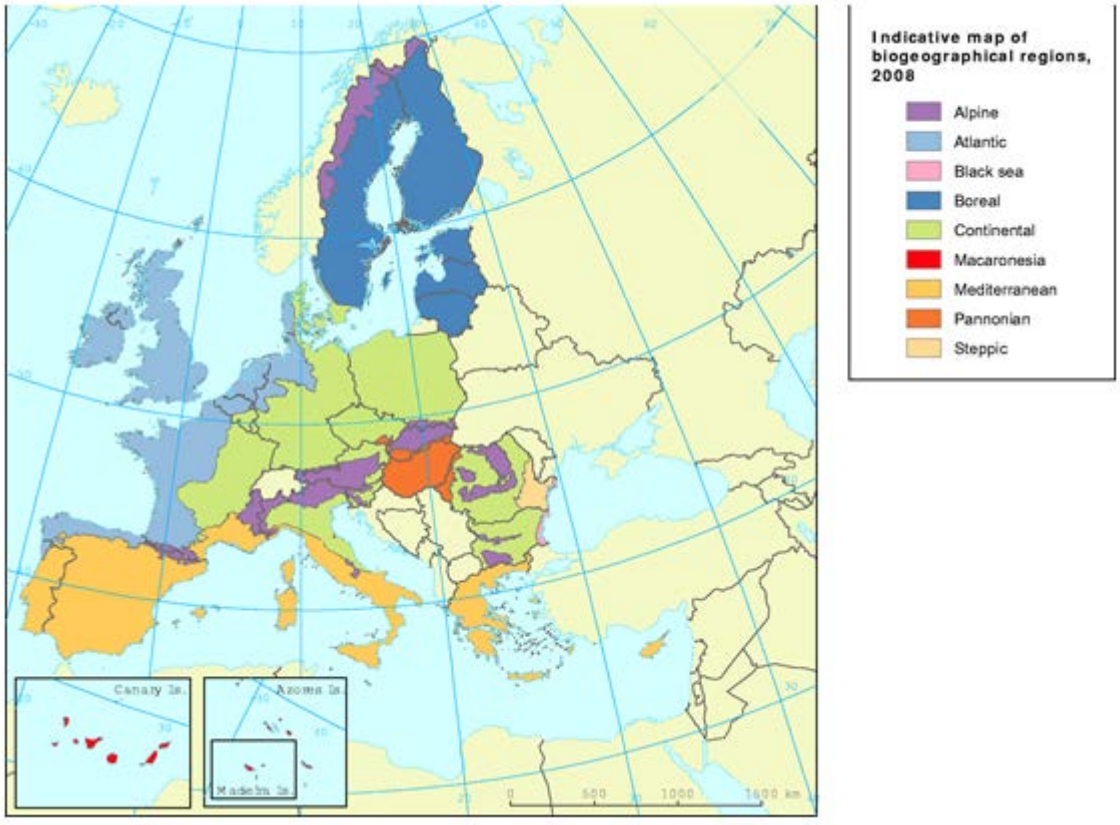
4.3 Concepts for the ecological zonation of Europe

In this project, it was claimed to collect data on dung organisms data from all of Europe. However, it was found that the existing data are very sparse and also unevenly distributed. According to the prior evaluation a comprehensive account of the 27 EU member states is not possible, since in many regions there is simply a lack of appropriate data. On the other hand, there are, mostly due to the activities of a single workgroup, regional priorities (e.g. southern France, including the alpine entry and slopes of the Pyrenees). Even assuming a much-improved data set, it will be virtually impossible to develop a fine zonation in Europe under these conditions. Instead, the zones are based largely on the distribution of the "classic" dung beetle families, especially Aphodiidae, Scarabaeidae, and Geotrupidae.

In any case, the zonation will focus on grassland sites, because they are the habitat for those dung and soil communities that may be exposed to VMPs. In addition, the distribution of soil organisms at crop sites was included in the analysis because they can be exposed when dung contaminated with VMPs is

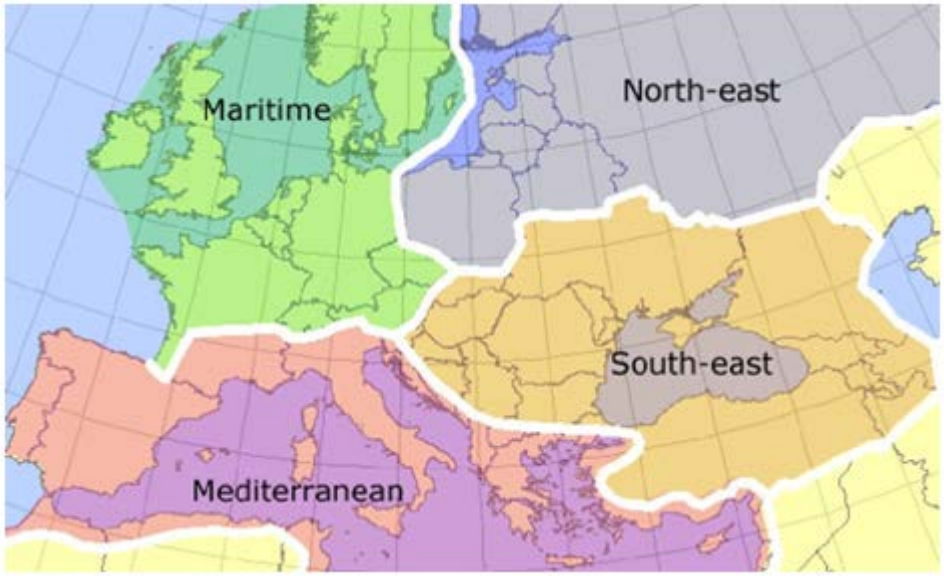
applied there. Because of experiences from laboratory, semi-field and field experiments the soil organisms being most sensitive to ivermectin are springtails (Collembola) (Jensen et al. 2003; 2009). Therefore, any zonation should focus on these arthropods. Because of the amount of available information and their role in the dung degradation, the distribution of earthworms is also taken into account when preparing such a zoning of Europe. Classification approaches focusing on these two soil organism groups are available, but do not cover the whole of Europe (see Figure 22; EFSA 2010b).

Figure 22: Map of the biogeographical regions of the EU 27 (EEA 2009)



The results of a recently completed project for monitoring potential effects of genetically modified plants (GMPs) on non-target organisms (above or below ground) suggest, however, that due to the different factors that determine the distribution of above and belowground organisms, two different zoning approaches are necessary (Jaensch et al., 2011). While there is no recommendation given for soil organisms, the authors examined for above-ground living organisms existing EU regionalization concepts - and recommend as most appropriate approach, the map of European biogeographical regions (Figure 22; EEA 2009). At present, it is not decidable whether it is suitable also for dung organisms. Due to the poor data situation, it also could be a much simpler approach, e.g. using the biogeographical division as it is proposed by EPPO (2005) (Figure 23). Further studies should focus on this.

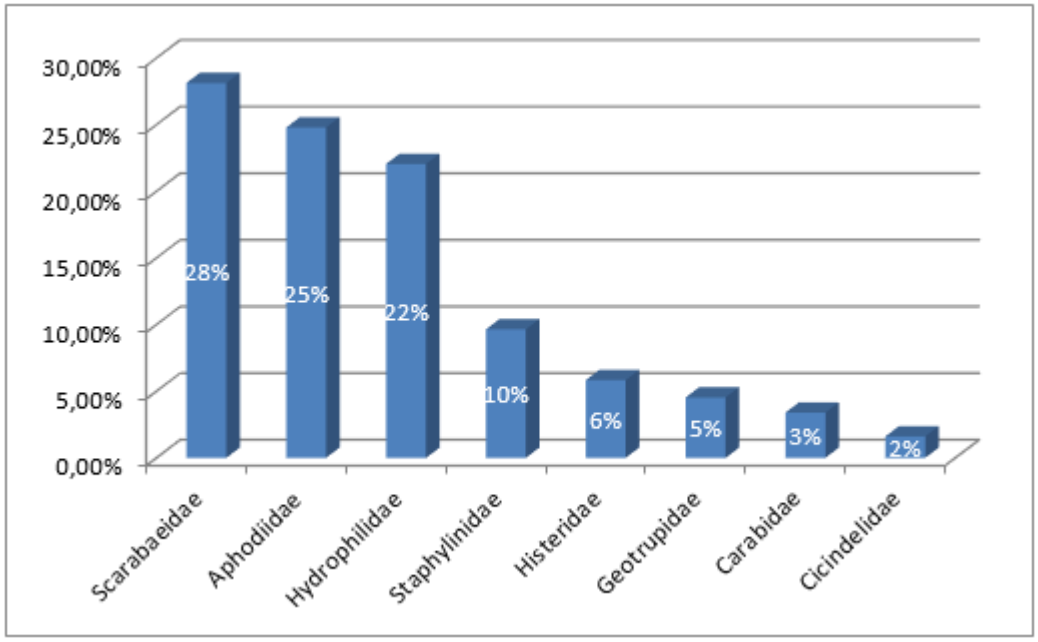
Figure 23: Zones with comparable climate conditions of Europe (EPPO 2005)



4.4 Distribution of dung-beetles in North America

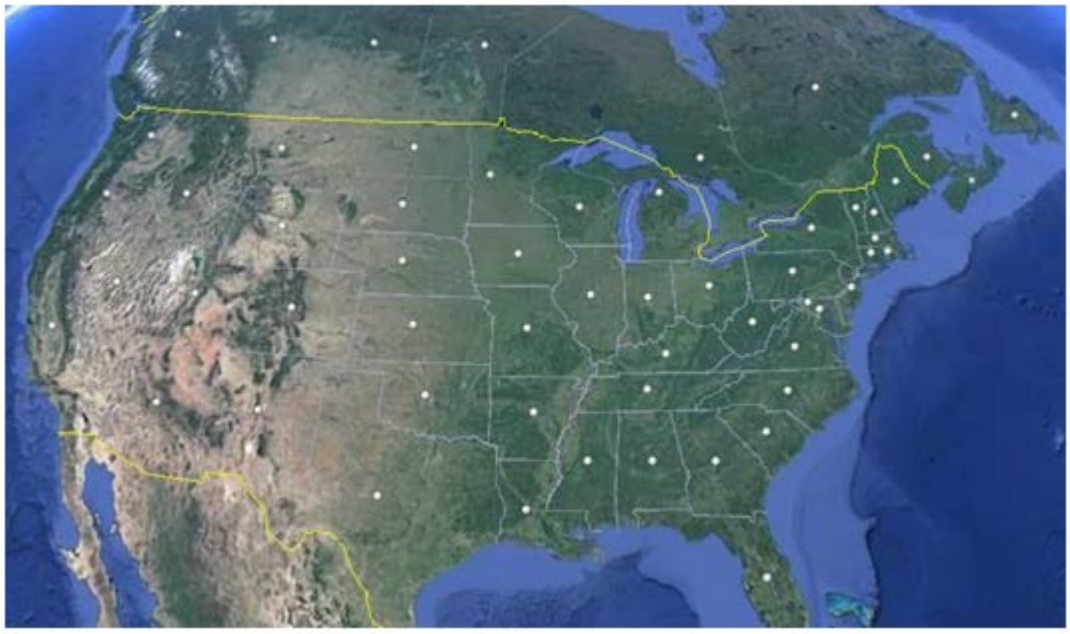
The distribution pattern of the most important groups of dung organism in Nord-America (many of these species have been introduced from Europe) is shown in the following maps, mainly based on data provided by Blume (1985). Unfortunately, no specific sample locations are given in this publication – only the federal states in which a species was found. However, in order to get a general overview on the biogeography of dung organisms in North America this information might be suitable – but surely more work is needed here. Figure 24 shows the distribution of dung beetle data on a family level (Blume 1985; Floate 2011), indicating that in contrast to Europe here Scarabaeidae seem to be more frequent than Aphodiidae. However, the difference is not very large.

Figure 24: Family based distribution pattern of dung organism abundance from North-America according to the project database



Actually, the distribution on the family level reveals similar patterns: dung beetles occur all over North America except the Northern half of Canada and parts of the Rocky Mountains (other gaps are probably caused by the lack of sampling activities) (Figure 25-Figure 29).

Figure 25: Distribution of the sampled dung organisms according to the project database from North-America



Most sample points present geographically not specified locations but rather wide spread regions such as federal states

Figure 26: Locations of the previously existing collection points of species from the Coleoptera family Geotrupidae from North-America according to the project database

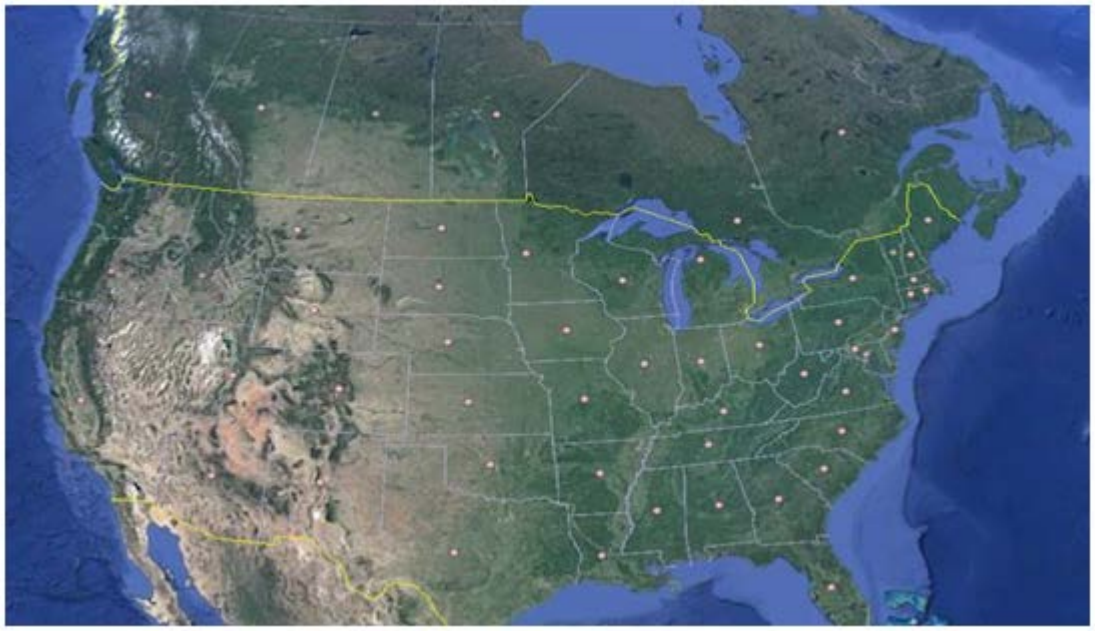


Figure 27: Locations of the previously existing collection points of species from the Coleoptera family Aphodiidae from North-America according to the project database

Figure 28: Locations of the previously existing collection points of species from the Coleoptera family Hydrophilidae from North-America according to the project database

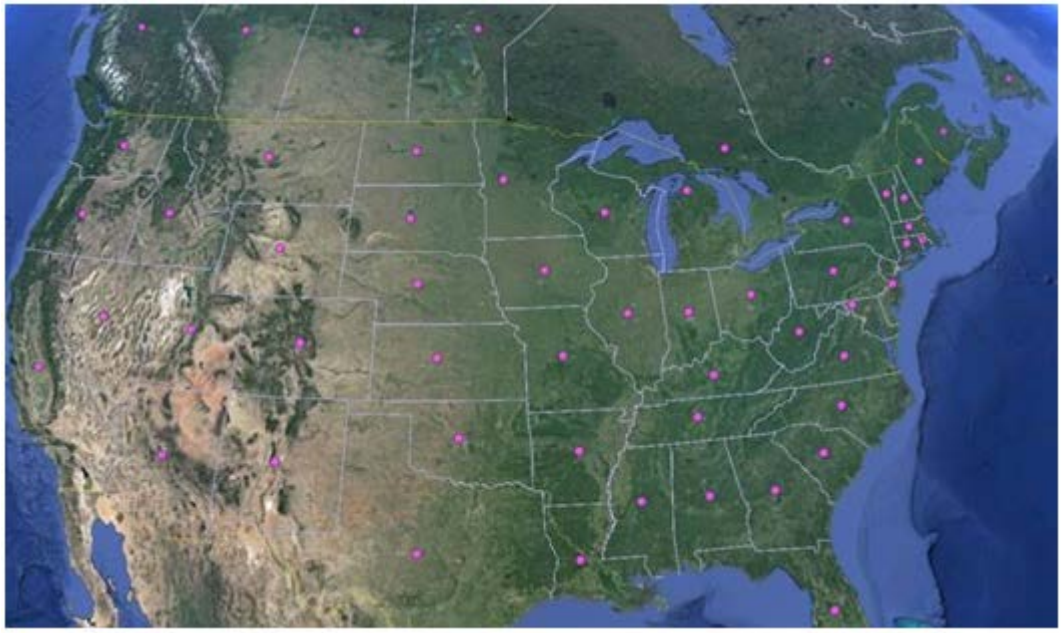


Figure 29: Locations of the previously existing collection points of species from the Coleoptera family Staphylinidae from North-America according to the project database

5 WP I: Description of different routes of exposure of parasiticides including new developments in VMP applications

Originally, it was planned to combine the identified and characterized European dung organism communities (see chapter 3) with exposure patterns of VMPs. Due to the lack of data this work could not be complete. Accordingly, it is difficult to formulate recommendations how exposure scenarios currently used in the risk assessment of parasiticides such as ivermectin could be improved. Therefore, the current state of the discussion will be documented and a prelimited assessment will be made.

In the beginning, it should be clarified what "non-target-organisms" are: they are the opposite to "target-organisms", i.e. those animals, which are harmful to livestock e.g. cattle, pigs, sheep etc.). Therefore, in the context of this project all dung- and soil organisms should be seen as "non-target-organisms". In practice, it is virtually impossible to test all possible applications of the used VMPs. Therefore, a prioritization has to be made. This prioritization is based on the one hand on taxonomic, biogeographical and ecological information of organism communities (what happens where?) and on the other hand on the sensitivity of these organisms known from laboratory tests and the current literature. The question of the sensitivity is the main topic in the following chapter. Firstly, however, information on the agricultural application of VMPs (amount, abundance, type of farm animal, exposure route etc.) have to be compiled.

5.1 Consumption and application of VMPs (exposure sources)

Consumption (that is amount) and application (how much at which farming animal) of the most important VMPs in Europe has to be known for any kind of risk assessment. Unfortunately, there is no corresponding database. Therefore, we received a list of the European approved drugs including the application type from the sponsor (Table 2). According to the sponsor this list is only a necessary but not sufficient condition for the quantified estimation of exposure of dung organisms, because any information of the usage amount of the products is missing. Therefore, further evaluation is not yet possible.

Table 2: List of those veterinary parasiticides (products) registered in the European Union including type of application and farm animals to be treated

Product name	Pharmaceutical form	Type of application	Species
Alfamectin	Powder	Oral	Pigs
Animec 8.7 mg/g oral paste	Paste	Oral	Horse
Bimectin Fluke	Solution for injection	Subcutaneous	Cattle
Chanectin Injektion	Solution for injection	Subcutaneous	Cattle
Chanectin Pour-On 0.5%	Solution	Onto the skin	Cattle
Closamectin Pour-On	Solution	Onto the skin	Cattle
Diapec P Gel	Paste	Oral	Horse
Diapec R	Solution for injection	Subcutaneous	Cattle
Diapec S	Solution for injection	Subcutaneous	Pigs
Ecomectin 1% Injektion	Solution for injection	Subcutaneous	Cattle, pigs, sheep
Ecomectin cattle Pour-on	Solution	On the back	Cattle
Equell	Paste	Oral	Horse
Equimax	Gel	Oral	Horse
Equimax Tabs	Chewable tablet	Oral	Horse

Product name	Pharmaceutical form	Type of application	Species
Equimectin	Gel	Oral	Horse
Eqvalan Duo	Paste	Oral	Horse
Eqvalan-paste ad us. vet.	Paste	Oral	Horse
Eraquell	Paste	Oral	Horse
Eraquell Tabs, 20mg chewing tablet	Chewable tablet	Oral	Horse
Fermectin Injektion	Solution	Subcutaneous	Pigs
Furexel	Paste	Oral	Horse
Furexel Combi	Paste	Oral	Horse
Hippomectin 12 mg/g oral gel	Gel	Oral	horse
Ivermectin Entwurmung 12 mg/g-oral gel	Gel	Oral	horse
Ivermectin Virbac 18,7 mg/g orale paste	Paste	Oral	horse
Iverpour Pour-On solution 0.5%	Solution	Onto the skin	Cattle
Ivertin Cattle	Solution for injection	Subcutaneous	Cattle
Ivomec	Solution	Subcutaneous	Cattle, pigs, sheep
Ivomec F	Solution for injection	Subcutaneous	Cattle
Ivomec Maximizer 100mg	Bolus	Oral	Sheep
Ivomec Maximizer 200mg	Bolus	Oral	Sheep
IVOMEC Pour-On	Solution	Onto the skin	Cattle, deer
Ivomec premix	Medicated feedingstuff	Oral	Pigs
Ivomec S/0,27%	Solution for injection	Subcutaneous	Pigs
IVOMEC SR Bolus	Bolus	Oral	Cattle
Ivomec-P	Paste	Oral	Horse
Ivomec.S	Solution for injection	Subcutaneous	Pigs
Medimec Pour-On solution 0.5%	Solution	Onto the skin	Cattle
Noromectin Injection	solution for injection	Subcutaneous	Cattle, pigs
Noromectin Pour-on	Solution	On the back	cattle
Noromectin premix, 0,6 g/100g	Medicated feedingstuff	Oral	Pigs
Overtin Injectable	Solution	Subcutaneous	Cattle
Paramectin Injection	Solution	Subcutaneous	Cattle, pigs
Paramectin horse	Paste	Oral	Horse
Paramectin Pour-on	Solution	On the back	Cattle
Qualimec 1% Injection	Solution	Subcutaneous	Cattle, sheep, pigs
Qualimec cattle Pour-on	Solution	On the back	Cattle
Sumex Injection	Solution	Subcutaneous	Cattle, pigs
Sumex Pour-On solution 0.5%	Solution	Onto the skin	Cattle
Ursomectin 10 mg/ml solution	Injection	Subcutaneous	Cattle, pigs

Product name	Pharmaceutical form	Type of application	Species
Vectin 22,75mg chewing tablets	Chewable tablet	Oral	Horse
Vetimec 18.7 mg/g Oral paste	Paste	Oral	Horse
Virbamec pour on	Solution	On the back	Cattle
Virbamec, 10mg/ml solution	Solution for injection	Subcutaneous	Cattle
Virbamec-S, 10mg/ml solution	Solution for injection	Subcutaneous	Pigs
Wedemec R	Solution for injection	Subcutaneous	Cattle
Wedemec S	Solution for injection	Subcutaneous	Pigs

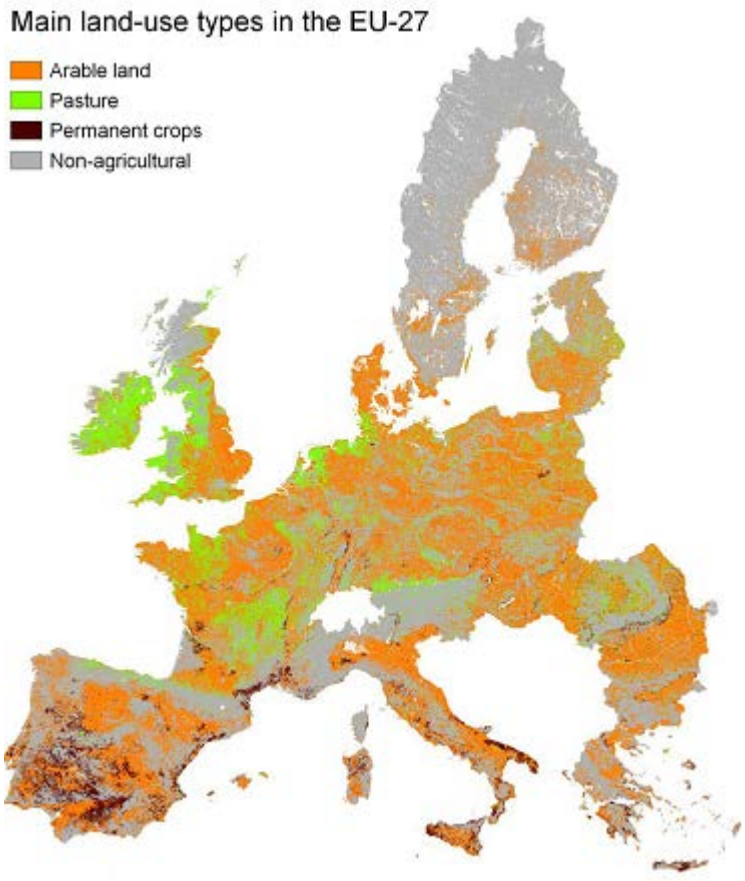
Source: German Federal Environment Agency

5.2 Farm animals to be treated

Information of the quantity and occurrence of the major farming animals (cattle, horse, sheep) and the respective treated pasture management of target species will be compiled here.

In a first step, the distribution of different forms of land use in Europe has been investigated (Figure 30). As a result, grassland is the dominant land use in major parts of France, almost all of Ireland, west England and Netherlands and parts of northern Germany, Poland and the Baltic States. It has to be noted that also in other parts of Europe extensive grazing is performed (for example: Spain, Denmark or Romania). Non-agricultural areas, marked in grey in Figure 30 (mostly mountains) are not used for grazing and therefore an impact on dung organism communities by VMP is highly unlikely.

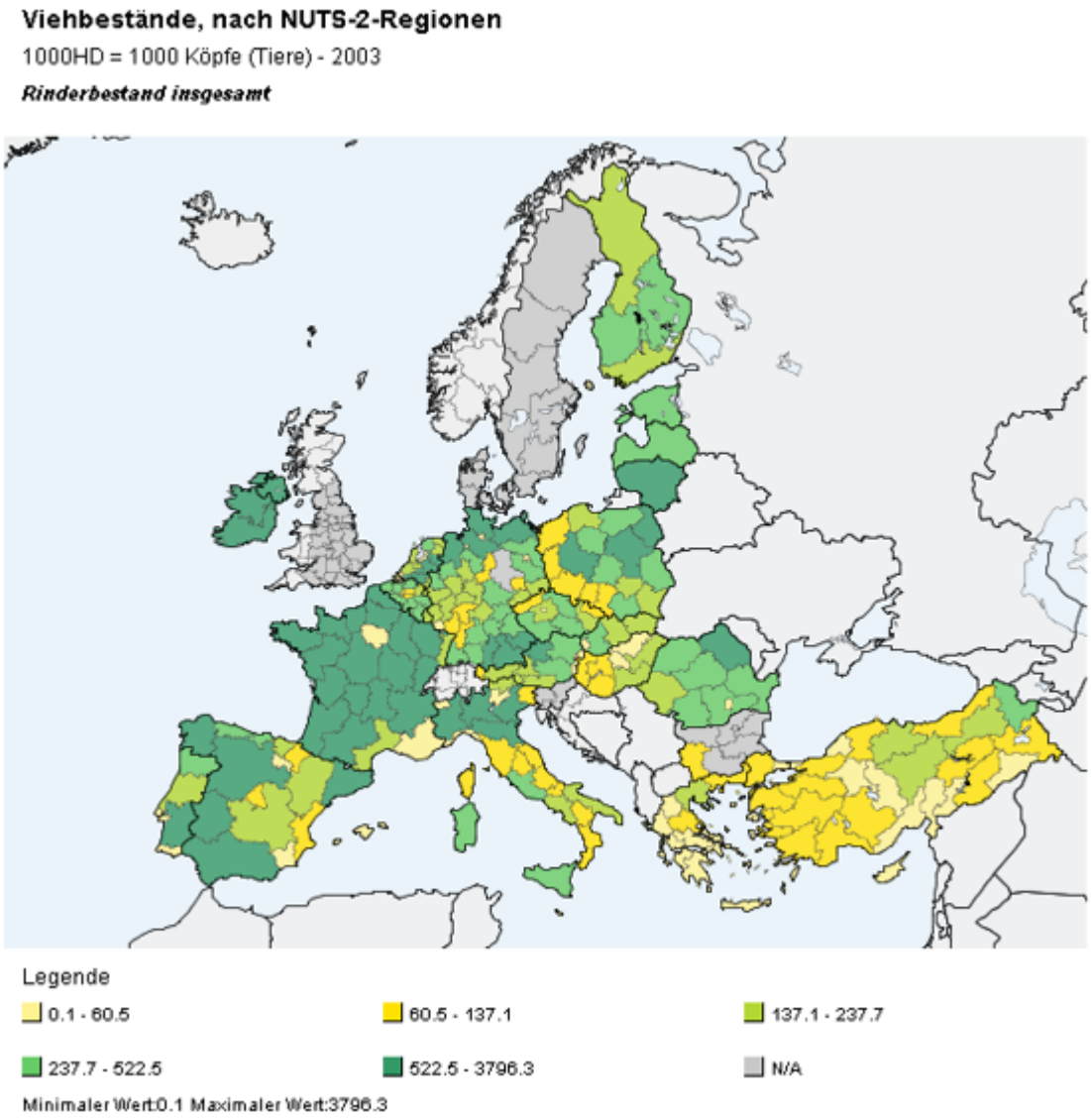
Figure 30: Distribution of the most important land use forms in Europe (EFSA 2010a)



In a next step, descriptions (source: EURO Stat) of the occurrence of farm animals in European regions were used. As an example, Figure 31 and Figure 32 show the amount of cattle and sheep stocks in Europe. All data on the amount and distribution of livestock in each European member state come from surveys carried out on farms in the months of November or December of each year.

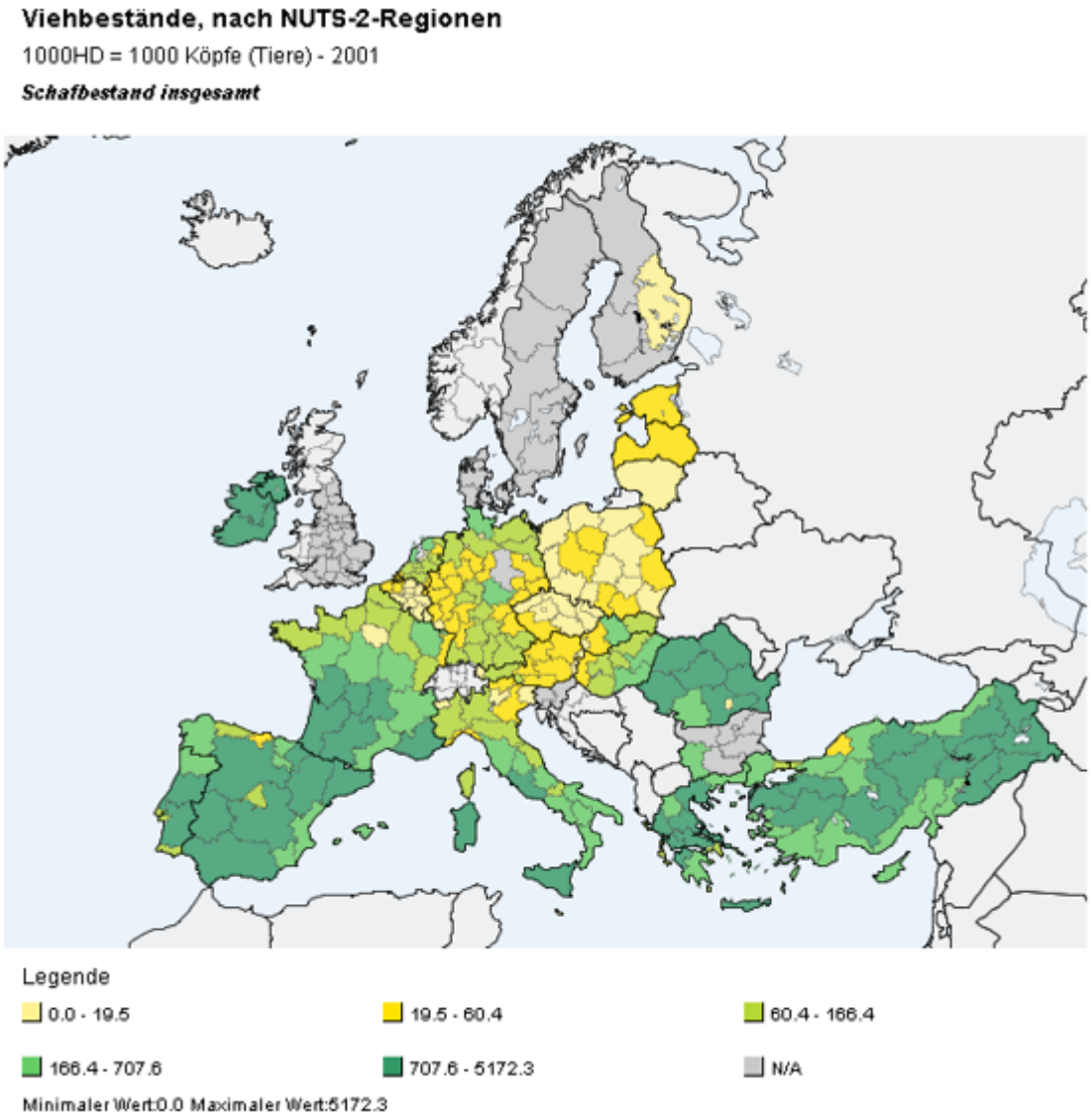
When comparing the different maps, it is obvious that high numbers on livestock in the data base do not correspond to the distribution of grasslands, i.e. the main land use for pasture farming (for example the description of Spain in Figure 30 and Figure 31/Figure 32). These differences have to be investigated in further studies; for example in terms of harmonizing the different data bases or on spatial remuneration. Moreover, an extension to (at least) the horse stock would be useful.

Figure 31: Data on the distribution of cattle in the NUTS-2 Regions of Europe



Source: Euro-Stat: http://epp.eurostat.ec.europa.eu/tgm/mapToolClosed.do?tab=map&init=1&plugin=1&language=de&pcode=tgs00045&toolbox=legend; Ansicht 20.04.2011

Figure 32: Data on the distribution of cattle in the NUTS-2 Regions of Europe



Source: Euro-Stat: http://epp.eurostat.ec.europa.eu/tgm/mapToolClosed.do?tab=map&init=1&plugin=1&language=de&pcode=tgs00045&toolbox=legend; Ansicht 20.04.2011

As part of the assessment of the livestock inventory, an assessment of the entry of VMPs in the environment was conducted in the ERAPharm project (Schneider et al. 2007). The authors performed an extensive literature review on the occurrence of bovine animals, swine, sheep and poultry (each analogous to the EUROSTAT classification divided into various age and weight classes) in Europe. This information was connected with data on excretion rates and environmental parameters (climate and soil properties, slope, proximity to the nearest water, etc.). The results were assigned to maps, focusing on risks to the aquatic environment caused by VMPs. Based on this data set18 distinct regions in Europe were identified (Table 3).

Table 3: List of land use classes (CORINE) potentially affected by VMP

Land use class	CORINE Class[1]	Nr
Arable land	Non-irrigated arable land	12
	Permanently irrigated land	13
	Rice fields	14
Grassland	Pastures	18
Mixed areas	Annual crops associated with permanent crops	19
	Complex cultivation patterns	20
	Land principally occupied by agriculture, with significant areas of natural vegetation	21
Marginal	Natural grasslands	26
Areas	Moors and heathland	27
	Sparsely vegetated areas	32
Land use class	CORINE Class1	Nr
Arable land	Non-irrigated arable land	12
	Permanently irrigated land	13
	Rice fields	14
Grassland	Pastures	18
Mixed areas	Annual crops associated with permanent crops	19
	Complex cultivation patterns	20
	Land principally occupied by agriculture, with significant areas of natural vegetation	21
Marginal	Natural grasslands	26
Areas	Moors and heathland	27
	Sparsely vegetated areas	32
Land use class	CORINE Class1	Nr
Arable land	Non-irrigated arable land	12

For details see Schneider et al. (2007)

Although this data set is not directly representative for the present project (e.g. here no work has been done regarding dung organism communities of chicken dung), several conclusions are obvious

- The land use classes to be considered are probably limited to four: No. 18 (= pasture scenario) as well as Nos. 12-14 (cropland-scenarios). In individual cases the land use class No. 26 (e.g. horse breeding in Dutch national parks; Lahr personal communication) seems to have certain importance.
- Since it will not be possible in the foreseeable future to create a correspondingly detailed characterization of dung organism communities (if there are several different ones) in Europe, a regionalization of Europe into 18 land use classes would not make sense.

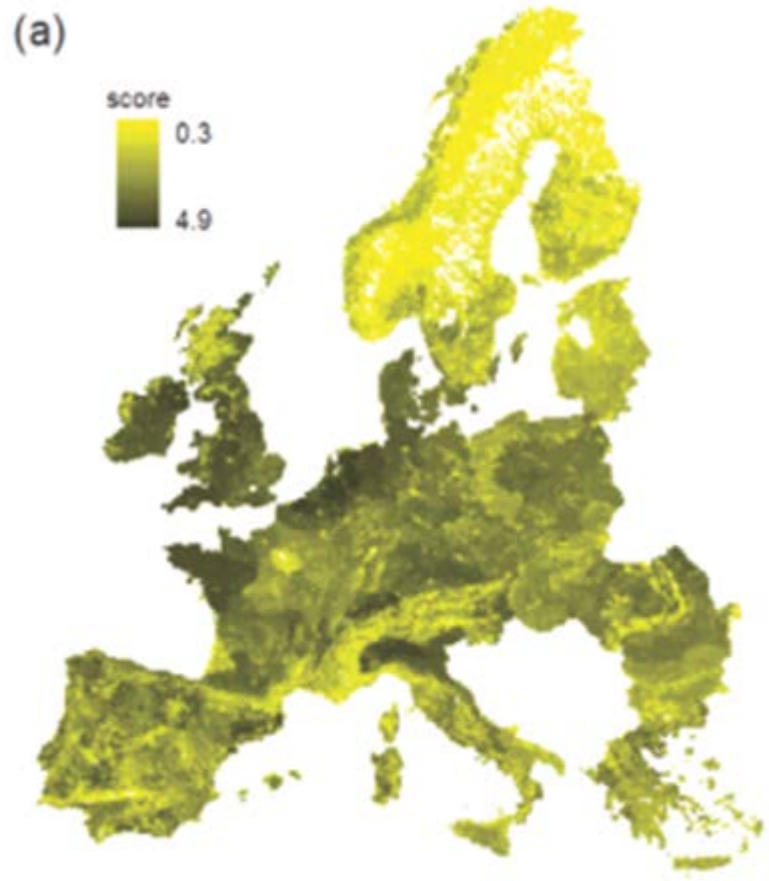
[1] European Environment Agency; Copenhagen

The main conclusion of Schneider et al. (2007) concerns the significant environmental risk through the use of VMPs on large regions of Europe. These are Ireland, nearly all of England, northwest of France (specially the Bretagne), large parts of Belgium, the Netherlands, north of Germany and Denmark. In southern Europe Catalonia as well as the Italian Padan Plain are potentially under the risk of environmental pollution with VMPs. This distribution matches with the occurrence of grassland (not everywhere: see for example Italy and Denmark; A2) as the dominant land use (EFSA 2010a). Even if it is not possible to compare results of this list with the threatened dung or soil organism community, a brief impression of the potential problem could be given.

Finally, further assessments of individual exposure routes are presented below:

- Way of utilization, for example grassland and agricultural crop land: these descriptions are as detailed as possibly listed in the current report
- Dung organism communities: The identification of related communities has also been reported; according to current knowledge, it is mainly based on the distribution of several dung beetle families. In case of the soil organism communities only distribution patterns of collembola and earthworm could be presented (unfortunately only for some EU member states).
- EU regions: For a clarification of this issue a minimum amount of data is required. Since this minimum is not available no further details on EU regionalization can be given today. Any identified regions must also be verified with administratively defined regions, where data on livestock numbers and the resulting amount of dung are available. The implementation of these requirements is currently not possible.
- Agricultural criteria such as treatment of livestock (long-term vs. short-term treatment) or animal breeding (pasture vs. stabling, this issue could eventually be modeled). For a proper conclusion on this issue, more data on livestock farmers or agricultural chambers is required. At least the bigger EU-member states should be represented in this test, which is beyond the scope of this project.

Figure 33: Distribution of the “tendency scores” for VMP loads (TM)



Schneider et al. 2007; the darker the colour the higher the amount of dung produced by farm animals (not differentiated by species).

In summary, it should be noted that a rough estimate of the hazard potential of dung communities of organisms through VMPs in Europe should be possible on a longer term with the used maps. Apart from the data gaps, quantified information about prescribed and used amount of VMPs, grouped into product, target species, time period, etc. are missing. The sponsor should question the possibility to investigate on those issues in a further study. The content of this chapter is described in detail in the Annex (Chapter 16). Based on a literature review (in particular the results of the EU FP6 project ERA-Pharm) and information provided from national (UBA) and international (EFSA) agencies the usage of VMP in Europe is mapped. The main outcome of this exercise, published by Schneider et al. (2007), is that due to the use of VMP an increased environmental risk is possible in the following regions: Ireland, most of England, Northwest France (especially the Bretagne), Belgium, the Netherlands, Northern Germany, Denmark, Catalonia and the Po-valley in Italy. This distribution is similar (but not equal) to the distribution of grasslands in Europe.

6 WP I: Identification of the most relevant and most sensitive species of dung communities, which might be affected by antiparasiticides

6.1 Discussion of the protection goal:

In addition to the description of the contract, in the first meeting between the sponsor and the partners of this project the protection goal "biodiversity" was defined in the context of the environmental risk assessment of VMPs (see discussion during the last workshop of the Aveiro group in Berlin (UBA 2009)). In the VICH guideline GL 6 (2000), which provides basic guidance (i.e. phase I) for the accreditation of VMPs in Europe, the protection goal is not given. This gap was closed by the VICH guide line GL 38 (2004), which describes phase II of the risk assessment. Below the key phrases of section 2.1 of this document are given in abbreviated form:

The overall target of the assessment is the protection of ecosystems. The aim of the guidance provided in Phase II (and in Phase I) is to assess the potential for VMP to affect non-target species in the environment, including both aquatic and terrestrial species. The taxonomic levels tested are intended to serve as surrogates or indicators for the range of species present in the environment. Impacts of greatest potential concern are usually those at community and ecosystem function levels, with the aim being to protect most species. However, there may be a need to distinguish between local and landscape effects. Unfortunately, the protection objectives in the relevant "Guidance Document" (EMA 2008) are not further specified. Similar phrases are known from other EU-documents such as the recently modified guidelines for biocides (EC 2009; EFSA 2010c) or the draft of the Soil Frameworks Directive (EU 2006; see also the wording of paragraph 2 of the German Federal Soil Protection Act (BbodSchG 1998). In addition, any protection goal should also be in line with the Ecosystem Service approach (MEA 2005; Elmquist et al. 2009). In summary, the protection goals for the environmental compartment dung in the context of the use of VMPs like Ivermectin could be defined as the protection of two features of the dung organism communities:

1. Their structural diversity (i.e. biodiversity), e.g. the species composition
2. Their functional diversity (i.e. the benefits), e.g. the dung degradation

Primarily, in this chapter it was planned to quantify the sensitivity of the dung organism communities in several regions, e.g. by measuring their biodiversity on a species level as well as on their ecological groups, as a basis for further actions. Referring to the last statement beetles could be divided in "Tunnelers" and "Dwellers" (Hanski & Cambefort 1991) and earthworms into "Anecics", "Epigeics" and "Endogeics" (Bouché 1977). A differentiation of the whole soil organism community according to their body size in Micro-, Meso- and Macrofauna is possible, but does not give much more information since it is too broad (Swift et al. 1979). In general, it should be possible to identify and classify traits which could affect the sensitivity of dung- and soil organisms towards VMP (e.g. recovery ability, depth distribution, etc.). Based on this information a vulnerability analysis could be performed (De Lange et al. 2009). This classification should be used to identify indicator species or groups which are suitable for the evaluation of the biological quality of dung or soil. However, such an evaluation is only possible if reference conditions for the structural or functional diversity at a given site are known (Römbke & Breure 2005). Especially in the last step it is important to identify information gaps, because even for the well-studied VMP ivermectin there are whole groups of dung and soil organisms with insufficient knowledge of their sensitivity (e.g. snails). However, it is important to note that the current knowledge on ivermectin toxicity to dung and soil organisms (among others) has been well compiled in recent publications (e.g. Boxall et al. (2005); Floate et al. (2004); Jochmann et al. (2010)). Especially the review of Lumaret et al. (2012), in which the partners of this project were involved, summarizes the state of knowledge on the effects of ivermectin on the diversity of dung and soil organism communities very well. When using such data, the interaction of the effects of VMPs as well as the influence of other biotic and abiotic stressors has to be taken into account.

6.2 Tests with dung organisms: Literature data

Laboratory tests

In the following, the results of laboratory tests with ivermectin and with standard ecotoxicological test species (e.g. *Aphodius constans*, *Scathophaga stercoraria* or *Folsomia candida*) are presented in Table 4 and Table 5, respectively. Here not only the most sensitive species of each group is presented but also all relevant organisms that need to be covered in an environmental risk assessment for a VMP. Therefore, not all previously performed laboratory tests with ivermectin are listed, but only those which actually have been used in the EU project ERAPharm for the ERA of ivermectin (Liebig et al. 2010).

Table 4: Terrestrial effect studies with dung organisms

Test organism	Test method	Effectconcentration a)	Reference
Musca autumnalis (dung fly)	OECD (2008a)	EC50 21 d, emergence rate = 4.65 µg/kg dung fw	Römbke et al. (2010c)
Scathophaga stercoraria (dung fly)	OECD (2008a)	LC50 28 d = 20.9 µg/kg dung fw NOEC 28 d, development time = 0.84 µg/kg dung fw	Römbke et al. (2009)
Scathophaga stercoraria	Specific test design (acute toxicity)	LC50 48 h, larvae = 36 µg/kg dung fw EC50 3-4 w., emergence = 1.0 µg/kg dung fw	Strong and James (1993)
Aphodius constans (dung beetle)	OECD (2010)	LC50 21 d = 176 µg/kg dung fw LC50 21 d = 880 µg/kg dung dw NOEC 21 d, larval survival = 320 µg/kg dung dw	Hempel et al. (2006)
Aphodius constans (dung beetle)	OECD (2010), modified	LC50 21 d = 100 µg/kg dung fw b) LC50 21 d = 590 µg/kg dung dw	Lumaret et al. (2007)

see Liebig et al. 2010; a) All effect concentrations refer to nominal concentrations

It appears that dung fly organisms show the highest sensitivity against ivermectin, whereas beetles are much less sensitive. The inclusions of additional species or test endpoints in the literature does not change this statement considerably (Lumaret et al. 2012). In addition (and this is an experience made in tests with other chemicals too), sublethal endpoints such as biomass or reproduction should be preferred to mortality. Other parameters such as morphological changes are often difficult to be recognized for various reasons (e.g. Floate & Coghlin 2010). In addition, it could also be stated that the results shown in Table 4 are very robust. In the case of the fly tests this statement is based on two international ring tests (Römbke et al. 2009; 2010). In the case of the dung beetle tests, evidence for the good reproducibility and repeatability of the test with *Aphodius constans* was found in interlaboratory comparison tests (i.e. work performed in parallel at the University of Montpellier and at ECT GmbH, including the results of unpublished studies run for UBA in the last years). However, in these recent studies it could also be shown that the existing dung beetle test with *Aphodius constans* could clearly be improved by changing the test endpoints (e.g. instead of larval survival the hatching success of adults) or by increasing the test duration (Römbke & Scheffczyk 2010).

Field studies

In the already mentioned literature on the effects of ivermectin on dung and soil organisms (Lumaret et al. 2012) various field studies are listed too. Unfortunately, it is virtually impossible to evaluate most of these studies, since they differ greatly in execution, duration, endpoints as well as the details of exposure to ivermectin in toto. It should also be considered that in all studies on the toxicity of ivermectin, the influence of other factors on the endpoint dung organism community, e.g. avoidance or attraction of formulated substances (Floate 1998b) or, generally, the community's natural changes due to, for example, climatic factors (Errouissi et al. 2004) is often not included in the publications. Therefore, only two field studies will be presented as examples in the following.

The first publication which focused on "diversity" as a parameter in studies with dung organisms was provided by Krüger & Scholt (1998a, b). Two cattle herds at two different time points (first in the dry, then a year later in the rainy season) from South Africa were used on an area of approximately 80 ha. One herd was treated with ivermectin, the other one served as the control. Dung insect communities were sampled one month before and three months after treatment. Diversity parameters were calculated using multivariable statistics. After ivermectin treatment a significant decline in species diversity and a significant increase in the dominance of a single species were observed. In another sampling 12 month after and just before the second treatment no difference between the control and the treated herd was found. Again, one and three months after the second treatment no effects related to the treatment with more Ivermectin were found, although a random sampling after application showed strong effects on Hydrophilid larvae or the puppets of Scarabaeidae and Diptera. The results of this complex study suggest that the strength of the ivermectin influence depends on several factors, i.e. not only to the VMP itself, but also on the prevailing climatic conditions and the spatial extent of the treatment and the number of shares held in a herd of grazing animals.

As a second example a study is presented which was performed near Madrid (Spain) in the framework of the EU project ERAPharm (Römbke et al. 2010b). Cattle dung with four different concentrations (plus a positive and a negative control) of ivermectin was exposed for 28 days (impact test) and 86 days (degradation test). Ivermectin did not have an effect on micro-arthropods, sampled directly under each dunghill. In contrast, strong and long-lasting effects were detected on dung-inhabiting diptera larvae during the whole test phase. Dung beetles showed lower but species-specific reactions, leading to shifts in species dominance (specifically affected: *Volinus distinctus*). However, it has to be kept in mind that only adult beetles were collected, which tent to be less sensitive than larvae. In addition, adult dung beetles were attracted by the spiked dung and therefore show high abundance even on dung with high ivermectin concentrations (in fact their number was higher than in the negative control (Errouissi & Lumaret 2010)). The dung of untreated animals was degraded significantly faster than that containing ivermectin.

In the same study in Spain the effects of ivermectin on Staphylinidae were also studied, revealing quite complex interaction patterns (Figure 34). When comparing the total number of Staphylinidae in the treated dung compared with the control, there were no direct negative effects (Förster et al. 2011). This result is in-line with other studies performed with these predatory arthropods (Madsen et al 1990; Floate et al. 2002.). Many predaceous staphylinid beetles, with the exception of the parasitoid Aleocharines, do not spend their larval stage within the dung pad ecosystem may partly explain the lack of negative response to ivermectin residues. However, measuring another endpoint might have been useful: This could be the rate of emergence from fly pupae in case of Aleocharines by taking into account that in the present study no adverse effect were postulated for Aleocharines, due to a high number of sampled adult specimens; but, as all treated dung pads lacked fly pupae, no treated dung pad is going to be able to function as reproduction substrate of colonizing *Aleochara* beetles.

Experiences made in the four field studies (Work Package II)

According to our results (see Chapter 16 for details), the use of ivermectin has a long-term effect on various groups of dung organisms, in particular flies - most notably the Sphaeroceridae and Sepsidae. Even dung excreted 28 days after application of this VMP, containing ivermectin at concentrations as low as 0.01 - 0.05 mg/kg d.w., is highly toxic for various fly groups at all test sites. Actually, due to the study of an extra treatment only performed at the Lethbridge site it is highly likely that this effect does last at least 56 days after application of ivermectin, if not longer. Ivermectin also negatively impacts the emergence of dung beetles from treated cattle dung, but in this case the effect is mainly limited to the first two weeks after the application of ivermectin. In addition, the impact is not restricted to species of the family Aphodiidae (i.e. “typical” dung beetles but species of the families Hydrophilidae and, to a lesser extent, Ptilidae (both often overlooked) were also impacted. Staphylinid beetles and parasitic wasps show an intermediate sensitivity: less than various flies but more than most dung beetles. This result was not expected from the relatively few studies with rove beetles found in the literature.

In general, field studies provide additional information about indirect effects, but their evaluation deeply depends upon the sampling design and the data set quality of specific faunal groups. Hence, one should focus on long-term trials and worst-case scenarios. This may include (1) to increase the distance between the studied dung pads to avoid rapid migration from untreated dung pads (of the control group or from outside the study site), (2) to conduct long-term trials, which take place in a landscape of enhanced ivermectin usage and (3) to choose endpoints which are of great importance for the reproduction and, thus, the population stability of specific faunal groups, such as times of activity or the ability of recovery.

Figure 34: Principal Response Curves (PRCs) for the effect of ivermectin on the Staphylinid species community in dung pads, which were exposed on a pasture near Madrid

PRCs were separately conducted for (a) all taxa, (b) only carnivorous taxa and (c) only omnivorous taxa. Presented are the canonical coefficient of the different treatments at each sampling date after exposure in the field and the species weights of all taxa. Treatments: control (full diamond), T7 (open diamond), T2 (full square), T3 (open square), T4 (full triangle), spiked (open triangle), Hopp, pers.comm.

6.3 Tests with soil organisms: Literature data

Looking at soil organisms Collembola are clearly the most sensitive group, while the representatives of soft-bodied invertebrates (earthworms, enchytraeids) react significantly less to ivermectin. Very little effects were observed on predatory mites (Römbke et al. 2010a) and nematodes (Grønvold et al. 2004). The last statement should be considered with caution because only the results of one study are available so far. Recent results from multi-species tests indicate that Collembola are significantly more stressed by ivermectin in case predatory mites are present in the same test vessels (Jensen & Scott-Fordsmand 2012). However, in more complex semi-field or field studies effects on soil organism groups or their feeding activity were only found at high concentrations (Förster et al. 2011; Römbke et al. 2010b).

Table 5: Terrestrial effect studies with soil organisms

Test organism	Test method	Effectconcentration a)	References
Eisenia fetida (earthworm)	OECD 222 (2004c) (artificial soil, TOC 3.6%)	NOEC 28 d, biomass = 5.0 mg/kg dw NOEC 56 d, reprod. = 2.5 mg/kg dw EC50 56 d, reprod. = 5.3 mg/kg dw	Römbke et al. (2010a)
Eisenia fetida (earthworm)	subchronic earthworm test (artificial soil)	NOEC 28 d, biomass = 12 mg/kg dw LC50 28 d = 315 mg/kg dw	Halley et al. (1989a)
Eisenia fetida (earthworm)	OECD 207 (1984) (artificial soil)	NOEC 14 d, biomass = 4 mg/kg dw LC50 14 d = 15.8 mg/kg dw	Gunn & Sadd (1994)
Enchytraeus crypticus (Enchytraeidae)	ISO 16387 b, (field soil: TOC 1.6%)	NOEC 28 d, reprod. = 3.0 mg/kg dw EC50 28 d, reprod. = 36 mg/kg dw LC50 28 d > 300 mg/kg dw	Jensen et al. (2003)
Folsomia candida (Collembola)	ISO 11267 (1999) (artific. soil: TOC 3.6%)	NOEC 28 d, reprod. = 0.3 mg/kg dw EC50 28 d, reprod. = 1.7 mg/kg dw	Römbke et al. (2010a)
Folsomia fimetaria (Collembola)	ISO 11267 (1999), (field soil: total carbon 1.6%)	NOEC 28 d, reprod. = 0.3 mg/kg dw EC50 28 d, reprod. = 1.7 mg/kg dw LC50 28 d = 8.4 mg/kg dw	Jensen et al. (2003)

See Liebig et al. 2010; a) Effect concentrations refer to nominal concentrations

At all sites (but in different intensities) significant reductions of the number of earthworms and springtails could be found at several ivermectin concentrations and sampling dates (for details see Chapter 16). However, despite an indication that ivermectin was responsible for these differences, a clear concentration-effect relationship could not be identified, so overall the effect of ivermectin on

these two soil organism groups was certainly weaker than that on the dung decomposing groups. Despite these noticeable detrimental effects on dung (and partly soil) arthropods, ivermectin overall did not significantly hamper dung degradation at all test sites. As discussed above, this indicates that dung pat degradation, at least at the later stages, is more a function of the activity of earthworms, which were only rarely affected by ivermectin, and physical deterioration, rather than biological degradation by coprophilic insects. Obviously, (extreme) weather conditions may have influenced the outcome of these studies at some sites, e.g. via affecting the abundance of certain important groups of dung insects (such as in Montpellier, where dung beetles were much rarer than in previous years).

6.4 Summary of this chapter

As indicated previously the biological and ecological information on many dung (and also soil) organisms is not sufficient (or so widely scattered) that detailed statements regarding the autecological properties determining their sensitivity towards ivermectin or other VMP are difficult to make. For both groups, there are some exceptions (e.g. the classification of dung beetles (in dwellers, tunnelers, and rollers (Hanski & Cambefort 1991)) or earthworms (in epigeics, endogeics, anecics (Bouché 1977)), respectively. When combining these classifications, focusing on the handling of dung (beetles, especially their larvae) or the vertical distribution in the soil (earthworms) with their feeding behavior, the respective exposure of these invertebrates to ivermectin is determined – and thus the effects this VMP might have. Thus, the following conclusions can be drawn for these two groups:

Beetles: At our study sites, mainly soil dwellers (usually relatively small species of the family Aphodiidae) were found. They spend their whole development in the dung or in the uppermost soil layer. Both larvae and adults of these species feed also on dung fibres directly or on microbes filtered from the dung, meaning that this group is surely more exposed to ivermectin than tunnelers or rollers in the short-term (in the long-term, the difference might be not that large because of the persistence of ivermectin in the buried brood-balls). On the other hand, due to their body size their impact on dung degradation is only limited, while species belonging to the two other groups are often larger, i.e. are more able to influence dung degradation. Thus, when selecting test species, representatives of both dwellers and tunnelers should be used. While an OECD guidance document is already available for *Aphodius constans*, a guideline for an *Onthophagus* species is still not available (a ring test is expected to be finished in 2014).

Earthworms:
Epigeic worms are mainly exposed to ivermectin since they are living not only close to the surface but they are also feeding directly on the dung. Endogeic worms prefer to feed on soil in the uppermost mineral layer, meaning that their exposure is usually very limited. Finally, deep-burrowing (i.e. anecic) species are regularly feeding on the soil surface as well, but it seems that they prefer plant material (e.g. leaves) which are drawn into their burrows. Own experiences in the field studies indicate that in fact epigeic worms are more often significantly reduced compared to worms of other ecological groups. This result is in-line with the selection of the epigeic lumbricids *Eisenia fetida* or *Eisenia andrei* as test species. However, regarding the identification of "worst-case" scenarios there is further action needed.

- Firstly, Römbke & Scheffczyk (2010) have shown that the existing dung beetle test with A. constans should be modified (at least in a way that chronic endpoints and a longer test duration could be used as part of a tiered test strategy) – for details see Chapter 8.
- Secondly, based on the results of our field studies it is recommended to investigate in detail whether (and in addition to the test with O. taurus) further beetle groups (e.g. hydrophilidae, Ptilidae) could be used as ecotoxicological test species. Currently, our results confirm that they should be included when studying dung organisms in field studies. However, whether it is suitable (and practical) to increase the number of laboratory test species needs further research.

- Thirdly, the status of the beetle family Staphylinidae should be re-considered. Due to their quite broad ecological role (mainly predatory, but also fungal feeders) they have not been included in laboratory testing and field monitoring. Actually, a standard test with a partly predatory, partly parasitic Aleochara-species has already been proposed, which is usually used in the area of pesticide registration (Samsoe-Petersen 1987).
- Despite the fact that there are hints as early as in the early nineties of the last century (Schaper & Liebisch 1991, Floate 1998a) it could only recently be confirmed that flies of the family Sepsidae are more sensitively reacting to ivermectin than the two fly standard test species (Blanckenhorn et al. 2013a, b). However, the individual species within this family show tremendous differences in sensitivity, meaning that it is very difficult right now to recommend one specific species for becoming a standard test species. In any case, this family (plus, looking at the results of our field studies), the Sphaeroceridae should be included in the list of groups monitored in field studies.
- Probably the same as stated above for the Sepsidae and Sphaeroceridae is true for parasitic wasps since at Zurich and (partly) Montpellier significant effects on these organisms were found. This is in line with the few data found in the literature (e.g. Floate & Fox 1999). However, handling and determination of these (usually very small) wasps is difficult. Therefore, it is recommended to include this group in field studies. In parallel, and again there is a parallel to the Sepsidae, laboratory studies should be performed in order to clarify whether a wasp species (and which) could be useful for laboratory tests. Right now, it seems that this is quite a task due to their specific biology.

However, according to the literature and our own experiences there are organism groups which today cannot be recommended for the risk assessment of VMP. This is true for all organism groups listed in Table (i.e. those related closely to the dung compartment) not mentioned so far (with two exceptions, see below). Usually, the reasons for this evaluation is that they are too rare and too difficult to handle or to determine. In addition, usually no information is available regarding their sensitivity to VMP. The exceptions are the nematodes, both living in dung and soil, and the mites. Regarding Nematoda, it is very difficult to make general statements on such a diverse taxonomic and ecological group. In fact, they are usually handled in soil ecotoxicology on the level of trophic groups which might be a suitable approach also for other stressors. Despite the fact that an ISO test has recently been standardized (ISO 2010), (very few) laboratory tests had been performed so far – and the data available indicates a low sensitivity towards ivermectin (Grønvold et al. 2004). Finally, since both target- and non-target species belong to this group (and it is often not really clear which species is what) further investigations regarding the taxonomy and ecology of these organisms are recommended before using them for the ERA of VMP. A comparable statement is possible for mites, especially the predatory gamasids, which are the most important predators in many soils (Usher 1985). However, their low sensitivity towards VMP (Römbke et al. 2010a) combined with the lack of experience related to dung pats does not allow their immediate use in the ERA of VMP.

In the context of soil tests with VMP an additional recommendation is possible: as could be shown the performance of multiple-species tests adds relevant information: in the few examples known so far (Jensen & Scott-Fordsmand 2012) the effect values determined in these tests are by an order of magnitude lower than those determined in single species tests, meaning that in this case a direct influence on the ERA of (in this case) ivermectin did occur: based on these results a risk was identified which was not visible beforehand.

Finally, the question has to be discussed which effects the inclusion of further organism groups would have on the current practice of ERA of VMP. On the level of laboratory tests probably not much since in the existing tests (or soon to be modified, i.e. the beetle tests) already toxicity can be identified. The only exception might be the inclusion of sepsids because their sensitivity could be higher by an order of magnitude. However, the selection of the most appropriate species, the standardization of test methods and the testing of further VMP than ivermectin are urgently needed before a final decision

could be made. On the level of field tests, the usage of those organism groups used here would surely influence the ERA of VMP in more direct ways:

- Monitoring the diversity and abundance of a broad range of organism groups would increase the probability of identifying sensitive species, minimizing at the same time the chance to overlook effects. This could easily happen since at different sites with different climatic factors, soil properties, landuse histories (in short: with different ecological conditions) different dung and soil organisms groups are important (see Chapter 16). In addition, the determination of realistic effect values (e.g. an EC10 (field) or a NOEC (field) would be possible.
- However, probably more importantly, the study of various organism groups in the field, especially for a long period of time (see our results: the use of dung collected 28 days after application of the VMP was probably not sufficient) would provide much information regarding the ecological relevance of the effects which will be observed after the usage of many VMP. For example, is there a potential for recovery? Will some species or groups be replaced by others? Do functionally important species occur during the whole season or year? Would it be possible to avoid reproduction cycles of dung organisms by shifting application dates?

For these reasons, it is surely recommended to perform field studies when a risk for the dung and/or soil organism community is indicated based on the results of laboratory tests. In addition, there is another reason why the performance of field tests is important for the performance of an ERA for VMP: Only on this level of investigation (and despite some important insights when running multi-species laboratory tests) the interaction of the VMP with the abiotic (e.g. the climatic factors affecting the transport and, more importantly, the degradation of the VMP in dung and soil) and biotic (e.g. the influence of competition or predation within the dung and soil organism communities exposed) could be taken into account. One indication of these other factors is the observation that the duration of dung degradation did differ at our four study sites. Another indicator for these factors is the different composition of the dung organism communities, i.e. the (potential) influence of large scarabaeid beetles on dung degradation in Montpellier. However, this example does also show that the performance and interpretation of field studies could be difficult: due to an unusual dry spring time when starting the study in Montpellier, the number of beetles caught was very low, meaning that neither effects of ivermectin on these species nor the impact of these species on dung degradation could be clearly specified.

In order to repeat the lessons learned it could be stated that:

- Laboratory: few changes (inclusion of sepsid flies; use of multi-speies tests) are needed
- Field: studies do provide necessary and relevant information regarding the ERA of VMP
- No quantification of these changes on the outcome of the ERA of VMP is possible today, since the amount of information, completely based on tests with one VMP so far, is too low.

7 WP I: Verification of existing models for the risk assessment of parasiticides related to dung fauna communities

Based on the desire to have one more option on higher levels of the environmental risk assessment of VMPs besides very expensive tests (especially field studies) it has been proposed to use models for the evaluation of the effects of VMP on dung organisms (Vale & Grant 2002; Boxall et al 2007). This proposal is based on experiences made in the context of assessing side effects of pesticides on non-target arthropods (Jepsen et al 2005; see also the review of modeling in the EU pesticide registration, Galic et al 2010) or the possible effects of genetically modified organisms on arthropods (e.g. Squire et al 2003). Currently a working group from the University of Aarhus (Denmark) attempts to develop a suitable model called “ToxDung” for regulatory practice (Axelsen & Jensen, personal note). This model was introduced on an expert talk in Montpellier in April 2011. Its construction and its properties are briefly described below.

Two populations, one control (= in untreated dung pads) and a treatment (= dung pads containing the VMP) are compared with each other. It is assumed that these VMP are toxic for dung organisms (in most cases flies and/or beetles). Otherwise no need for a "Higher-tier testing" would occur. To simulate different appeals or settlements depending on the age of the dung pad it is assumed that pads which have been in the field for a different time (fresh, medium old, old) are available for dung organisms. The respective concentration of the VMPs on the one hand depends on the (at least in this stage of the development of the model) constant application rate, on the other hand on the degradation of the VMP (already in the farm animals and the storage time of the dung). Several treatments can be simulated per season.

Three “species” are used as representatives of the populations to be protected, which primarily differ in the length of their life cycle (short, medium, long). In addition, life stages were separated (for example larvae, adult females searching for places for oviposition…) where it is assumed that the VMP does not cause any changes in behavior. Abundance as well as biomass of any “species” was observed on a determined area (for example one hectare) during their life cycle and activity time. From these variables, the reaction of the respective "species" in the two dung pad groups (control, treatment) is to be modeled over at least one season. In later stages of development, the model could be refined, for example, as follows:

- The ecosystem is getting more complex (e.g. by extrapolation to the landscape level)
- More species of different trophic levels (predator, parasitoid) are included
- The behavior (e.g., walking) of the "species" is also simulated

Generally, the approach of J. Jensen is suitable to serve as a basis for modeling the effects of VMPs on dung organisms. However, there are several open issues still to be considered:

- How to describe exposure issues on a fine scale?

This is likely to be the easiest step, because the fate and behavior of each new VMP in the environment is routinely required when assessing the risk of such a chemical.

- Which of the three "species" are used in the model estimation?

Data on the effects are likely to be performed with standard species used in laboratory tests. This criterion considerably restricts the selection. Therefore, at maximum the two-fly species and the two-beetle species are included. Recent developments (eg, the high sensitivity of Sepsidae) are not (yet) considered.

- Is ecological information of the individual species available?

Some of the test species are considered to be well known (especially the two-fly species). However, even for them detailed data on their "normal" population development is lacking, especially regarding their variability in space (regional?) and time (seasons?).

- Can ecological data be extrapolated from laboratory tests to field studies?

Because it's not known which factors determine the toxicity of VMP in the field, this problem can only be solved by using "safety/assessment factors".

Two examples showing how modeling could work in principle is given below (Lumaret et al. 2010). In short, they combine information on the time and frequency of treatments with different VMP and the occurrence of dung organisms, in Great Britain and Southern France, respectively.

Table 6: Tabular comparison of the application dates of certain parasiticides and the times of activity or reproduction of individual dung beetle species

Treatment type*	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Doramectin					b			c		c	e	
Eprinomectin				b			b	b		e	c	
Fenbendazole				d	a	a	a		a		a	a
Ivermectin		a	a	b	a	b	b		a	d	b	
Morantel				k								
Moxidectin									k			
Oxfendazole			e	e								
Permethrin							k					
Times of activity or reproduction of dung beetles**												
Copris lunares												
O. taurus												
O. vacca												
Aph. corpus												
Aph. constans												
G. mutator												
Tr. vernalis												

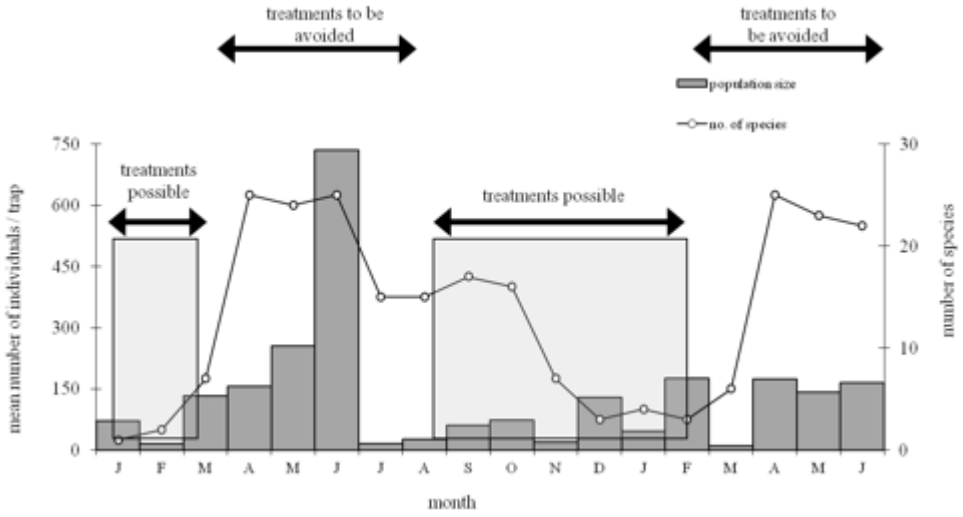
* Percentages of treated group for each type of treatment: a (1-10%); b (11-20%); c (21-30%); d (31-40%); e (41-50%); …; k (100%)

** Period of activity (light grey); period of reproduction (dark grey)

Source: Lumaret 2010

Table 6 shows very large differences for the substances listed here. Some of them are relatively rarely used (and if, only for a short time – up to one month) but when, a high percentage (up to 100%) of farm animals is treated (e.g. Morantel, Moxidectine, permethrine). On the contrary, VMP such as ivermectin could be applied at several months, usually with less than 50% of animals treated. It should also be noted that VMP could be applied almost through the whole year (only January no treatment is announced). It should also be noted that in several months (most obviously: April) several VMP could be used at the same time. Probably, not all of these treatments will be applied at the same place to the same animals on a regular basis, but at least the possibility has to be taken into account. No information is available about the robustness of these application (and thus exposure) data. This is often a problem because to our knowledge there are no central databases listing this information in a way that it could be used for risk assessment purposes. It is assumed that usually only indirect evidence could be gained by looking at data from VMP producers or shops: when and how much is sold. In the lower part of Figure 35, the times of activity and reproduction of seven dung beetle species is given. Without going into details there is a wide overlap: at times of low VMP usage, these beetles are not active or do not reproduce – and vice versa. So, it is difficult to imagine how VMP treatments and the protection of these species could be organized in terms of mutual exclusion in time.

Figure 35: Comparison of the abundance and biodiversity (=number of species) of dung beetles and the treatment periods of farm animals in Southern France



Source: Lumaret 2010

In the second example actually the same approach is visualized, comparing the treatment periods of farm animals with abundance and biodiversity (= number of species) of dung beetles over time. In this case, however, the authors went one step further and indicated, in which periods treatments are possible: between September and end of February/mid-of-March. It should be noted that, probably due to climatic factors, this period is not fix (not surprisingly). This is quite a long time – and in our view it is not really justified: in September, October and, maybe, in early November abundance of dung beetles is low but number of species is still high. Summarising the experiences made in these two studies such an approach might work, but a lot biological information on the species level is needed. Such an amount might be available for some "charismatic" dung beetles in some regions, but this is surely not true for almost all other species of the dung organism community in most regions. So, while such comparisons are an interesting approach in most cases it will not be a useful tool for the Environmental Risk Assessment of VMP since detailed data on the dung organism community are not available.

Since it was not planned to develop a model as part of this project, at least a list of criteria that should be considered in such a development are provided in the following:

- Substance-related data (which substance properties affect the outcome?): Characterizing of the VMPs, in particular the environmental behavior (eg DT values in dung and soil)
- Application rates, depending on livestock and time
- Ecotoxicological laboratory test results, especially for dung and soil organisms, including any kind of ecotoxicological effects of VMPs from the same activity class
- Location based data (i.e. which region the model is going to be used?): Region (coordinate, landscape space, land use, soils, etc.)
- Climate (such as time-differentiated temperature and precipitation sequence)
- Spatial and temporal patterns differentiated application of the VMP, also involving the application of other (comparable?) VMPs
- Biological characterization of the species, such as data on taxonomy, trophic position (food), movement or migration options, reproductive behavior and recovery options
- Knowledge on the biogeography (geographically and temporally separated) of the selected species in the region
- Information of other factors regarding the influences of VMPs, especially additional stressors of natural (interaction with other species) as well as anthropogenic (pesticides pressure from adjacent fields) origin

It should be noted that these criteria aimed primarily on dung organisms, but in principle (and based on the results of laboratory tests) also soil organisms should be involved.

8 WP I: Verification of existing risk assessment approaches (analysis of deficits) for VMP and formulation of new approaches, in particular regarding evaluation criteria and methods

8.1 Analysis of deficits of the existing ERA of VMP

The current risk assessment approach for VMP has several deficits. Most of them were already discussed at the meetings of the "informal" Aveiro-Group. This group, consisting of experts from governmental agencies, industry and universities, met several times in the years between 2009 and 2011 (it got its name since the first meeting was held at the University of Aveiro, Portugal). In the meantime, some results of these discussions have been published (Jochmann et al. 2012; Adler et al. 2013). Taking these publications into consideration, the main deficits addressed in this report are the following (please note that several of these issues were already, at least partly, discussed in Chapter 6 and 7).

The test strategy itself: Currently, testing the effects of a VMP on dung and soil organisms is described in detail only for some basic laboratory tests (Phase II Tier A). In case the results of the single species tests with dung organisms indicate that a risk cannot be excluded there is no guidance how to proceed in Phase II Tier B. Actually, there is only a note saying that "regulatory guidance should be sought". Obviously, there is an urgent need to provide a tiered test strategy for such cases in which the risk quotient is still > 1 after Tier A (including a refinement of the PEC). For soil organisms the situation is basically the same: no tests are specified for Tier B. However, for these organisms the situation is less problematic since a risk is more rarely identified than for dung organisms.

The tests used so far: In the respective VICH document (VICH 2004) dung fly and dung beetle larvae are listed as test organisms in Phase II, Tier A, which have to be performed according to standards (OECD 2008a; OECD 2010). According to new experiences it seems that not only a test with one more beetle species from the ecological group of tunnelers but also other very sensitive dung organisms such as sepsid flies are missing in VMP testing. In addition, the existing dung beetle test, focuses on an acute endpoint (larval mortality), which means that it is probably not very sensitive. Since parasiticides have usually much higher effects on arthropods than on annelids a test with a soil arthropod is missing (e. g. using collembolans).

The role of dung (and soil) biodiversity in higher-tier testing: Despite the fact that no guidance is given how to proceed with the ERA of a VMP in Phase II Tier B when a risk to dung and soil organism communities cannot be excluded it is clear that the structure and function of these communities are the main protection goals. So far, no proposal has been put forward how these goals can be addressed technically, but there are two ideas which might be useful:

- The modeling of the effects of VMP on dung and soil organism communities. Using dung organisms as an example it is laid down in Chapter 7 of this report that data availability, especially on the ecology of the potentially affected species, is not sufficient for promoting this option in the near future
- The performance of field studies, studying the structure of these communities under real conditions (e.g. like pesticides are tested in earthworm field studies (ISO 1999b))

8.2 Proposal for a new test strategy

It should be noted that this proposal has already been published (Adler et al. 2013). Therefore, only an abbreviated version is presented here. Parasiticides used in animals reared on pasture always enter Phase II Tier A because in Phase I question 16 the VICH guideline (VICH 2000) explains: "VMPs that are ecto- and/or endoparasiticides used in pasture should advance directly to Phase II to address spe-

cific areas of concern, e.g. dung fauna." Therefore, parasiticides used for food animals reared on pasture have to undergo a tailored risk assessment in Phase II regardless if they are exceeding the trigger value in Phase I (Predicted Environmental Concentration in soil ≥100 µg/kg) or not.

Available test methods with dung fauna for Phase II Tier A testing

So far, for dung organisms one standard test guideline and one test guidance document are available for testing in Phase II, Tier A (OECD 2008a; OECD 2010). Despite some performance problems, mainly because the dung beetle species *Aphodius constans* (ecologically speaking, a dweller) cannot be tested in laboratory mass cultures (Römbke et al. 2007), these tests are used frequently for regulatory purposes within the European Union. Robust results have been published for the environmental risk assessment of well-known parasiticides such as ivermectin (Liebig et al. 2010). However, results of toxicity tests using *A. constans* may not be extended to other ecological groups of dung beetles, like tunnelers or rollers. Possible test strategies for an appropriate scenario beyond Tier A in accordance to the VICH guideline are described and discussed in the following mainly for dung beetles only (Table 7), but in principle the same considerations made in the different tier approaches are applicable for dung flies, too.

Table 7: Tiered dung beetle test approach: exposure test scenarios and properties of the established dung beetle larvae test (OECD 2010) and the two newly developed sublethal tests

Scenario	Test method	Development stage	Endpoint	Description
Tier A	larvae test (OECD 2010)	Larvae	mortality: LC50 morphology: NOEC, EC50	laboratory, 21 d, spiked or excreted dung, homogenized dung is used. 20 replicates per treatment, one larva test per test vessel.
Tier B1	elongated larvae test (Römbke et al. 2012)	larvae (start) to adults	mortality: LC50 development, pupation, hatch and hatching rate: NOEC, EC50	laboratory, 10 weeks (max. 70 d), spiked or excreted dung. Start as described above. After 21 d, transfer of the treated dung on LUFA St. 2.2 standard soil in new test vessels until beetles will hatch.
Tier B2 (repellent substance)	adult reproduction test (Römbke et al. 2012)	adults (start) via eggs (F1) to larvae (F1)	mortality: LC50 development, reproduction, NOEC, EC50	laboratory, 21 d, spiked or excreted dung. Start with 20-30 adult beetles per test vessel. Contaminated dung is used on top of non-contaminated LUFA St. 2.2 soil. 2-4 replicates per treatment.

ECX – effect concentration x%; NOEC – no observed effect concentration, (d – days). Note that in case excreted dung is used as test substrate residue analysis is required

Proposals for approaches In Phase II Tier B

In principle, the following test characteristics should be considered for a Tier B dung organism study. The life stages to be investigated include: eggs, larvae, pupae, imagines (adult), and parameters with a number of endpoints:

- Mortality (number of dead/alive larvae or pupae or adults compared to the primarily inserted number of eggs or larvae or adults)
- Development (e.g. developmental time and rate of eggs or larvae or pupae, hatching success of larvae from eggs and adults from pupae, stagnation or delay in hatching and pupation, time to first hatch from eggs or pupae, malformations)
- Reproduction (e.g. number of eggs, eggs per female, percentage of fertilized eggs, sex ratio)
- Behavioral effect (in the case of a repellent mode of action of the test compound), either directly or indirectly via reproductive endpoints
- Duration of test: parental generation, more generations

In the case of dung beetles, two new methods with the established test species *A. constans* are currently under development: The Elongated Larvae Test and the Adult Reproduction Test named following Tier B1 and Tier B2. They are described more in detail in Adler et al. (2013). At this point another possibility already used in the testing strategy for plants can be mentioned (EMA 2011): In case several results from comparable endpoints studied with different dung fauna insects are available they can be used for species sensitivity distribution (SSD) calculations. In general, all data used in the SSD calculation need to meet the general quality requirements of VMP risk assessment. Usually, a minimum set of 10 different species of dung organisms has to be tested, including dung beetles and dung flies. However, for dung fauna insects only very few standardised test systems with different dung beetles and dung flies are available. Therefore, the mathematical calculation of a SSD has turned out to be not possible for the dung compartment so far.

Dung Fauna Test Methods For Test Strategies Beyond Phase II Tier B

Field Tests: Tier C

In theory, in-door or out-door semi-field methods, either with introduced organisms or with the natural community, could be used in this tier (Table 8). Such methods have been used already in tests with parasiticides and soil organisms (e.g. Boleas et al. 2005; Jensen et al. 2009; Förster et al. 2010). However, up to now it was not possible to keep and expose dung organisms in such test systems. Field studies cover all life stages of dung organisms (mainly beetles and flies) as well as the decomposition of dung pads and the degradation of the active ingredient, verified by chemical analysis of the test item. Such studies have been performed many times, mainly with ivermectin or related substances, but since they differ methodologically the comparability of results is usually low (e.g. Wardhaugh & Mahon 1991; Floate et al. 2002; Webb et al. 2007, 2010; Iwasa et al. 2008; Römbke et al. 2010b). Therefore, experts proposed basic considerations for a harmonised approach of field testing (Jochmann et al. 2010). In this publication, all issues relevant for the performance of a field study for the assessment of the effects of a parasiticide on dung organisms are listed.

Table 8: Exposure test scenarios proposed for tiered dung beetle test approach (ECx – effect concentration x%

Scenario	Test method	Development stage	Endpoint	Description
Tier C	field tests	all stages including adults, eggs, larvae and pupae in field simultaneously	dung decomposition species diversity, abundance, NOEC, EC10	field study including changes in exposure (degradation of test compound) and immigration, 4-12 weeks depending on the half-life-values of the test substance, spiked or excreted (preferred) dung.
Tier D	modelling	all stages	species diversity, abundance	First approaches are made, more knowledge of the taxonomy, biology and ecology of the dung fauna community is needed

NOEC – no observed effect concentration, d – days. Note that in case excreted dung is used as test substrate residue analysis is required

8.3 Evaluation of the number of available test species with VMP:

According to current knowledge, dung flies and dung beetles are usually the most sensitive test species for VMP in general and ivermectin in particular. Therefore, it does make sense to focus on these groups (and chronic endpoints) in Tier A.

Dung flies:

Status quo: Standard test with two species (S. stercoraria; M. autumnalis) available (OECD 2008a).

Outlook: Development of a sepsid test is recommended because of the high sensitivity of these flies (Blanckenhorn et al. 2013a, b). In addition, it has to be studied whether other fly species can be used in the standard test methods in order to cover regional differences in the fly communities in Europe. After implementation of the proposed changes the available test species are representative for the dung fly communities in Europe. However, it has to be discussed (and studied) whether the test battery could be improved by developing a test with parasitic wasps (known for their high sensitivity) (Floate & Fox 1999).

Dung beetles:

Status quo: Standard test for one species (*A. constans*) available (OECD 2010); currently a second species (*O. taurus*) is studied in an international ring test (Römbke & Scheffczyk 2010).

Outlook: After inclusion of a second species into the OECD Guidance Document, which belongs to another ecological group than *A. constans*, the test battery for dung beetles is probably representative for the European dung beetle communities. However, as in the case of dung flies, it should be checked whether then same test could be performed with different species in order to cover regional differences in the structure of beetle communities.

Soil organisms:

Status quo: Standard tests with four species are available (*Eisenia andrei* / fetida, *Enchytraeus crypticus*, *Folsomia candida*, *Hypoaspis aculeifer*) (OECD 2004a, b; OECD 2009; OECD 2008b).

Outlook: In the case of soil organisms it could already be proven that the same test method could be used for different species (e.g. for different species of the enchytraeid *genus Enchytraeus* or the collembolan *genus Folsomia*). Further collembolan species from other biogeographic regions are currently under revision whether they are suitable as test species, e.g. under Mediterranean conditions (Bandow et al. 2013). In addition, the testing of several microarthropods together in a simple laboratory microcosm seems to be possible – and in such a test the sensitivity seems to be increasing compared to single-species tests (Jensen et al. 2009). However, there are possibilities to increase the number of potential test species due to the following reasons:

- Standardization of a test with predatory beetles, most probable from the family Staphylinidae. Experience and even a draft guideline are available for the species *Aleochara bilineata* (Candolfi et al. 2000)
- Further standardization of the existing test (ISO 2010), in particular regarding an increase of the number of test species. Some nematodes are parasites of livestock, meaning that they are targets of VMP such as ivermectin. Interestingly, the few available test data for nematodes do not show high sensitivity of the nematode test species *Caenorhabditis elegans* (Grønvold et al. 2004). Therefore, further investigation on nematode sensitivity towards VMP are necessary
- Standardisation of a snail test (ISO 2006)
- Especially for Mediterranean sites it might be useful to develop a test with isopods, since at dry and warm sites these organisms can play a role such as earthworm species in temperate regions (Drobne 1997; Jänsch et al. 2005; Van Gestel 2012). However, due to the lack of information on the effects of VMP on these organisms further research is needed before considering them for the ERA of VMP (see also chapter 6.4)

Assuming that the proposals made here are implemented in existing risk assessment schemes for VMP it should be possible to cover many parts of Europe in terms of soil organisms.

8.4 Analysis of the use of dung organism communities as an endpoint in higher-tier studies

Despite the fact that the number of species in the laboratory increased considerably during the last years it is clear that the protection goal biodiversity cannot adequately be covered by such tests alone. Because of the complexity and variability (in space and time) of the structure of dung and soil organism communities semi-field or field studies are necessary (the option whether a change in assessment factors is helpful in this context is discussed in detail by Adler et al. (2013)). However, so far there are not many experiences available regarding the use of the endpoint biodiversity in higher-tier studies with VMP. The most urgent aim of further research is the definition of reference values, i.e. to define which diversity of a community is „normal“ in a certain region and time. While this is (almost) possible for soil organism communities in some parts of Europe (see chapter 2.3) the available information regarding dung organism communities is not yet sufficient (see chapter 2.2).

Obviously, there is an urgent need to learn more about the composition of dung organism communities in Europe as well as their variability in space and time, even without the influence of VMP. Research at as many as possible field sites, the usage of standardized sampling and evaluation procedures and the setting-up of a central database are the most important activities in this context.

In order to summarise the discussion in chapter 6 and the arguments laid down in this chapter (not to mention the lack of real alternatives in the area of higher-tier testing- see chapter 7) it is recommended to perform field studies when a risk for the dung and/or soil organism community is indicated based on the results of laboratory tests.

9 WP I: Assessment of existing risk management and risk mitigation measures for VMP and formulation of new approaches

9.1 Introduction and background

In parallel to the evaluation of the environmental risk assessment of VMP a systematic evaluation of the state-of-the-art of risk mitigation measures for VMP and the dung compartment was performed. This work was only possible due to the fact that in parallel to this project the ECT GmbH (in cooperation with the Fraunhofer-Gesellschaft Schmallenberg) was involved in another UBA project which focused on this issue (Liebig, M., Knacker, T., Wenzel, A. & Hahn, T. 2009): Entwicklung von wirksamen Maßnahmen zur Verringerung des Umweltrisikos von Tier- und Humanarzneimitteln. UBA-Vorhaben; FKZ-Nr. 3709 65 40; see also Liebig et al. 2014). The main results regarding VMP are summarised here. Please note that in Subchapter 9.4 an evaluation of these RMM has been added which was not part of the original UBA report (Liebig et al. 2009).

When seeking authorization for placing medicinal products on the market within the European Union (EU) applicants have to provide environmental risk assessments (ERAs) according to VICH (2000, 2004) complemented by EMA (2008) for veterinary medicinal products (VMP). If the ERA of a VMP indicates an unacceptable risk to the environment, i.e. the risk quotient (RQ) consisting of the ratio PEC (predicted environmental concentration) to PNEC (predicted no effect concentration) is equal or larger than 1, and/or the risk-benefit balance is negative, i.e. the therapeutic benefit is outweighed by risks to the environment, safety or efficacy, the authorization can be refused (Directive 2001/83/EC, with further amendments). An exemplary performance of an ERA for a VMP is provided by Liebig et al. (2010). Risk mitigation measures (RMMs) can be applied to improve the prevention and protection of the environment and, in case of risk indication within the ERA (i.e. RQ ≥ 1), after Phase II Tier B in order to reduce or manage the risk.

Applicants and competent authorities are interested in a set of recommended and appropriate RMMs from which, when considering a specific product, the adequate RMMs can be chosen. The existing guidelines for ERAs of VMP (EMA, 2008, 2012) as well as the guidelines for preparing the summary of product characteristics (SmPC; EC, 2006, 2009a) provide a rather limited number of exemplary RMMs.

The EMA guideline in support of the VICH guidelines on the environmental risk assessment lists criteria that RMMs need to fulfill.

The EMA reflection paper on risk mitigation measures (EMA 2012) provides a list of RMMs fulfilling those criteria and a list of RMM which are not considered to fulfill those criteria. For the mitigation measures aiming to protect dung fauna, not all relevant information on agricultural practice and on dung fauna was available to assess those measures. It was for example not fully clear, if not treating animals on the same pasture in successive seasons was really helpful to mitigate effects on dung fauna.

In principle, RMMs can be related to the whole life-cycle of a pharmaceutical product. The objective of this study is to propose a catalogue of RMMs which may serve applicants and competent authorities as a useful source of appropriate and efficient RMMs to be applied within marketing authorization procedures. To this end, existing and new RMMs based on modified exposure models are evaluated using criteria related to, amongst others, efficiency, practicability and compatibility with the European and/or national law.

The general search for RMMs in scientific literature revealed one paper (Montforts et al., 2004) where the authors provided specific precautions for disposal beyond those recommended by the SmPC guideline (EC, 2006). Adler et al. (2008) identified 22 product groups containing 109 different active ingredients for which RMMs should be applied to reduce the environmental concentrations to acceptable levels. According to Adler et al. (2008) the RMMs can be separated into three categories:

- Short-term measures; e.g., improved disposal and sewage treatment techniques, refusal of spreading of contaminated dung
- Mid-term measures; e.g., modified risk perception and risk communication of producers and consumers of medicinal products
- Long-term measures; e.g., decisions that foster the concept of sustainable pharmacy

However, most of the short-term and all mid-term and long-term measures are not appropriate to be applied as RMM within marketing authorization procedures. This is due to the fact that marketing authorization procedures and RMMs applied within such procedures are product specific, but the above listed measures are not.

9.2 Legal boundaries for the application of RMM for VMP

1. **The problem of the addressee:** The addressee of the authorization to market pharmaceuticals is the pharmaceutical industry (marketing authorization holder). If an unacceptable environmental risk was identified within the marketing authorization procedure (i.e. RQ ≥ 1), which could be reduced due to RMM (RQ < 1), the marketing authorization holder receives the permission with the condition of RMM. But the addressee of the RMM in practice is in most cases not the marketing authorization holder itself, but the user of the VMP. Numbering
2. **Legal consequences:** If a RMM does not have the character of a recommendation (label), contempt of the RMM should have legal consequences (penalty). Neither a control of the RMM nor a penalty is provided by Directive 2001/82/EC (EC 2001) or the AMG (2005).

Concerning VMP and their residues, the application of slurry on agricultural land is the main entrance pathway into the environment. The RMM for the use of VMP should be consistent with the relevant laws to become an acceptable part of the agricultural practice. Therefore, the relevant legislation for the management of farm-produced fertilizers, regardless of the use of VMP, are summarised in the following using the example of the German legislation.

The European Nitrate Council Directive 91/676/EEC (EC 1991) aims to protect water quality across Europe by preventing nitrates from agricultural sources polluting ground and surface waters and by promoting the use of good farming practices. The Nitrate Council Directive (EC 1991) is implemented in German law as “Düngeverordnung” (DüV 2007). Relevant regulations regarding nitrogen fertilization management are laid down in Appendix III of the Directive, for example:

- Periods when fertilization is prohibited
- Minimum storage capacity for livestock manure (at least 6 months, use only when the crop needs nutrients) according to national administrative regulations and local ordinances
- Rules to control the spread of nutrients near water or on slopes, to reduce the risk of contamination, e.g. by immediate incorporation of the applied slurry into the soil and under consideration of nitrogen (N) balance between nitrogen added to the soil (e.g. mineral fertilizer, livestock manure, etc.), and nitrogen removed from the soil in crops. The prevention of excessive levels of nutrients on farmland is a binding principle of the Good Agricultural Practices as laid down in the DüV (2007). A breach of this requirement would be considered as an administrative offence
- Limit of 170 kg nitrogen per hectare per year (in justified exceptional cases higher amounts are possible upon application)

In cases of agricultural soil use, the obligation of the German Federal Soil Protection Act (BBodSchG 1998) to protect or restore the functions of the soil on a permanent sustainable basis shall be fulfilled by good agricultural practice including site-specific management of agricultural land. Besides the introduction of environmental quality standards for VMP in surface and groundwater, reducing nitrates is an integral part of the Water Framework Directive 2000/60/EC (EC 2000). This directive confirms

that nitrate concentrations must not exceed the trigger value of 50 mg L-1, as laid down in the Drinking Water Directive 98/83/EC (EC 1998) and the Nitrate Council Directive (EC 1991).

9.3 Presentation of the main results

For a successful application/implementation of the RMM the following criteria should be fulfilled by the RMM:

- Exposure of the drug to the environment is effectively reduced (effectiveness)
- Measure has a long-lasting effect (sustainability)
- Effectiveness of the measure is verifiable (verifiability), e.g. by means of re-assessment of exposure taking the measure into account
- Measure is explicitly directed to appropriate addressee (addressing)
- Action is in accordance with Good Agricultural Practice (practicality, for VMP)
- The measure is proportionate (proportionality principle)
- Measure is consistent with the relevant law(s) (legitimacy)

In addition, the RMMs should be in accordance with the principles of GAP ("Good agricultural practice"); see also EMA (2012).

When assessing the compiled RMMs according to the above described evaluation criteria and considering legal boundaries given by German laws a list of nineteen reasonable and appropriate RMMs is proposed and shown in Table 9. This list resulted from excluding those RMMs which did not fulfill all of the a.m. criteria, or, as far as possible, RMMs were adapted in such way that the criteria were generally fulfilled. For example, the criteria "addressing" was not considered in all compiled RMMs from existing VMP. Therefore, in all RMMs proposed in Table 9 the addressee, i.e. the professional group that is in control of the specific constraint, was included. Some RMMs, whether compiled or derived from exposure models, with analogous content were combined by harmonizing and improving the wording in order to fulfill the criteria. For example, the existing precautions for disposal "should not enter surface waters" and "should not enter the environment" were combined (with others) resulting in the precaution for disposal D-01 (Table 9). Consequently, the RMMs shown in Table 9 are an aggregation of existing RMMs with derived RMMs as shown above. The list is divided into five categories which address the following precautions for VMP: disposal, use in aquaculture, use in intensively reared animals, use in pasture animals, and combined use in intensively reared and pasture animals. It should be noted that this list is considerable longer than the one given in the "Reflection paper on risk mitigation measures related to the environmental risk assessment of veterinary medical products" (EMA 2012), mainly because it is more detailed. In addition, according to EMA (2012), only the RMM D-01, U-22, U-23 are fully compliant with the criteria given in VICH-TGD (EMA 2008), while U-11, U-14 and U-24 are not. However, as discussed by Liebig et al. (2014), it is stated in the EMA document (2012) that the RMM given there have been used as examples which do not cover all potential situations, meaning that additional mitigation measures for specific cases have to be considered.

Table 9: Catalogue of proposed appropriate and effective risk mitigation measures (RMMs) for VMP which may support applicants and competent authorities

Precautions for the disposal	
D-01	The user (e.g. veterinarian or livestock owner) has to ensure that any unused product or waste materials derived from the product, such as empty containers, do not contaminate water courses, surface waters or other parts of the environment. Veterinary pharmaceutical products must not be disposed of via sewage but should be disposed of preferentially via local return systems for hazardous waste. If disposed with household waste it should be taken care that no misuse of these wastes could occur.
D-02	The user (e.g. veterinarian or livestock owner) has to ensure that any unused product or rests of the dip do not contaminate water courses, surface waters or other parts of the environment. Dips must not be disposed of via sewage but should be disposed of via local return systems for hazardous waste.
Precautions for the use in aquacultures	
U-01	Constraint to the user (fish owner): Prior to use of the product a discharge certificate is required from the relevant authority for release of this product into the aquatic environment.
U-02	Constraint to the user (fish owner): Use only if the flow rate of untreated waters allows for an x-fold dilution of the volume of treated water before discharge into surface waters. Where the appropriate dilution of treated water cannot be achieved the farm must have a discharge process to limit the release of product into the environment to within the parameters described. This can be achieved by the use of holding tanks and ponds, discharge lagoons and biofilters to clean treated water. Where this applies the user must monitor the discharge concentration to ensure the parameters are not exceeded.
Precautions for the use in intensively reared animals	
U-11	Constraint to the farmer: Before spreading slurry (manure) from treated animals, it has to be stored for at least x day/months.
U-12	Constraint to the farmer: Slurry (manure) from treated animals must not be spread on areas where run-off could occur (slope > 10%).
U-13	Constraint to the farmer: Slurry (manure) from treated animals must only be spread on arable land if x-fold diluted with slurry (manure) from untreated animals.
U-14	Constraint to the farmer: When spreading slurry (manure) from treated animals onto arable land a safety margin of x meters to the water's edge has to be maintained.
U-15	Constraint to the farmer: When spreading slurry (manure) from treated animals onto arable land the maximum nitrogen spreading limit must not exceed X kg N ha-1 yr-1.
U-16	Constraint to the farmer: Slurry (manure) from treated animals must only be spread on arable land in X portions of the maximum nitrogen spreading limit with minimum time intervals of Y days.
U-17	Constraint to the farmer: Slurry (manure) from treated animals must not be spread on soils with an organic C-content < x%.
U-18	Constraint to the farmer: After spreading of slurry (manure) from treated animals, soil must be ploughed to a depth of at least x cm (>5 cm).

Precautions for the use in pasture animals	
U-21	Constraint to the veterinarian/animal holder: Strategic treatment of stock is only allowed after the fly or dung beetle season in autumn or in early spring.
U-22	Constraint to the animal holder: Animals [animal group] from free-range husbandry must be kept indoor during treatment and X days following treatment.
U-23	Constraint to the animal holder: During treatment and X hours/days following treatment animals [animal group] must be kept away from watercourses.
U-24	Constraint to the animal holder: [Product] is toxic to dung organism (flies, beetles). Therefore, animals [animal group] must not be kept on the same pasture every season.
Precautions for the use in intensively reared and pasture animals	
U-31	Constraint to the veterinarian/animal holder: Only treat affected animals [animal group] when required. For correct diagnosis and development of an appropriate treatment schedule a veterinarian should be consulted. Faecal worm (worm egg) counts can be used as indicator as to whether treatment is needed or not.
U-32	Constraint to the user of the product: During use of the teat dip or spray dripping residues are must be collected and disposed of separately (cf. special precautions for the disposal, SmPC section 6.6).
U-33	Constraint to the farmer: Dirty water must only be spread with a maximum spreading rate of x L (< 50000) ha-1 onto arable land or pastures.

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9.4 Evaluation of the proposed RMM

In the following, the RMM listed in Table 9 are discussed in order to clarify their usability for the protection of dung (and soil) organism communities. It is NOT the aim of this report to discuss whether these RMM are useful for other protection goals. Since it is not practical to discuss all 33 RMM listed in Table 9 according to the eight evaluation criteria it will done the other way round: for each of the eight criteria it will be discussed which RMM (and, if possible, how much) fulfill the respective criterion. As a general guidance how these criteria have to be interpreted it will be asked which RMM could be suitable for the protection of dung and soil organism communities in general (including remarks on its respective practicability). Due to the overall focus of this project it is NOT possible to discuss all potential legal implications of the performance of an RMM (for a general discussion on this issue see Subchapter 9.3).

First of all, however, it has to be stated that most of the proposed RMM are on purpose NOT relevant for the protection goals this report focuses on. In details, this means:

- RMM D-01 and D-02 focus on precautions for the disposal of VMP. They are not relevant specifically for dung and soil communities since they will always act on a very local scale, probably not affecting dung organisms at all and soil organisms only for a short period of time
- RMM U-01 and U-02 focus on aquaculture. Thus, they are not relevant for dung and soil organism communities by definition

Out of the remaining RMM U-11 to U-33, being divided into three scenarios IR (= use in intensively reared animals), P (used in pasture animals) and a mixture of IR and P, several RMM address explicitly disposal issues (U-32 and U-33) or the protection of the aquatic compartment (U-12, U-14, U-23).

Are the proposed RMM in agreement with the Good Agricultural Practice?

The authors assume that this is true for all RMM listed in Table 9. It can be questioned whether these RMM are practical or not, but there is one issue which should be kept in mind: To the knowledge of the authors, nobody has investigated so far whether spreading the allowed amount of manure (measured as kg N/ha/y) could affect dung or soil organism communities. These RMM were introduced to protect the groundwater or the function of the soil as an agricultural production factor. The type of and the concentration of a specific VMP in this manure could differ quite considerably, meaning that sticking to this RMM does not mean that dung and soil organism communities are "automatically" protected.

In any case, the impact of these RMM addressing the potential exposure of dung and soil organism communities should be investigated in detail whether they are protective for these organisms. The easiest way to ensure that the protection goals are fulfilled would be to avoid spreading manure into the environment as long as there are measurable concentrations of VMP in this manure. Whether this state would be reached by simply storing the manure as long as this aim is reached (which surely depends mainly on the respective VMP and the storage conditions) or by specific measures cannot be predicted. However, we strongly recommend to base the decision whether spreading is possible not only on residue analysis but also on biological tests. Knowing the concentration of a VMP alone does not necessarily mean that effects especially on dung organisms can be excluded, since the interaction between manure properties and the VMP (i.e. its availability) do also play a role. Whether such biological tests are practical on the farm level can be doubted– but maybe testing each VMP in different manure types may give an indication on its toxicity to, in particular, dung flies. Surely, research is needed here.

By exclusion, this leaves just the following RMM to be discussed in detail: U-21, U-22, U-24.

These RMM do address (more or less clear) dung organism communities, mainly be referring to dung flies and beetles. We assume that these RMM, without saying it explicitly, also refer to soil organisms (they probably have not mentioned because they are considered to be less sensitive to VMP than dung organisms which is mostly true).

Are these RMM efficient and practical?

U-21: No, it is not efficient. As the authors have discussed in Chapter 7, there is no specific season of activity of dung organisms (such as early spring or autumn). Different species are active at different times of the year – and this pattern does also change in different regions. While the general idea of protecting dung organism communities via this RMM, there is simply not enough knowledge on the biology and ecology of dung organisms to put into practice.

U-22: Yes, it could be efficient and practical, but this depends on the issues discussed above for comparable RMM for the IR scenario (U-11 to U-18). Assuming that the necessary waiting time has been defined by appropriate (chemical and biological) tests exposure of dung and soil organism communities could be avoided.

U-24: Maybe. This depends very much on the properties of the VMP, i.e. how persistent it is in dung and soil as well as how strong the effects on organisms were after the first application. In this context, it must be reminded that even a single application of VMPs such as ivermectin can have very detrimental effects, especially on dung insects. In other words: a decrease in the application frequency of VMPs is in general helpful for the protection of dung and soil organisms. However, it depends very much on the toxicity of the specific VMP whether such a measure is sufficient or not.

Do the proposed RMM consider specific properties of a VMP (here: ivermectin)?

No, not really. Indirectly the property of persistence is addressed, for example via the duration of the storage time. However, after using ivermectin on pasture animals, an effect on dung and soil organisms cannot be excluded.

Does the RMM have different effects on individual dung organism?

To the knowledge of the authors this question has not been addressed in research, yet.

Are general measures for the protection of the biodiversity in agricultural regions helpful?

Probably, yes. In accordance with protection measures for other organisms or ecosystem compartments, there are potentially other RMM which might be helpful for dung and soil organism communities as well. Examples include:

- Set-up of management strategies specifically aimed for the protection of biodiversity in agro-ecosystems (so far, such measures are mainly aimed at birds and insects, such as carabid beetles, butterflies or, especially, bees); e.g. no applications at times of high activity such as foraging (EFSA 2013)
- Promoting keeping some parts of the agricultural landscape free of VMP usage, e.g. stripes of grassland around pastures with treated livestock

However, this would only help generalist species associated to dung such as predatory staphylinid beetles which could search for alternative food sources, not "true" dung organisms (i.e. those depending on the dung in terms of food or reproduction) such as dung flies or dung beetles;

- General promotion of keeping farm animals without chemical treatments as much as possible

It should be noted that this discussion is beyond the specific experiences of (most of) the authors of this report.

Are there proposals for further RMM, including detailed ideas on how to integrate these new RMM aiming to protect dung organism communities, in the current risk mitigation practice?

No. At this stage of our research we do not have further proposals. However, one obvious strategy in this context is to first determine if the VMP treatment is needed. For example, in Canada animals are treated with VMP when going onto pasture in the spring, even in areas were parasite burdens traditionally have been low (Floate, pers. Comm.).

In this context RMM U-31 should be mentioned, specifying the identification of "appropriate treatment schedules"; i.e. the usage of VMP should always be justified clearly.

9.5 References to chapter 1 - 9

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10 WP2: Aims of the practical part (four field studies), in connection with the information provided in WP1

As listed already in Chapter 1 this project had two main aims:

a) Collection of knowledge about the effects of ivermectin on the diversity of dung fauna, soil invertebrates and plants (including the identification of knowledge gaps)

b) Implementation of the new information into existing risk assessment and risk management schemes for ivermectin and other parasiticides. Alphabetical

Basic consideration when planning the practical work of this project was the common understanding that any risk assessment is performed in order to address two protection goals: both the function and the structure of the (dung) ecosystem have to be protected. When talking about structure in fact the biodiversity of the dung organism community is meant as defined by the 1992 United Nations Earth Summit in Rio de Janeiro: "biodiversity" is "the variability among living organisms from all sources, including, 'inter alia', terrestrial, marine, and other aquatic ecosystems, and the ecological complexes of which they are part: this includes diversity within species, between species and of ecosystems" (UNCED 1992).

Based on this common understanding about the protection goals the aims of the project could be specified as follows:

1. Does the use of ivermectin cause any long-term effect on dung fauna biodiversity?
   In particular, how long stays the “toxic” impact of the dung?
2. Is there a difference between the sensitivity of the species tested in standard laboratory tests and those species found in the four field studies? In other words, are the model species used in the laboratory representative for the communities in the field?
3. Is there evidence of recovery of dung organism populations (if possible, can internal (intrinsic) recovery be distinguished from immigration (“re-colonization”))?

In this context the ecological properties of the dung and soil organisms have to be taken into account (e.g. how quickly they can recover).

These aims will be addressed in the following five chapters, i.e., the methods and results used in the four field tests are presented in detail. The information gained at each study site is presented separately, using the format of a scientific paper. Only in the case of the soil organisms the results from all four sites are already integrated in one chapter. Due to the fact that all methods used were performed in a similar way at the four study sites, which differed considerably in their ecological conditions, detailed guidance could be given in terms of test performance and standardization. This information is compiled in the format of a Standard Operation Procedure (SOP) which has been added as Annex to this report.

After these five chapters, the methods and results used in the four field tests as well as the relevant information presented in the chapters of WP I will be compiled and discussed. When doing so, the main question is whether field studies could be a relevant extension part of the current test strategy for the ERA of VMPs (VICH 2004). This discussion is based on the proposals made by Jochmann et al. (2011) and Adler et al. (2013). Based on this bulk of information, recommendations will be presented how such a new test strategy could look like.

Finally, the following questions will be addressed:

4. Is it possible to use own results and literature data to address more general questions:

- Do any risk mitigation measures exist that can guarantee dung fauna biodiversity?
- Is there a possibility for a sustainable pasture management by using parasiticides?

Thus, by combining our knowledge on the effects of IVM on the structure and function of dung and soil communities with existing and potentially new risk mitigation measures (RMM) it will be possible to understand which role RMM can play in the future ERA of IVM and related VMPs.

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11 WP2: Non-target effects of ivermectin residues on structure and function of coprophilous communities of arthropods in a grassland: southern France

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ABSTRACT:

In the face of continuing loss of biodiversity, studies on ecosystem functioning are increasingly important for understanding the relationship between biodiversity and functions. The maintenance of pasturelands' quality is due in part to the coprophilous communities which degrade dung and contribute to ecological functions such as the nutrient recycling. Many veterinary medicinal products (VMPs), as ivermectin (IVM) found unchanged in faeces, can impact the coprophilous community and consequently the functioning. In Europe and North America, the environmental risk of VMPs is addressed in an authorization process and a standardized approach is needed for national authorities, industry, and consultants to complete the requirement for higher tier studies (particularly field studies) with VMPs. The present study examines the IVM level of residues in dung after the administration to animals, its degradation rate over time in natural conditions and the response of the coprophilous community to the presence of IVM in dung. IVM was still excreted in faeces 28 days after administration. Its degradation in the field was quick in the first three months and then slowed down, but IVM was still detectable 13 months after deposition of pats. Although dipterans were particularly sensitive to IVM, other groups like dung beetles, staphylinids and parasitic wasps were also affected. Despite an impact on the structure of the coprophilous community, degradation of dung was not significantly affected. This work revealed the difficulty to address a functional loss, as adults (less sensible to IVM) contribute to the functioning despite the higher mortality of their larvae.
Key words: dung, beetles, flies, ecotoxicology, biodiversity

11.1 Introduction

In the European Union and North America, the environmental risk of veterinary medical products (VMP), and in particular, parasiticides is addressed in an authorization process. This process is based on guidelines published by the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH), which is a trilateral program to harmonize technical requirements for these drugs in Europe, Japan, and the United States. The Environmental Risk Assessment (ERA) allows a tiered approach. In Phase I (VICH, 2000), general aspects regarding use and exposure are handled. In Phase II, ecotoxicological test requirements are specified (VICH, 2004). An ERA of VMP for dung fauna is required if the substance acts as a parasiticide for the treatment of pasture animals. In Tier A of Phase II, studies are done to assess the non-target effects (if any) of fecal-excreted parasiticides on dung beetles and flies. If a risk is identified, additional studies are required (Tier B) to characterize the nature and extent of the non-target effects using representative non-target organisms as bioassays. However, further information on Tier B studies (and beyond) for dung organisms are missing in the guidelines. In fact, the only advice given on how to proceed beyond Tier A is a statement in the (VICH, 2004) guideline: "Regulatory guidance should be sought on appropriate studies." In the "Revised Guideline on Environmental Impact Assessment for Veterinary Medicinal Products" (EMA 2008) no further information on higher tier-testing of dung organisms is given.

Numerous studies have assessed the effects of VMPs on dung organisms, both in the laboratory and in the field within the last 25 years (e.g. Lumaret et al. 2012). However, these studies have been performed using different methods, on different insects, and with different VMPs species. A standardized approach is lacking, but is needed for use by the European Medicines Agency (EMA) as well as national authorities, industry, and consultants to complete the VICH requirement for higher tier studies (particularly those in the field) with VMPs.

In order to address this problem, the German Federal Environmental Agency (UBA) sponsored a project which had, among others, the aim to perform field studies with a model VMP (i.e. ivermectin) in different ecological regions in Europe and North America, using the structure and function of dung and soil organisms as assessment endpoints. The practical work was based on the recommendations compiled by Jochmann et al. (2011) and was performed in the Mediterranean (South France), Continental (Central Switzerland), Atlantic (The Netherlands) region of Europe and in the Prairie (Western Canada) region of North America. In each of these four studies the same questions were addressed:

1. Does the use of ivermectin cause any effect on dung fauna biodiversity?
2. Does ivermectin affect the degradation of the dung?

Besides answering these questions practical recommendations concerning the practicability and the informational value of the recommendations of Jochmann et al. (2011) will be given.

In this contribution, the test performed at a grassland site near Montpellier (France) is described, which used methods generally comparable to those used for the tests performed in Wageningen (The Netherlands; Lahr et al., this report), Zurich (Switzerland, Blanckenhorn et al., this report) and Lethbridge (Canada) (Floate et al., this report).

11.2 Specific situation

Face to the statement of biodiversity loss, a particular attention increased on the role played by the biodiversity in the ecosystems functioning and on the consequences of species loss on this functioning (Costanza et al., 1997; Chapin et al., 2000). Several studies showed that biodiversity was crucial as it increases the resilience of the systems (Kinzig et al., 2001; Hooper et al., 2005; Balvanera et al., 2006; Cardinale et al., 2006). The ecosystem functioning is determined by the relative species abundance, their functional traits at specific and community levels and by inter and intra-specific interactions

(Walker, 1992; Chapin et al., 1997; Loreau et al., 2001; Naeem & Wright, 2003; Qin et al., 2003; Wohl et al., 2004; Kirwan et al., 2009; Gallardo et al., 2011; Philpott et al., 2012). The positive Biodiversity-Ecosystem Functioning (BEF) (Naeem & Wright, 2003; Gravel et al., 2011) relationship can be attributed to mechanisms like complementarity effects, in which case more species use up available resources more completely (Fridley, 2001; Loreau et al., 2001). Furthermore, it has long been suggested that communities which are more diverse in species or functional groups have greater stability against environmental perturbations based on the diversity-stability hypothesis (Johnson et al., 1996; Hooper et al., 2005). Under changing conditions, the redundancy of functionally similar species or the compensation by better-adapted species may buffer ecosystem processes (Hooper et al., 2005). The attention toward the role of biodiversity was crystalized by the MEA (Millennium Ecosystem Assessment) (MEA, 2005) and then the TEEB (The Economics of Ecosystems and Biodiversity) (Sukhdev & Kumar, 2008). The notion of ecosystem services was created to focus on the role of the biodiversity on functions benefiting to humans. One of the aspects of this notion is the necessity to have a better understanding of the BEF relationships. Indeed, in conservation biology it appears crucial to understand the role of each player in a community to assess the resilience of ecological functions. The dung community seems well suited to study interspecific competition and coexistence of species because such microhabitat is well delimited and constitutes a micro-ecosystem (Mohr, 1943). In grazed pastures, coprophilous communities play an important role in maintaining pasture fertility and productivity by removing the herbivore dung (Stevenson & Dindal, 1987; Lumaret & Kadiri, 1995). At this moment, few studies concern BEF relationships in such communities (Slade et al., 2011; Beynon et al., 2012; Braga et al., 2013).

One angle to study this BEF relationships is to analyse the impact of a disturbance on both the community structure and the ecosystem functioning. Face to the increasing number of disturbances that diversity has to cope with, it is important to assess the impact on the ecological functions carried out by this diversity. Species show different sensitivities to disturbance. It may result changes in species abundance which depend on the degree of disturbance and the level of resilience of the different species. During the 1980s, the field of veterinary medical products (VMPs) was revolutionized by the introduction of endectocides with a strong activity against both ectoparasites and endoparasites (Campbell, 1989; Kornis, 1995). Such compounds were extensively and increasingly used in veterinary medicine and agriculture. Many VMPs as ivermectin (IVM) are found unchanged in faeces and thus can impact the coprophilous community (Sommer et al., 1992; Andrew & Halley, 1996; Alvinerie et al., 1999; Lumaret et al., 2012). IVM is routinely used as a reference substance in ecotoxicological standard tests performed by veterinary pharmaceutical firms to get marketing authorizations (Römbke et al., 2007; Römbke et al., 2010a; Blanckenhorn et al., 2013a). The pharmacokinetic excretion profile of IVM in cattle dung is well-known (Sommer et al., 1992; Sommer & Steffansen, 1993; Canga et al., 2009; Fernandez et al., 2009; Krogh et al., 2009; Celestina et al., 2010; Liebig et al., 2010; Forster et al., 2011; Iglesias et al., 2011) and many works (mostly laboratory experiments) showed the high toxicity of this compound to many invertebrates (mainly Diptera and Coleoptera) (Kruger & Scholtz, 1995; Kruger & Scholtz, 1997; Errouissi et al., 2001; Taylor, 2001; Hempel et al., 2006; Lumaret et al., 2007; Römbke et al., 2010c; Forster et al., 2011; Gonzalez-Canga, 2012; Lumaret et al., 2012; Blanckenhorn et al., 2013b). Dung beetles and fly larvae (especially sepsids, sphaerocerids and most Cyclorrhapha) appear to be highly sensitive to IVM residues in faeces (Madsen et al., 1990; Cook, 1991; Schaper & Liebisch, 1991; Wardhaugh et al., 1996; Boxall et al., 2002; Boxall et al., 2004). Most works concerned a single species at the same time and not the whole community and its functioning in the field (Römbke et al., 2010b).

In case a risk is identified in lower tier tests (i.e. non-target organisms are affected at field relevant concentrations) of the environmental risk assessment required according to the VICH guidelines (VICH 2004), higher tier tests (mesocosm or field studies) have to be performed. The aim of this study is: 1) to develop and evaluate a robust methodology for estimating the risk due to VMPs in the context of a

higher tier test; 2) to assess IVM effects on both community structure and dung degradation; 3) to correlate structural data from diverse treatments with the level of ecological function carried out; 4) to have a better understanding of the BEF relationships within coprophilous communities.

11.3 Material and methods

11.3.1 Site

Experiments were conducted in spring and summer 2011 (from May till September) in Saint-Martin de Londres (SML), a subhumid Mediterranean climate site, 35 km north of Montpellier (43°48'39N, 3°44'35E, elevation 250 m, Hérault, France). It was regularly grazed in spring by about 150 Aubrac heifers which provided abundant and predictable trophic resources for dung invertebrates which constituted complex assemblages (Lumaret et al., 1992; Errouissi, 2003). This site corresponded to an herbaceous garrigue dominated by Brachypodium retusum, Quercus ilex and Thymus vulgaris. The substratum consisted of dry and fissured clay soil on hard limestone. The annual average temperature was 13.9 [illegible] (Errouissi & Lumaret, 2010).

11.3.2 Cattle

28 one-year-old Aubrac heifers, 361± 36 kg on average (live weight), were used in this trial for an IVM administration and dung collect. Animals were kept in field conditions during the whole experimental period in a farm in SML. No additional feed was supplied except hay and fresh water. The animals were not pregnant and were not treated with any pharmaceuticals for at least 100 days before faeces were collected.

11.3.3 Administration of IVM

IVM was pour-on administered to cattle. According to the manufacturer's recommendations, animals received 500 µg.kg−1 body weight doses (1mL by 10 kg body weight of Ivomec® bovine pour-on, Merial, France).

11.3.4 Faecal sampling and set up

Control dung (about 100 kg) was collected prior to administration. Faecal samples were collected at 3, 7, 14 and 28 days after IVM pour-on application (D+3 until D+28). The dung collected from all animals was mixed thoroughly for each treatment, packaged in plastic bags, labelled and frozen at –20 °C and defrosted just before use. Seven subsamples of approximately 50 g each were taken from each treatment for further analyses (water content, mineral content and IVM concentration). Initial water content ranged from 81 to 85% (82.5% on average) (oven-drying for 24 h at 100°C). Ash content varied from 11 to 15% of dry weight (12.4% on average) (muffle furnace; 500°C for 12 h). In total, five different treatments were obtained: control, D+3, D+7, D+14, D+28. For all treatments, IVM residues were analyzed in initial dung post-administration and at different dates (1, 2, 6, 9 and 13 months) after the dung pats were deposited in the field.

11.3.5 Analytical procedure for the determination of the antiparasitic agent ivermectin in cattle dung

Reagents and equipment

Acetonitrile of HPLC-gradient grade (>99.9%) was supplied by VWR international (Radnor, Pennsylvania, USA). High purity water was prepared by a Milli-Q water purification system (Millipore, Milford, MA, USA). N-methylimidazole (99% purity), triethylamine (99% purity), trifluoracetic anhydride (99% purity) and trifluoracetic acid (99% purity) were supplied by Sigma-Aldrich (Steinheim, Germany). The standard substances ivermectin (CAS RN: 70288-86-7, 96% purity) and doramectin (CAS RN: 117704-25-3, 90% purity) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). For extraction, a Vortex Genius 3 shaker (IKA, Staufen, Germany), a rotary shaker Swip KS-10, (Bühler, Tü-

bingen, Germany), and an ultrasonic bath Sonorex Super RK255H (Bandelin electronic, Berlin, Germany) were used. As a centrifuge a Rotanta 460 R (Hettich, Tuttlingen, Germany) was used. Syringe filters (PTFE, 0.45 µm, 13 mm) were supplied by Wicom GmbH (Heppenheim, Germany). Solid phase extraction cartridges (Strata C-18-E, 500 mg, 55 µm, 70 Å) were purchased from Phenomenex (Torrance, California, USA).

Standard solutions

All standard solutions of doramectin and ivermectin were prepared in acetonitrile and stored at -18 °C. Stock solutions were made by dissolving 2.5 µg ivermectin or doramectin in 25 mL acetonitrile. These solutions were used to prepare ivermectin working standard solutions of 2000 and 100 µg/L, as well as doramectin working standard solutions of 2000 and 200 µg/L. With these solutions 9 calibration standards covering the relevant concentrations were prepared on daily basis.

Extraction and clean-up of the dung samples

The extraction procedure was mainly based on an adapted and optimized method as described by Litskas et al. (2010). Dung samples were homogenised. After determining the water content of the different sample series, a total dry matter of about 0.6 g for cattle dung was weight into polypropylene-vials. Cattle dung stored in the field were then moistened up to a water content of about 85%. The remoistened samples were kept at room temperature for 24 h. The initial dung samples already had a water content of about 82%.

Internal standard doramectin dissolved in 25 mL acetonitrile was added in an amount near that expected in the sample. The suspension was kept for 15 min in an ultrasonic waterbath, 30 min on a mechanical shaker at room temperature at 450 rpm and again for 15 min in the ultrasonic water bath. Subsequently, the sample was centrifuged for 30 min at 2000 x g and 22 °C. For the cattle dung samples stored in the field 10 mL of each supernatant were directly transferred to polypropylene-vials. For the fresh dung samples the extracts were cleaned up with an additional solid phase extraction (SPE). For this, 20 mL of the solution were diluted with 66.6 mL water and 66.6 µL triethylamine. The SPE cartridges were conditioned with 10 mL acetonitrile and 10 mL acetonitrile/water (3:7, v/v). Subsequently, the samples were extracted with a C18-SPE-cartridge (500 mg, 55 µm, 70 Å) at a flow rate of 3 mL min-1. The extraction was followed by a washing step with 12 mL acetonitrile/water (1:1, v/v) at a flow rate of 8 mL min-1. With 5 mL of acetonitrile the analyte was eluted under gravity into a polypropylene-vial.

The solvent was evaporated under a gently stream of nitrogen at 55 °C to complete dryness. For reconstitution 1000 µL acetonitrile were added to the sample. It was vortexed for 2 min, kept in an ultrasonic bath for 10 min, kept for 30 min on a mechanical shaker at 450 rpm, vortexed again for 30 s, and put again in the ultrasonic bath for 5 min. Finally, it was again kept for 30 min on a mechanical shaker at 450 rpm. After filtration (0.45 µm, PTFE) 700 µL of the solution were transferred into a HPLC-vial for the derivatization step.

Derivatization with trifluoracetic anhydride

The sample was derivatized according to an adapted procedure developed by Berendsen et al. (2007). First, 100 µL of N-methylimidazole/acetonitrile (1:1, v/v) were added to 700 µL of the reconstituted and filtered sample, followed by 50 µL of triethylamine. Subsequently, 100 µL of trifluoracetic anhydride/acetonitrile (1:1, v/v) were added. Finally, 50 µL of trifluoracetic acid were given into the vial. After each addition of reagent the closed HPLC vial was shaken for at least 5 seconds. To finish the derivatization reaction the closed HPLC-vials were kept for 30 minutes at 60°C in an oven.

High performance liquid chromatography with fluorescence detection (HPLC-FLD)

The determination with the HPLC-FLD was carried out within the first 48 hours after the derivatization. Chromatographic separation and determination was performed on an Agilent 1200 HPLC system

(Agilent, Santa Clara, California, USA) consisting of a degasser (G1322A), a quaternary pump (G1311A), an autosampler and injection unit (G1329A), a column thermostat (G1316A) and a fluorescence detector (G1321A). The gradient elution was performed using a mobile phase of water (A) and acetonitrile (B) at a flow rate of 0.3 mL min−1 with the following gradient: 0–47 min, 60-100% B; 47–52 min, 100% B; 52–53 min, 100-60% B; 53-60 min, 60% B. The injection volume was 20 µL and the analytes were separated on a 150 mm × 2.1 mm i.d. 3 µm particle size, Dionex (Sunnyvale, California, USA) Acclaim PolarAdvantadge II C18-Column. The column temperature was 30 °C. The fluorescence detection was carried out at an excitation wavelength of 364 nm and an emission wavelength of 463 nm.

Figures of merit

The limit of detection (LOD) and limit of quantification (LOQ) values were determined with the calibration method on the basis of DIN 32645(2008). The LOD for dung samples from medicated cattle was 5.1 µg / kgdw and the LOQ 12.4 µg / kgdw. All data of the extractions with an inadequate recovery of the internal standard (<80% and >120%) were assorted. For the remaining samples the mean recovery of the internal standard doramectin was 99.1% (RSD 10.2%) for the dung samples stored in the field and 101.3% (RSD 8.9%) for the fresh or initial dung samples with the additional SPE-clean-up.

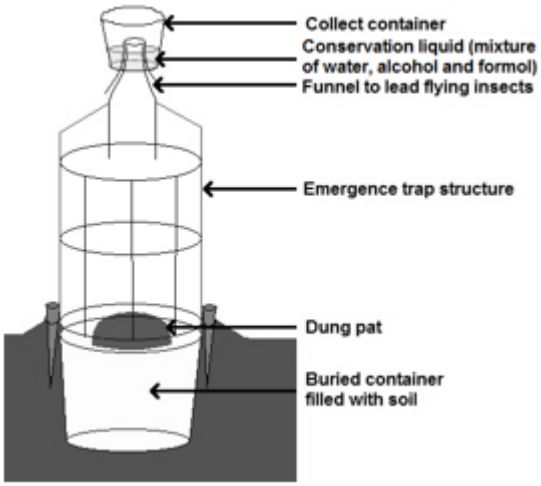
11.3.6 Field experiment

Defrosted dung samples from different treatments were deposited in the field to assess separately the IVM effect to diversity (emergence traps) and functioning (pats degradation).

Structure

Containers (capacity 7L, 25 cm high, Ø 15 cm) were buried to their rim in the soil and filled with soil. Dung pats (about 800 g defrosted dung) were deposited at the surface of each container organized in line at least 2 m apart along the fence of the grazed area to avoid cattle disturbance. Dung pats were left free to be colonized by insects for one, two and three weeks, then emergence traps were set up to collect insects as they emerge (Figure 36). Ten replicates per treatment were done for the one-week colonization period, and five replicates for the other cases. Weekly collections were made in 2011 for about 3 months, between late spring and summer. At the end of this period, containers were removed and the soil was sieved in the laboratory to collect coprophilous invertebrates which remained in the soil beneath pats.

Figure 36: Emergence trap to collect insects from dung pats



All invertebrates were stored in alcohol (95%) before determination at Paul Valery University in Montpellier. Dung beetles were identified at species level (Dellacasa, 1988; Baraud, 1992; Dellacasa,

1995)). Staphylinids were identified at the genus level by Tronquet (Tronquet, 2001) and separated in two size classes (small and large). Flies were identified at family level by Blanckenhorn's team (Switzerland). Wasps were identified at the family level by Delvare.

Function

Dung pats sets corresponding to each treatment were deposited in line at least 2 m apart along the fence of the pasture to avoid cattle disturbance, following a replicated sequence of 5 treatments (control and 4 dates post-administration). To facilitate their identification, pats were labelled and deposited on mesh nettings (2*2 cm). At each sampling date after deposit (1, 2, 6, 9 and 13 months), pats were collected into plastic bags, ground with a blender, weighed and sub-samples were oven-dried for at least 48h at 100°C. Approximately 50 g were heated in a muffle furnace at 500°C for 12 h to determine the ash content. Calculation gave the percentage of organic matter per pat, depending on the treatment and time after deposit. Ash content was used as a proxy of invertebrate activity: the more the burying activity was important, the more the quantity of soil incorporated into the pat was high, resulting in higher ash content. Five replicates per treatment and for each sampling date were used.

11.3.7 Statistical analyses

Residues: to assess the IVM loss in dung pats over time depending on initial treatments (IVM concentrations in fresh dung after administration to cattle), a two-ways Anova was processed on IVM concentration as a function of sampling day and months post deposit.

Diversity: to assess the IVM effect on diversity, Kruskal-Wallis analyses and exact Mann-Whitney pairwise tests were carried out on the number of individuals and families collected according to different treatments. For the Sepsidae family, a t-test was performed to test whether the mean number of individuals in control pats was significantly different from treated ones.

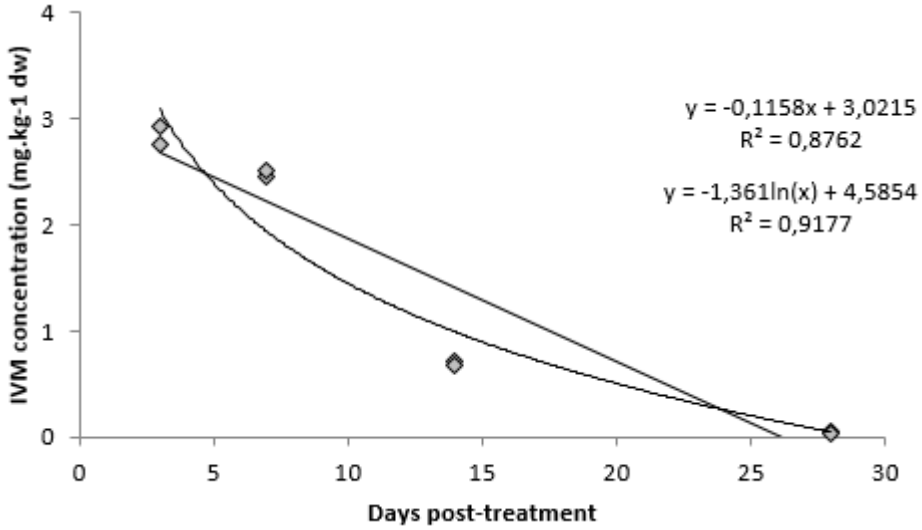
Functioning: assessing the ecosystem functioning consisted in the measure of the remaining weight of dung pats over time and treatments. Ancovas were performed using time as a covariable and treatment as a fixed factor. Kruskal-Wallis analyses were carried out on dry weight of pats and their organic and mineral contents among treatments at each sampling month, completed by exact Mann-Whitney pairwise comparisons.

11.4 Results

11.4.1 IVM residues

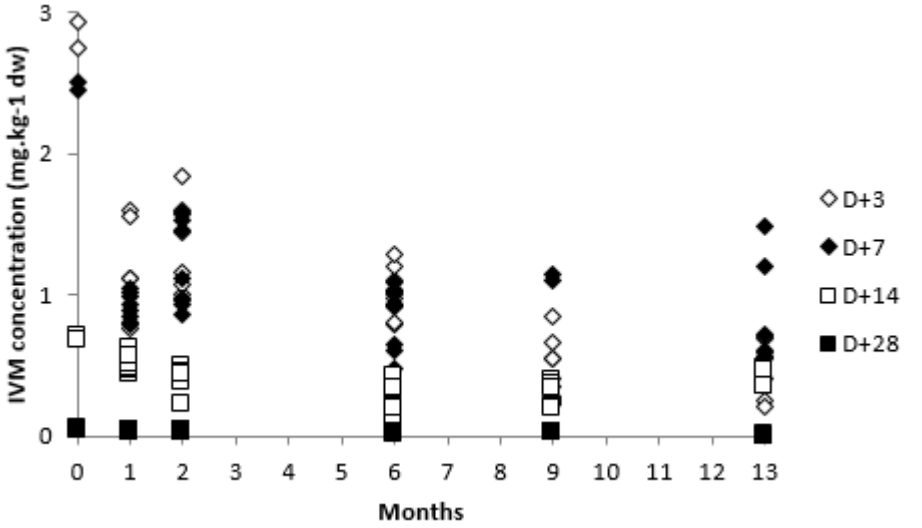
The highest initial ivermectin concentration in dung collected from animals was obtained 3 days post-administration (DPA) (2.845 mg.kg-1 dw), close to the concentration value at 7 DPA (2.480 mg.kg-1 dw). The 14 DPA concentration value (0.692 mg.kg-1 dw) was 4 times lower than the peak of elimination. After 28 days, IVM was still detectable (0.049 mg.kg-1 dw). The excretion profile of IVM over time corresponded rather to a Log regression (r2 = 0.918) than a linear regression (r2 = 0.876) (Figure 37).

Figure 37: Ivermectin excretion profile over time



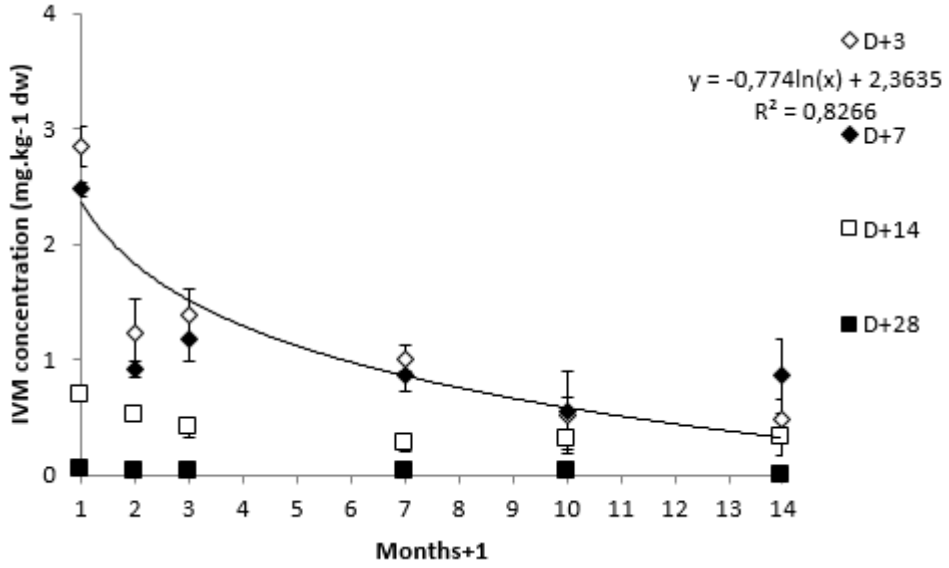
For each treatment, two measures of IVM concentration in the dung were obtained from chemical analysis. The IVM concentration over time is linear (R2=0.8762) or logarithmic (R2=0.9177).

Figure 38: Ivermectin degradation in faeces over time (individual values)



Months in the field post-deposit are on the abscissa and IVM concentration on the ordinate. Dung samples were collected for each treatment and at different times post-deposit and chemically analyzed to obtain IVM concentration.

Figure 39: Ivermectin degradation in faeces over time (mean values)



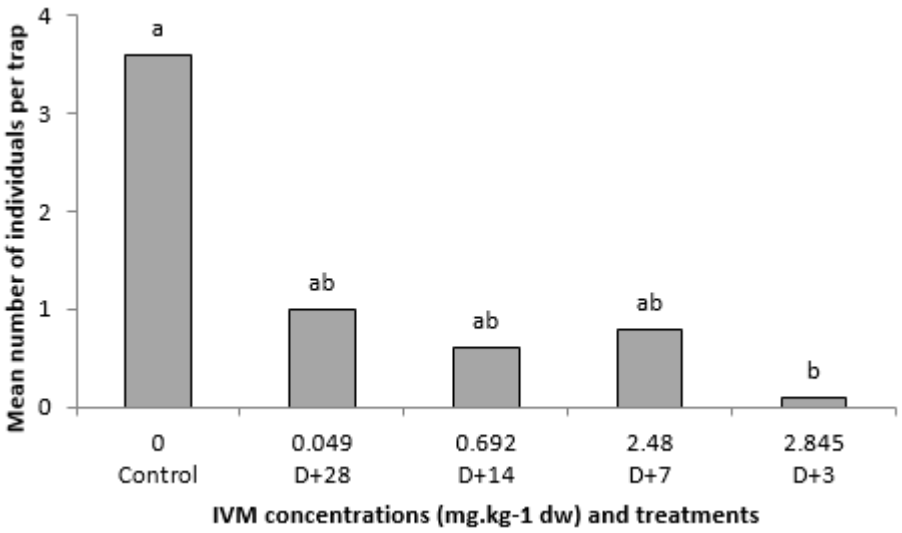
Months in the field post-deposit are on the abscissa and IVM concentration on the ordinate. Dung samples were collected for each treatment and at different times post-deposit and chemically analyzed to obtain IVM concentration. Means for each treatment and date post-deposit were calculated and reported in this graph. A logarithmic regression was realized for the D+3 IVM concentrations over time.

The degradation of IVM in dung pats deposited in the field followed a Logarithmic curve for all the treatments with an asymptote in the decay (Figure 37Figure 39). A quick decrease of ivermectin concentration in dung was observed in the first months, with a DT50 for the two highest initial concentrations (3 and 7 DPA) obtained after 2-3 months post deposit, followed by a slower decrease. For all treatments except the 28 DPA one, IVM was still detectable in dung pats after 13 months. Using time post-administration (days) and time of deposit in the field (months) as continuous covariates, a two-ways Anova showed that time was significant. IVM concentration decreased with increasing days post-administration. Time of deposits (months) was also significant. The interaction between the initial IVM concentrations and the time of degradation of IVM was significant, meaning non-parallel decay lines.

11.4.2 Structure: Effects on dung beetles

After a one-week colonization of dung pats, 52 new emerged dung beetles in total were collected, belonging to the Aphodiidae [(*Aphodius fimetarius* (L.), *Otophorus haemorrhoidalis* (L.), *Emadus quadriguttatus* (Herbst))] and Scarabaeidae (*Caccobius schreberi* (L.), *Onthophagus vacca* (L.)). Although the difference between all treatments was not significant (H=5.002, p=0.287), emergence was significantly lower in D+3 compared to the control (p=0.033) (Figure 40). More beetles have emerged in the control than in other treatments.

Figure 40: Mean number of Aphodiidae and Scarabaeidae collected per trap and extracted from soil under pats, according to IVM initial concentration in dung for a one-week colonisation

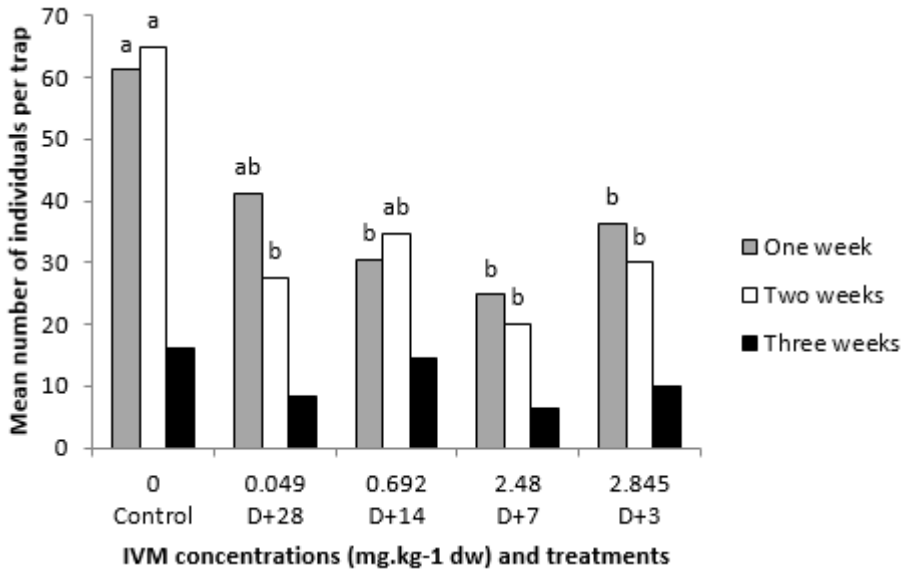


The IVM concentration is expressed in mg.kg-1 dw. Mann-Whitney pairwise comparisons were processed between treatments; letters indicate significant differences.

11.4.3 Structure: Effects on Diptera

Dipterans were collected from dung pats from treated cattle (pour-on) freely colonized during 1, 2 and 3 weeks. After a one-week colonisation, 3682 dipterans were collected from the ten replicates of all treatments (50 pats in total), belonging to 6 Nematocera (1741 individuals) and 12 Brachycera families (1941 individuals). A significant effect of treatments was observed on brachyceran emergence ($H=11.11$, $p=0.025$). More individuals emerged significantly in control than from pats collected 3, 7 and 14 days post-administration (D+3 exact $p=0.035$; D+7 exact $p=0.003$; D+14 exact $p=0.009$) (Figure 41).

Figure 41: Mean number of brachycerans collected per trap, according to IVM initial concentration in dung and the time of colonisation (1, 2 and 3 weeks post-deposit)



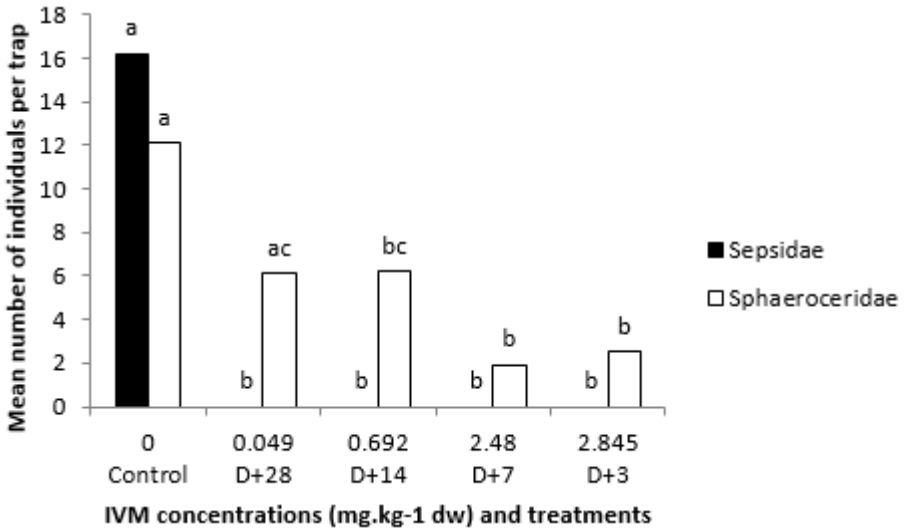
The IVM concentration is expressed in mg.kg-1 dw. Mann-Whitney pairwise comparisons were processed between treatments for each time of colonisation; letters indicate significant differences.

For the two-week colonisation, 1549 dipterans were collected from 5 replicates of all treatments (25 pats in total; 886 brachycerans and 663 nematocerans). The number of brachycerans which emerged was higher in control compared with all treatments, except for D+14 (D+3 exact p=0.024; D+7 exact p=0.008; D+28 exact p=0.024) (Figure 41). The difference was significant (H=9.757, p=0.045).

After a three-week consecutive colonisation, fewer dipterans (382 in total) which emerged from 5 replicates of all treatments were collected (25 pats in total; 274 brachycerans and 108 nematocerans). Difference between control and treated was not significant, even if more individuals emerged from control (Figure 42).

Going further in the identification of one-week dipteran, sepsids emerged exclusively from control pats even from pats collected four weeks after the pour-on administration (t=4.379 p=0.002) (Figure 43). Sphaerocerids were also highly affected by IVM (H=18.47, p=0.001), with a significant difference in number of individuals which emerged from control dung compared with dung collected at D+3, D+7 and D+14 (2-3 times more in control) (D+3 exact p<0.001; D+7 exact p<0.001; D+14 exact p=0.022) (Figure 44).

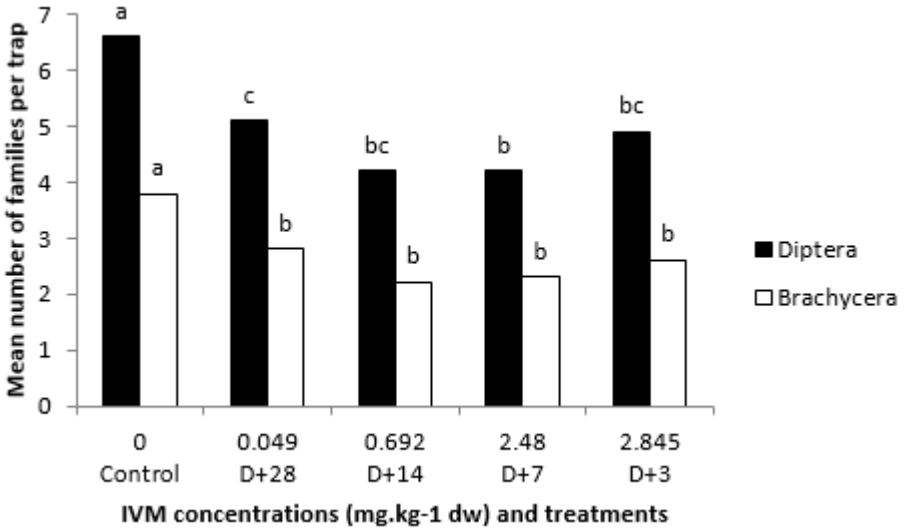
Figure 42: Mean number of sepsids and sphaerocerids collected per trap, according to IVM initial concentration in dung for a one-week colonisation



The IVM concentration is expressed in mg.kg-1 dw. Mann-Whitney pairwise comparisons were processed separately for each family between treatments; letters indicate significant differences.

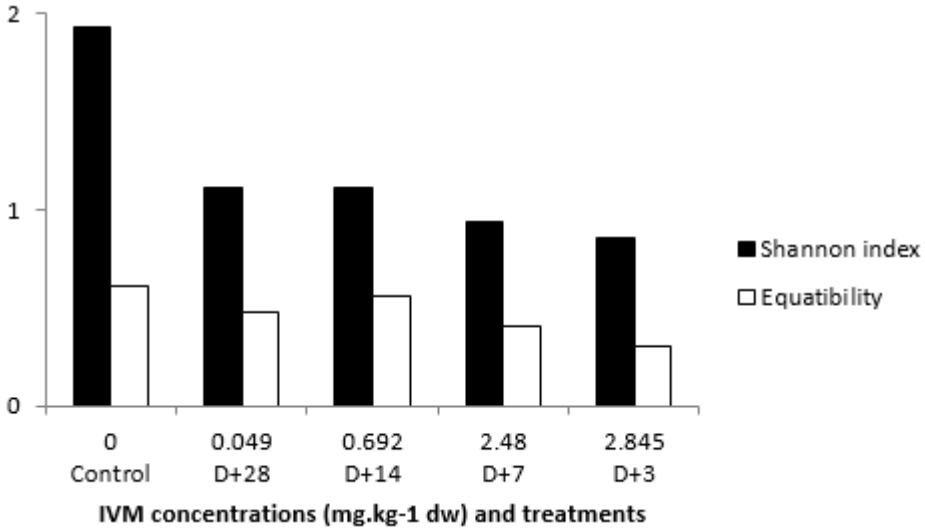
Significant differences were observed in total number of dipteran families depending on time (control and treatments post-administration) (H=13.83, p=0.008). The number of nematoceran families did not change significantly with treatment, contrary to brachycerans whose the number of families was significantly higher in control compared to different times post-administration (D+3 exact p=0.010; D+7 exact p<0.001; D+14 exact p<0.001; D+28 exact p=0.010) (Figure 43). The Shannon-Weaver index on brachyceran diversity reflects the strong impact of IVM with a higher index value for control compared to values obtained for D+3 and D+7 (Figure 44).

Figure 43: Mean number of dipteran (brachycera + nematocera) and brachyceran families collected per trap, according to IVM initial concentration in dung for a one-week colonisation



Only families with more than one individual were considered. Mann-Whitney pairwise comparisons were processed between treatments separately for dipteran and brachyceran; letters indicate significant differences.

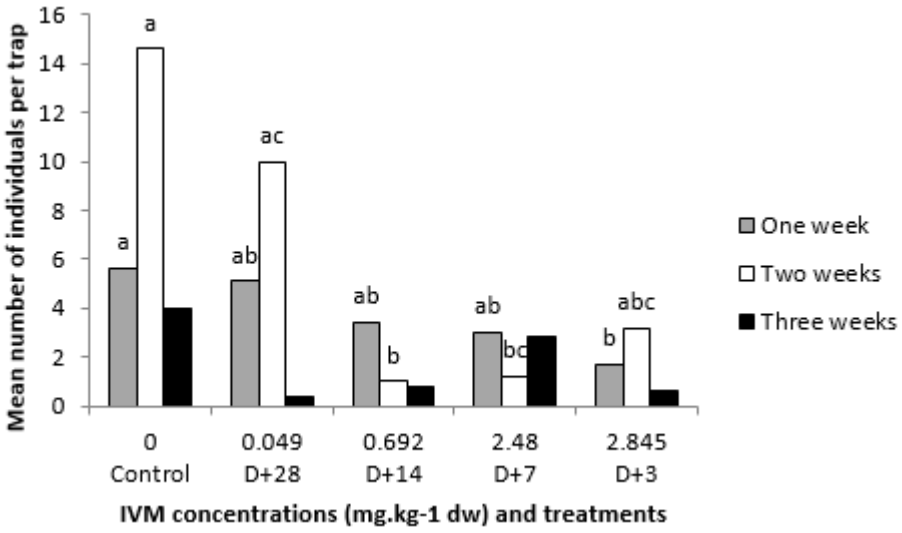
Figure 44: Shannon index and equitability (evenness) for brachyceran according to IVM initial concentration in dung for a one-week colonisation



11.4.4 11.4.4 Structure: Effects on Staphylinidae

The rove beetles' diversity was measured exhaustively for two replicates (10 pats). Staphylinids were roughly separated in two groups, small and large species. Small species are mostly parasites of dipteran pupae while large ones are opportunistic predators. After a one-week colonisation, the large rove beetles were represented by a very small number of individuals. Seven genera were collected in total, four of them (5 species) being more likely associated with dung (Ontholestes, Oxypoda, Philonthus and Xantholinus genera). As regards the small individuals, 14 species belonging to 9 genera were collected in total. Eleven species belonging to six genera (Acrotona, Anotylus, Atheta, Monotoma, Tinotus and Trichiusa) were generally considered as part of coprophilous community.

Figure 45: Mean number of staphylinids collected per trap, according to IVM initial concentration in dung and the time of colonisation (1, 2 and 3 weeks post-deposit)



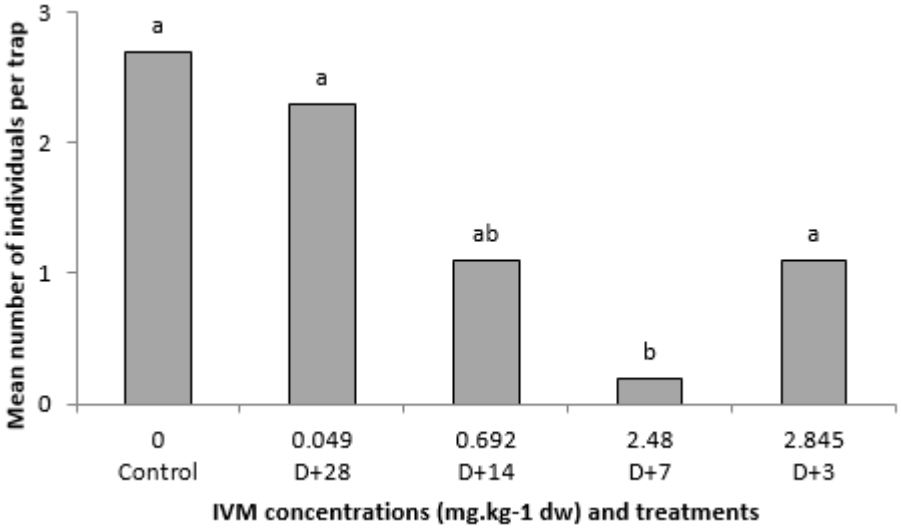
The IVM concentration is expressed in mg.kg-1 dw. Mann-Whitney pairwise comparisons were processed between treatments for each time of colonisation; letters indicate significant differences.

After a one-week colonisation, 188 new emerged small rove beetles in total were collected from the ten replicates of the five treatments (control and four dates post-administration; 50 pats in total). There was no significant difference among treatments in spite of a tendency to obtain more staphylinids in control. The higher was the IVM concentration, the less was the number of rove beetles; the difference between D+3 and control was significant (D+3 exact p=0.040) (Figure 45). After a two-week colonisation period, 150 newly emerged rove beetles were collected from pats (5 replicates; 25 pats in total). The difference between treatments was significant (H=13.76, p=0.008). Individuals emerged in a significant higher number from control than from 7-14 treated dung (D+7 exact p=0.008; D+14 exact p=0.008; D+28/D+14 exact p=0.040) (Figure 45). After a three-week colonisation, emergences were reduced, with 43 rove beetles in total (5 replicates; 25 pats in total). No significant difference was observed between treatments even if the numbers of rove beetles in control were higher than in D+3, D+14 and D+28 pats (Figure 45).

11.4.5 Structure: Effects on Hymenoptera (parasitoid wasps)

The hymenopteran diversity was measured exhaustively for two replicates (10 pats). After a one-week colonisation, individuals belonging to 18 families were identified. Species of the Pteromalidae family were of particular importance with many parasitoids wasps of flies. This family was represented by two genera, Pachycrepoideus (subfamily Pteromalinae) and Spalangia (subfamily Spalangiinae). Attention was focused on the Spalangia genus. From the ten replicates (5 treatments; 50 pats in total), 74 Spalangia individuals were collected. Difference between treatments was significant (H=10.73, p=0.030). Fewer wasps emerged significantly in D+7 than in control (D+7 exact p=0.004; D+7/D+3 exact p=0.032; D+7/D+28 exact p=0.016) (Figure 46).

Figure 46: Mean number of Spalangia wasps collected per trap, according to IVM initial concentration in dung for a one-week colonisation



Mann-Whitney pairwise comparisons were processed between treatments; letters indicate significant differences.

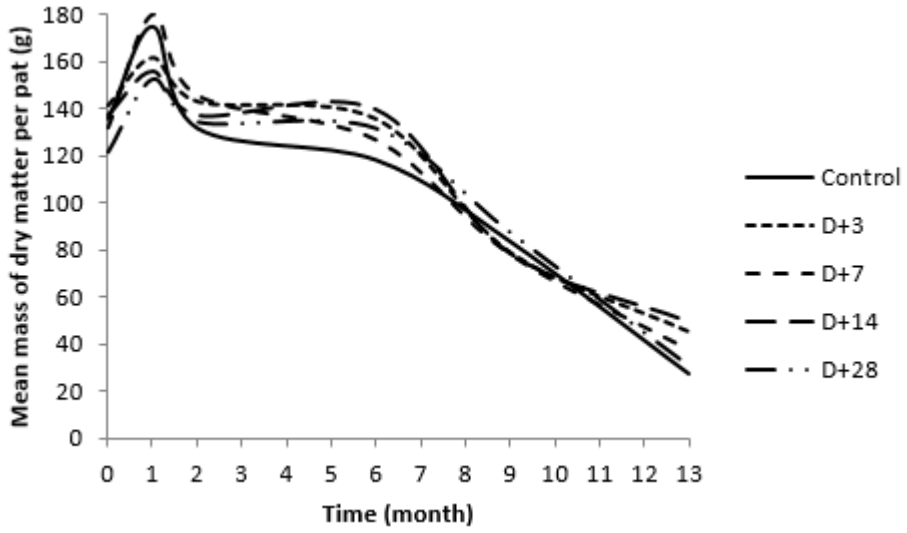
11.4.6 Function: Effects on dung decomposition

Assessing the ecosystem functioning consists in measuring the remaining weight of dung pats according to time and treatments. This weight can be expressed as the remaining dry matter (in mass). However, to measure more accurately the dung degradation, we focused on the remaining organic matter in percentage of the initial organic matter. The measure of the percentage of the mineral part of a pat compared with its dry weight at a sampling date can assess the activity of invertebrates according to time and treatments.

Variation with time of dry weight of pats

For all treatments, whatever was the IVM concentration, as well as for controls, the apparent dry weight of pats one month after their deposit in the field was higher than initial (Figure 47). This peak was due to the incorporation of mineral particles inside the pats in relation with the activity of beetles and earthworms. A part of soil excavated by dung beetles when digging their pedotrophic nests was inserted within pats, which artificially increased their dry weight. The estimate of this additional mineral fraction can be considered as a good indicator of the biological activity of organisms involved in the degradation of pats. Between months 2 and 6, the plateau noticed in all the curves corresponded to the dry period (July to November) which slowed down the activity of soil invertebrates. This period stopped with the advent of rains in autumn. Differences among treatments were not significant.

Figure 47: Variation over time of the mean dry weight of pats (in grams) according to time of collection of dung after IVM administration to animals

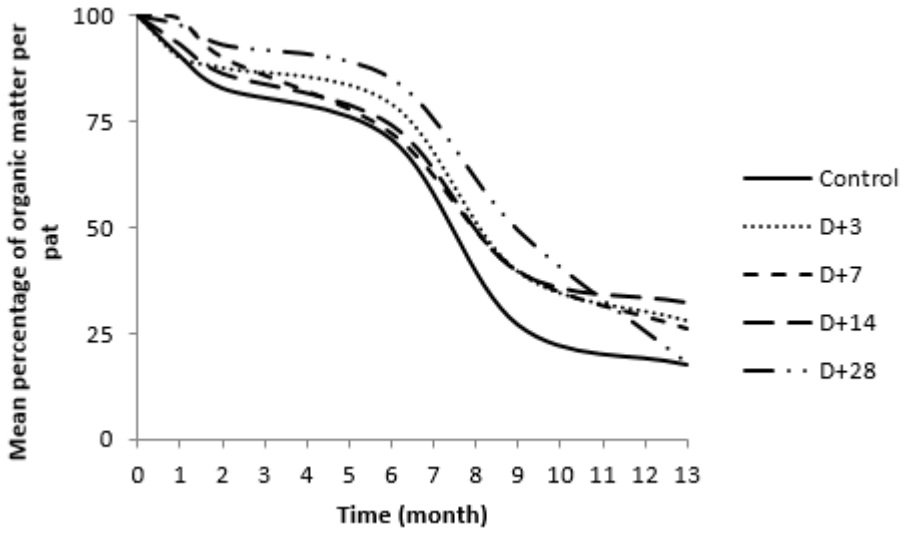


Means for each treatment and date post-deposit were calculated and reported in the graph to get the profile of degradation of dung pats over time depending on treatment.

Variation with time of the organic matter content of pats (free ash dry weight)

Processing a covariance analysis on remaining organic weight once mineral content was excluded for the pour-on formulation, neither treatment ($F=1.897$, $p=0.115$) nor the interaction between time and treatment ($F=0.709$, $p=0.588$) were significant (Figure 48). That means that for each treatment the slopes of organic matter variation according to time were not significantly different. Time alone was significant ($F=470.180$, $p<0.001$). Eliminating the non-significant interaction, there was no main effect of treatment ($F=1.915$, $p=0.112$) but a time effect ($F=474.643$, $p<0.001$). No significant difference among treatments for each sampling month was observed ($p>0.05$).

Figure 48: Reduction over time of the mean dry organic matter (free ash) percentage of pats according to time of collection of dung after IVM administration to animals

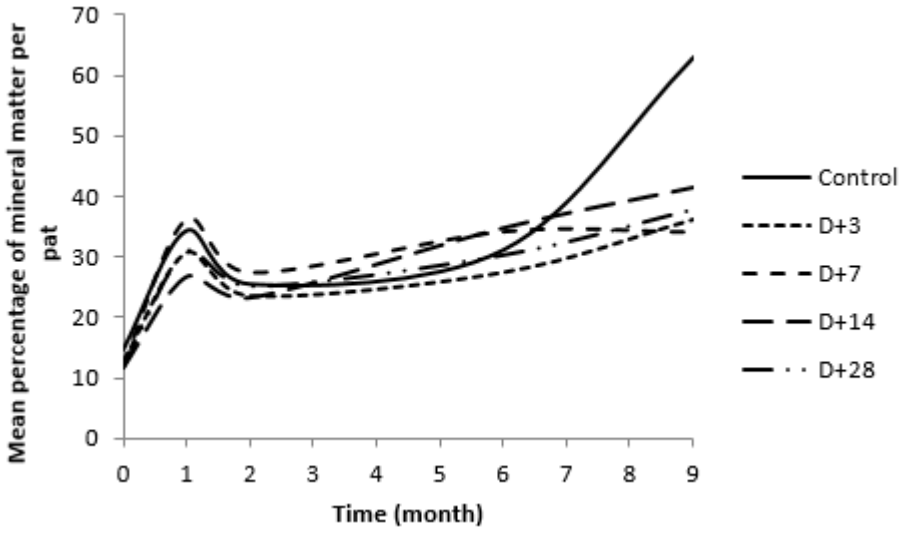


This mean percentage of dry organic matter (compared with the initial weight of pats deposited in the field) for each treatment and each time post-deposit is on the ordinate.

Variation with time of the mineral matter content of pats (ash dry weight)

For the mineral part of dung, expressed as a percentage of the dry weight of pats at sampling times, the covariance analysis revealed a significant interaction between time and all treatments ($F=3.088$, $p=0.019$). Time effect was very significant ($F=34.529$, $p<0.001$), while treatment was only significant at $p<0.1$ ($F=2.162$, $p=0.079$). The increase over time of mineral part in dung was significantly slower for dung collected at days D+3, D+7 and D+28 after administration than for control ($p<0.05$) (Figure 49). Removing the interaction, time was significant ($F=31.842$, $p<0.001$) contrary to treatment as a main effect ($F=1.994$, $p=0.101$). When treatments were considered independently, the main effect of D+3, D+14 and D+28 treatments were noticed ($t=-2.590$, $p=0.011$; $t=-1.998$, $p=0.048$; $t=-2.155$, $p=0.034$ respectively), with more soil particles incorporated in control dung. After nine months exposure, difference among treatments was significant ($H=10.21$, $p=0.037$) with less mineral matter in pats of D+3, D+7 and D+28 treatments than in control ($p<0.05$).

Figure 49: Variation over time of the mean mineral content of pats (%) according to time of collection of dung after IVM administration



This mean percentage of dry mineral matter per pat for each treatment and each time post-deposit is on the ordinate

11.5 Discussion

11.5.1 IVM residues

The peak of IVM excretion in faeces occurred between 3 and 7 days post-administration (DPA) (i.e. 2.845 and 2.480 mg.kg-1 dw respectively). The excretion peak was in the high range and slightly longer than shown in previous studies where the IVM excretion peak was observed about 3–5 days after application with maximum concentrations of 0.2–4.2 mg.kg−1 (dw) (Alvinerie et al., 1999; Edwards et al., 2001; Boxall et al., 2004; Fernandez et al., 2009; Römbke et al., 2010b). Our results are in accordance with the recent study of Iglesias et al. (2011) who found high concentration one week after administration. Although the IVM concentration was low at 28 DPA (0.049 mg.kg-1 dw), such concentration was not null which indicates a long time of IVM excretion in dung.

Our study gives a good overview of IVM degradation in dung over time, with IVM still detectable in pats exposed to a wide range of climatic conditions over a full year (sun, rain, frost…). Previous studies demonstrated that IVM in dung and soil degrades rather slowly in winter and/or under laboratory conditions (half-lives 3-8 months) or rapidly in summer (half-life 7-14 days) (Halley, Nessel and Lu, in (Campbell, 1989; Lumaret et al., 2012). Our results showed that DT50 was obtained after 2-3 months for the D+3 and D+7 concentrations, a time longer than expected for a summer period. The IVM degradation we observed was more rapid than found by Suarez et al. (2003), with a DT50 of up to 180 days after dung pats deposit. The profile of IVM degradation could be attributed first to a rapid photodegradation between May and August (corresponding to a dry summer under Mediterranean climate) followed by a slow microbial activity. Sommer and Steffansen (1993) suggested that photodegradation had minimal effect on IVM degradation since there were no differences in persistence between crust and core in dung pats. The persistence of IVM in dung can be explained by its tight tie to organic matter in faeces and soil and its low leaching by rain water (Halley et al., 1989). Harrowing pastures was shown to increase the surface of dung exposed to sunlight which may enhance photodegradation and increase aerobic microbial IVM degradation in soil faeces mixtures (Wislocki et al., 1989; Halley et al., 1993).

11.5.2 Structure

Dung breeders were affected by IVM with lower emergences of dung beetles and dung flies. The diversity of dung beetles was particularly low both in species and individuals, contrary to observed for flies. Few beetles emerged, due to an unusual dry spring season during the experiment. After pupation, the new generation of dung beetles was less active due to the dryness of soil and most beetles were waiting at the adult stage in their pedotrophic nests. The very small number of dung beetles we obtained has reduced the scope of the conclusions. Beetle abundance depends largely on abiotic factors (Koskela & Hanski, 1977). In their study, Palestrini, Barbero & Rolando (1998) explained that the low abundance they observed was probably due both to abundant rainfalls and to the small size of artificial dung pats they used (1.4 kg fw). In our experiment, we used lighter pats of about 1 kg fw which could be partly responsible of a low colonisation. The very small number of adult beetles which emerged from D+3 dung pats can be explained by the high IVM concentration in dung (2.845 mg.kg-1 dw) (Fig. 74). Kruger and Scholtz (1997) observed that after an injection of 200 µg IVM per kg, adult emergence of *Euoniticellus intermedius* and *Onitis alexis* was reduced during the 2-7 days post-administration. Kruger and Scholtz (1998) concluded that large scale impact of IVM is likely to depend on several factors like climatic conditions, the spatial scale of administration and the number of animals treated. Evidence of the toxic effects of excreted IVM on larval Scarabaeidae was provided with higher mortality and inhibition in their development (Strong et al., 1996). The IVM concentrations we obtained in the first two weeks (2.845-0.692 mg.kg-1 dw) were higher than found in another field study where Euoniticellus fulvus larvae died with an IVM concentration of 0.16 mg.kg-1 (dw) and their development was delayed at 0.06 mg.kg-1 (Lumaret et al., 1993). In a laboratory test on Aphodius constans, the median lethal concentration (LC50) was 0.88–0.98 mg.kg-1 (dw) of dung (Hempel et al., 2006). Despite the low number of individuals we obtained, the initial IVM concentrations in dung could indicate an impact on most species. According to Römbke et al. (2010b), the sensitivity of most dung beetles corresponded to a lowest observed effect concentration (LOEC) and a no observed effect concentration (NOEC) of 0.81 and 0.78 mg IVM kg−1 dung (dw), respectively. In the case of Aphodius fimetarius which was the dominant species we obtained, the LC50 and NOEC were 0.5 and 0.3 mg IVM kg-1 dung (dw), respectively (Lumaret et al., 2012). Since abiotic factors have strong impact on emergence, it leads to some difficulties to compare between years and thus to observe longer effects of VMPs on the whole population. On the basis of juvenile survival data, a model designed by Wardhaugh et al. (2001) predicts that beetle activity in the next generation emerged from treated dung is likely to be reduced by as much as 25–35%. Effects vary according to the time of drug administration and should be greatest when administration occurs soon after the emergence of a new generation of insects. Multivoltine species are likely to be more affected than univoltine, due to their relatively brief period of egg-laying (Wardhaugh et al., 2001). The estimates under more realistic farming conditions predict maximum cumulative mortalities of <25% on an individual farm with a certainly higher value when focusing on the toxicity of a single pat from a treated animal (Sherratt et al., 1998). However, the models developed did not consider sub-lethal effects such as a reduction in reproductive abilities and species interactions, such as competition and density dependent effects. The extent to which predicted population losses may be compensated by immigration is also widely unknown and would deserve further study.

Flies were more impacted than dung beetles and confirmed observations done on the abundance of dung fly larvae impacted (NOEC < 0.31 mg.kg-1 (dw)) compared to adult dung beetles (Römbke et al., 2010b). A difference can be noticed between brachycerans (more sensitive; lower number of individuals and families) and nematocerans. Offspring of Neomyia cornicina (Diptera) was very sensitive to ivermectin with no emergence at IVM concentrations of 0.16 and 0.06 mg.kg-1 (dw) (Lumaret et al., 1993). Sepsids were particularly affected by IVM, with no emergence during the first 28 days post-administration (Fig. 76), as previously demonstrated by Blanckenhorn et al. (2013b). Sepsids are good candidates for standardized laboratory tests performed to assess the side effects of IVM administration to cattle (Blanckenhorn et al., 2013a). Our field results strengthen the recommendation of their suitability as indicators of pharmaceutical residues effects on fly communities. Sphaerocerids play a

similar role in the first week post-administration period to assess dipteran structural modifications due to IVM administration (Fig. 76). A two-week colonisation period was as efficient as a single one-week period to collect similar number of dung flies. On the contrary, after a three-week colonisation period few individuals were collected because of the quick emergence of most of flies which flew away before the emergence traps were set up.

IVM impacted higher trophic levels, e.g. rove beetles and parasitoid wasps. The higher number of staphylinids collected from the two-week colonisation pats indicated that this time of colonisation was more relevant to test the IVM effects on this group (Fig. 79). After a free three-week colonisation period, few individuals were collected in emergence traps because of the quick emergence of most small staphylinids (below three weeks according to (Ashe, 1990; Hanley & Goodrich, 1994; Goodrich & Hanley, 1995; Hu & Frank, 1995; Echegaray Wilson, 2012) which emerged before the traps were set up. Results of emergence of rove beetles from the 3-14 days post-administration pats and freely colonized for one and two weeks demonstrated IVM impact which could be explained by both direct and indirect effects: 1) direct effects to species that were reported to feed exclusively on cattle dung (Hinton, 1944; Skidmore, 1991; Fincher, 1992; Hu & Frank, 1995); 2) indirect effects due to the mortality of their prey and hosts (mostly flies).

Parasitic micro-hymenopterans from the same trophic level than rove beetles were similarly affected by IVM concentrations in dung (Fig. 80). That is the case of Pteromalidae species whose hosts (brachycerans) were strongly impacted by IVM administration to cattle. Floate and Fox (1999) found similar results in Canada with species of the same family (Muscidifurax zaraptor (Kogan and Legner)).

VMPs excreted by livestock into the environment can thus deeply disturb the composition of the cattle dung insect community at the landscape scale and likely affect the function and ecosystem services of the dung community as a whole. A special attend toward the response diversity would be necessary since the species diversity that can perform similar ecosystem functions but have different capacities to respond to disturbance, provide greater resilience to the entire system (Elmqvist et al., 2003; Mori et al., 2013). More than their functional ability, assessing their variation in response to different disturbances and environmental changes is thus crucial otherwise even a small disturbance could result in loss of species from the same functional group and thus in functional loss.

11.5.3 Function

The IVM impact on dung pat degradation was not as strong as expected, emphasizing the difficulty to assess this impact on functioning, mostly due to the high variability among pats (Iglesias et al., 2011). Römbke et al. (2010b) found that the decomposition of dung pats was significantly affected when IVM initial concentration was 0.78 mg.kg−1 (dw) due to the impact of IVM on dung fly and dung beetle larvae. Under Mediterranean conditions, dung beetles are the main agents responsible for the disappearance of dung (Lumaret & Kirk, 1991; Lumaret et al., 1992). Contrary to their larvae, the relatively low sensitivity to IVM of adult beetles (both dwellers and tunnelers) which carry out the immediate function of dung degradation may hide the long-term environmental risk on the diversity and the reduction of offspring. The effect of IVM in dung degradation depends on the season and the dung beetle guilds, as dwellers are mostly active in wet periods and tunnelers in dry periods. Function carried out by dwellers (mostly Aphodiidae species) can be more affected than by tunnelers as their larvae develop inside dung pats and contribute to their degradation. High IVM concentration can directly affect degradation. Tunnelers dig deep pedotrophic nests under fresh pats and they fill them rapidly from the surface. IVM contained in dung stored in burrows is protected from photodegradation and is degraded slowly in dark (Lumaret et al., 1993). IVM stored in nests can affect larval development of tunnelers but not the dung degradation as adults do not discriminate treated and untreated dung and contribute to the same level to the function. A higher proportion of tunnelers compared to dwellers could thus lead to more difficulties to find significant effects of IVM on the dung degradation. Differences in

climatic conditions from one year or season to the next could induce a higher variability in degradation than the activity of organisms. The very dry conditions in spring 2011 reduced both dung beetle activity and dung degradation. Usually total degradation occurs in less than 6 months in temperate areas or wet seasons (Aarons et al., 2004; Lee & Wall, 2006; Cruz et al., 2012) and in more than one year under Mediterranean conditions (Lumaret & Kadiri, 1995). Under normal conditions (absence of IVM), dryness reduces pats attractiveness and restricts colonisation (rapid crust formation) which resulted in a small number of emergence. Degradation functions carried out by insects are thus weakened. Beynon et al. (2012) suggested that short-term dung decomposition is not influenced by species richness under normal conditions. Under perturbation, thanks to specific difference in sensitivity to IVM, the ecosystem functioning can be maintained due to high richness assemblages. Beynon et al. (2012) showed that within the tunnelers group, species redundancy allowed to maintain ecosystem functioning in spite of the high sensitivity of Onthophagus similis which revealed the importance of other species less affected.

The peak of mineral content observed after one month is in accordance with soil excavated to the surface by the beetles (Brown et al., 2010). Although the soil loss could be firstly considered wrongly in the sense of services, it is very likely that the tunneling activity by large dung beetles but also by earthworms contribute to increase water infiltration and reduce surface water runoff (Blanchart et al., 2004; Brown et al., 2010). By burrowing dung pellets at different depths in the soil, tunnelers contribute to organic matter incorporation which could influence the soil chemical composition with in particular an increase of the N content (Rodriguez et al., 2005). It is the first study which evaluated the soil incorporation into dung pats over time. After nine months, the higher mineral content in control pats validates our hypothesis of using the mineral content as an indicator of invertebrate activity. Under Mediterranean conditions, 9 months appeared to be the more relevant time for the use of this indicator. Dung beetles, as a diverse group, have been already proposed as an invertebrate cost-effective focal taxon for biodiversity research and conservation, especially in context of disturbance (Spector, 2006; Gardner et al., 2008). Furthermore, both large beetles (tunnelers) and earthworms can be classified as ecosystem engineers because they directly or indirectly affect the resources availability through modifications of their physical environment (Jones et al., 1994; Lavelle et al., 1997) and are mostly responsible of the mineral incorporation in pats (O'Hea et al., 2010). By their vertical transport activity (mineral and organic matter), tunnelers carry on their integument spores of telluric fungi and microorganisms which stimulate microbial mineralization activity (Breymeyer et al., 1975; Lussenhop et al., 1980). To strengthen the role of these large beetles, Braga et al. (2013) have shown their higher ability to spread large seeds. The mineral content indicator can thus evaluate their integrative activity and assess the quality of ecosystem functioning particularly in a disturbing context like VMPs administration.

Acknowledgement

We thank Dr. Delvare for his help in identifying parasitoid wasps, Mr. Tronquet in identifying rove beetles and Maëllia in sorting individuals. We would like to thank P. Teisserenc for supplying cattle for the experiment. The work described here was sponsored by the German Federal Environmental Agency (Project-No. FKZ 371063412).

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12 WP2: Non-target effects of ivermectin residues on structure and function of coprophilous communities of arthropods in a grassland: Swiss pre-Alps (Switzerland)

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ABSTRACT

Veterinary pharmaceutical residues can cause severe damage to the dung ecosystem. Over the past 25+ years, numerous studies have assessed the effects of Veterinary Medicinal Products (VMPs) on dung organisms in the laboratory and the field, however using different methods, insects and VMPs. A standardized approach is much needed and actually mandated by an authorisation process aimed at harmonizing technical requirements for such drugs in Europe, Japan, and North America. In a coordinated project sponsored by the German Federal Environmental Agency (UBA), we performed parallel field studies with one particular widely used model VMP, the parasiticide ivermectin, in the Mediterranean (South France), Continental (Central Switzerland), Atlantic (The Netherlands) regions of Europe and the Prairie (Western Canada) region of North America, to assess its effect on the composition (structure) of the dung community and its function in terms of dung degradation. Here we report the results of this international field ring test for a grassland site near Zurich, Switzerland, which was conducted from May to December 2011 using dung from Montpellier (F). There were 5 treatments with ivermectin concentrations of 0 (control), 0.049 (excreted on Day 28), 0.692 (D14), 2.480 (D7), 2.845 (D3) mg*kg-1 dry weight (measured post-hoc after passing through the treated cattle). We used 10 replicate dung pats of 0.5 kg per treatment. We found that the species richness and biodiversity (Shannon index) of the dung decomposing community of beetles (Coleoptera), flies (Diptera) and parasitoid wasps (Hymenoptera) decreased with ivermectin concentration in our structural experiment. However, in the functional test using the same dung the breakdown of dung pats contaminated with ivermectin was not more retarded than in the control. Parallel pitfall traps verified the ambient presence of most insect groups that emerged from our experimental dung pats, although numbers were not always proportional. Results at the other sites were qualitatively similar, demonstrating that such a field test assessing the entire dung community is robust despite strongly varying environmental circumstances. (320 words)

Key words: dung, beetles, flies, ecotoxicology, biodiversity

12.1 Introduction (note edits)

In the European Union and North America, the environmental risk of veterinary medical products (VMP), and particularly parasiticides, is addressed in an authorization process. This process is based on guidelines published by the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH), which is a trilateral program to harmonize technical requirements for these drugs in Europe, Japan, and the United States. This Environmental Risk Assessment (ERA) permits a tiered approach. In Phase I (VICH 2000), general aspects regarding use and exposure are handled. In Phase II, ecotoxicological test requirements are specified (VICH 2004). An ERA of VMP for dung fauna is required if the substance acts as a parasiticide for the treatment of pasture animals. In Tier A of Phase II, studies are done to assess the non-target effects (if any) of parasiticides excreted via faeces on dung-living (or -decomposing) beetles and flies. If a risk is identified, additional studies are required (Tier B) to characterize the nature and extent of the non-target effects using representative non-target organisms as bioassays. However, further information on Tier B studies (and beyond) for dung organisms are missing in the guidelines. In fact, the only advice given on how to proceed beyond Tier A is a statement in the VICH (2004) guideline: "Regulatory guidance should be sought on appropriate studies." In the "Revised Guideline on Environmental Impact Assessment for Veterinary Medicinal Products" (EMA 2008) no further information on higher tier-testing of dung organisms is given.

Numerous studies have assessed the effects of VMPs on dung organisms, both in the laboratory and in the field within the last 25 years (e.g. Lumaret et al. 2012). However, these studies were performed using different methods, on different insects, and with different VMPs. A standardized approach is lacking, but is much needed by the European Medicines Agency (EMA) as well as national authorities, industry, and consultants to complete the VICH requirement for higher tier studies (particularly those in the field) with VMPs.

In order to address this problem, the German Federal Environmental Agency (UBA) sponsored a project aimed at performing field studies with one particular model VMP (i.e. ivermectin) in different ecological regions in Europe and North America to assess the entire structure (i.e. the composition) and function (i.e. the dung degradation) of dung and soil organisms as endpoints. The experimental work was based on the recommendations compiled by Jochmann et al. (2012) and was performed in the Mediterranean (South France), Continental (Central Switzerland), Atlantic (The Netherlands) regions of Europe and in the Prairie (Western Canada) region of North America. In each of these four studies the same questions were addressed:

1. Does the use of ivermectin excreted in cattle dung cause any effect on dung fauna biodiversity?
2. Does ivermectin affect the degradation of the dung?

Here we report on the overall results of this international field ring test. Besides answering the above questions, practical recommendations concerning the practicability and the informational value of the recommendations of Jochmann et al. (2012) will be given.

In this contribution, the test performed at a grassland site near Zurich (Switzerland) is described, which used methods generally comparable to those used for the tests performed in Wageningen (The Netherlands; Lahr et al., this report), Montpellier (France, Tixier et al., this report) and Lethbridge (Canada) (Floate et al., this report).

12.2 General Description of the Swiss field site

The Swiss study was performed on a pasture (a farm until 1979) near the University of Zurich's Irchel campus (altitude ca. 550 m), which lies at the fringes of the city of Zurich in the canton of Zurich (47.37° N, 8.55° E), in the Swiss pre-Alps (voralpines Mittelland). Agriculture and cattle / milk cow pastures are common and interspersed with forest and human settlements. Zurich has a temperate

climate, with a long-term average temperature of 8.5°C, and –0,5°C in January (coldest month) and 17.6°C in July (warmest month). There are on average 88 frost days (December to February), with the last frost typically in early April and the first frost in late October. Average rainfall is ca. 1100 mm, spread out evenly over the months, with a bit more precipitation in summer (August with 133 mm being the wettest) than in winter (January with 67 mm being driest). Relative humidity lies between 73% (July) and 85% (December).

12.3 Materials and Methods

The Swiss study was conducted from 23 May (when dung was first brought out) to December 2011 (last measurement of the functional, degradation part). Frozen dung from the Montpellier (F) site was used (see Chapter 11).

12.3.1 Analytical procedure for the determination of the antiparasitic agent ivermectin in cattle dung

Reagents and equipment

Acetonitrile of HPLC-gradient grade (>99.9%) was supplied by VWR international (Radnor, Pennsylvania, USA). High purity water was prepared by a Milli-Q water purification system (Millipore, Milford, MA, USA). N-methylimidazole (99% purity), triethylamine (99% purity), trifluoracetic anhydride (99% purity) and trifluoracetic acid (99% purity) were supplied by Sigma-Aldrich (Steinheim, Germany). The standard substances ivermectin (CAS RN: 70288-86-7, 96% purity) and doramectin (CAS RN: 117704-25-3, 90% purity) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). For extraction, a Vortex Genius 3 shaker (IKA, Staufen, Germany), a rotary shaker Swip KS-10, (Bühler, Tübingen, Germany), and an ultrasonic bath Sonorex Super RK255H (Bandelin electronic, Berlin, Germany) were used. As a centrifuge a Rotanta 460 R (Hettich, Tuttlingen, Germany) was used. Syringe filters (PTFE, 0.45 µm, 13 mm) were supplied by Wicom GmbH (Heppenheim, Germany). Solid phase extraction cartridges (Strata C-18-E, 500 mg, 55 µm, 70 Å) were purchased from Phenomenex (Torrance, California, USA).

Standard solutions

All standard solutions of doramectin and ivermectin were prepared in acetonitrile and stored at 18 °C. Stock solutions were made by dissolving 2.5 µg ivermectin or doramectin in 25 mL acetonitrile. These solutions were used to prepare ivermectin working standard solutions of 2000 and 100 µg/L, as well as doramectin working standard solutions of 2000 and 200 µg/L. With these solutions 9 calibration standards covering the relevant concentrations were prepared on daily basis.

Extraction and clean-up of the dung samples

The extraction procedure was mainly based on an adapted and optimized method as described by Litskas et al. (2010). Initial dung samples were homogenised. A total amount of about 3 g was weight into polypropylene-vials. The initial dung samples had a water content of about 82%.

Internal standard doramectin dissolved in 25 mL acetonitrile was added in an amount near that expected in the sample. The suspension was kept for 15 min in an ultrasonic waterbath, 30 min on a mechanical shaker at room temperature at 450 rpm and again for 15 min in the ultrasonic water bath. Subsequently, the sample was centrifuged for 30 min at 2000 x g and 22 °C. The extracts of the fresh dung samples were cleaned up with an additional solid phase extraction (SPE). For this, 20 mL of the supernatant were diluted with 66.6 mL water and 66.6 µL triethylamine. The SPE cartridges were conditioned with 10 mL acetonitrile and 10 mL acetonitrile/water (3:7, v/v). Subsequently, the samples were extracted with a C18-SPE-cartridge (500 mg, 55 µm, 70 Å) at a flow rate of 3 mL min-1. The extraction was followed by a washing step with 12 mL acetonitrile/water (1:1, v/v) at a flow rate of 8 mL min-1. With 5 mL of acetonitrile the analyte was eluted under gravity into a polypropylene-vial. The

solvent was evaporated under a gently stream of nitrogen at 55 °C to complete dryness. For reconstitution 1000 µL acetonitrile were added to the sample. It was vortexed for 2 min, kept in an ultrasonic bath for 10 min, kept for 30 min on a mechanical shaker at 450 rpm, vortexed again for 30 s, and put again in the ultrasonic bath for 5 min. Finally, it was again kept for 30 min on a mechanical shaker at 450 rpm. After filtration (0.45 µm, PTFE) 700 µL of the solution were transferred into a HPLC-vial for the derivatization step.

Derivatization with trifluoracetic anhydride

The sample was derivatized according to an adapted procedure developed by Berendsen et al. (2007). First, 100 µL of N-methylimidazole/acetonitrile (1:1, v/v) were added to 700 µL of the reconstituted and filtered sample, followed by 50 µL of triethylamine. Subsequently, 100 µL of trifluoracetic anhydride/acetonitrile (1:1, v/v) were added. Finally, 50 µL of trifluoracetic acid were given into the vial. After each addition of reagent the closed HPLC vial was shaken for at least 5 seconds. To finish the derivatization reaction the closed HPLC-vials were kept for 30 minutes at 60°C in an oven.

High performance liquid chromatography with fluorescence detection (HPLC-FLD)

The determination with the HPLC-FLD was carried out within the first 48 hours after the derivatization. Chromatographic separation and determination was performed on an Agilent 1200 HPLC system (Agilent, Santa Clara, California, USA) consisting of a degasser (G1322A), a quaternary pump (G1311A), an autosampler and injection unit (G1329A), a column thermostat (G1316A) and a fluorescence detector (G1321A). The gradient elution was performed using a mobile phase of water (A) and acetonitrile (B) at a flow rate of 0.3 mL min−1 with the following gradient: 0–47 min, 60-100% B; 47–52 min, 100% B; 52–53 min, 100-60% B; 53-60 min, 60% B. The injection volume was 20 µL and the analytes were separated on a 150 mm × 2.1 mm i.d. 3 µm particle size, Dionex (Sunnyvale, California, USA) Acclaim PolarAdvantadge II C18-Column. The column temperature was 30 °C. The fluorescence detection was carried out at an excitation wavelength of 364 nm and an emission wavelength of 463 nm.

Figures of merit

The limit of detection (LOD) and limit of quantification (LOQ) values were determined with the calibration method on the basis of DIN 32645(2008). The LOD for dung samples from medicated cattle was 5.1 µg / kgdw and the LOQ 12.4 µg / kgdw. All data of the extractions with an inadequate recovery of the internal standard (<80% and >120%) were assorted. For the remaining samples the mean recovery of the internal standard doramectin was 101.3% (RSD 8.9%) for the initial dung samples.

12.3.2 Structural, dung insect community composition experiment

We had 5 ivermectin treatments: Day 0 = untreated control, plus dung collected on Days 3, 7, 14, 28 after ivermectin treatment of the cattle. Corresponding ivermectin concentrations measured post-hoc were 0, 0.049 (D28), 0.692 (D14), 2.480 (D7), 2.845 (D3) mg*kg-1 dry weight, respectively. There were 10 replicate dung pats of 0.5 kg per treatment, arranged in two systematically randomized 5x5 arrays with the pats being spaced 6 m apart from each other (Figure 50a). Pats were placed onto ca. 5 l of soil contained in a plastic bowl, the bottom of which was perforated to allow water to drain while preventing soil organisms from escaping (Figure 50b). The bowl was dug into the ground with its rim level with the surface. Pats were left out for 7 days to be populated by dung insects. Thereafter each bowl with the soil and the dung were transferred into an emergence container placed in an open shed nearby (Figure 50c). The emergence containers had only one exit leading into an ethanol-filled bottle to capture all emerging insects. Conditions in the emergence containers were moist, as they were largely sealed (Figure 50d). Capture bottles were harvested (i.e. exchanged) three times a week (Monday, Wednesday, and Friday) for the next 3 months (first emergent on 6 June 2011). Emergence was checked again in spring of 2012 to capture hibernated insects. Flies were stored in ethanol in the

emergence bottles (or other containers) until they were sorted, counted and identified at some later date.

All captured beetles (Coleoptera), flies (Diptera, both Brachycera and Nematocera) and wasps (Hymenoptera) were identified to varying taxonomic levels, mostly to the genus level. Per pat we computed three measures describing biodiversity of a community, the number of taxa (taxon richness), number of individuals (abundance), and the Shannon index of diversity, which combines information about richness and abundance (Pielou 1974). In the final analysis, the taxonomic levels of the identified groups differ, some being species, others subsuming genera or even families; however, this should not introduce systematic bias. Taxon richness (emerged numbers) and the Shannon diversity index were analyzed using regression against the (continuous) effective ivermectin concentration corresponding to the sampling days as given above. We analogously analyzed taxon richness (emerged numbers) of the various subtaxa in response to ivermectin concentration.

Of a total of 36 taxa (genera) of beetles (Coleoptera), flies (Diptera) and Hymenopteran parasitoids obtained and identified, only those that emerged with at least 15 individuals in total from any dung pat (Table) were deemed common enough for this analysis. (Note that we necessarily expect the very sensitive species only to emerge from the dung pats with little to no ivermectin.) Whenever necessary, we binned rare taxa post-hoc into higher taxonomic units to reach these numbers.

Figure 50: Depiction of the Zurich field site of the (a, b) emergence (structural study), the emergence trap (c) and (d) methodology, and the (e, f) dung degradation (functional) study

12.3.3 Functional, dung degradation experiment

Performance

We used the same dung and 5 ivermectin treatments (Day 0 = untreated control, 3, 7, 14, 28). There were 5 replicate pats of 0.5 kg per treatment, arranged sequentially along a fence close to the pasture, with the pats being spaced 3-4 m apart from each other (Figure 50e). There were 5 time points to be sampled 1, 2, 3, 5 & 7 months after deposition, requiring a total of 5 (replicates) x 5 (treatments) x 5 (time points) = 125 pats. Pats were placed onto coarse cloth mesh allowing insects and earthworms to go through but permitting picking up the drying pats for drying and weighing (Figure 50f).

From each pat, a 50 g sample was weighed, oven-dried for 24 h at 105 °C, and then reweighed to determine wet weight. The dry dung sample was then finely ground and a 1 g subsample was placed into a weighed silica crucible. The subsample was weighed, heated in a muffle furnace to 500 °C for 5 h and allowed to cool overnight for a total time of ca. 24 h in the muffle furnace. Subsamples then were transferred from the muffle furnace to an oven at 55 °C for a further 24 h and then reweighed to determine the ash content.

Statistical analysis

Dung degradation over time as a function of ivermectin concentration was analyzed in two ways. We first estimated the best-fit function to the data by model fitting, testing linear, quadratic, cubic (which would pick up any sigmoid shape), exponential and logarithmic functions. The fit was judged by the r2 value and by the Akaike Information Criterion (AIC), the latter adjusting for the number of estimated parameters K of the functions used (K= 1 for most functions except quadratic (2) and cubic (3): Burnham & Anderson 2001). This procedure was applied equally to the entire data and to individual data sets of the different sites (and ivermectin concentrations).

For significance testing across ivermectin treatments and/or sites, ANCOVA of the pat dry (or ash) weights (log10-transformed or not) against month (both continuous variables in absolute values; i.e. grams vs. months) was used, including ivermectin treatment (and site) as fixed factors. The fitting exercise told us that using regular GLM or ANCOVA, which always assumes linear fits, is appropriate because the linear fit was close to being best. Retarded dung decomposition at higher ivermectin concentrations would in this analysis show as a time (i.e. month) by ivermectin concentration interaction (likewise for decomposition differences among sites).

12.3.4 Pitfall traps

Four pitfall traps were positioned at the outside corners of one of the 5x5 arrays for the structural experiment (i.e. distance of > 30 m apart) to determine the composition of the activity of insects at the study site during the time that pats were exposed in the field. The pitfall traps were roofed (to prevent filling up with rain water), filled with non-toxic salt water, and were left out for 7 days and then emptied. A total of 7 monthly samples (May – November 2011) with 4 replicates each, were taken. All beetles (Coleoptera), flies (Diptera) and wasps (Hymenoptera) found in the pitfall traps were identified to various taxonomic levels, mostly to the genus level. The counts were tabulated. No statistical tests were performed.

12.4 Results

12.4.1 Residue analysis

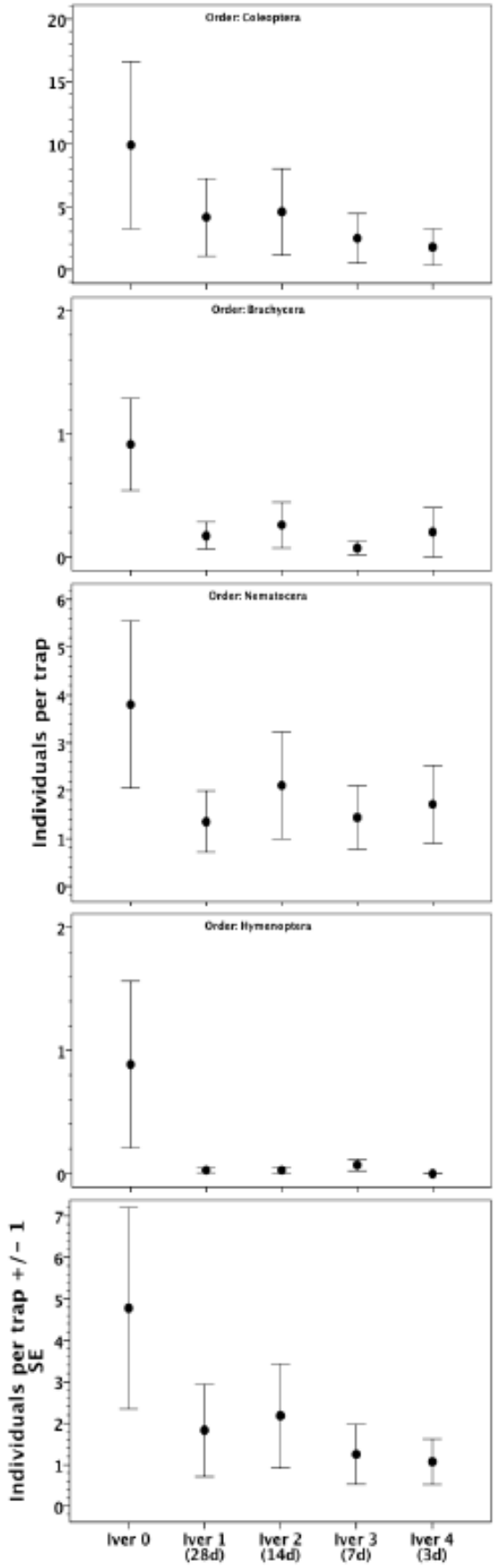
The highest initial ivermectin concentration in dung collected from animals was obtained 3 days post-administration (DPA) (2.845 mg.kg-1 dw), close to the concentration value at 7 DPA (2.480 mg.kg-1 dw). The 14 DPA concentration value (0.692 mg.kg-1 dw) was 4 times lower than the peak of elimination. After 28 days, IVM was still detectable (0.049 mg.kg-1 dw). The excretion profile of IVM over time

corresponded rather to a Log regression (r2 = 0.918) than a linear regression (r2 = 0.876). For details, see Chapter 11 (Tixier, this report).

12.4.2 Structural, dung insect community composition experiment

Ca. 3700 beetles, flies and wasps belonging to 36 taxa were counted and identified (mostly) to the genus level (Table 10). Some of the taxa with low numbers were binned into higher taxon groups in Table 10. Overall, ivermectin reduced dung insect richness and biodiversity (Figure 51), as generally more insects emerged from the uncontaminated control dung pats. However, the effect of ivermectin was not as gradual as expected, as even dung sampled after 28 days with low concentrations of ivermectin (0.049 mg*kg-1 dry weight = Ivermectin 1 in Table 10 & Figure 51) resulted in significantly lower numbers. Importantly, the overall Shannon index declined significantly with ivermectin concentration in the dung. Of the 22 taxa (largely genera) of insects with sufficiently high numbers so they could be individually analyzed. 18 showed negative regression coefficients indicating lower numbers at higher ivermectin concentrations, of which 9 were statistically significant (Table 10).

Figure 51: Individuals per trap (+ / - one std.) at the five concentrations (including the control)



From top to bottom: Coleoptera, Brachycera, Nematocera, Hymenoptera and all groups together. IVM (mg /kg d.w.): Iver 0 (0); Iver 1 (0.049); Iver 2(0.692); Iver 3 (0.248); Iver 4 (0.2845)

Table 10: Mean number ± SD of individuals emerged per pat (n = 10 replicates per ivermectin treatment) for various beetle, fly and wasp groups (genera)

Order	Family	Genus	Total emerged	mean Iver0	± SD	mean Iver1 (28)	± SD	mean iver2 (14)	± SD	mean Iver3 (7)	± SD	mean iver4 (3)	± SD	r	t	P
Coleoptera	Carabidae	5 spp	7	0,2	± 0.42	0,1	± 0.32	0,2	± 0.63	0	± 0	0,1	± 0.32			
	Hydrophilidae	Cercyon	80	5,2	± 2.04	1,8	± 1.32	0,9	± 1.73	0,1	± 0.32	0	± 0	-0,627	5,579	<0.001
	Monotomidae	Monotoma	6	0,2	± 0.63	0	± 0	0,3	± 0.95	0,1	± 0.32	0	± 0			
	Ptiliidae	Nephanes	25	0,5	± 0.71	0,8	± 1.62	0,6	± 1.26	0,4	± 0.7	0,2	± 0.42	-0,167	1,172	0,247
	Scarabaeidae	Aphodius	15	0,9	± 2.51	0,5	± 0.97	0,1	± 0.32	0	± 0	0	± 0	-0,236	1,684	0,099
	Staphylinidae	>3 spp	16	0,7	± 1.25	0,3	± 0.48	0,3	± 0.67	0,3	± 0.48	0	± 0	-0,228	1,62	0,112
	Staphylinidae1	Anothylus	335	22,2	± 27.45	3,2	± 3.68	4,6	± 4.6	2,1	± 1.52	1,4	± 1.26	-0,324	2,376	0,022
	Staphylinidae2	Atheta	2026	80,7	± 44.56	38	± 35.8	42,3	± 45.84	24,1	± 18.13	17,5	± 14.01	-0,441	3,4	0,001
	Staphylinidae3	Autalia	17	0,2	± 0.63	0,6	± 1.07	0,3	± 0.48	0,2	± 0.42	0,4	± 0.52	-0,048	0,336	0,728
	Staphylinidae4	Cordalia	63	2,4	± 1.96	0,5	± 0.97	2	± 3.37	0,7	± 0.95	0,7	± 1.34	-0,207	1,464	0,15
	Staphylinidae5	Neobisnius / Othius	98	2,3	± 3.3	1,5	± 2.51	3,2	± 6.73	1,8	± 2.78	1	± 1.05	-0,103	0,719	0,476
	Staphylinidae6	Plathyste-tus	70	3,8	± 3.52	2,7	± 5.25	0,3	± 0.67	0,2	± 0.42	0	± 0	-0,405	3,066	0,004

Order	Family	Genus	Total emerged	mean Iver0	± SD	mean Iver1 (28)	± SD	mean iver2 (14)	± SD	mean Iver3 (7)	± SD	mean iver4 (3)	± SD	r	t	P
Diptera: Brachycera	Anthomyidae	3 spp	17	1,6	± 2.55	0,1	± 0.32	0	± 0	0	± 0	0	± 0	-0,27	1,938	0,059
	Hybotidae		9	0,1	± 0.32	0,2	± 0.42	0,5	± 1.08	0,1	± 0.32	0	± 0			
	Muscidae	3 spp	3	0,3	± 0.48	0	± 0	0	± 0	0	± 0	0	± 0			
	Phoridae	Megaselia	45	0,6	± 0.7	0,8	± 1.14	1,3	± 2.75	0,4	± 0.84	1,4	± 1.58	0,048	0,335	0,738
	Psilidae		1	0,1	± 0.32	0	± 0	0	± 0	0	± 0	0	± 0			
	Sepsidae	Sepsis	29	2,8	± 7.51	0,1	± 0.32	0	± 0	0	± 0	0	± 0	-0,171	1,206	0,234
	Sphaeroceridae	5 spp	9	0,9	± 1.6	0	± 0	0	± 0	0	± 0	0	± 0			
Diptera: Nematocera	Cecidomyiidae1	Lestodiplo-sini	47	1,2	± 1.62	2,1	± 2.69	0,8	± 1.14	0,6	± 0.7	0	± 0	-0,365	2,718	0,009
	Cecidomyiidae2	Micromya	14	0,9	± 1.6	0,1	± 0.32	0,1	± 0.32	0,1	± 0.32	0,2	± 0.42	-0,166	1,167	0,249
	Cecidomyiidae3	Monardia	24	1,3	± 1.83	0,4	± 0.7	0,2	± 0.42	0,3	± 0.67	0,2	± 0.42	-0,236	1,685	0,099
	Chironomidae	Smittia (?)	376	14,4	± 17.14	5,3	± 4.95	9	± 12.63	5,4	± 6.75	3,5	± 4.4	-0,245	1,75	0,087
	Sciaridae1	Bradysia	242	7,9	± 8.49	2,2	± 3.19	4,6	± 6.95	3	± 3.3	6,5	± 10.7	-0,003	0,022	0,983
	Sciaridae2	Lycoriella	76	3,1	± 2.77	0,5	± 0.53	0,8	± 1.48	1	± 1.15	2,2	± 3.74	0,007	0,047	0,963
	Sciaridae3	Scatopsci-ara	52	1,6	± 1.58	0,2	± 0.42	1,4	± 1.17	1,1	± 1.66	0,9	± 2.51	-0,004	0,029	0,977

Order	Family	Genus	Total emerged	mean Iver0	± SD	mean Iver1 (28)	± SD	mean iver2 (14)	± SD	mean Iver3 (7)	± SD	mean iver4 (3)	± SD	r	t	P
Hymenop-tera	Braconidae		13	1,3	± 1.49	0	± 0	0	± 0	0	± 0	0	± 0	-0,317	2,315	0,025
	Eucoilidae		48	4,8	± 4.52	0	± 0	0	± 0	0	± 0	0	± 0	-0,354	2,62	0,012
	5 spp		10	0,1	± 0.32	0,2	± 0.42	0,2	± 0.42	0,3	± 0.95	0	± 0			
Total			3773													
Shannon diversity overall				**1,82**	**± 0.25**	**1,47**	**± 0.36**	**1,59**	**± 0.33**	**1,42**	**± 0.36**	**1,39**	**± 0.29**	**-0,314**	**2,288**	**0,027**

The last columns give the estimated correlation r (and significance) of abundance with the actual ivermectin concentration based on regression

12.4.3 Functional, dung degradation experiment

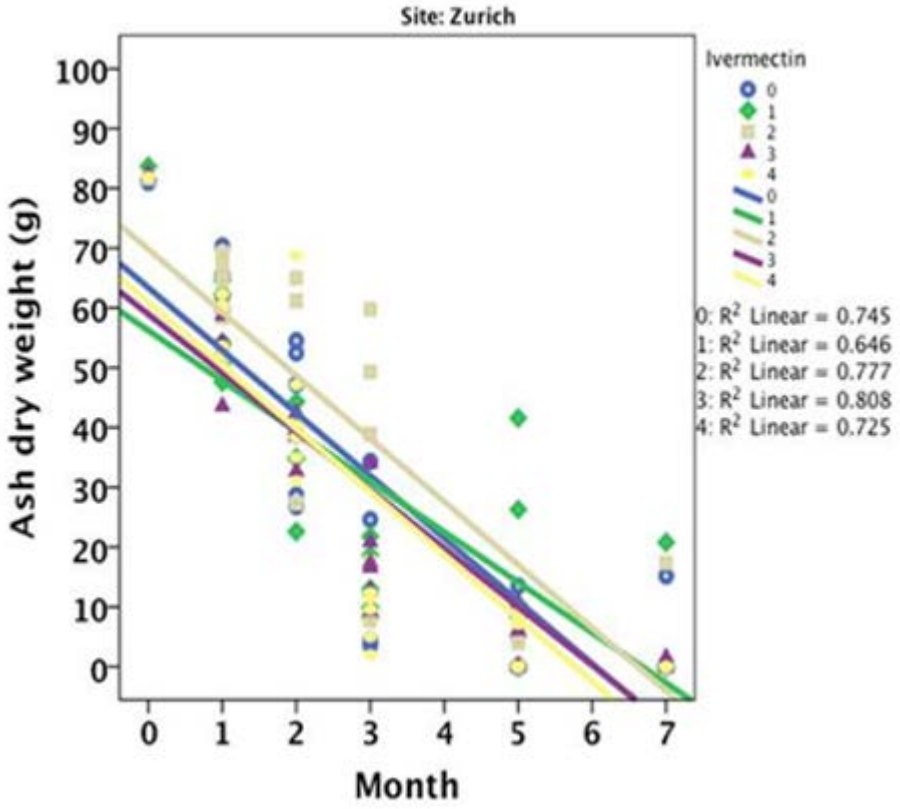
In general, the exponential fit to the dung decomposition data came out best with the lowest AIC(c) value, although the linear and logarithmic fits were close (i.e. the evidence ratio difference is <2). Therefore, using linear regression in our tests below is appropriate. (Note that the fitted model parameters have a standard error, analogous to the slope of the regression line, which can in principle also serve to see or test for differences between treatments, if only pair-wise.)

ANCOVA indicates that the interaction between time (month) and ivermectin treatment (concentration) is not significant, so it could be dropped from the model (Figure 52; Table 11). This is also true when only analyzing the first two months, when primarily insect activity (rather than earthworms, Collembola or physical factors) would contribute to the breakdown of the dung. Thus, Ivermectin does not seem to affect overall dung decomposition, despite significant reductions of the decomposing insect community (Table 11).

Table 11: ANCOVA of remaining dry dung pat weight against time (month), for various ivermectin concentrations plus a control

Ash dry weight (g)					**log10 (Ash dry weight (g))**			
Source	**df**	**Mean Square**	**F**	**P**	**df**	**Mean Square**	**F**	**P**
Corr. Model	3	9678.27	77.92	<0.001	3	17.147	140.586	<0.001
Intercept	1	34282.42	275.99	<0.001	1	52.343	429.159	<0.001
Month	1	8343.53	67.17	<0.001	1	13.896	113.933	<0.001
Ivermectin	1	11.01	0.09	0.767	1	0.025	0.209	0.648
Month * ivermectin	1	30.52	0.24	0.621	1	0.191	1.57	0.213
Error	79	124.213			129	0.122		
Total	83				133			
R Squared = 0.738					R Squared = 0.760			

Figure 52: Regression plot of ash dry weight (in g; top) as a function of time in the field (month), reflecting natural dung degradation, for the five ivermectin treatments (cf. Figure 51 for classification)



12.4.4 Pitfall traps

Over 7 months, from May to November 2011, our pitfall traps caught a total of 2700 beetles, flies and hymenoptera belonging to 80 taxonomic groups (largely families), not all being strictly coprophilous. As could be expected, beetles were most common, especially rove beetles (Staphylinidae), some of which are parasitoids and predators of dung insects. We caught a substantial number of flies and wasps as well (Table 12; Figure 52). Numbers peaked in summer and declined in autumn, as could be expected.

Table 12: Organisms caught in the pitfall traps

Family	Genus	May	June	July	Aug	Sept	Oct	Nov
Coleoptera (beetles)								
Carabidae	Abax	0	0	1	0	0	0	0
	Bembidion	0	1	0	0	0	0	0
	Harpalus ?	0	1	0	0	0	0	0
	Olistophus ?	5	0	0	0	0	0	0
	Pterostichus	5	6	1	1	1	1	0
	Zabrina/Zabrus	0	6	1	1	0	0	0

Family	Genus	May	June	July	Aug	Sept	Oct	Nov
Chrysomelidae	Chaetochnema	3	3	2	1	0	12	0
	Lythraria	5	6	18	14	32	41	7
Curculionidae	(Apioninae)	5	6	2	0	0	0	1
	Sitona	1	1	2	1	0	2	0
Dermestidae	Dermetes	1	0	0	0	0	0	0
Elateridae	Agrypnus	3	1	0	0	0	0	0
Histeridae	Hister	0	0	0	1	0	0	0
Hydrophilidae	Cercyon	8	2	1	13	0	0	0
	Megasternum	1	1	0	6	2	0	0
	Sphaeridium	0	0	0	1	0	0	0
Lathridiidae	??	0	6	1	2	2	0	0
Leiodidae	??	1	0	0	0	0	0	0
Nitidulidae	Carpophilus	0	1	0	0	0	0	0
	Pria	0	0	0	1	0	0	0
Ptiliidae	Achrotrichis	11	5	3	13	0	0	0
Scarabaeidae	Aphodius	6	1	0	0	0	1	1
	Hoplia	0	7	0	0	0	0	0
	Onthophagus	24	5	2	2	0	0	0
Scolytidae	Lymantor	10	28	2	0	0	0	0
Silvanidae	?	0	0	0	0	0	1	1
Staphylinidae	Anotylus	763	165	92	30	11	0	0
	Atheta	105	39	65	25	7	2	0
	Autalia	2	3	1	0	0	0	0
	Carpelimus	0	0	0	1	0	0	0
	Cordalia	0	0	0	1	0	0	0
	Gyrohypnus	0	0	0	1	0	0	0
	Heterothops	0	0	8	0	0	0	0
	Megarthrus	2	0	0	0	0	0	0
	Ocypus	0	6	1	4	3	0	0
	Omalium	0	0	0	0	0	1	1

Family	Genus	May	June	July	Aug	Sept	Oct	Nov
	Othius	0	0	1	0	0	0	0
	Oxypoda	2	0	0	0	0	0	0
	Philonthus	12	6	5	4	2	1	0
	Plathystetus	3	0	0	0	0	0	0
	Tachyporus	0	0	2	0	0	0	0
	Xantholinus	0	0	0	0	0	0	3
	??	1	0	0	0	0	0	0
Total		**979**	**306**	**211**	**123**	**60**	**62**	**14**
Diptera: Brachycera (flies)								
Calliphoridae		0	0	0	1	0	0	0
		0	0	0	0	1	0	0
Drosophilidae		2	13	4	0	1	0	0
Lonchaidae		0	0	1	0	0	0	0
Muscidae		2	1	2	0	2	6	1
Phoridae		2	12	2	2	2	1	1
Sarcophagidae		2	0	0	3	0	0	0
Scathophagidae		1	0	0	0	0	1	2
Sepsidae		78	7	7	3	14	0	0
Sphaeroceridae		305	47	44	12	11	2	1
Syrphidae		0	0	0	1	0	0	0
??		0	4	1	19	0	0	0
Total		**392**	**84**	**61**	**41**	**31**	**10**	**5**
Diptera: Nematocera (flies)								
Anisopodidae		1	0	0	0	0	0	0
Cecidomyiidae		10	2	1	2	1	0	0
Ceratopognidae		0	0	2	1	1	0	0
Chironomidae		32	2	0	3	0	0	0
Psychodidae		19	1	2	2	2	0	0
Scatopsidae		3	0	1	2	1	0	0

Family	Genus	May	June	July	Aug	Sept	Oct	Nov
Sciaridae		0	4	1	0	0	1	0
Total		65	9	7	10	5	1	0
Hymenoptera (wasps)								
Bethylidae		1	0	0	0	0	0	0
Braconidae	Alysiinae	2	1	0	0	0	0	0
	??	0	1	2	0	1	1	0
Ceraphronidae	A	5	1	0	0	0	0	0
	B	0	22	0	0	0	0	0
	C	0	1	0	0	0	0	0
	??	0	0	2	1	0	0	0
Diapriidae	A	1	0	0	0	0	0	0
	B	4	6	0	0	0	0	0
	C	1	4	0	0	0	0	0
	D	0	6	0	0	0	0	0
	??	2	1	5	2	0	0	0
Eucolidae		3	0	0	0	1	1	0
Formicidae		27	22	12	45	5	1	0
Mymaridae		0	2	0	0	0	0	0
Platygastridae		0	0	1	0	0	0	0
Scelionidae		1	0	10	3	1	0	0
??		0	3	4	1	3	1	1
Total		**47**	**70**	**36**	**52**	**11**	**4**	**1**

12.5 Discussion (Zurich)

The Zurich experimental part indicates that ivermectin does negatively affect the dung decomposing community of beetles (Coleoptera), flies (Diptera, both Brachycera and Nematocera) and (largely parasitoid) wasps (Hymenoptera) in that it reduced the number of emerged insects in our structural experiment. Nevertheless, this did NOT substantially affect dung degradation over time in the parallel functional test using the same dung, as the break-down of dung pats contaminated with ivermectin was not detectably retarded. The reasons for this lack of congruence of both experiments here are not clear (cf. Römbke et al. 2010). It could be that dung pat degradation in Switzerland at least at the later stages is more a function of earthworm activity, which are common at the study site (and which were only rarely affected by ivermectin (see Chapter 15; Römbke et al.). Pitfall traps that were run parallel with the emergence (structural) experiment verified the ambient presence of most insect groups that

emerged from our experimental dung pats, albeit numbers were not always proportional (e.g. Sepsid flies). However, some knowingly common taxa were not at all represented in the emergence data (notably Scathophaga stercoraria), pointing to effects of the particular experimental procedure we used to catch emerging insects.

Our approach to obtain dung with naturally degraded ivermectin concentrations by collecting dung from cows treated with ivermectin at different time points worked well, as the residual ivermectin concentrations measured post-hoc by chemical analysis in the Montpellier dung excreted on days 3, 7, 14 & 28 after treatment (used in the Montpellier, Wageningen and Zurich experiments) indeed decreased as expected. The resulting concentration intervals were not as evenly spaced as desirable (see Figure 37 in the Montpellier report, Chapter 11), or when spiking dung instead, but this is a minor problem when analyzing ivermectin in a continuous fashion using regression. The pros and cons of this vs. the (less natural) spiking approach are clear, and our study here yielded roughly similar results to those obtained by Jochmann (2011) with spiked dung (unpublished data; cf. also Montpellier study unpublished material).

In our structural (emergence) experiment, the effect of ivermectin was not as gradual as expected (Figure 51). Even the dung sampled after 28 days, which had low concentrations of ivermectin (0.049 mg*kg-1 dry weight), resulted in significant reductions of dung insects in Zurich. Such stepwise responses are expected for the very sensitive taxa such as sepsids (Floate 1998a,b; Blanckenhorn et al. 2013a), but not necessarily in general, because overall emergence data (such as the Shannon index) necessarily average over sensitive, not so sensitive and insensitive insect groups. Such a stepwise response was not obtained in general by Jochmann et al. (2011).

Acknowledgement

The work described here was sponsored by the German Federal Environmental Agency (Project-No. FKZ 371063412).

12.6 References to chapter 12

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13 WP2: Non-target effects of ivermectin residues on structure and function of coprophilous communities of arthropods in a grassland: The Netherlands

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ABSTRACT

Once excreted by livestock, fresh dung attracts many species of dung flies and dung beetles. Some of these insects enhance dung degradation by their burrowing and fragmenting activities. It is well known that anti-parasitic substances administered to livestock, in particular the widely used macrocyclic lactones such as ivermectin, negatively affect the dung insect fauna when they are excreted. In the European Union and North America, the environmental risk of parasiticides and other veterinary medicines is addressed in an authorization process based on guidelines published by the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH). However, at present, there is no guidance on executing higher tier field tests. In order to address this problem, the German Federal Environmental Agency (UBA) sponsored field studies with a model VMP (i.e. ivermectin) in different ecological regions in Europe and North America. The practical work was performed in the Mediterranean (South France), Continental (Central Switzerland), Atlantic (The Netherlands) regions of Europe and in the Prairie (Western Canada) region of North America. In this paper the results from The Netherlands are reported. The study was conducted at a grassland site near the town of Wageningen. Dung from cattle topically treated with ivermectin was collected on Day 0 (pre-treatment), Day 3 post-treatment (average ivermectin concentration 2.84 mg/kg d.w.), Day 7 (2.48 mg/kg d.w.), Day 14 (0.69 mg/kg d.w.) and Day 28 (0.05 mg/kg d.w.). In a structural experiment, pats were left in the field for one week during May and emergence of adult insects was monitored for 3 months in the laboratory. Ivermectin negatively impacted the emergence of various groups of dung flies, most notably from the families Sphaeroceridae and Sepsidae. There was no recovery of the total number of flies up to Day 28 (0.05 mg ivermectin/kg dung dry weight). Ivermectin also had a negative effect on the emergence of dung beetles. However, there was no significant effect in the dung from Day 28 (0.05 mg/kg). In a second, functional experiment the dung was left in the field until November and dung weight was monitored. The treatments did not have an effect on the degradation rate of cattle dung in the study. The DT50 of organic matter was c. 2 months in all treatments. It is concluded that the study design is suitable to evaluate the effects of parasiticides on dung fauna structure and dung degradation under field conditions in higher-tier testing for risk assessment. However, (extreme) weather conditions during the experiments may interfere with the abundance of certain important groups of dung insects.

Key words: dung, beetles, flies, ecotoxicology, biodiversity

13.1 Introduction

In the European Union and North America, the environmental risk of veterinary medical products (VMP), and in particular, parasiticides is addressed in an authorization process. This process is based on guidelines published by the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH), which is a trilateral program to harmonize technical requirements for these drugs in Europe, Japan, and the United States. The Environmental Risk Assessment (ERA) allows a tiered approach. In Phase I (VICH 2000), general aspects regarding use and exposure are handled. In Phase II, ecotoxicological test requirements are specified (VICH 2004). An ERA of VMP for dung fauna is required if the substance acts as a parasiticide for the treatment of pasture animals. In Tier A of Phase II, studies are done to assess the non-target effects (if any) of fecal-excreted parasiticides on dung beetles and flies. If a risk is identified, additional studies are required (Tier B) to characterize the nature and extent of the non-target effects using representative non-target organisms as bioassays. However, further information on Tier B studies (and beyond) for dung organisms are missing in the guidelines. In fact, the only advice given on how to proceed beyond Tier A is a statement in the VICH (2004) guideline: "Regulatory guidance should be sought on appropriate studies." In the "Revised Guideline on Environmental Impact Assessment for Veterinary Medicinal Products" (EMA 2008) no further information on higher tier-testing of dung organisms is given.

Numerous studies have assessed the effects of VMPs on dung organisms, both in the laboratory and in the field within the last 25 years (e.g. Floate et al., 2005; Lumaret et al. 2012). However, these studies have been performed using different methods, on different insects, and with different VMPs species. A standardized approach is lacking, but is needed for use by the European Medicines Agency (EMA) as well as national authorities, industry, and consultants to complete the VICH requirement for higher tier studies (particularly those in the field) with VMPs.

In order to address this problem, the German Federal Environmental Agency (UBA) sponsored a project which had, among others, the aim to perform field studies with a model VMP (i.e. ivermectin) in different ecological regions in Europe and North America, using the structure and function of dung and soil organisms as assessment endpoints. The practical work was based on the recommendations compiled by Jochmann et al. (2012) and was performed in the Mediterranean (South France), Continental (Central Switzerland), Atlantic (The Netherlands) regions of Europe and in the Prairie (Western Canada) region of North America. In each of these four studies the same questions were addressed:

1. Does the use of ivermectin cause any effect on dung fauna biodiversity?
2. Does ivermectin affect the degradation of the dung?

Besides answering these questions practical recommendations concerning the practicability and the informational value of the recommendations of Jochmann et al. (2012) will be given.

In this contribution, the test performed at a grassland site near Wageningen (The Netherlands) is described, which used methods generally comparable to those used for the tests performed in Montpellier (France; Tixier et al., this report), Zurich (Switzerland; Blanckenhorn et al., this report) and Lethbridge (Canada; Floate et al., this report).

13.2 General Description of the Region

The experiment in The Netherlands was conducted in a pasture at Unifarm, the experimental biological farm of Wageningen UR, situated c. 500 m from the Wageningen campus (Figure 53). Wageningen is a town of c. 30,000 inhabitants. The experimental site was situated just north of the town in an area with arable land and grasslands intertwined. The site is close to a major ecological transfer zone.

History:

The field itself had three houses on it until 2006. In that year the houses were demolished and all hard materials were removed. A layer of black earth from the adjacent area was placed on top of the soil and the field was seeded with two biological varieties of English rye grass. Since that time the field has remained grassland. It was mowed several times a year. Fertilizers and pesticides have never been used in the field.

Figure 53: Aerial photograph of the Wageningen study site (Circle)

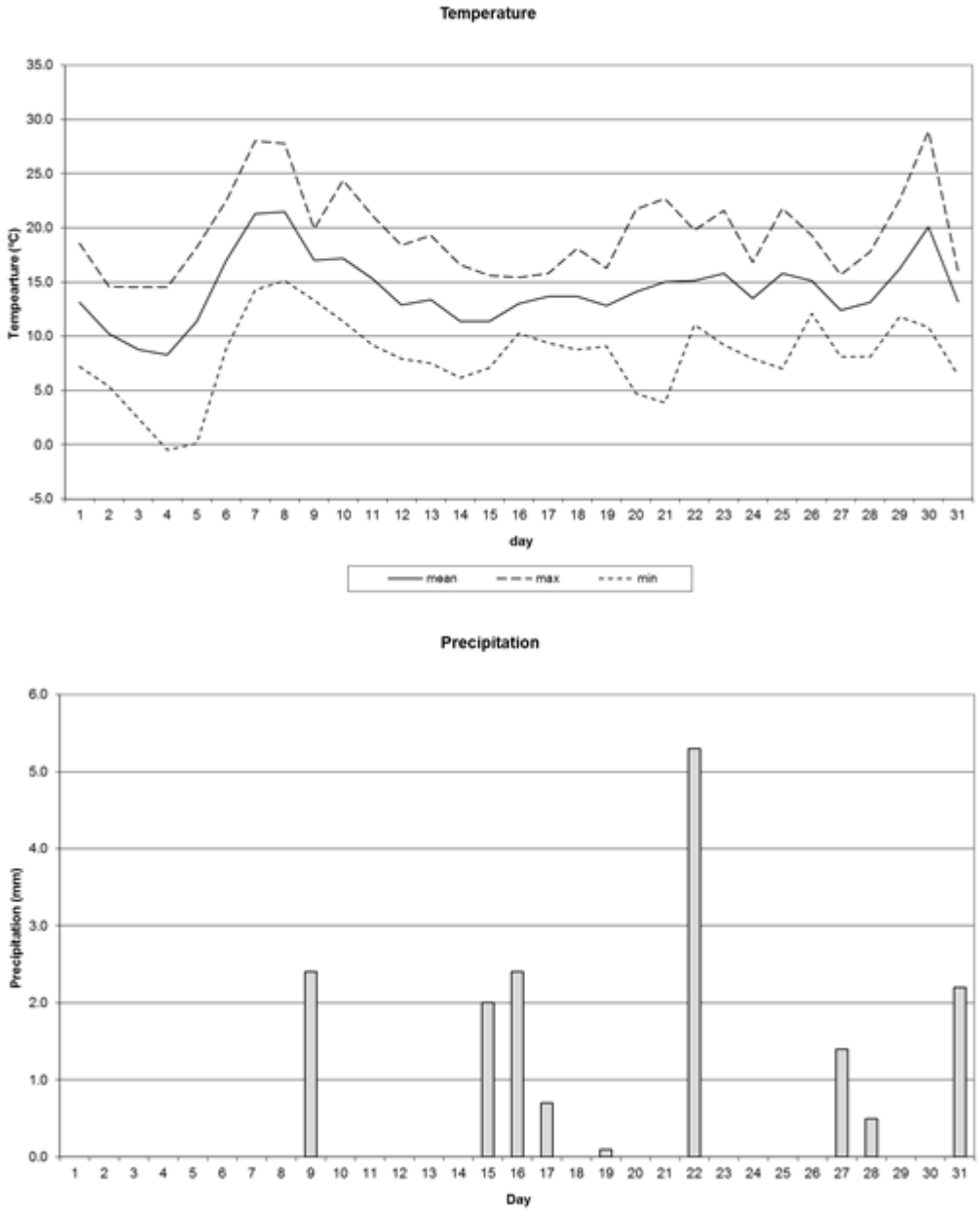
Lahr, pers.comm.

Meteorological conditions:

The experimental site has a temperate climate, characteristic of the Atlantic meteorological zone of northwestern Europe, i.e., relatively cool summers and mild, humid winters. Data on the experimental period were obtained from the meteorological station at Haarweg (Wageningen UR) just west of Wageningen. During 2011 annual total precipitation 2011 was 846 mm and annual average temperature 2011 was 10.5 °C.

Figure 54 shows the temperature and precipitation respectively during May 2011. During the week of the structural experiment (20-27 May) the weather was rather warm, maximum daily temperatures were above 20 °C on four out of seven days that week. It was a dry week, only one shower occurred, 5 mm after 2 days. The following period, when only the functional experiment continued in the field, was relatively cold and wet (June-August). In the fall of 2011 temperatures gradually dropped and rainy periods alternated with dry spells (September-November).

Figure 54: Daily temperatures and precipitation at the Haarweg meteorological station in Wageningen, The Netherlands, during May 2011



13.3 Materials and Methods

Experimental design

The experiment was conducted with dung prepared by the University of Montpellier in April 2011 (for details, see Lumaret et al. 2013). Cattle were treated with pour-on formulation of ivermectin at 0.5 mg/kg body weight. Dung was collected from the animals at Day 0 (pre-treatment control) and at Days 3, 7, 14, 28. The dung was stored in a fridge at -20 C and transported by car in frozen state to The Netherlands where it was also kept in a fridge until the start of the experiments.

In the spring 2011 the field experiments were started. Dung was thawed the night before and on the morning of 20 May the dung of each treatment was mixed and pats of 500 g were made by hand and

placed in the field. Parallel experiments were conducted to assess the structural and functional response to the ivermectin treatments. Wire mesh cages were placed over pats to exclude birds, but still allowed for colonization by insects (see Figure 54).

Study performance

Structure - For the structural part of the experiment, dung pats were left one week in the field on a plate with a layer of approximately 2 cm moist sand for colonisation by dung fauna. Small holes in the bottom of the plates allowed for drainage. Each ivermectin treatment was replicated ten times (Days 0, 3, 7, 14, 28 = 50 pats). On 27 May, after 7 days in the field, the pats of this sub experiment were collected and transported to the laboratory. In the laboratory each dung pat was placed in a specially designed emergence trap (See Figure 55) that captured any flying and crawling insects emerging from the dung in conservation solution. The pats were kept for more than three months in the traps. Emergent insects were collected at regular intervals, preserved in 70% ethanol, and later identified and enumerated. The experiment was terminated at 1 September. At that time emergence of adult insects had stopped. Careful examination of the dung and the underlying sand revealed only a few beetle larvae.

Function - The functional sub experiment was designed to test the effect of ivermectin residues on dung degradation. Twenty-five replicated pats were made for each treatment (Days 0, 3, 7, 14, 28 = 125 pats) and placed outdoors on May 20. Methods for preparation and placement of the function pats were the same as described for the structure pats, except that function pats were placed on netting (ca. 25 x 25 cm, mesh width 8 to 10 mm), which was in direct contact with the soil (Figure 56). Use of the netting facilitated recovery of pats from the field, but did not impede biological activity at the dung-soil interface by for example earthworms.

To measure changes in their weight over time, five 'function' pats per treatment were removed from the field and brought to the laboratory at five sampling times (T1-5): on June 16 (c. 1 month exposure), July 15 (c. 2 months exposure), August 24 (c. 3 months exposure), September 29 (c. 4 months exposure) and November 24 (c. 6 months exposure). Dung dry weight was determined by gravimetric determination of weight loss of the whole dung pat. A balance with a precision of 0.001g was used. Fresh weight was assessed immediately after collection of dung pads in the field. Dry weight was determined after drying the whole dung pad at 105±5°C for at least 24 hours. Samples were allowed to cool down in an exicator filled with silica gel for 45 minutes before re-weighing. Total organic matter was determined by gravimetric determination of sample weight loss on a balance with a precision of 0.001g. A randomized sub sample of the dry dung pads of approximately 3 g was taken and put in a ceramic crucible. The sample was then heated in a furnace at 550°C for at least 3 hours. After cooling down in an excicator filled with silica gel for 45 minutes samples were re-weighed.

Seasonal occurrence of dung fauna - Pitfall traps were operated at the study site to determine the composition of insects active at the study site before, during after the time that pats were exposed in the field (Figure 55). Pitfalls were self-made. Each trap comprised a plastic ring (diameter 30cm, height 15 cm) with a plastic bottom buried with the lip of the trap level with the soil surface. The trap held a preservative (4% formaldehyde solution) and was easily removed to recover insects collected during the trap period. A wire screen (2 cm grid) over the mouth of each trap supported a dung bait. Baits comprised fresh cattle dung (200g) and were replaced once every three to four days. Baits were made in advance and frozen until needed. Traps were operated from April 27 to May 9 (continuously) and once a month during one week between May 20 and September 15. Insects recovered from traps were stored in 70% ethanol and later sorted, counted and identified.

Figure 55: Pictures from the structural experiment in The Netherlands

From left clockwise: study site with wiring cages above the dung pats, individual dung pat on a plate with sand, emergence traps in the laboratory, and pitfall trap in the field.

Figure 56: Picture from the functional experiment in The Netherlands: Dung pat laid out on a net in the field

Analytical procedure for the determination of the antiparasitic agent ivermectin in cattle dung.

Reagents and equipment - Acetonitrile of HPLC-gradient grade (>99.9%) was supplied by VWR international (Radnor, Pennsylvania, USA). High purity water was prepared by a Milli-Q water purification

system (Millipore, Milford, MA, USA). N-methylimidazole (99% purity), triethylamine (99% purity), trifluoracetic anhydride (99% purity) and trifluoracetic acid (99% purity) were supplied by Sigma-Aldrich (Steinheim, Germany). The standard substances ivermectin (CAS RN: 70288-86-7, 96% purity) and doramectin (CAS RN: 117704-25-3, 90% purity) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). For extraction, a Vortex Genius 3 shaker (IKA, Staufen, Germany), a rotary shaker Swip KS-10, (Bühler, Tübingen, Germany), and an ultrasonic bath Sonorex Super RK255H (Bandelin electronic, Berlin, Germany) were used. As a centrifuge a Rotanta 460 R (Hettich, Tuttlingen, Germany) was used. Syringe filters (PTFE, 0.45 µm, 13 mm) were supplied by Wicom GmbH (Heppenheim, Germany). Solid phase extraction cartridges (Strata C-18-E, 500 mg, 55 µm, 70 Å) were purchased from Phenomenex (Torrance, California, USA).

Standard solutions - All standard solutions of doramectin and ivermectin were prepared in acetonitrile and stored at 18 °C. Stock solutions were made by dissolving 2.5 µg ivermectin or doramectin in 25 mL acetonitrile. These solutions were used to prepare ivermectin working standard solutions of 2000 and 100 µg/L, as well as doramectin working standard solutions of 2000 and 200 µg/L. With these solutions 9 calibration standards covering the relevant concentrations were prepared on daily basis.

Extraction and clean-up of the dung samples - The extraction procedure was mainly based on an adapted and optimized method as described by Litskas et al. (2010). Dung samples were homogenised. All dung samples taken from the field experiment have been dried in an oven after sampling, which did not have a significant effect on ivermectin concentration. After determining the water content of the different sample series a total dry matter of about 0.6 g for cattle dung was weight into polypropylene-vials. Cattle dung stored in the field were then moistened up to a water content of about 85% for cattle dung. The remoistened samples were kept at room temperature for 24 h. The initial dung samples already had a water content of about 82%. Internal standard doramectin dissolved in 25 mL acetonitrile was added in an amount near that expected in the sample. The suspension was kept for 15 min in an ultrasonic waterbath, 30 min on a mechanical shaker at room temperature at 450 rpm and again for 15 min in the ultrasonic water bath. Subsequently, the sample was centrifuged for 30 min at 2000 x g and 22 °C.

For the cattle dung samples stored in the field and for the soil samples 10 mL of each supernatant were directly transferred to polypropylene-vials. For the fresh dung samples the extracts were cleaned up with an additional solid phase extraction (SPE). For this, 20 mL of the solution were diluted with 66.6 mL water and 66.6 µL triethylamine. The SPE cartridges were conditioned with 10 mL acetonitrile and 10 mL acetonitrile/water (3:7, v/v). Subsequently, the samples were extracted with a C18-SPE-cartridge (500 mg, 55 µm, 70 Å) at a flow rate of 3 mL min-1. The extraction was followed by a washing step with 12 mL acetonitrile/water (1:1, v/v) at a flow rate of 8 mL min-1. With 5 mL of acetonitrile the analyte was eluted under gravity into a polypropylene-vial. The solvent was evaporated under a gently stream of nitrogen at 55 °C to complete dryness. For reconstitution 1000 µL acetonitrile were added to the sample. It was vortexed for 2 min, kept in an ultrasonic bath for 10 min, kept for 30 min on a mechanical shaker at 450 rpm, vortexed again for 30 s, and put again in the ultrasonic bath for 5 min. Finally, it was again kept for 30 min on a mechanical shaker at 450 rpm. After filtration (0.45 µm, PTFE) 700 µL of the solution were transferred into a HPLC-vial for the derivatization step.

Derivatization with trifluoracetic anhydride - The sample was derivatized according to an adapted procedure developed by Berendsen et al. (2007). First, 100 µL of N-methylimidazole/acetonitrile (1:1, v/v) were added to 700 µL of the reconstituted and filtered sample, followed by 50 µL of triethylamine. Subsequently, 100 µL of trifluoracetic anhydride/acetonitrile (1:1, v/v) were added. Finally, 50 µL of trifluoracetic acid were given into the vial. After each addition of reagent the closed HPLC vial was shaken for at least 5 seconds. To finish the derivatization reaction the closed HPLC-vials were kept for 30 minutes at 60°C in an oven.

High performance liquid chromatography with fluorescence detection (HPLC-FLD) - The determination with the HPLC-FLD was carried out within the first 48 hours after the derivatization. Chromatographic separation and determination was performed on an Agilent 1200 HPLC system (Agilent, Santa Clara, California, USA) consisting of a degasser (G1322A), a quaternary pump (G1311A), an autosampler and injection unit (G1329A), a column thermostat (G1316A) and a fluorescence detector (G1321A). The gradient elution was performed using a mobile phase of water (A) and acetonitrile (B) at a flow rate of 0.3 mL min−1 with the following gradient: 0–47 min, 60-100% B; 47–52 min, 100% B; 52–53 min, 100-60% B; 53-60 min, 60% B. The injection volume was 20 µL and the analytes were separated on a 150 mm × 2.1 mm i.d. 3 µm particle size, Dionex (Sunnyvale, California, USA) Acclaim PolarAdvantadge II C18-Column. The column temperature was 30 °C. The fluorescence detection was carried out at an excitation wavelength of 364 nm and an emission wavelength of 463 nm.

Figures of merit - The limit of detection (LOD) and limit of quantification (LOQ) values were determined with the calibration method on the basis of DIN 32645 (2008). The LOD for dung samples was 5.1 µg / kgdw and the LOQ 12.4 µg / kgdw. All data of the extractions with an inadequate recovery of the internal standard (<80% and >120%) were assorted. For the remaining samples the mean recovery of the internal standard doramectin was 96.8% (RSD 10.8%) for the dung samples stored in the field and 109.4% (RSD 2.7%) for the initial dung samples with the additional SPE-clean-up.

Statistical design and analysis

The dung pats of the structural and functional sub experiments were jointly placed in the field in a randomized block design. Forty cages (5x1m) were placed in seven rows of five cages. Distances between cages were c. 1m between the short ends of the cages and 3m between the sides. Each cage held five dung pats at distances of approximately 0.5 m. Two rows were used for the structural experiment. Under each of the ten cages in these rows one pat from each ivermectin treatment was placed. Hence, each of the ten cages replicated every treatment of the experiment. The remaining five rows were used for the functional sub experiment. Here, each cage in each row held five similarly treated dung pats, i.e., with the same ivermectin concentration and each row contained five cages with the five treatments (Day 0, 3, 7, 14, 28). At each of the five sampling times mentioned before (T1...T5), one dung pat from each cage in the functional experiment was sampled.

Fly and beetle counts from the structural experiment were very variable indeed with, for most species, a large number of zeroes along with a few sometimes relatively large counts. This may be due to the fact that dung pats are colonized by only few individuals that each produce many eggs that later emerge as adults. Thus, a dung pat that is not colonized by a particular species of dung insect may yield a ‘zero’ whereas a dung pat that is colonized by one individual of the same species may produce for example 15 adults. Only for species with relatively few ‘zeroes’ the counts themselves could be analyzed. For this purpose we used a generalized linear model employing the negative binomial distribution. The negative binomial distribution was used instead of the Poisson because there was sometimes heavily overdispersion in the data. The likelihood ratio test was used to test for pairwise differences. The block effect could unfortunately not be included in this analysis because this gave spurious significant but unrealistic differences due to high number of zeroes.

A logistic decay model, i.e. $y = \alpha/[1 + \exp(-\beta(\log(t) - \mu)]$, a sigmoid, was fitted to OM weights observed at time points T0=0, T1…T5 using nonlinear regression. The time t was the number of days since the start of the experiment. Note that log(t) is used as an argument since the resulting curve is flat at T0=0; it also gives a much better fit than with t as an argument. The null hypothesis of common parameters β and µ was tested against the alternative that these parameters are different for each concentration. This was tested using an F test. Note that in both the null model and the alternative model separate parameters α are used for each concentration.

To assess the breakdown of organic matter, an exponential decay model, i.e. $y = \alpha \exp(-\beta t)$, was fitted to OM weights observed at time points T1…T5 using nonlinear regression. The time t was the number of days since the start of the experiment. The null hypothesis of a common exponential decay parameter β was tested against the alternative that the decay parameter is different for each concentration. This was tested using an F test. Note that in both the null model and the alternative model separate parameters α are used for each concentration.

13.4 Results

13.4.1 Residues in dung

The average initial concentrations (T0) are the same as described in Chapter 11 (Tixier et al., this report):

Day 3 – 2.84 mg/kg d.w.

Day 7 – 2.48 mg/kg d.w.

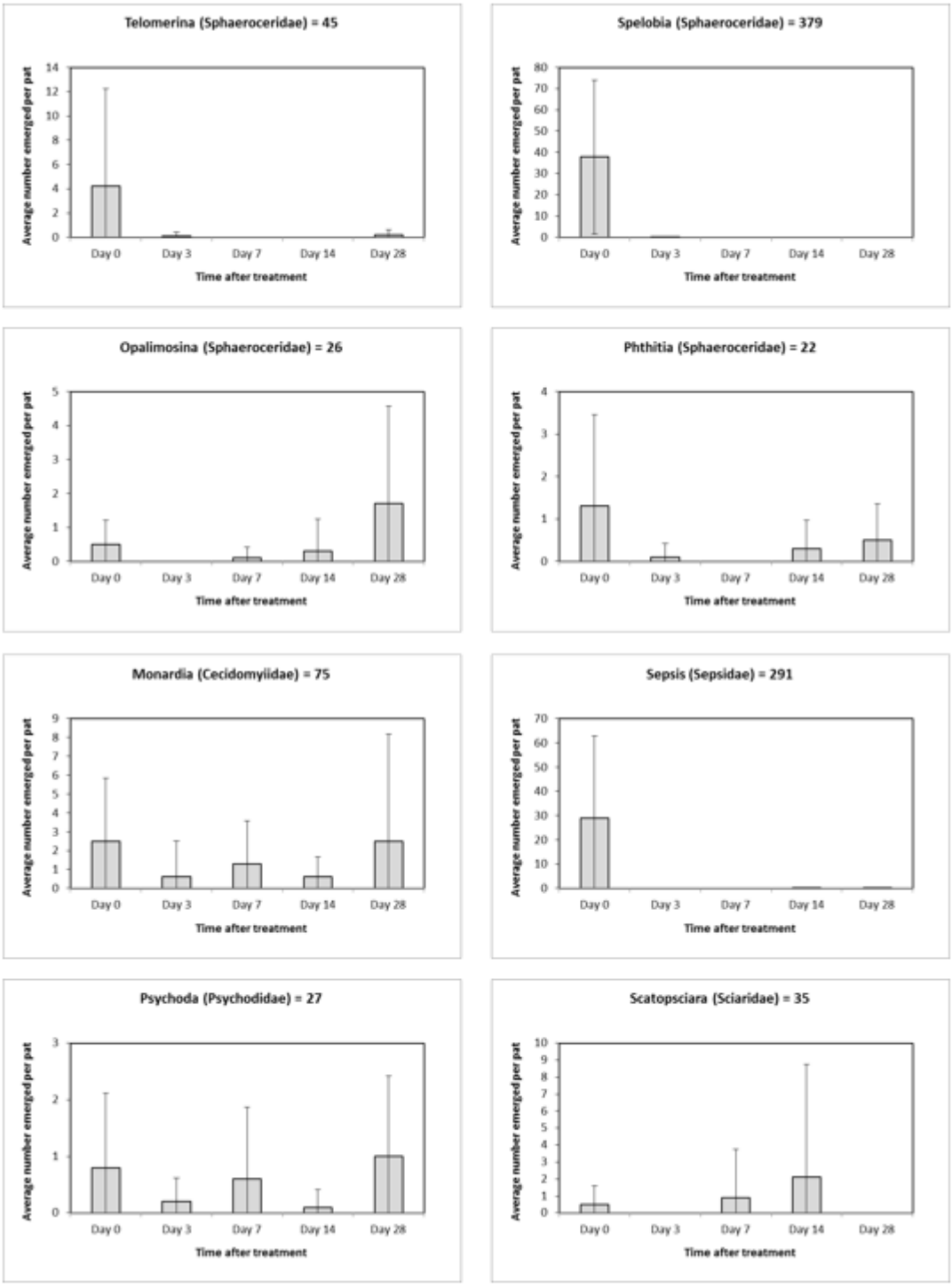
Day 14 – 0.69 mg/kg d.w.

Day 28 – 0.05 mg/kg d.w.

13.4.2 Emergence of dung flies

Flies were identified up to the genus level. A list of the groups that emerged from the dung during the emergence period of three months is given in Annex 13.1 to this chapter. Many groups of flies were only found sporadically and their numbers fluctuated erroneously. For these groups statistical analysis to detect any negative effects was not possible. For approximately one third of the groups the numbers were higher and an analysis could be performed. Figure 57 shows the average numbers of the most important fly groups and Figure 58 shows the total number of flies. The results of the statistical analysis are given in Table 13.

Figure 57: Average number of individuals (± S.E.) of different groups of flies emerged from dung collected at different time intervals following ivermectin treatment of cattle

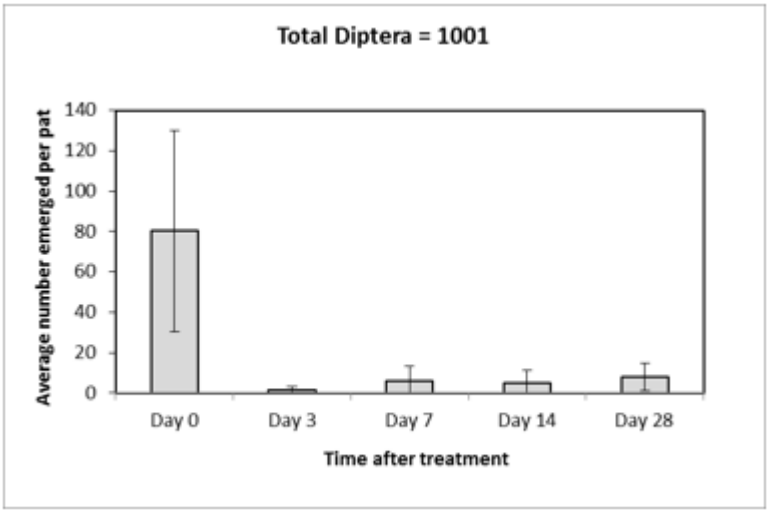


Day 0 (pre-treatment), Day 3 post-treatment (average ivermectin concentration 2.84 mg/kg d.w.), Day 7 (2.48 mg/kg d.w.), Day 14 (0.69 mg/kg d.w.) and Day 28 (0.05 mg/kg d.w.). The total number of individuals emerged during the whole experiment is indicated in the title of each graph.

The most affected were flies of the genus Telomerina, Spelobia (both Sphaeroceridae) and Sepsis (Sepsidae). For these groups numbers were suppressed in all treatments, without apparent recovery in the dung collected 28 days after application of ivermectin to the cattle. Also affected were Opalimosina (Sphaeroceridae) and Phthitia (both Sphaeroceridae), but these groups recovered on Day 7 and Day

14 respectively. Monardia (Cecidomyiidae), Psychoda (Psychodidae) and Scatopsciara (Sciaridae) were not significantly affected. The Sphaeroceridae as a whole were also significantly reduced without recovery in the Day 28 treatment and the same was observed for the family of Hybotidae and the total number of flies (see Table 13 and Figure 58).

Figure 58: Average number of individuals (± S.E.) of total flies (Diptera) emerged from dung collected at different time intervals following ivermectin treatment of cattle



Day 0 (pre-treatment), Day 3 post-treatment (average ivermectin concentration 2.84 mg/kg d.w.), Day 7 (2.48 mg/kg d.w.), Day 14 (0.69 mg/kg d.w.) and Day 28 (0.05 mg/kg d.w.). The total number of individuals emerged during the whole experiment is indicated in the title.

Table 13: Results of the structural experiment

Species	Family	Treatment effect compared to control (Day 0)			
		Day 28	Day 14	Day 7	Day 3
Flies (Diptera)					
Telomerina (Sphaeroceridae)	Sphaeroceridae	0.005	0.000	0.000	0.002
Spelobia (Sphaeroceridae)	Sphaeroceridae	0.000	0.000	0.000	0.000
Opalimosina (Sphaeroceridae)	Sphaeroceridae	0.145	0.605	0.177	0.024
Phthitia (Sphaeroceridae)	Sphaeroceridae	0.313	0.145	0.003	0.030
Coproica (Sphaeroceridae)	Sphaeroceridae	0.033*	0.141	0.033*	0.033*
Monardia (Cecidomyiidae)	Cecydomyiidae	1.000	0.134	0.465	0.134

Species	Family	Treatment effect compared to control (Day 0)			
Sepsis (Sepsidae)	Sepsidae	0.000	0.000	0.000	0.000
Psychoda (Psychodidae)	Psychodidae	0.779	0.060	0.731	0.156
Scatopsciara (Sciaridae)	Sciaridae	0.157	0.510	0.783	0.157
Total	Sphaeroceridae	0.000	0.000	0.000	0.000
Total	Cecydomyiidae	0.961	0.172	0.496	0.072
Total	Hybotidae	0.019	0.005	0.019	0.000
Total	Chironomidae	1.000	0.283	0.160	0.283
Total flies		0.000	0.000	0.000	0.000
Beetles (Coleoptera)					
Otophorus haemorrhoidalis_L	Aphodiidae	0.008	1.000	0.000	0.000
Agrilinus ater De Geer	Aphodiidae	0.406	0.406	0.002	0.159
Agrilinus scybalarius Fabr	Aphodiidae	0.000	0.018	0.291	0.000
Esymus pusillus Herbst	Aphodiidae	1.000	0.391	0.139	0.139
Calamosternus granarius L	Aphodiidae	0.110	0.110	0.277	0.570
Teuchestes fossor L	Aphodiidae	0.000	0.018	0.113	0.113
Planolinus uliginosus Harold	Aphodiidae	0.654	0.654	0.306	0.306
Euorodalus coenosus Panzer	Aphodiidae	0.306	0.313	0.306	0.306
Cryptopleurum sp	Hydrophilidae	0.239	0.204	0.489	0.489
Cercyon sp.1	Hydrophilidae	0.348	0.002	0.445	0.004
Cercyon sp.2	Hydrophilidae	0.207	0.161	0.047	0.000
Cercyon sp.3	Hydrophilidae	0.700	0.707	1.000	1.000
Oxytelidae sp.	Oxitaelidae	0.071	0.496	0.071	0.406

Species	Family	Treatment effect compared to control (Day 0)			
Total	Aphodiidae	0.192	0.192	0.000	0.000
Total	Hydrophilidae	0.852	0.003	0.035	0.000
Total	Scarabaeidae	1.000	0.239	0.239	1.000
Total dung beetles (without staphylinids)		0.383	0.027	0.000	0.000
Total Coleoptera		0.355	0.035	0.000	0.000

The table provides the p-value of a pairwise comparison using a generalized linear model (GLM) of the number of species emerged in an ivermectin treatment (Day 28, 14, 7 and 3) with the number emerged from the control dung (Day 0). Significant differences are shaded grey. *Analysis based on presence/absence only

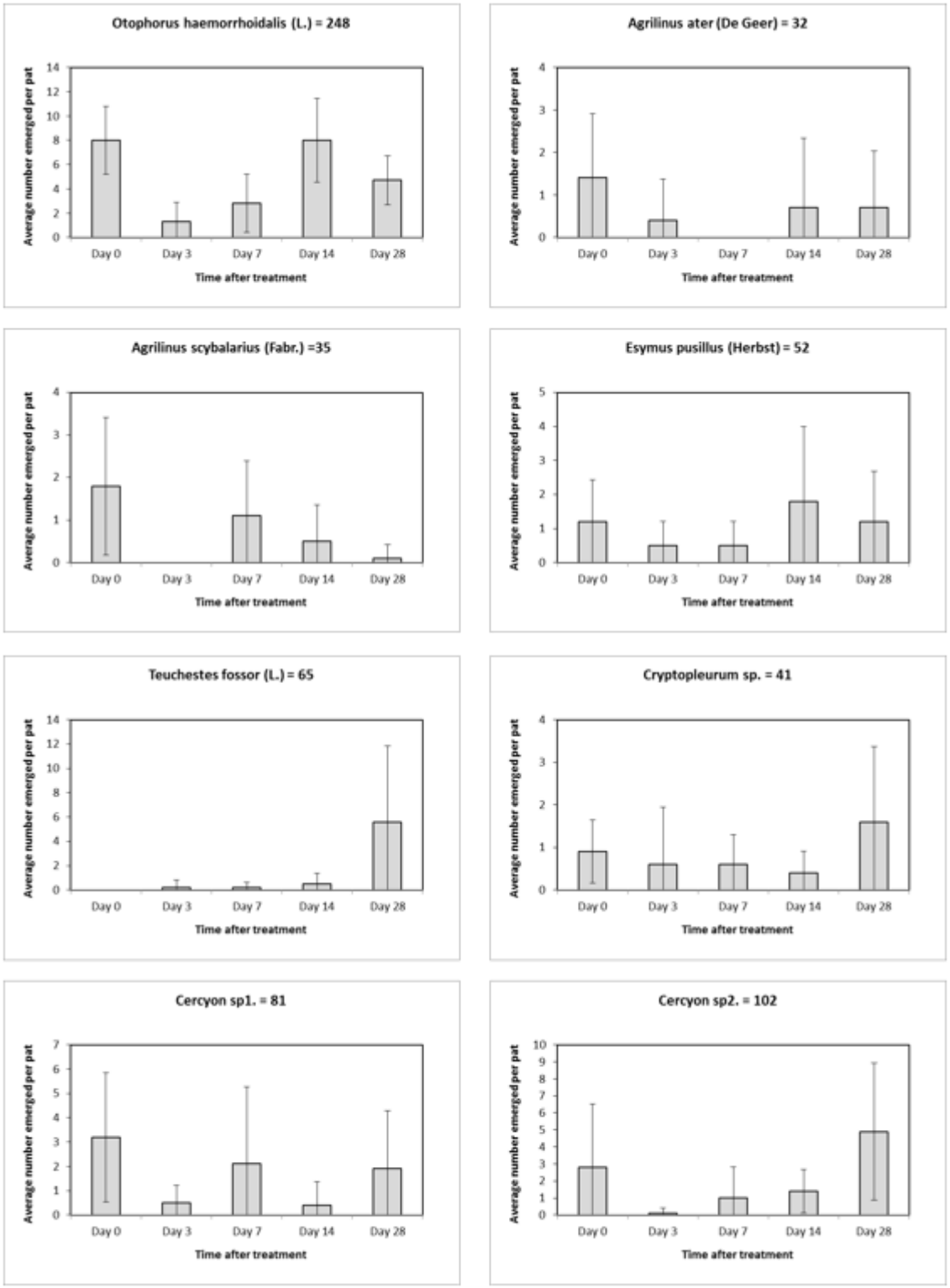
13.4.3 Emergence of dung beetles

The beetle species identified from the emergence experiments can be found in Annex 2. The dung beetle families of Aphodiidae and Hydrophilidae were represented by several species whereas only one species of Scarabaeidae was encountered. A few staphylinid beetles also emerged from the dung, but their number was low. As for the flies, the numbers of dung beetles were highly variable and as a consequence statistical analysis proved useful for only a few species and groups. These are shown in Figure 59.

Significant reductions of average numbers in the treatments were observed for *Otophorus haemorrhoidalis*, *Agrilinus ater*, *Agrilinus scybalarius*, *Teuchestes fossor* (all Aphodiidae), *Cercyon sp.1* and *Cercyon sp.2* (both Hydrophilidae) (Table 13). Recovery at lower ivermectin concentrations was not as clear cut as for affected groups of flies. Some species seemed to recover but decreased again in lower concentrations (O. haemorrhoidalis, A. ater, A. scybalarius, Cercyon sp.1). Others were only affected in the lower concentrations (T. fossor). Not all species were equally sensitive to the treatment. *Esymus pusillus, Calamosternus granarius, Planolinus uliginosus, Euorodalus coenosus* and *Cryptopleurum sp.* were not significantly affected (Table 13).

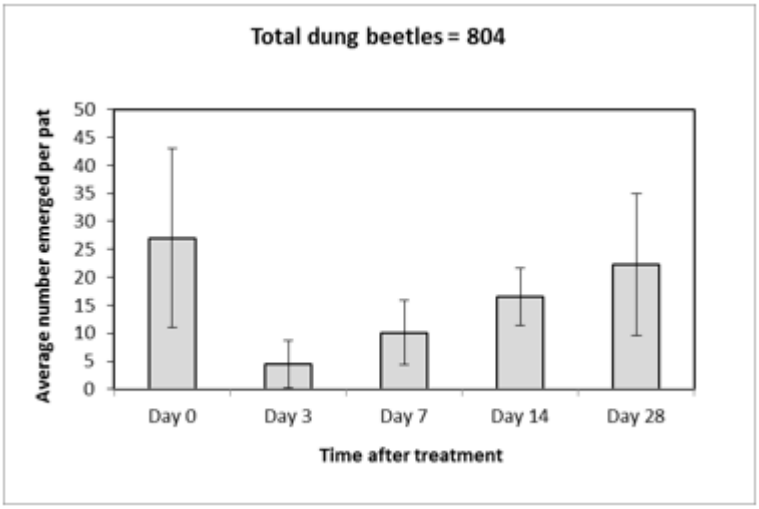
Negative effects could also be detected at higher taxonomic levels. Total numbers of Aphidiidae and Hydrophilidae were significantly reduced and recovers in the Day 14 and Day 28 treatments respectively. The total number of Scarabaeidae was not significantly altered. Both the total number of Coleoptera and of dung beetles (Figure 60) was affected in the dung of Day 3, 7 and 14, but not at Day 28.

Figure 59: Average number of individuals (± S.E.) of different groups of beetles emerged from dung collected at different time intervals following ivermectin treatment of cattle



Day 0 (pre-treatment), Day 3 post-treatment (average ivermectin concentration 2.84 mg/kg d.w.), Day 7 (2.48 mg/kg d.w.), Day 14 (0.69 mg/kg d.w.) and Day 28 (0.05 mg/kg d.w.). The total number of individuals per species emerged during the whole experiment is indicated in the title of each graph.

Figure 60: Average total number (± S.E.) of dung beetles (coleoptera without Staphylinidae) emerged from dung collected at different time intervals following ivermectin treatment of cattle

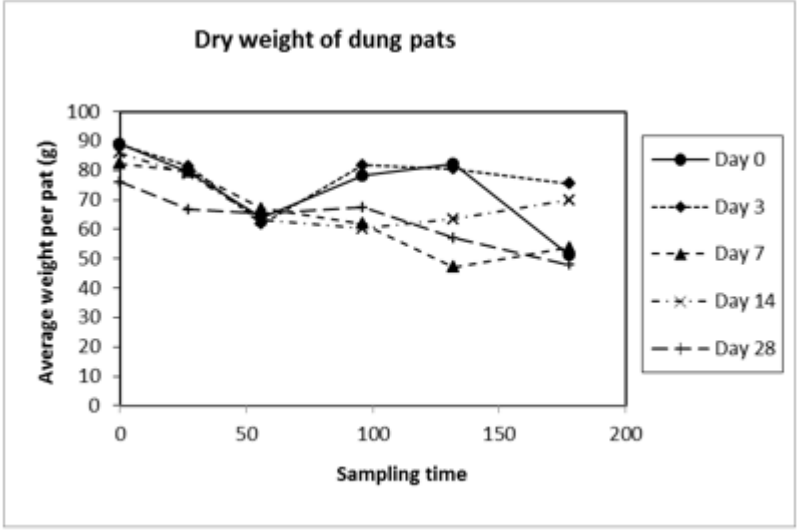


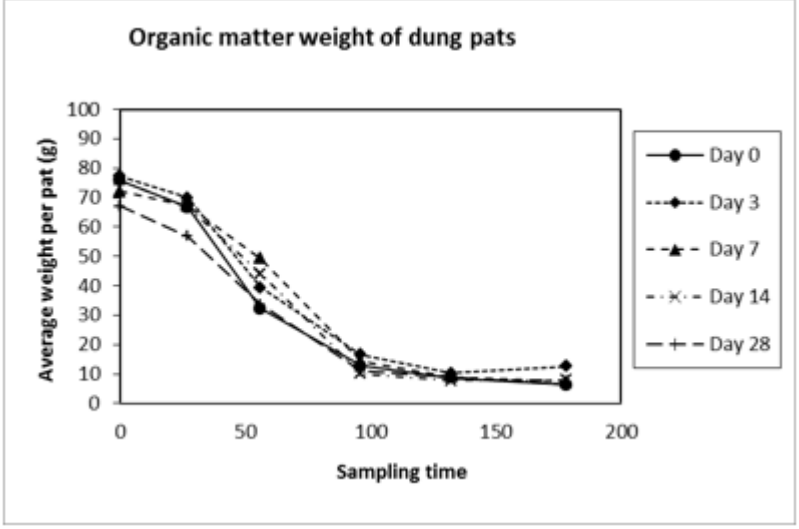
Day 0 (pre-treatment), Day 3 post-treatment (average ivermectin concentration 2.84 mg/kg d.w.), Day 7 (2.48 mg/kg d.w.), Day 14 (0.69 mg/kg d.w.) and Day 28 (0.05 mg/kg d.w.). The total number of individuals per species emerged during the whole experiment is indicated graph.

13.4.4 Dung breakdown

The total average dry weight and total average organic matter weight per dung pat are shown in Figure 60. It can be clearly seen that dry weight decreases over time, but that disappearance is not complete after 6 months (Figure 60). In the field it was observed that the dung structure of the pats was replaced by soil, apparently through the action of earthworms and other animals. The dry weight of this cow dung does therefore not seem a good indicator of its breakdown. The breakdown of organic matter starts slowly, probably because of the dry conditions during spring. The weight of the total organic matter per dung pat, however, shows an almost perfect sigmoidal disappearance (Figure 60), but ivermectin treatments hardly differ. This is confirmed by statistical analysis. The null hypothesis of common parameters β and μ could not be rejected ($F=1.72$, $p=0.101$), i.e., there is no significant effect of ivermectin treatment on organic matter breakdown. An objection to the F test used is that the residual variance is not constant, e.g. the variance at time point T2 is much larger than at time point T1. Therefore, a weighted nonlinear regression, with different weights for the different time points which are estimated from the variance at each time points, was also performed. Since there are no replicates at time point T0=0, the weight for this time points was taken to be equal to the weight for time point T1. This analysis gave very similar results. Again, the null hypothesis of common parameters is not rejected ($F=1.18$, $p=0.316$). Estimates of the parameters and time points are provided in Table 16. The estimated parameters allow estimation of the time at which specific percentages of OM weight have decayed. Such time points are given in Table 14, both for the separate curves as well as for the common curve.

Figure 61: Average dung pat dry weight and average dung pat organic matter weight for different ivermectin treatments





Day 0 (pre-treatment), Day 3 post-treatment (average ivermectin concentration 2.84 mg/kg d.w.), Day 7 (2.48 mg/kg d.w.), Day 14 (0.69 mg/kg d.w.) and Day 28 (0.05 mg/kg d.w.).

Table 14: Estimated parameters for a logistic decay model of dung organic matter breakdown

Parameter	Estimate	s.e.
β	-2.5320	0.0869
μ	4.07877	0.02676
$\alpha(Day\ 0)$	75.615	1.317
$\alpha(Day\ 3)$	79.164	1.339
$\alpha(Day\ 7)$	75.731	1.308
$\alpha(Day\ 14)$	75.952	1.317
$\alpha(Day\ 28)$	65.179	1.249

Day 0 (pre-treatment), Day 3 post-treatment (average ivermectin concentration 2.84 mg/kg d.w.), Day 7 (2.48 mg/kg d.w.), Day 14 (0.69 mg/kg d.w.) and Day 28 (0.05 mg/kg d.w.).

Table 15: Time (in days) needed for the breakdown to a given percentage remaining organic matter in cattle dung with different concentrations ivermectin

Percentage	Day 0	Day 28	Day 14	Day 7	Day 3	Mean	Common
50.0	55.6	55.6	56.8	65.7	61.8	59.1	59.1
25.0	85.6	89.5	86.1	96.5	97.6	91.1	91.2
12.5	119.3	129.2	118.7	129.8	138.9	127.2	127.4
10.0	131.7	144.1	130.6	141.8	154.1	140.5	140.7

Day 0 (pre-treatment), Day 3 post-treatment (average ivermectin concentration 2.84 mg/kg d.w.), Day 7 (2.48 mg/kg d.w.), Day 14 (0.69 mg/kg d.w.) and Day 28 (0.05 mg/kg d.w.).

13.4.5 Pitfall traps

In the period between May and September, 26485 animals were caught in the pitfall traps, mainly Diptera, but also dung beetles, Staphylinidae, Formicidae, other Hymenoptera, Araneae and mites (Table 16). While the majority of the caught organisms are coprophilous (specifically most of the Diptera and a majority of the beetles), there are also organisms living mainly in the soil (e.g. most mites). Most rove beetles (Staphylinidae) are predators. Depending on the organism group, numbers peaked in summer (e.g. Diptera), did not change during the sampling period (Coleoptera) or were most often caught in spring or autumn (Formicidae). Due to the high number of animals, taxonomic determination is still on-going.

Table 16: Numbers of individuals caught in pitfall traps in the period between May (continuously) and September (one week per month) from eight organism groups caught

Month	Diptera	Staphy-li-nidae	Coleop-tera	Formi-cidae	Araneae	Other Hy-men.	Acari
May I	566	111	345	343	253	6	2
May II	6135	184	300	130	178	3	91
May III	2451	314	315	99	262	17	64
June	3596	189	123	49	166	10	91
July	2950	122	376	190	541	44	57
August	1253	157	525	243	337	40	27
September	2271	155	487	142	205	113	217
Sum	**19222**	**1232**	**2471**	**1196**	**1942**	**233**	**549**

Time (in days) needed for the breakdown to a given percentage remaining organic matter in cattle dung with different concentrations ivermectin.

13.5 Discussion (limited to Dutch Atlantic Zone)

13.5.1 Details

The total number of flies that emerged from the dung pats was rather low, i.e., 1001 specimens in the whole experiment. For example, not a single specimen of Scathophaga stercoraria emerged from the pats although it is often observed on cow dung in the surroundings of Wageningen (personal observation, J. Lahr). The reason for this observation is not exactly clear but several possibilities come to mind. It could be due to weather conditions. Many dung flies are known to hibernate during the summer. Perhaps hibernation started early in 2011 because the spring was relatively dry. It could also be due to the study site. However, in a similar experiment performed with pony dung the year before in early June, some 880 fly specimens emerged on average from every untreated control dung pat, compared to an average of 80 individuals per cow dung pat in the present experiment (Lahr et al., 2013). So enough flies may occur in the area. Another possibility is that freezing of the dung prior to the field experiments made the dung less attractive by reducing the release of the most volatile odorants. This remains to be investigated and this would be useful information for any recommendations to be given for the performance of such field studies. Due to the lack of detailed taxonomic information on the very high number of dung organisms, other insects and (few) mites caught in the pitfall traps it is not possible yet decide whether the whole dung organism community was collected in the emergence experiment. The results of the cow dung studies reported here are quite similar to those in the previous tests with pony dung (Lahr et al., 2010, 2011). Dung insects are affected by ivermectin in dung contaminated by routine treatments of livestock, especially dung flies, but there is no effect of ivermectin on the rate of organic matter breakdown in dung in the field. Sphaeroceridae (lesser dung flies) were the most abundant fly family and they were affected by both treatments. Thus, they can be a suitable indicator group for effects of parasiticides.

13.5.2 Summary

- Ivermectin also negatively impacts the emergence of dung beetles from treated cattle dung. The total number of dung beetles recovers at Day 28

- The treatments do not have an effect on the degradation rate of cattle dung in the study (DT50 organic matter is c. 2½ months)
- The study design is suitable to evaluate the effects of parasiticides on dung fauna structure and dung degradation under field conditions in higher-tier testing for risk assessment
- However, (extreme) weather conditions during the experiments may interfere with the abundance of certain important groups of dung insects

13.5.3 Outlook

Future higher tier field experiments with ivermectin and dung from cattle should include dung samples taken 56 days after treatment or even later.

13.6 References to chapter 13

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Annex 13.1

Occurrence of dung flies

Identification by W. Blanckenhorn (University of Zurich).

- *Microchrysa*
- *Telomerina (Sphaeroceridae)*
- *Spelobia (Sphaeroceridae)*
- *Opalimosina (Sphaeroceridae)*
- *Trachyopella (Sphaeroceridae)*
- *Coproica (Sphaeroceridae)*
- *Ceroptera (Sphaeroceridae)*
- *Phthitia (Sphaeroceridae)*
- *Monardia (Cecidomyiidae)*
- *Oligotrophidi fem. (Cecidomyiidae)*
- *Micromya (Cecidomyiidae)*
- *Heteropezini (Cecidomyiidae)*
- *Lestodiplosini (Cecidomyiidae)*
- *Ocydromia (Hybotidae)*
- *Drapetis (Hybotidae)*
- *Ceratopognidae*
- *Chaetocladius (Chironomidae)*
- *Prosmittia (Chironomidae)*
- *Heterotanytarsus (Chironomidae)*
- *Mesocricotorpus (Chironomidae)*
- *Chironomidae fem.*
- *Sepsis (Sepsidae)*
- *Psychoda (Psychodidae)*
- *Scatopsciara (Sciaridae)*
- *Megaselia (Phoridae)*
- *Delia (Anthomyiidae)*
- *Chalcidoidea Hebecnema (Muscidae)*
- *Hapleginella (Chloropidae)*

Annex 13.2

Occurrence of dung beetles

Identification by J.-P. Lumaret (University of Montpellier).

Family Aphodiidae:

- *Agrilinus ater (De Geer)*
- *Agrilinus scybalarius (Fabr.)*
- *Aphodius fimetarius (L.)*
- *Calamosternus granarius (L.)*
- *Chilothorax distinctus (Müller)*
- *Chilothorax lineolatus Ill.*
- *Chilothorax sticticus (Panzer)*

- *Colobopterus erraticus (L.)*
- *Esymus pusillus (Herbst)*
- *Euorodalus coenosus (Panzer)*
- *Otophorus haemorrhoidalis (L.)*
- *Oxyomus silvestris (Scop.)*
- *Planolinus uliginosus (Harold)*
- *Teuchestes fossor (L.)*
- *Trichonotulus scrofa (Fabr.)*

Family Scarabaeidae:

- *Onthophagus similis Scriba*

Family Hydrophilidae:

- *Cercyon sp 1*
- *Cercyon sp 2*
- *Cercyon sp 3*
- *Cryptopleurum sp.*
- *Sphaeridium sp1.*

Staphylinoidea:

- *Family Oxytelidae*

14 WP2: Non-target effects of ivermectin residues on structure and function of coprophilous communities of arthropods in a grassland: Western Canada

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ABSTRACT

In the European Union and North America, the environmental risk of veterinary medical products (VMP), and parasiticides in particular, is addressed in an authorization process under the purview of the European Union. This process requires the development of a standardized protocol to assess the non-target effects of VMPs to dung-breeding arthropods (structure) and dung degradation (function) for dung voided by treated livestock. In a coordinated project sponsored by the German Federal Environmental Agency (UBA), we performed parallel field studies with the parasiticide, ivermectin, in the Mediterranean (South France), Continental (Central Switzerland), Atlantic (The Netherlands) regions of Europe and the Prairie (Western Canada) region of North America. Results reported here are limited to the latter study.

The Canadian study was performed as two experiments, one each in 2011 (function, structure) and 2012 (structure). Each experiment assessed the effect of six treatments. The ivermectin concentrations associated with these treatments in 2011 were: 0 (control), 0.015 (excreted on Day 56), 0.065 (D28), 0.341 (D14), 7.675 (D7) and 5.029 (D3) mg ivermectin/kg dung dry weight. Ivermectin concentrations were not measured in 2012, but were assumed to be similar. For the 'function' part of the study, results from 2011 did not detect an effect of residues on dung degradation. The function aspect of the study was not repeated in 2012. For the 'structure' part of the study, results from 2011 indicated that residues suppressed numbers of the beetle *Sphaeridium lunatum* (in treatments D3 and D7), of the fly *Coproica mitchelli* (D3, D7), and of sphaerocerid flies (D3). Results from 2012 indicated that residues suppressed numbers of ptiliid beetles (D3, D7), C*. mitchelli* (D3, D7, D14, D28), Sepsis flies (D3, D7, D14, D28, D56), an unidentified fly 'L' (D3, D7) and staphylinid beetles 'B' (D3, D7). Pitfall traps operated in parallel with the experiments verified the ambient presence of most insect groups that emerged from our experimental dung pats, although numbers were not always proportional. Results at study sites in other countries were qualitatively similar, demonstrating that such a field test to assess the entire dung community is robust despite strongly varying environmental circumstances.

Key words: dung, beetles, flies, ecotoxicology, biodiversity

14.1 Introduction

In the European Union and North America, the environmental risk of veterinary medical products (VMP), and in particular, parasiticides is addressed in an authorization process. This process is based on guidelines published by the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH), which is a trilateral program to harmonize technical requirements for these drugs in Europe, Japan, and the United States. The Environmental Risk Assessment (ERA) allows a tiered approach. In Phase I (VICH 2000), general aspects regarding use and exposure are handled. In Phase II, ecotoxicological test requirements are specified (VICH 2004). An ERA of VMP for dung fauna is required if the substance acts as a parasiticide for the treatment of pasture animals. In Tier A of Phase II, studies are done to assess the non-target effects (if any) of fecal-excreted parasiticides on dung beetles and flies. If a risk is identified, additional studies are required (Tier B) to characterize the nature and extent of the non-target effects using representative non-target organisms as bioassays. However, further information on Tier B studies (and beyond) for dung organisms are missing in the guidelines. In fact, the only advice given on how to proceed beyond Tier A is a statement in the VICH (2004) guideline: "Regulatory guidance should be sought on appropriate studies." In the "Revised Guideline on Environmental Impact Assessment for Veterinary Medicinal Products" (EMA 2008) no further information on higher tier-testing of dung organisms is given.

Numerous studies have assessed the effects of VMPs on dung organisms, both in the laboratory and in the field within the last 25 years (e.g. Lumaret et al. 2012). However, these studies have been performed using different methods, on different insects, and with different VMPs species. A standardized approach is lacking, but is needed for use by the European Medicines Agency (EMA) as well as national authorities, industry, and consultants to complete the VICH requirement for higher tier studies (particularly those in the field) with VMPs.

To address this problem, the German Federal Environmental Agency (UBA) sponsored a project which had, among others, the aim to perform field studies with a model VMP (i.e. ivermectin) in different ecological regions in Europe and North America, using the structure and function of dung and soil organisms as assessment endpoints. The practical work was based on the recommendations compiled by Jochmann et al. (2012) and was performed in the Mediterranean (South France), Continental (Central Switzerland), Atlantic (The Netherlands) region of Europe and in the Prairie (Western Canada) region of North America. In each of these four studies the same questions were addressed:

1. Does the use of ivermectin cause any effect on dung fauna biodiversity?
2. Does ivermectin affect the degradation of the dung?

Besides answering these questions practical recommendations concerning the practicability and the informational value of the recommendations of Jochmann et al. (2012) will be given. In this contribution, the test performed at a grassland site near Lethbridge (Canada) is described, which used methods generally comparable to those used for the tests performed in Wageningen (The Netherlands; Lahr et al., this report), Zurich (Switzerland; Blanckenhorn et al., this report) and Montpellier (France; Tixier et al., this report).

14.2 General Description of Region

The Canadian study was performed at Lethbridge in the province of Alberta. Lethbridge is a city of ca. 90,000 located at an elevation of 929 m. It is located ca. 75 km north of the United States border and ca. 100 km east of the Rocky Mountains. The region surrounding Lethbridge is part of the province's Mixedgrass Natural Subregion. The subregion is characterized with rolling and hummocky till plains and level lacustrine areas dominated by Dark Brown Chernozem soils (NRC 2006). Weather records (1981-2010) for Lethbridge identify an annual precipitation of 395 mm with the majority of precipitation occurring in May (54.5 mm) and June (84.4 mm). Mean daily air temperatures for January and

July are -5.3 and 18.4 oC, respectively. Per month, daily mean wind speeds average 12.9 to 18.5 km/hr. Lethbridge has an average of 124 frost-free days per year with an average date for first fall frost of September 18 and an average date for last spring frost of May 17 (http://www1.agric.gov.ab.ca/$department/deptdocs.nsf/all/sag6301). An estimated 85% of the subregion is planted to annual crops (e.g., wheat, barley, canola) with about 5% of the land being irrigated (NRC 2006). Pastured cattle and horses are common on properties near Lethbridge.

14.3 Materials and Methods

The Canadian study comprised two experiments, one each in 2011 and 2012. In 2011, low recovery of insects in Trial 1 led to a second trial at the same site. The experiment in 2012 was performed using dung from a different group of cattle treated a year after the cattle treated for Experiment 1. Thus, Experiment 1 (two trials) and Experiment 2 (one trial) were independent, replicated experiments. Experiment 1 included a 'structure' (species diversity) component for both Trials 1 and 2, but only Trial 1 included a 'function' (dung degradation) component. Experiment 2 included a structure component, but did not include a function component.

14.3.1 Analytical procedure for the determination of the antiparasitic agent ivermectin in cattle dung

Reagents and equipment

Acetonitrile of HPLC-gradient grade (>99.9%) was supplied by VWR international (Radnor, Pennsylvania, USA). High purity water was prepared by a Milli-Q water purification system (Millipore, Milford, MA, USA). N-methylimidazole (99% purity), triethylamine (99% purity), trifluoracetic anhydride (99% purity) and trifluoracetic acid (99% purity) were supplied by Sigma-Aldrich (Steinheim, Germany). The standard substances ivermectin (CAS RN: 70288-86-7, 96% purity) and doramectin (CAS RN: 117704-25-3, 90% purity) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). For extraction, a Vortex Genius 3 shaker (IKA, Staufen, Germany), a rotary shaker Swip KS-10, (Bühler, Tübingen, Germany), and an ultrasonic bath Sonorex Super RK255H (Bandelin electronic, Berlin, Germany) were used. As a centrifuge a Rotanta 460 R (Hettich, Tuttlingen, Germany) was used. Syringe filters (PTFE, 0.45 µm, 13 mm) were supplied by Wicom GmbH (Heppenheim, Germany). Solid phase extraction cartridges (Strata C-18-E, 500 mg, 55 µm, 70 Å) were purchased from Phenomenex (Torrance, California, USA).

Standard solutions

All standard solutions of doramectin and ivermectin were prepared in acetonitrile and stored at 18 °C. Stock solutions were made by dissolving 2.5 µg ivermectin or doramectin in 25 mL acetonitrile. These solutions were used to prepare ivermectin working standard solutions of 2000 and 100 µg/L, as well as doramectin working standard solutions of 2000 and 200 µg/L. With these solutions 9 calibration standards covering the relevant concentrations were prepared on daily basis.

Extraction and clean-up of the dung and soil samples –

The extraction procedure was mainly based on an adapted and optimized method as described by Litskas et al. (2010). Initial dung samples were homogenised. A total amount of about 3 g was weight into polypropylene-vials. The initial dung samples had a water content of about 82%. Internal standard doramectin dissolved in 25 mL acetonitrile was added in an amount near that expected in the sample. The suspension was kept for 15 min in an ultrasonic waterbath, 30 min on a mechanical shaker at room temperature at 450 rpm and again for 15 min in the ultrasonic water bath. Subsequently, the sample was centrifuged for 30 min at 2000 x g and 22 °C. For the fresh dung samples the extracts were cleaned up with an additional solid phase extraction (SPE). For this, 20 mL of the supernatant were diluted with 66.6 mL water and 66.6 µL triethylamine. The SPE cartridges were conditioned with 10 mL acetonitrile and 10 mL acetonitrile/water (3:7, v/v). Subsequently, the samples were extracted

with a C18-SPE-cartridge (500 mg, 55 µm, 70 Å) at a flow rate of 3 mL min-1. The extraction was followed by a washing step with 12 mL acetonitrile/water (1:1, v/v) at a flow rate of 8 mL min-1. With 5 mL of acetonitrile the analyte was eluted under gravity into a polypropylene-vial. The solvent was evaporated under a gently stream of nitrogen at 55 °C to complete dryness. For reconstitution 1000 µL acetonitrile were added to the sample. It was vortexed for 2 min, kept in an ultrasonic bath for 10 min, kept for 30 min on a mechanical shaker at 450 rpm, vortexed again for 30 s, and put again in the ultrasonic bath for 5 min. Finally, it was again kept for 30 min on a mechanical shaker at 450 rpm. After filtration (0.45 µm, PTFE) 700 µL of the solution were transferred into a HPLC-vial for the derivatization step.

Derivatization with trifluoracetic anhydride

The sample was derivatized according to an adapted procedure developed by Berendsen et al. (2007). First, 100 µL of N-methylimidazole/acetonitrile (1:1, v/v) were added to 700 µL of the reconstituted and filtered sample, followed by 50 µL of triethylamine. Subsequently, 100 µL of trifluoracetic anhydride/acetonitrile (1:1, v/v) were added. Finally, 50 µL of trifluoracetic acid were given into the vial. After each addition of reagent the closed HPLC vial was shaken for at least 5 seconds. To finish the derivatization reaction the closed HPLC-vials were kept for 30 minutes at 60°C in an oven.

High performance liquid chromatography with fluorescence detection (HPLC-FLD)

The determination with the HPLC-FLD was carried out within the first 48 hours after the derivatization. Chromatographic separation and determination was performed on an Agilent 1200 HPLC system (Agilent, Santa Clara, California, USA) consisting of a degasser (G1322A), a quaternary pump (G1311A), an autosampler and injection unit (G1329A), a column thermostat (G1316A) and a fluorescence detector (G1321A). The gradient elution was performed using a mobile phase of water (A) and acetonitrile (B) at a flow rate of 0.3 mL min−1 with the following gradient: 0–47 min, 60-100% B; 47–52 min, 100% B; 52–53 min, 100-60% B; 53-60 min, 60% B. The injection volume was 20 µL and the analytes were separated on a 150 mm × 2.1 mm i.d. 3 µm particle size, Dionex (Sunnyvale, California, USA) Acclaim PolarAdvantadge II C18-Column. The column temperature was 30 °C. The fluorescence detection was carried out at an excitation wavelength of 364 nm and an emission wavelength of 463 nm.

Figures of merit

The limit of detection (LOD) and limit of quantification (LOQ) values were determined with the calibration method on the basis of DIN 32645 (2008). The LOD for dung samples was 5.1 µg / kgdw and the LOQ 12.4 µg / kgdw. All data of the extractions with an inadequate recovery of the internal standard (<80% and >120%) were assorted. For the remaining samples the mean recovery of the internal standard doramectin was 101.3% (RSD 8.9%) for the initial dung samples.

14.3.2 Experiment 1 (2011)

Experiment 1 was performed at the Lethbridge Research Centre, which abuts the eastern boundary of the City of Lethbridge. The study site (49°41'25.46"N; 112°46'26.15"W) was a grassy mowed area immediately adjacent to a small pasture and ca. 50 m from two cattle feedlots holding ca. 50 cattle (Figure 62). The pasture and feedlots are used yearly to house various breeds of cattle, varying with the research needs of the Centre's scientists. The pasture was seeded to tame grasses many years prior to the study. It may also have been chemically fertilized but, if so, not for several years prior to the study. This site has been successfully used in previous research to study aspects of dung insect ecology (Floate 1998a; Floate 1998b; Floate and Gill 1998; Floate et al. 2002; Tiberg and Floate 2011).

Collection and storage of dung.

Dung was collected from untreated cattle (Day 0) the week of March 7. Cattle comprised a group of seven Holstein steers (558 kg ave. body weight) maintained on a diet of hay and housed in common pens. On March 15, the steers were treated with a topical formulation of ivermectin (Ivomec® pour-on for cattle; Lot: NF50140) at the recommended dose (500 mcg ivermectin/kg body weight). Dung subsequently was collected from the treated animals 3, 7, 14, 28 and 56 days post-application. For each collection date, fresh dung (<3 h old) from multiple pats was placed in large plastic bags and then in plastic pails (11 liter capacity; Product no. 723, ProWestern Plastics Ltd., St Albert, Alberta; 1 bag per pail). Bags were sealed and lids placed on the pails to prevent desiccation during storage and to allow pails to be stacked. Pails were held at -20°C until used. As advised by Jochmann et al. (2011), sufficient dung was collected to allow for the experiment to be repeated if necessary.

Structure (Trials 1 & 2)

To assess the effect of residues on dung-breeding insects, dung was thawed, thoroughly mixed, and then formed into pats of a standard shape and volume (0.5 litre) using a plastic mold. Pats were placed on a layer of sand (1 cm in depth) in polystyrene plastic (Styrofoam®) plates (23 cm in diameter) (Figure 62). Ten such pats were made for each treatment (Days 0, 3, 7, 14, 28, 56 = 60 pats) and placed outdoors in a randomized grid. Wire mesh cages were placed over pats to exclude birds, but still allowed for colonization by insects. Small holes in the bottom of the plates allowed for drainage.

Pats were exposed in the field for 7 days for Trial 1 (June 9 – 16) and for 6 days for Trial 2 (June 29 – July 5). After exposure, plates and their associated pats were brought indoors and placed in separate insect emergence cages held at about 22°C. Cages were 11 litre pails of the same type as used to freeze dung, but fitted with fine mesh sleeves (Figure 62). Cages were examined weekly for insect emergence. Adult insects observed in cages were removed, stored in 70% ethanol and later sorted, counted and identified. To reduce insect mortality due to desiccation, distilled water (50 ml) was added to each plate every second week. After a period of about 8 weeks, no further adult insects were observed and pats were broken apart. Careful examination of the dung and the underlying sand revealed only a few beetle larvae.

Pitfall traps were operated at the study site to determine the composition of the activity of insects at the study site during the time that pats were exposed in the field (Figure 62). Each trap comprised two plastic pails (1 L capacity), one nested inside the other, buried with the lip of the trap level with the soil surface. The outer pail prevented the hole from collapsing. The inner pail held a preservative (propylene glycol formulated in a commercial product sold as nontoxic antifreeze) and was easily removed to recover insects collected during the trap period. A wire screen (25 mm grid) over the mouth of each trap supported a dung bait and excluded rodents and birds. The bait was suspended below the grid, being held in position by a small wire (i.e., a 'twist tie'). Baits comprised fresh cattle dung (ca. 75 g) wrapped in two layers of cheesecloth. Baits were made in advance and frozen until needed. For Trial 1, 5 traps were operated from June 1 – 7 (not rebaited). For Trial 2, 10 traps were operated June 23 – July 4 (rebaited twice). Insects recovered from traps were stored in 70% ethanol and later sorted, counted and identified.

To test for an effect of treatment on insect emergence from cages, statistical analyses were arbitrarily restricted to taxa represented by at least 50 individuals. This step was taken to eliminate taxa for which analyses were unlikely to detect effects of ivermectin, if present, due to the low number of individuals collected (Type II errors). To avoid underestimating the effect of residues on insects developing in the dung of treated cattle, insects that were adults before pats were placed in cages were excluded from analyses. These insects were identified by their emergence in cages before they could have completed egg-to-adult development. The progeny of these colonizers typically emerged several

weeks later, providing a clear separation between the two generations. In the current study (Experiments 1 and 2), 'colonists' mainly comprised Scarabaeidae and Staphylinidae. These same methods have been used in previous studies that have assessed the insecticidal activity of faecal residues in cattle dung (Floate 1998b; Floate et al. 2002).

For each taxon, ANOVA tests were used to assess the effect of treatment (Day 0, 3, 7, 14, 28, 56) on the recovery of insects. The non-normality of the data could not be corrected with standard transformations. Hence, data were rank-transformed prior to analyses. As a precaution against low statistical power, all analyses were performed with a critical P value of 0.05, modified with sequential Bonferroni corrections (Rice 1989).

Function (Trial 1 only)

To test the effect of ivermectin residues on dung degradation, 25 replicated pats were made for each treatment (Days 0, 3, 7, 14, 28, 56 = 150 pats) and placed outdoors on June 9 in a randomized grid. Methods for preparation and placement of the function pats were the same as described for the structure pats, except that function pats were placed on plastic netting (ca. 25 x 25 cm, mesh width 8 to 10 mm), which was in direct contact with the soil (Figure 62). Use of the netting facilitated recovery of pats from the field, but did not impede biological activity at the dung-soil interface.

To measure changes in their weight over time, five 'function' pats per treatment were removed from the field in 2011 on July 6 (ca. 1 month exposure), August 4 (ca. 2 month exposure) and October 17 (ca. 4 month exposure). In 2012, a further five 'function' pats per treatment were removed from the field on April 27 (ca. 10 month exposure) and June 19 (ca. 12 month exposure). Function pats were not removed from the field between October and April as during this period the ground was frozen and (or) covered by snow. From each pat, a 50 g sample was weighed, oven-dried for 24 h at 105 °C, and then reweighed to determine wet weight. The dry dung sample was then finely ground and a 1 g subsample was placed into a weighed silica crucible. The subsample was weighed, heated in a muffle furnace to 500 °C for 5 h and allowed to cool overnight for a total time of ca. 24 h in the muffle furnace. Subsamples then were transferred from the muffle furnace to an oven at 55 °C for a further 24 h and then reweighed to determine the ash content. To test for an effect of time in field and treatment, a 2-way ANOVA was used with ash weight content as the dependent variable (critical P = 0.05). Because they were normally distributed within and across treatments, data were not transformed prior to analyses.

Figure 62: From Canadian study: a) site used for Experiment 1 (2011); b) 'structure' pat used to test effect of residues on insect diversity; c) insect emergence cage used to recover insects from structure pats; d) pitfall trap with dung bait used to monitor insect activity during period that structure pats were exposed in the field; e) 'function' pat used to test effect of residues on dung degradation; f) site used for Experiment 2 (2012)

The white 'objects' in Figure 62f are insect emergence cages into which structure pats are being placed after being exposed in the field.

14.3.3 Experiment 2 (2012)

The format for Experiment 2 was essentially identical to that used for Experiment 1 with the following changes. The study site was located about 2 km west of Lethbridge on native grassland on private property (49°42'27.69"N; 112°56'23.96"W) (Figure 62). t has never been chemically fertilized and cattle have not grazed at the site for 10+ years. The closest pasture with cattle was ca. 600 m distant. The breed, age and treatment of these cattle is unknown. Dung was collected from untreated cattle (Day 0) the week of March 12. Cattle comprised a group of seven Holstein cows (1,077 kg ave. body weight) maintained on a diet of barley silage and housed in common pens. On March 20, the cows were treated with a topical formulation of ivermectin (Ivomec® pour-on for cattle; Lot: NF531213) at the recommended dose (500 mcg ivermectin/kg body weight). Dung subsequently was collected from the treated animals 3, 7, 14, 28 and 56 days post-application. For the structure component of the experiment, pats were exposed from May 30 – June 11. Pitfall traps were operated from May 29 – June 12, and were rebaited three times during this period. The effect of treatment on dung degradation (function) was not assessed.

14.4 Results

14.4.1 Chemical analyses.

For Experiment 1, ivermectin residues (mg ivermectin / kg dung dry weight) were not detected in dung of untreated cattle; i.e., Day 0. For dung from treated cattle, detected levels of ivermectin residues at the time of excretion were: Day 3 (5.02870), Day 7 (7.67508), Day 14 (0.34092), Day 28 (0.06458) and Day 56 (0.01531). For Experiment 2, samples of fresh dung that were sent for analyses were misplaced. Thus, no residue analyses were performed on these samples.

14.4.2 Pitfall traps

Collections with pitfall traps documented high numbers of coprophilous insects present at study sites in 2011 and 2012 when experiments pats were exposed to insect colonization (Table 17). In 2011, a total of 40 taxa were recovered. The most abundant taxa included the dung beetles *Aphodius erraticus, A. granarius and A. vittatus* (Scarabaeidae), *midge* (Chironomidae), *fungus gnats* (Sciaridae), the small dung fly *Coproica mitchelli* (Sphaeroceridae) and an unidentified fly species (Dip. D). The same complex of species was generally present in both Trial 1 and Trial 2 of Experiment 1. In 2012, the most abundant taxa included the dung beetles *A. erraticus* and *Onthophagus nuchicornis*, two species of rove beetles (Staphylinidae), the small dung fly C. mitchelli, and an unidentified fly species (Dip. D). Adjusting for differences in the number of trap days (Site 1: 5 traps x 20 days = 100 trap days; Site 2: 10 traps x 14 days = 140 trap days), the complex of coprophilous insects at the two sites appeared to be generally similar

14.4.3 Effect of treatment on 'structure'.

For Experiment 1, insects recovered from emergence cages were combined across Trials 1 and 2 to increase the likelihood of detecting treatment effects. With 10 pats/treatment used in each trial, combining the two trials allowed for a sample size of 20 pats/treatment. Dung used in both trials originated from the same set of collections. Structure pats for both trials were exposed for colonization by insects at the same site, but separated in time by ca. 3-4 weeks.

A total of 40 taxa were recovered from samples combined across the two trials that comprised Experiment 1 (Table 18), but only 12 taxa were sufficiently common (i.e., 50+ individuals) for analyses (Table 19). Treatment effects were detected for three of these latter taxa. Compared to dung from untreated cattle (Day 0): i) fewer Sphaeridium lunatum (Hydrophilidae) were recovered from dung of cattle treated 3 and 7 days previously, ii) fewer Sphaeroceridae (Diptera) were recovered from dung of cattle treated 3 days previously, and iii) fewer C. mitchelli were recovered from dung of cattle treated 3 and 7 days previously. These findings may underestimate the actual duration of effect. For example, significantly fewer S. lunatum were recovered in treatments for Day 28 and Day 56 compared to Day 0. However, no significant difference was detected between Day 14 and Day 0. Thus, differences detected in later treatments with lower levels of ivermectin (i.e., Day 28, Day 56) were attributed to other factors.

Table 17: Insects recovered in pitfall traps operated during the period that structure pats were exposed in the field for Experiment 1 in 2011 (5 traps) and Experiment 2 in 2012 (10 traps)

ORDER Family Genus species	Experiment 1 Trial 1 (June 1 to 7)	 Trial 2 (June 23 to July 4)	Experiment 2 (May 29 – June 12)
COLEOPTERA (beetles)			
Curculionidae	2	0	0
Histeridae	1	4	16
Hydrophilidae			
Sphaeridium bipustulatum	2	48	14
Sphaeridium lunatum	8	10	40
Sphaeridum scarabaeoides	3	15	7
Ptiliidae (Coleop. B)	1	3	84
Scarabaeidae			
Aphodius distinctus	2	0	10
Aphodius erraticus	107	380	806
Aphodius fimetarius	13	33	11
Aphodius fossor	6	4	3
Aphodius granarius	138	25	49
Aphodius haemorrhoidalis	0	14	11
Aphodius prodromus	48	2	93
Aphodius vittatus	132	27	39
Onthophagus nuchicornis	0	4	401
Staphylinidae			
Staphylinidae sp. A	30	28	44
Staphylinidae sp. B	1	53	276
Staphylinidae sp. C	13	27	127
Coleoptera sp. C	0	5	0
DIPTERA (flies)			
Ceratopogonidae	0	0	7

ORDER Family Genus species	Experiment 1 Trial 1 (June 1 to 7)	 Trial 2 (June 23 to July 4)	Experiment 2 (May 29 – June 12)
Chironomidae	261	105	33
Unidentified sp. A	0	2	1
Unidentified sp. B	0	0	0
Sarcophagidae			
Ravinia spp.	0	14	15
Scatophagidae			
Scatophaga stercoraria	62	85	56
Sciaridae			
Lycoriella sp.	20	317	22
Sepsidae			
Sepsis spp.	12	40	69
Sphaeroceridae			
Coproica mitchelli (Dip A)	47	348	692
Unidentified (Dip E)	22	73	24
Unidentified Diptera			
Diptera sp. B	5	3	6
Diptera sp. C	20	70	37
Diptera sp. D	119	518	136
Diptera sp. F	3	1	1
Diptera sp. G	10	0	0
Diptera sp. I	30	4	0
Diptera sp. J	0	1	J
Diptera sp. L	0	0	12
HYMENOPTERA (wasps)			
Eucoilidae			
Unidentified (Hym C)	3	70	29
Unidentified (Hym D)	3	9	0

ORDER Family Genus species	Experiment 1 Trial 1 (June 1 to 7)	 Trial 2 (June 23 to July 4)	Experiment 2 (May 29 – June 12)
Unidentified (Hym E)	3	44	13
Unidentified (Hym F)	0	9	0
Mymaridae (Hym B)	3	9	10
Pteromalidae (Hym A)	1	13	9

Table 18: Insects recovered from structure pats in Experiment 1 in 2011 (two trials) and Experiment 2 in 2012

ORDER Family Genus species	Experiment 1 Trial 1	 Trial 2	 TOTAL		Experiment 2
COLEOPTERA (beetles)					
Histeridae	2	1	3		6
Hydrophilidae					
Sphaeridium bipustulatum	4	1	5		6
Sphaeridium lunatum	124	49	173		2
Sphaeridum scarabaeoides	108	6	114		8
Ptiliidae (Coleop. B)	571	70	641		706
Scarabaeidae					
Aphodius distinctus	2	0	2		1
Aphodius erraticus	9	5	14		3
Aphodius fimetarius	71	5	76		48
Aphodius fossor	7	14	21		5
Aphodius granarius	149	15	164		41
Aphodius haemorrhoidalis	0	0	0		12
Aphodius prodromus	4	0	4		0
Aphodius vittatus	136	10	146		27
Onthophagus nuchicornis	1	0	1		38
Staphylinidae					

ORDER Family Genus species	Experiment 1 Trial 1	Trial 2	TOTAL		Experiment 2
Staphylinidae sp. A	148	48	196		130
Staphylinidae sp. B	48	9	57		2004
Staphylinidae sp. C	57	7	64		29
Coleoptera sp. C	0	0	0		1
DIPTERA (flies)					
Ceratopogonidae	0	0	0		242
Chironomidae	9	2	11		41
Unidentified sp. A	0	0	0		10
Unidentified sp. B	0	0	0		36
Sarcophagidae					
Ravinia spp.	0	3	3		9
Scatophagidae					
Scatophaga stercoraria	0	0	0		16
Sciaridae					
Lycoriella sp.	4	1	5		14
Sepsidae					
Sepsis spp.	2	3	5		115
Sphaeroceridae					
Coproica mitchelli (Dip A)	38	21	59		547
Unidentified (Dip E)	107	21	128		9
Unidentified Diptera					
Diptera sp. B	7	0	7		0
Diptera sp. C	1	0	1		62
Diptera sp. D	2	1	3		18
Diptera sp. F	2	0	2		0
Diptera sp. G	0	96	96		0
Diptera sp. L	0	0	0		119

ORDER Family Genus species	Experiment 1 Trial 1	Trial 2	TOTAL		Experiment 2
HYMENOPTERA (wasps)					
Eucoilidae					
Unidentified (Hym C)	40	8	48		12
Unidentified (Hym E)	1	0	1		0
Pteromalidae (Hym A)	3	0	3		4
Mymaridae (Hym B)	3	1	4		1
TOTAL	**1660**	**397**	**2057**		**5860**

Within experiments, taxa represented by 50+ individuals (highlighted) were used in analyses to test for an effect of ivermectin treatment on insect recovery (see Table 19 and Table 20).

Table 19: Experiment 1. Analyses performed on taxa represented by 50+ individuals for collections combined across Trials 1 and 2. Data are mean (± SE) number of individuals recovered per structure pat (n = 20 pats/treatment).

Taxa	Days post-application that dung was collected from treated animals						F 5, 114	P-value
	Day 0	Day 56	Day 28	Day 14	Day 7	Day 3		
Ptiliidae (Coleop. B)	8.2 ± 2.2	1.7 ± 0.6	5.2 ± 2.0	2.2 ± 0.8	2.0 ± 0.9	13.0 ± 6.4	2.656	0.026*
Staphylinidae sp. A	2.1 ± 0.4	3.4 ± 2.5	0.7 ± 0.2	1.3 ± 0.3	0.7 ± 0.2	1.8 ± 0.5	2.229	0.056
Sphaeridium lunatum	3.1 ± 0.7a	0.6 ± 0.2b	1.3 ± 0.4b	1.7 ± 0.5ab	1.0 ± 0.4b	1.1 ± 0.3b	4.508	0.001
Aphodius granarius	0.8 ± 0.2	0.5 ± 0.2	1.4 ± 0.5	1.8 ± 0.4	1.1 ± 0.3	2.7 ± 0.7	2.421	0.040*
Aphodius vittatus	2.0 ± 1.1	0.4 ± 0.2	2.0 ± 0.9	0.7 ± 0.3	1.2 ± 0.1	1.2 ± 0.4	1.132	0.347
Sphaeroceridae (Dip. E)	4.3 ± 2.5a	1.8 ± 1.0a	0.1 ± 0.1b	0.1 ± 0.1b	0.2 ± 0.1ab	0.1 ± 0.1b	5.026	<0.001
Sphaeridum scarabaeoides	0.4 ± 0.2	0.8 ± 0.3	3.8 ± 1.9	0.6 ± 0.3	0.0 ± 0.0	0.3 ± 0.1	3.161	0.010*
Diptera sp. G	0.9 ± 0.6	1.5 ± 1.2	0.4 ± 0.3	0.3 ± 0.2	1.7 ± 0.6	0.0 ± 0.0	1.467	0.206
Aphodius fimetarius	0.4 ± 0.2	0.5 ± 0.2	0.6 ± 0.3	1.1 ± 0.4	0.8 ± 0.3	0.6 ± 0.2	0.988	0.428
Staphylinidae sp. C	0.7 ± 0.2	0.4 ± 0.1	0.7 ± 0.2	0.5 ± 0.2	0.6 ± 0.2	0.5 ± 0.2	0.182	0.969
Coproica mitchelli (Dip. A)	0.9 ± 0.3a	1.5 ± 0.6a	0.2 ± 0.1ac	0.3 ± 0.2ab	0.1 ± 0.1bc	0.1 ± 0.1bc	4.371	0.001
Staphylinidae sp. B	0.3 ± 0.2	0.3 ± 0.2	1.0 ± 0.4	0.7 ± 0.2	0.3 ± 0.1	0.5 ± 0.2	1.287	0.274

Analyses performed on taxa represented by 50+ individuals for collections combined across Trials 1 and 2. Data are mean (± SE) number of individuals recovered per structure pat (n = 20 pats/treatment). * not significant after critical P-value adjusted with sequential Bonferroni corrections.

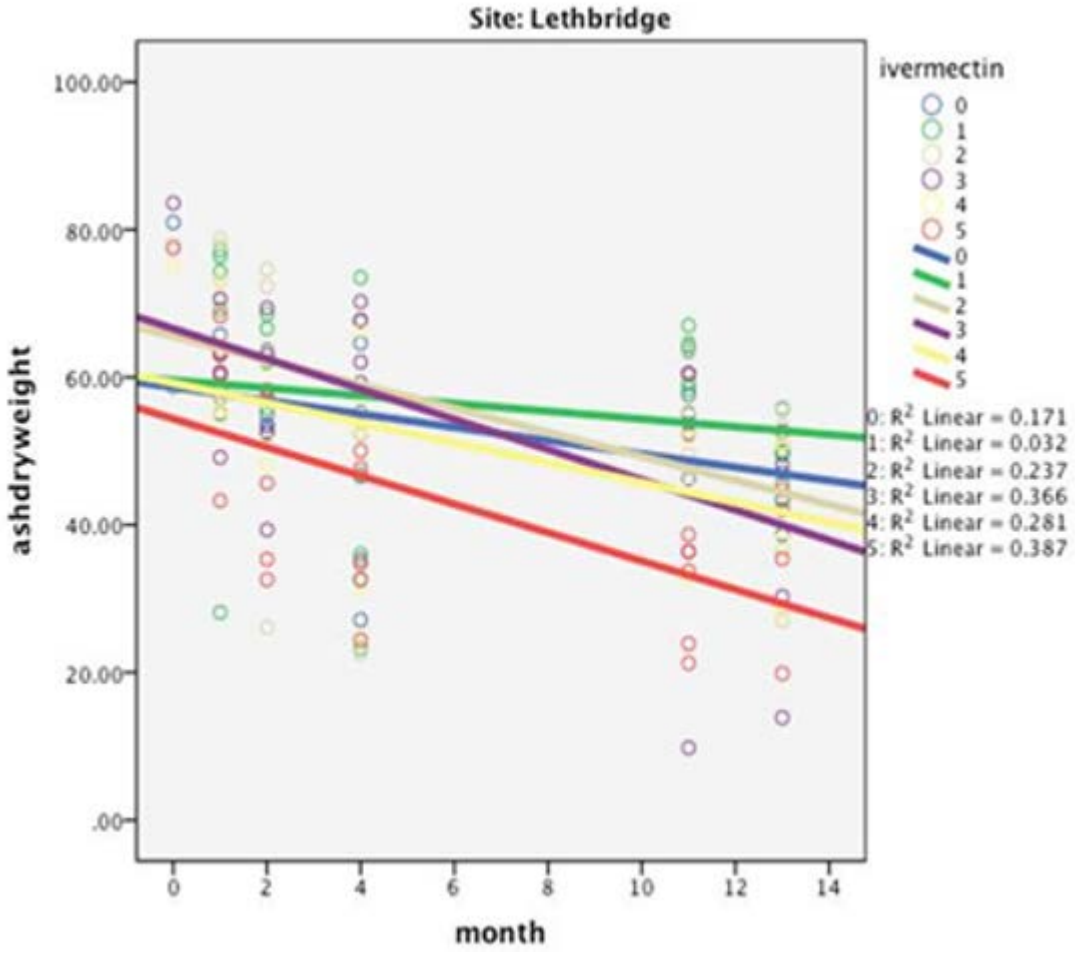
Table 20: Experiment 2. Analyses performed on taxa represented by 50+ individuals. Data are mean (± SE) number of individuals recovered per structure pat (*n* = 10 pats/treatment).

Taxa	Days post-application that dung was collected from treated animals						F 5, 52	P-value
	Day 0	Day 56	Day 28	Day 14	Day 7	Day 3		
Ptiliidae (Coleopt. B)	16.5 ± 5.8a	22.2 ± 11.0a	19.2 ± 8.3a	12.5 ± 6.7a	0.0 ± 0.1b	0.0 ± 0.0b	14.188	<0.001
Staphylinidae sp. A	2.9 ± 0.4	2.6 ± 0.9	3.5 ± 1.8	2.5 ± 0.8	0.8 ± 0.7	0.9 ± 0.5	1.380	0.247
Coproica mitchelli	35.7 ± 9.3a	18.9 ± 5.7a	0.1 ± 0.1b	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.0b	28.880	<0.001
Sepsis spp.	11.5 ± 5.4a	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.0b	19.775	<0.001
Diptera sp. L	4.8 ± 1.9a	1.9 ± 0.8ab	3.7 ± 1.0a	1.5 ± 0.6ab	0.0 ± 0.0b	0.0 ± 0.0b	8.273	<0.001
Ceratopogonidae	0.7 ± 0.7	0.1 ± 0.1	22.5 ± 15.3	0.7 ± 0.6	0.2 ± 0.2	0.0 ± 0.0	0.957	0.442
Diptera sp. C	3.2 ± 2.2	1.7 ± 1.1	0.4 ± 0.4	0.0 ± 0.0	1.0 ± 1.0	0.0 ± 0.0	2.771	0.027*
Staphylinidae sp. B	52.8 ± 16.9a	62.3 ± 12.8a	45.5 ± 11.7a	34.3 ± 9.4a	2.1 ± 0.6b	4.0 ± 0.8b	16.656	<0.001

Analyses performed on taxa represented by 50+ individuals. Data are mean (± SE) number of individuals recovered per structure pat (n = 10 pats/treatment). * not significant after critical P-value adjusted with sequential Bonferroni corrections.

In Experiment 2, 33 taxa were represented in collections from emergence cages (Table 18) for which eight taxa were sufficiently common to warrant analyses (Table 20). Treatment effects were detected for five of these latter taxa. Compared to dung from untreated cattle (Day 0): i) fewer Ptiliidae were recovered from dung of cattle treated 3 and 7 days previously, ii) fewer C. mitchelli were recovered from dung of cattle treated 3, 7, 14 and 28 days previously, iii) fewer Sepsis spp. (Sepsidae) were recovered from dung of cattle treated 3, 7, 14, 28 and 56 days previously, iv) fewer Diptera sp. L recovered from dung of cattle treated 3 and 7 days previously, and v) fewer Staphylinidae sp. B were recovered from dung of cattle treated 3 and 7 days previously.

Figure 63: Effect of treatment (ivermectin) and time (month) in field on dung degradation as measured by ash dry weight



Treatment codes are as follows: 0 = Day 0 (control), 1 = Day 56, 2 = Day 28, 3 = Day 14, 4 = Day 7, 5 = Day 3. Although dung degradation increased with time in field ($P < 0.0001$), no effect of treatment on dung degradation was detected ($P = 0.261$).

14.4.4 Effect of treatment on 'function'.

Results of the 2-way ANOVA test did not detect an effect of treatment on dung degradation ($P = 0.261$) nor an interaction effect between time in field and treatment ($P = 0.268$) (Figure 63). An effect was detected for time in field on dung degradation ($P < 0.0001$). For samples combined across treatments, the mean (± SE) ash dry weight was 75.6 (± 3.6) when pats were first placed in the field versus 41.7 (± 2.0) ash dry weight when the final set of function pats were removed 12 months later. These data indicated that the level of degradation for function pats during the study was 44.8%.

14.5 Discussion

14.5.1 Ivermectin residues

Residue levels of ivermectin in dung of treated cattle were generally comparable between European and Canadian studies. For D7, however, dung from Canada had a threefold higher IVM-concentration compared with that for Europe. Several factors may explain this difference. Peak excretion of residues following topical application of ivermectin occurs 2-3 days post-treatment, followed by a sharp decline to form a long tail that may persist for more than 4 to 6 weeks (e.g., Herd et al. 1996). Thus, collections on Day 6 versus Day 8, for example, can be expected to have different levels of residue. Residue levels also can be affected by diet. Peak excretion of residues was 0.36 mg kg−1 dung wet weight for grain-fed versus 0.09 for pasture-fed cattle treated with ivermectin in a subcutaneous formulation (Cook et al. 1996). Dung used for the Canadian study was collected from cattle maintained on a diet of hay, whereas dung used for the European studies was obtained from cattle maintained on pasture. Residue levels can further be affected by grooming behavior. In one study for which cattle were prevented from self-grooming and then treated topically with ivermectin, only 7% of the dose was recovered in their dung as parent compound versus 70% in dung of cattle allowed to lick themselves (Laffont et al. 2001). Cattle for the Canadian study were held in pens, which may have increased the likelihood of grooming among animals.

Results of chemical analyses documented the presence of ivermectin residues in dung of treated cattle, declining in a pattern consistent with previous studies. In dung of cattle treated with ivermectin in a topical formulation, Herd et al. (1996) reported concentrations of 18.5 and 0.04 mg ivermectin / kg dung dry weight in dung excreted 2 and 28 days post-treatment. In the current study, concentrations of 5.0 and 7.7 mg ivermectin / kg dung dry weight were detected in dung excreted 3 and 7 days post-treatment, declining to 0.06 mg ivermectin / kg dung dry weight in dung excreted 28 days post-treatment.

14.5.2 Effects of different sampling methods

Differences in insect recovery methods likely contributed to variation among study sites. In France, treatment pats were left in the field and covered with insect emergence cages after allowing time for colonization. This method likely enhanced the recovery of species that develop in soil beneath the pats. In Switzerland, The Netherlands and Canada, treatment pats were removed from the field after allowing time for colonization and placed in insect emergence cages indoors. In the Swiss study, the cages allowed for 'self-extraction' of insects to reduce labor requirements. However, the design of the cages may have allowed for multiple generations of some insect species to breed inside the cage to inflate their overall numbers in samples. Studies in Canadian and The Netherlands used cages of similar design for which insects were manually removed. Although this increased the labor requirement, it avoided potential concerns associated with the use of 'self-extracting' cages. Despite the variation in the methods employed to recover insects from treatment pats, general conclusions regarding the effect of ivermectin residues on dung structure was comparable.

14.5.3 Effects of ivermectin on dung organisms (limited to Canadian Prairie Zone)

The residues of ivermectin in the dung were associated with declines in the recovery of insects, particularly for treatments with the highest levels of residues; i.e., Days 3 and 7. For Experiment 1, declines were most evident for species of sphaerocerid flies (C. mitchelli, Dip. E). In Experiment 2, declines were again most evident for C. mitchelli, but also for Sepsis spp. The susceptibility of these two fly taxa to faecal residues of macrocyclic lactones has been documented in several previous studies at the Lethbridge Research Centre. For cattle treated topically with ivermectin, Floate (1998b) reported reduced emergence of Sepsis sp. in dung voided 1 – 12 wk post-application, and of C. mitchelli in dung voided 1 – 10 wk post-application. Floate et al. (2002) studied reductions of insects developing in

dung of cattle treated 1-4 weeks previously with topical applications of four macrocyclic lactone products. For cattle treated with either doramectin or ivermectin, reductions of Sepsis flies were observed at all weeks post-treatment. In a series of three experiments, Floate et al. (2008) examined insect emergence from dung of cattle treated with a topical application of doramectin up to 14 and 16 wk previously. Depending upon the experiment, reductions of C. mitchelli were observed for dung voided 8 – 12 wk post-application, 12 – 16 wk post-application, or not at all. For Sepsis sp., reductions were observed for dung voided 14 wk post-application, 12 – 16 wk post-application, or not at all.

In addition to their susceptibility, the reliability of Sepsis and Coproica as bioindicators of insecticidal residues can be attributed to their general abundance in cattle dung and wide-spread distribution. In cases were these species appear unaffected by residues, the data should be considered suspect unless proven otherwise. For example, the experiment in Floate et al. (2008) that failed to show an effect of treatment on Sepsis and C. mitchelli used cattle that had been treated with doramectin 12 wk prior to being again treated for use in the study. Although it was known that the pre-experimental treatment might introduce low levels of residue to control pats, the high toxicity of these low levels was not anticipated. Thus, by reducing number of these flies in the 'control' dung, the pre-treatment masked effects of the experiment treatment of doramectin. This finding illustrates the importance of using livestock with a known treatment history extending back for at least 4 months.

Low numbers of insects emerging from control pats likely resulted in an underestimate of the toxicity of ivermectin residues in Experiment 1, for which an average of 17 insects per pat was recovered (2 057 insects from 120 pats) (Table 18). By comparison and using similar methods, Floate (1998) recovered an average of 171 (16 445 insects from 96 pats in 1994) and 151 (18 180 insects from 120 pats in 1995) per pat during a 2-year study performed at the same site as Experiment 1. Furthermore, no flies in the genus Coproica, and only low numbers of flies in the genus Sepsis were recovered in experiments 1 and 2 (Table 18).

Given their abundance in pitfall traps at the site during exposure of structure pats, the absence of C. mitchelli in control dung for Experiment 1 was unexpected and suggested that the pats were unattractive to oviposition flies. This may reflect the diet of the cattle from which the dung was collected. Cattle in Experiment 1 were maintained on a diet of hay, which produced noticeably drier dung than that used in Experiment 2. Dung for the latter experiment was obtained from cattle maintained on a diet of barley silage. In an unrelated study, Tiberg and Floate (2011) compared attributes of dung from cattle maintained on a diet of hay supplemented with about 10% grain versus dung from cattle maintained on a diet of barley silage. They reported dung of hay-fed cattle to have a higher water content, in contrast to observations for the current study. More importantly, however, they found that dung of barley-fed cattle attracted more Sepsis flies, consistent with the hypothesis that dung of silage-fed cattle is more attractive as habitat for flies most suitable as bioassays of insecticidal residues in dung. Larger numbers of Sepsis and C. mitchelli were recovered in Experiment 2, which resulted in data better suited to detected insecticidal activity of residues.

14.5.4 Effect of ivermectin on dung pat function

No effect of residue on dung pat degradation (= function) was detected in Experiment 1 (2011). This result was not unexpected, because the insects shown to be affected in the current study were mainly small species of flies and beetles not known to be degraders of dung. Dung beetles present at the study site during the time that the function pats were initially placed in the field were mainly species of Aphodius (Table 17). Aphodiine species are not recognized as efficient degraders of dung relative to other species of dung beetles. Further, the delay in the start of the experiment due to weather may have meant the exposure of the pats occurred outside of the main breeding season for these species. The sole tunneling species of dung beetle detected at the site (Onthophagus nuchicornis) was present only in low number.

This finding emphasizes that it should not be concluded, a priori, that ivermectin residues in cattle dung will cause an appreciable delay in dung degradation. The process of degradation is not solely a function of insect activity but rather a process comprised of many factors that act alone and in concert. The diet of the animal affects the moisture content of its dung. Moisture content will affect the form (spread and thickness) of the pat when deposited. The shape affects the rate of dung pat desiccation, which will in turn affect its attractiveness to colonizing insects, rates of microbial activity, and disruption of the pat by the growth of vegetation from beneath. Although largely absent in the current study, earthworms also can be important agents of degradation. Overriding all of these factors are the direct and indirect effects of climate. The dry prairie climate of western Canada is not particularly conducive to earthworm activity, microbial activity or that of vegetation. However, all else being equal, dung pats degrade faster in the presence of insects than in their absence.

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Acknowledgement

The work described here was sponsored by the German Federal Environmental Agency (Project-No. FKZ 371063412).

15 WP2: Non-target effects of ivermectin residues on soil organisms at four grassland sites in France, Switzerland, The Netherlands and Canada

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ABSTRACT

In the European Union, the environmental risk of veterinary medical products (VMP), and particularly parasiticides, is addressed in an authorization process. Numerous field studies have assessed the effects of VMPs within the last 25 years), but these studies were performed using different methods, on different insects, and with different VMPs. In addition, most of them did not include soil organisms which might also be exposed to VMPs. Following the recommendations proposed by Jochmann et al. (2011) a field study with one particular model VMP (i.e. ivermectin) in different ecological regions in Europe (Switzerland, Netherlands, France) and Canada was performed to assess, among others, the diversity of soil organisms as endpoint. The ultimate question was whether it is useful to include soil organisms in field studies assessing side-effects of veterinary pharmaceuticals. Earthworms (Lumbricidae) and springtails (Collembola) were collected using standard sampling methods at different dates, one to twelve months after putting dung pats from treated cattle in the field (at Lethbridge, earthworms do not occur due to natural reasons). Three months after starting the study ivermectin concentrations below dung pats ranged between 0.02 to 0.03 mg/kg dw soil, while at later dates (five to seven months after starting the study) concentrations were almost always lower than 0.006 mg/kg dw soil. At the Zurich, Wageningen and Montpellier sites typical earthworm communities for such continental, Atlantic and Mediterranean regions were found: species-rich and with high abundance at the two former sites, but with few species and individuals at the latter site. Significant differences between earthworm abundance in toto (or the number of individual ecological groups) in soil under control or treated pats were determined in Wageningen one and three months after starting the study (usually at the two highest treatments). Three and five months after starting the study significant effects on earthworms were also found in Zurich, but at different treatments. At all four sites a diverse springtail community was found, but abundance was highly variable during the course of the study, probably caused by climatic factors. Significant differences occurred at all sites except Montpellier: one to five months after starting the study the total number of springtails, the number of individual age groups or of ecological groups was affected, but clear concentration-effect relationships were difficult to identify. Surely other factors than just ivermectin played a role. Summarizing the experiences made in this study it is recommended to include the study of soil organism in field studies assessing side-effects of veterinary pharmaceuticals.

Key words: dung, Lumbricidae, Collembola, ecotoxicology, biodiversity

15.1 Introduction

15.1.1 Background and aims of this study

In the European Union and North America, the environmental risk of veterinary medical products (VMP), and in particular, parasiticides is addressed in an authorization process. This process is based on guidelines published by the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH), which is a trilateral program to harmonize technical requirements for these drugs in Europe, Japan, and the United States. The Environmental Risk Assessment (ERA) allows a tiered approach. In Phase I (VICH 2000), general aspects regarding use and exposure are handled. In Phase II, ecotoxicological test requirements are specified (VICH 2004). An ERA of VMP for soil fauna is required if the substance acts as a parasiticide for the treatment of pasture animals. In Tier A of Phase II, studies are done to assess the non-target effects (if any) of fecal-excreted parasiticides on earthworms and springtails. If a risk is identified, additional studies are required (Tier B) to characterize the nature and extent of the non-target effects using representative non-target organisms as bioassays. However, further information on Tier B studies (and beyond) for soil organisms are missing in the guidelines. In fact, the only advice given on how to proceed beyond Tier A is a statement in the VICH (2004) guideline: "Regulatory guidance should be sought on appropriate studies." In the "Revised Guideline on Environmental Impact Assessment for Veterinary Medicinal Products" (EMA 2008) no further information on higher tier-testing of soil organisms is given.

Numerous studies have assessed the effects of VMPs on dung organisms, both in the laboratory and in the field within the last 25 years, but only few studies addressed potential effects of VMPs on soil organisms (e.g. Lumaret et al. 2012). Even these few studies have been performed using different methods, on different organisms, and with different VMPs. A standardized approach is lacking, but is needed for use by the European Medicines Agency (EMA) as well as national authorities, industry, and consultants to complete the VICH requirement for higher tier studies (particularly those in the field) with VMPs.

In order to address this problem, the German Federal Environmental Agency (UBA) sponsored a project which had, among others, the aim to perform field studies with a model VMP (i.e. ivermectin) in different ecological regions in Europe and North America, using the structure of, among others, soil organisms as assessment endpoints. The practical work was based on the recommendations compiled by Jochmann et al. (2012) and was performed in the Mediterranean (South France), Continental (Central Switzerland), Atlantic (The Netherlands) region of Europe and in the Prairie (Western Canada) region of North America. In each of these four studies the same questions were addressed: Does the use of ivermectin cause any effect on soil fauna biodiversity? Besides answering these questions practical recommendations, it will be discussed whether the recommendations of Jochmann et al. (2012) regarding the performance of VMP field studies are sufficient.

In detail, the following questions were studied in this part of the project:

- Are the sampling methods used suitable for this kind of study?
- Which soil invertebrate community does occur at the 4 field sites?
- Are these communities typical for the sites?
- Do the soil invertebrates under dung from treated cattle differ from those occurring below control dung pats?
- Is it useful to include the study of soil organisms in field studies assessing side-effects of veterinary pharmaceuticals?

15.1.2 Selection of the most appropriate soil organism groups

The soil is inhabited by a huge number of species from almost all major taxa. Therefore, in order to study the effects of a VMP occurring in dung pats on soil organisms a selection had to be made. In this process, five criteria were used:

1. Ecological relevance, i.e. do these organisms play an important role in grassland soils
2. Sensitivity towards ivermectin, i.e. is there evidence from laboratory or, better, field studies that ivermectin does affect these organisms
3. Availability of standard methods, i.e. are robust and generally accepted sampling and determination methods applicable
4. High diversity: Is the species composition (= structure) of a community diverse enough to be used as a measurement endpoint
5. Preferably: Occurrence at all four sites, i.e. the selected organisms are widely distributed and thus will be usable in different regions
6. Practicability i.e. is the effort for sampling reasonable

Based on these criteria, two groups were selected:

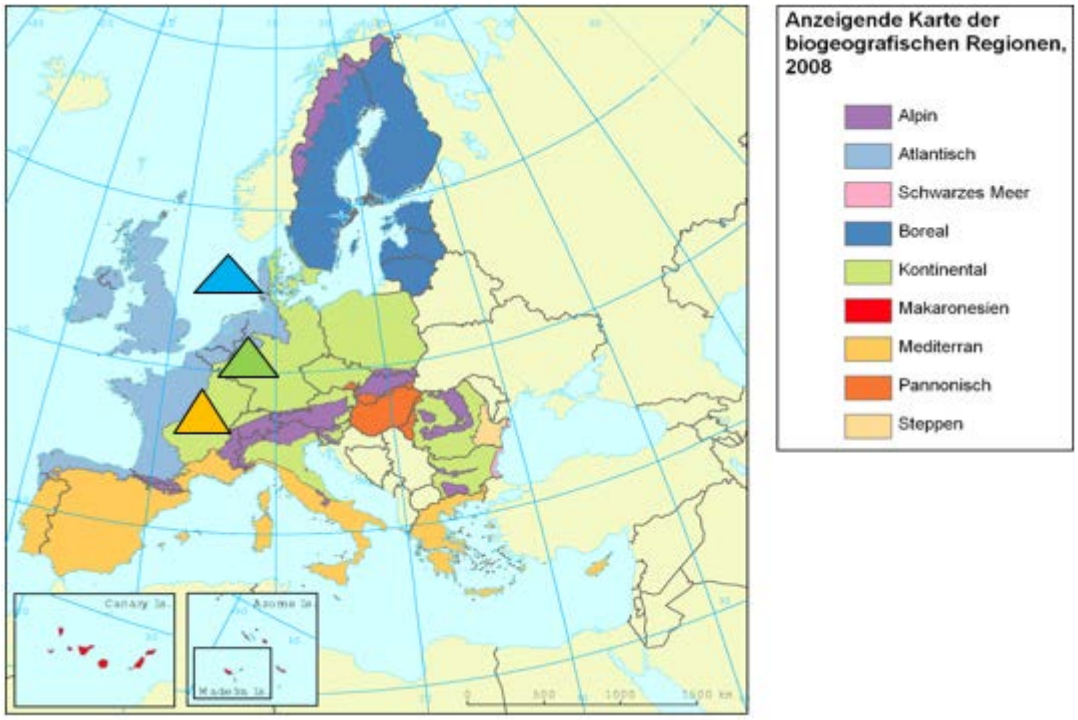
- Earthworms (Lumbricidae): They are doubtless at many sites ecologically very relevant, an ISO standard method is available (ISO 2006a) and they show a medium chronic sensitivity towards ivermectin (Römbke et al. 2010a). However, for natural reasons they do not occur in the prairies around Lethbridge, they do not show a very high diversity per site (about 10 species on average) and their sampling using hand-sorting is time consuming.
- Springtails (Collembola): These micro-arthropods were selected because they fulfill almost all criteria (e.g. a standard sampling method is available (ISO 2006b)). However, there ecological relevance at grassland sites is not very high, mainly because they occur in relatively low numbers at such sites (Hopkin 1997).

15.2 General Description of the Study Sites

The four study sites are located in different biogeographical regions of Europe (Figure 64) and North America (EEA 2009):

- Atlantic Region of Europe: Wageningen (The Netherlands)
- Continental Region of Europe: Zurich (Switzerland)
- Mediterranean Region of Europe: Montpellier (France)
- Prairie region of North America: Lethbridge (Canada)

Figure 64: Location of the three European study sites representing different ecological regions of Europe (EEA 2009)



Detailed descriptions of the four study sites are given in the previous chapters 11 – 14 (see also Figure 65Figure 68). An overview on the main site and soil parameters is given in Table 21. As expected, there are clear differences in mean annual temperature and precipitation, ranging from a cold and dry site (Lethbridge) to a slightly less cool but wet site (Zurich). The two other sites differ mainly in temperature (Montpellier is warmer than Wageningen), but less in precipitation. However, it has to be pointed out that these are average values which do not represent directly the conditions during the study. All four tests started at about the same time in spring and sampling of soil organisms was performed at different dates for up to one year. Land use and vegetation was quite similar at the four sites, but their history was not: Cattle were kept at all sites close to the actual study plots. Also the duration of cattle breeding differed between several decades in Lethbridge and five years in Wageningen. However, all study sites were surrounded by grasslands used as meadows.

Figure 65: Study site Montpellier

Figure 66: Study site Wageningen

Figure 67: Study site Zurich

Figure 68: Study site Lethbridge

Table 21: Overview on the main site and soil properties of the four study sites

Site / Soil Parameters	Montpellier	Zurich	Wageningen	Lethbridge
Coordinates	43°79'33.40 N; 3°73'18.75 O	47°23'44.87 N; 8°33'02.62 O	51°59'32.16 N; 5°39'39.82 O	49°41'25.46 N; 112°46'26.15 W
Landuse	Grass strip near crop site	Borderline of a meadow	Meadow (since 2006)	Meadow, used for cattle
Ann. precipit. (mm/y)	700	1123	846	365
Mean ann. temp. (°C)	13.0	7.9	10.5	5.8
pH (CaCl2 - method)	7.6	7.4	5.2	7.3
Organic matter (%)	3.1	4.6	2.9	6,2
Bulk density (g/L)	1149	1254	1449	987
WHCmax	48.0	47.6	34.2	60.7
Carbon	16.75	24.28	12,55	27.35
Nitrogen	1.646	3.018	1,009	2.747
C/N ratio	10.18	8.05	12.44	9.96
Soil texture	Silty loam	Clayey loam	Pure sand	Weakly clay loam
Clay < 0.002 mm	20.2	25.6	4.1	25.9
Silt 0.002 – 0.063 mm	56.1	38.7	9.5	36.2
Sand 0.063 – 2.000 mm	23.7	35.7	86.4	37.9

15.3 Materials and Methods

15.3.1 Experimental design

Dung was collected from untreated cattle (Day 0) in Montpellier (used for the three European studies) and in Lethbridge (used for the Canadian study) in early spring 2011 (for details of the cattle see chapter 11 - 14). Cattle were treated with a topical formulation of ivermectin (Ivomec® pour-on for cattle) at the recommended dose (500 mcg ivermectin/kg body weight). Dung subsequently was collected from the treated animals 3, 7, 14, and 28 days post-application (in Lethbridge, dung was collected after 56 days too). For each collection date, fresh dung (<3 h old) from multiple pats was placed in large plastic bags which were sealed to prevent desiccation during storage (at -20°C until use). As part of testing the effect of ivermectin residues on dung degradation, 25 replicated pats were made for each treatment (Days 0, 3, 7, 14, 28, (56) = 125 and 150 pats in Europe and Canada, respectively) and placed outdoors in a randomized grid. Each pat was put on a plastic netting (ca. 25 x 25 cm, mesh width 8 to 10 mm), which was in direct contact with the soil. Use of the netting facilitated recovery of pats from the field, but did not impede biological activity at the dung-soil interface. Five 'function' pats per treatment were removed from the field at differing dates at the four sites (see Table 22 for an overview) up to twelve months after exposure.

At the same dates, soil organisms were sampled at the places where the "function" pats had been removed: firstly, a soil core (2.5 cm diameter x 5 cm depth in Lethbridge but 5 cm diameter x 5 cm depth at the three other sites) was taken directly below the pat. Afterwards, a hole was dug into the soil (25 * 25 cm, with the place of the soil core at its center) and about 10 cm deep and the taken soil was sorted for earthworms by hand directly in the field (Figure 69). The soil cores were transported to the laboratory of ECT GmbH, where the micro-arthropods were extracted by heat extraction (i.e. via a Kempson apparatus) (Figure 70). All extracted arthropods as well as earthworms were fixed and stored in ethanol (70%). Taxonomic determination of earthworms (mainly Lumbricidae) was made using general keys (Bouché 1972; Sims & Gerard 1999; Blakemore 2002). Since at the Lethbridge site no earthworms occur (Reynolds 1996), only micro-arthropods were sampled at that site. Their determination is based on the "Synopses on Palaearctic Collembola" for the taxa Tullbergiinae (Zimdars & Dunger 1994), Symphypleona (Bretfeld 1999), Isotomidae (Potapow 2001) and Hypogastruridae (Thibaud et al. 2004). For all other groups the key of Schulz et al. (2003) was used. Only Collembola were determined on the species level; other micro-arthropods found in the same samples (mainly oribatid or gamasid mites) were counted on the group level.

Besides taxonomic differentiation both organism groups were also divided into three groups which differ in ecological, morphological and behavioral properties – but mainly concerning their depth distribution within the soil profile: in the case of earthworms epigeic (= litter dwellers), endogeic (= mineral soil inhabitants) and anecic (= vertical burrowers) can be distinguished (Bouché 1977). Since juvenile worms cannot be identified easily on the species level, for this kind of evaluation only adult worms are used. In addition, detailed assessment on the species level is performed only for abundance data (not for biomass), since the information gained is mostly the same. Collembola are classified accordingly as epigeic, hemiedaphic and euedaphic species (EFSA 2010b). These groups can be used as an additional endpoint for the assessment of the effects of VMPs on soil invertebrates.

Figure 69: Earthworm sampling: preparation of a hand sorting (site Montpellier)

Figure 70: Heat extraction of micro-arthropods in the laboratory

In the following, an overview on the sampling of invertebrates is given (Table 22). As already mentioned, no earthworm samples were taken at all in Lethbridge due to a known natural lack of these organisms at the sampling site. In addition, earthworms were only sampled once at Montpellier, because the soil was too dry for most of the study period. For the same reason the number of micro-arthropod samples is low at Montpellier too.

Table 22: Overview on the sampling design

Date	Earthworms			Microarthropods			
	Montpel-lier	Wage-ningen	Zurich	Leth-bridge	Montpel-lier	Wage-ningen	Zurich
			- 2011 -				
May	Start M.	Start W.	Start Z.		Start M.	Start W.	Start Z.
June	T1 M.	T1 W.	T1 Z.	Start L.	T1 M.	T1 W.	T1 Z.
July				T1 L.		T2 W.	T2 Z.
August		T2 W.		T2 L.		T3 W.	T3 Z.
September						T4 W.	
October			T2 Z.	T3 L.			T4 Z.
November		T3 W.			T2 M.	T5 W.	
December							T5 Z.
			- 2012 -				
April				T4 L.			
June	T2 M.			T5 L	T3 M.		

The worms and springtails found at each soil sampling occasion were evaluated separately:

- Abundance: mean number of individuals per treatment, calculated in total and for each taxon separately
- Biomass: mean biomass per treatment, calculated in total and for each taxon separately (not for springtails)
- Species composition: mean dominance spectrum of species per treatment

15.3.2 Analytical procedure for the determination of ivermectin in soil

Reagents and equipment

Acetonitrile of HPLC-gradient grade (>99.9%) was supplied by VWR international (Radnor, Pennsylvania, USA). High purity water was prepared by a Milli-Q water purification system (Millipore, Milford, MA, USA). N-methylimidazole (99% purity), triethylamine (99% purity), trifluoracetic anhydride (99% purity) and trifluoracetic acid (99% purity) were supplied by Sigma-Aldrich (Steinheim, Germany). The standard substances ivermectin (CAS RN: 70288-86-7, 96% purity) and doramectin (CAS RN: 117704-25-3, 90% purity) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). For extraction, a Vortex Genius 3 shaker (IKA, Staufen, Germany), a rotary shaker Swip KS-10, (Bühler, Tübingen, Germany), and an ultrasonic bath Sonorex Super RK255H (Bandelin electronic, Berlin, Germany) were used. As a centrifuge a Rotanta 460 R (Hettich, Tuttlingen, Germany) was used. Syringe filters (PTFE, 0.45 µm, 13 mm) were supplied by Wicom GmbH (Heppenheim, Germany). Solid phase extraction cartridges (Strata C-18-E, 500 mg, 55 µm, 70 Å) were purchased from Phenomenex (Torrance, California, USA).

Standard solutions

All standard solutions of doramectin and ivermectin were prepared in acetonitrile and stored at 18 °C. Stock solutions were made by dissolving 2.5 µg ivermectin or doramectin in 25 mL acetonitrile. These solutions were used to prepare ivermectin working standard solutions of 2000 and 100 µg/L, as well as doramectin working standard solutions of 2000 and 200 µg/L. With these solutions 9 calibration standards covering the relevant concentrations were prepared on daily basis.

Extraction and clean-up of the soil samples

The extraction procedure was mainly based on an adapted and optimized method as described by Litskas et al. (2010). Soil samples were homogenised and were sieved to <2 mm particle size. After determining the water content of the different sample series a total dry matter of about 3 g for soil was weight into polypropylene-vials. Soil samples stored in the field were then moistened up to a water content of about 50% for soil. The remoistened samples were kept at room temperature for 24 h. Internal standard doramectin dissolved in 25 mL acetonitrile was added in an amount near that expected in the sample. The suspension was kept for 15 min in an ultrasonic waterbath, 30 min on a mechanical shaker at room temperature at 450 rpm and again for 15 min in the ultrasonic water bath. Subsequently, the sample was centrifuged for 30 min at 2000 x g and 22 °C. For the soil samples 10 mL of the each supernatant were directly transferred to polypropylene-vials. The solvent was evaporated under a gently stream of nitrogen at 55 °C to complete dryness. For reconstitution 1000 µL acetonitrile were added to the sample. It was vortexed for 2 min, kept in an ultrasonic bath for 10 min, kept for 30 min on a mechanical shaker at 450 rpm, vortexed again for 30 s, and put again in the ultrasonic bath for 5 min. Finally, it was again kept for 30 min on a mechanical shaker at 450 rpm. After filtration (0.45 µm, PTFE) 700 µL of the solution were transferred into a HPLC-vial for the derivatization step.

Derivatization with trifluoracetic anhydride

The sample was derivatized according to an adapted procedure developed by Berendsen et al. (2007). First, 100 µL of N-methylimidazole/acetonitrile (1:1, v/v) were added to 700 µL of the reconstituted

and filtered sample, followed by 50 µL of triethylamine. Subsequently, 100 µL of trifluoracetic anhydride/acetonitrile (1:1, v/v) were added. Finally, 50 µL of trifluoracetic acid were given into the vial. After each addition of reagent the closed HPLC vial was shaken for at least 5 seconds. To finish the derivatization reaction the closed HPLC-vials were kept for 30 minutes at 60°C in an oven.

High performance liquid chromatography with fluorescence detection (HPLC-FLD)

The determination with the HPLC-FLD was carried out within the first 48 hours after the derivatization. Chromatographic separation and determination was performed on an Agilent 1200 HPLC system (Agilent, Santa Clara, California, USA) consisting of a degasser (G1322A), a quaternary pump (G1311A), an autosampler and injection unit (G1329A), a column thermostat (G1316A) and a fluorescence detector (G1321A). The gradient elution was performed using a mobile phase of water (A) and acetonitrile (B) at a flow rate of 0.3 mL min−1 with the following gradient: 0–47 min, 60-100% B; 47–52 min, 100% B; 52–53 min, 100-60% B; 53-60 min, 60% B. The injection volume was 20 µL and the analytes were separated on a 150 mm × 2.1 mm i.d. 3 µm particle size, Dionex (Sunnyvale, California, USA) Acclaim PolarAdvantadge II C18-Column. The column temperature was 30 °C. The fluorescence detection was carried out at an excitation wavelength of 364 nm and an emission wavelength of 463 nm.

Figures of merit

The limit of detection (LOD) and limit of quantification (LOQ) values were determined with the calibration method on the basis of DIN 32645 (2008). For all soil samples from medicated cattle a LOD of 0.9 µg / kg dw and a LOQ of 2.3 µg / kg dw was determined. All data of the extractions with an inadequate recovery of the internal standard (<80% and >120%) were assorted. For the remaining samples the mean recovery of the internal standard doramectin was 97.7% (RSD 10.1%) for the soil samples.

15.3.3 Statistical analyses

Data were analyzed in two ways:

ANOVA followed by Dunnett's or Williams test ($p \leq 0.05$, 1-sided smaller) was applied in case of normal distribution and homogeneous variances to determine statistically significant differences compared to the control. Normal distribution was tested by Kolmogorov-Smirnoff-test ($p \leq 0.05$) and homogeneity of the variance was tested by Bartlett's test ($p \leq 0.05$). In case of inhomogeneous variances Welch-t test for inhomogeneous variances with Bonferroni adjustment ($p \leq 0.05$, 1-sided smaller) was applied. The statistical software package ToxRat® (Professional Version 2.10.05) was used for these calculations.

In addition, data were analyzed as in the structural experiments with dung organisms; i.e. using simple linear regression of the number of taxa emerged (or, alternatively, the Shannon diversity index) as a function of the absolute ivermectin concentration. Specific planned pair-wise comparisons were also performed.

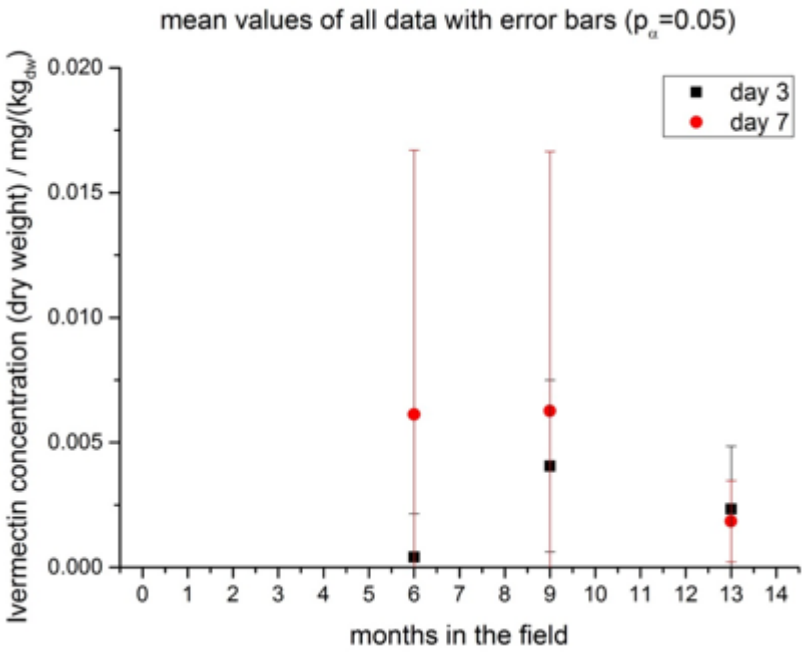
15.4 Results

15.4.1 Residue analysis in soil

Montpellier

Soil samples were taken three times in Montpellier, six, nine and thirteen months after starting the study. The concentration of ivermectin was only detectable in the D3 and D7 treatments. Both treatments did not differ and there was also no significant difference between the concentrations measured at the three dates: with the exception of the D3 treatment after six months, where almost no ivermectin could be detected, in all cases almost similar mean values between 0.002 and 0.006 mg ivermectin/kg soil dw were found (Figure 71).

Figure 71: Mean concentrations of ivermectin (two treatments: D3 and D7) and their standard deviation six, nine and thirteen months after start of the study in Montpellier



Wageningen

Soil samples were taken three times, two, three and seven months after starting the study. The concentration of ivermectin was detectable in the D3, D7 and, once, in the D14 treatments. At the first two sampling dates, concentrations of the D3 and D7 treatments were in the range of 0.005 to 0.02 mg/kg soil dw. Seven months after starting the study the concentrations of ivermectin in all treatments were about 0.001 mg/kg soil dw (Figure 71).

Zurich

Again, soil samples were taken three times, three, five and seven months after starting the study. At the first sampling, mean concentrations of D3 and D7 were determined as 0.02 and 0.03 mg/kg soil dw, respectively (Figure 73). Afterwards, the concentrations of ivermectin decreased to 0.003 to 0.008 mg/kg soil dw. At no date, significant differences between the concentrations of the two treatments did occur.

Lethbridge

Soil samples were only taken twice in Lethbridge, three and six months after starting the study. The concentration of ivermectin was only detectable in the D3 and D7 treatments. Both treatments did not differ and there was also no significant difference between the concentrations measured after three and six months: in all cases mean values between 0.003 and 0.007 mg ivermectin/kg soil dw were found (Figure 74).

Figure 72: Mean concentrations of ivermectin (three treatments: D3, D7, D14) and their standard deviation two, three and seven months after start of the study in Wageningen

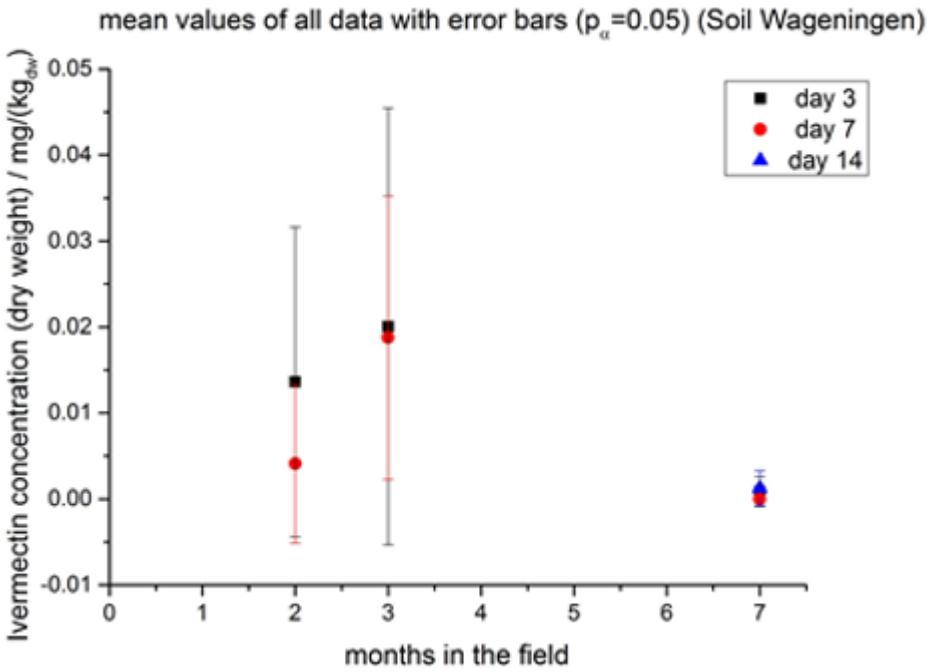


Figure 73: Mean concentrations of ivermectin (two treatments: D3, D7) and their standard deviation three, five and seven months after start of the study in Zurich

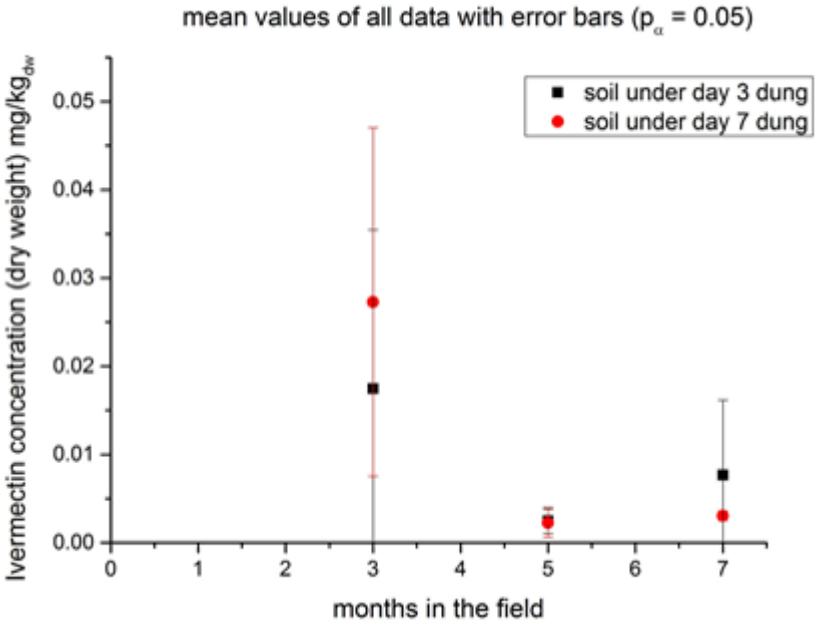
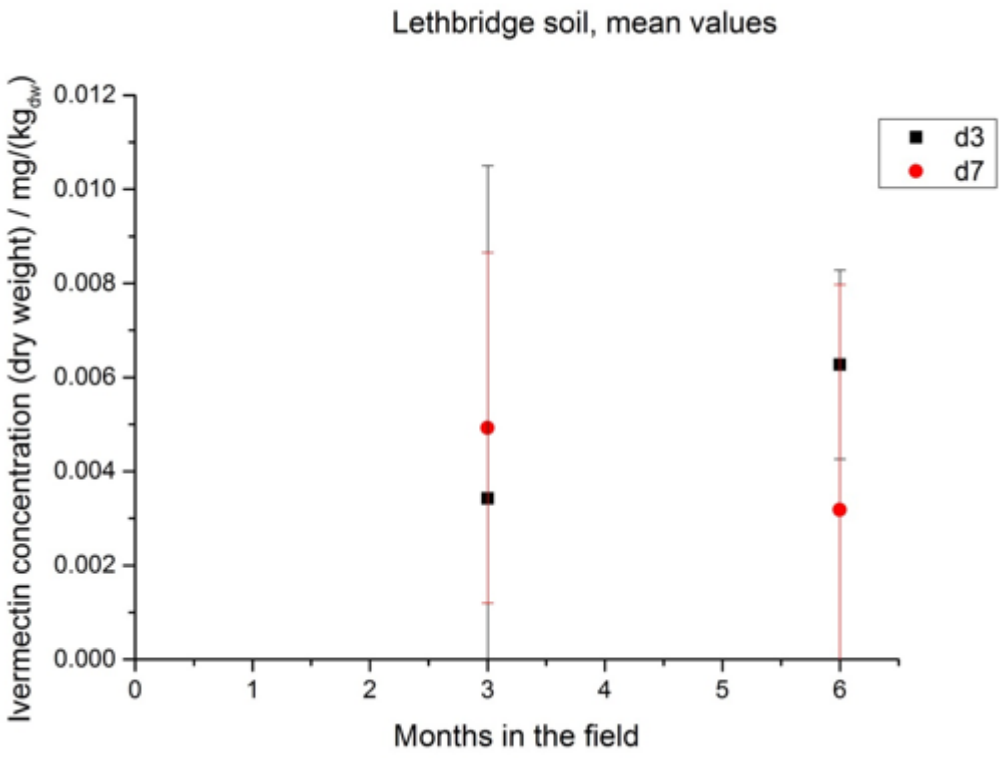


Figure 74: Mean concentrations of ivermectin (two treatments: D3 and D7) and their standard deviation three and six months after start of the study in Lethbridge



15.4.2 Species composition, abundance, and biomass of earthworms

Overview on the earthworm communities at the three sites

The diversity of the earthworm communities at the three study sites differed considerably (Table 23): between 2 and 11 earthworm species were found per site. As a first step, abundance and biomass are suitable assessment endpoints, either for all lumbricids, or separately for all juveniles and adults. By combining abundance and biomass numbers of all species belonging to the same ecological group data evaluation could be improved. The endpoint species composition could only be used for the Zurich site, since the number of species was too low at the other sites. As expected, the species used in standard laboratory tests, the compost worms Eisenia fetida and E. andrei, were not found in the field.

Table 23: List of earthworm species (Lumbricidae and Hormogastridae) found at the three study sites in France, The Netherlands and Switzerland

Species	Ecological Group	Montpellier	Wageningen	Zurich
Lumbricidae				
Allolobophora chlorotica	Endogeic		X	X
Aporrectodea sp.	Various	X	X	X
Aporrectodea caliginosa	Endogeic		X	X
Aporrectodea icterica	Endogeic			X
Aporrectodea longa	Anecic			X
Aporrectodea rosea	Endogeic			X

Species	Ecological Group	Montpellier	Wageningen	Zurich
Dendrobaena attemsi	Epigeic			X
Dendrobaena rubidus	Epigeic			X
Lumbricus spp.	Various		X	X
Lumbricus castaneus	Epigeic		X	X
Lumbricus rubellus	Epigeic		X	X
Lumbricus terrestris	Anecic			X
Octolasion spp.	Endogeic		X	X
Octolasion lacteum	Endogeic			X
Proctodrilus antipae	Endogeic		X	
Hormogastridae				
Vignysa teres	Endogeic	X		
Species number		2	6	11

One month after starting the study in the field (T 1; June 2011) in Montpellier, earthworms were sampled. Further samplings in November 2011 and June 2012 failed due to dry soil, i.e. no worms were found at all. Only two species could be found at this site: juvenile lumbricid worms belonging to the genus Aporrectodea and an adult worm which was genetically (using barcoding, COI) classified as *Vignysa teres* (also known as *V. popi* (Bouché 1972) (Hormogastridae, Oligochaeta). Control earthworm abundance was very low (10 ± 14 ind/m2).

Three samplings - one, three and six months after starting the study - were performed in Wageningen. At this site six species from five lumbricid genera were found, representing two of the three ecological groups (anecics are missing). Again, there are not enough species (and the individual numbers per species are too low too) to use community composition as an endpoint for the assessment of effects of ivermectin on earthworms. Control earthworm abundance was high, varying at the three dates between 198 and 627 ind/m2.

Two samplings – one and five months after starting the study - were performed in Zurich. With 11 lumbricid species from six genera it is the most species-rich study site, representing all three ecological groups. Therefore, species composition could be used for assessment purposes at this site. Control earthworm abundance was high, varying at the two dates between 288 and 378 ind/m2.

Effects of ivermectin on earthworms

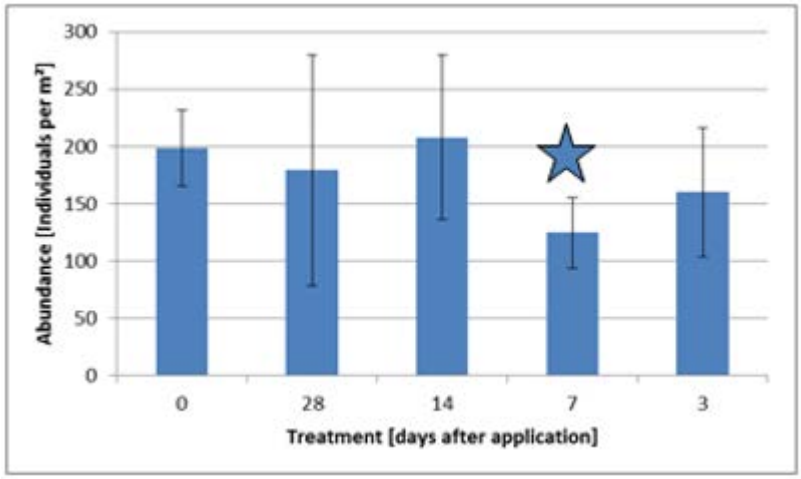
Assessment based an ANOVA:

In Montpellier, about ten earthworms per m2 were found at all treatments including the control, meaning that there was no significant difference in abundance. Earthworm biomass differed more between treatments (one to nine g fresh weight/m2), because the individual worms differed considerably in length and weight. However, no significant difference between treatments could be identified. Because of the lack of any effect for the whole group in combination with low diversity and low abundance, no further assessment was performed.

In Wageningen, statistical significant differences between the highest concentration (D 7) and the control (D 0) were found one month after the start of the study (T1) for total lumbricid (Figure 75) and total juvenile (Figure 76) abundance.

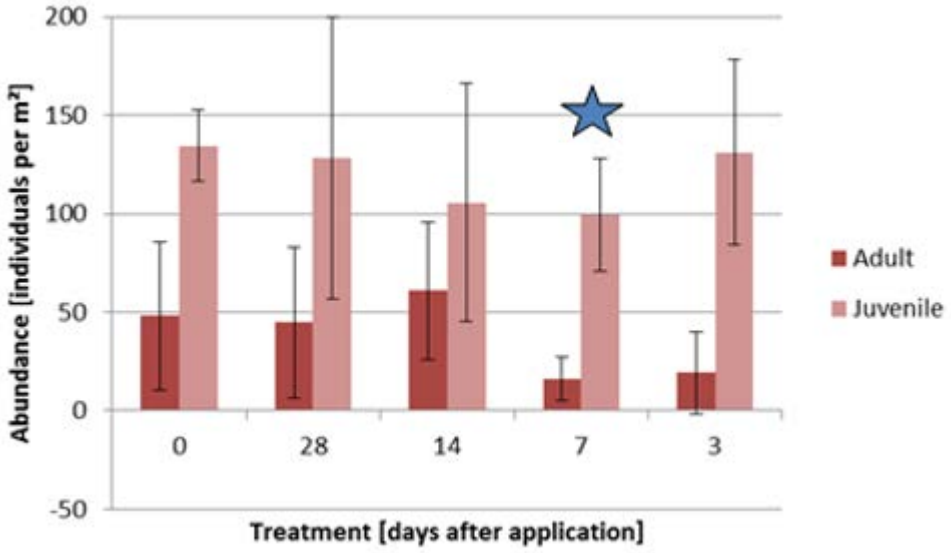
Significant differences between the two highest concentrations (D 7, D 3) and the control were detected at the same time (T1) for the number of epigeics (Figure 77). This effect lasted still after three and six months, but just for D7 (Figure 78 and Figure 79). In this case, epigeic biomass instead of abundance was affected. No other significant differences between control and treatments were found.

Figure 75: Total number of earthworms per square meter at the different concentrations one month after the start of the study in Wageningen



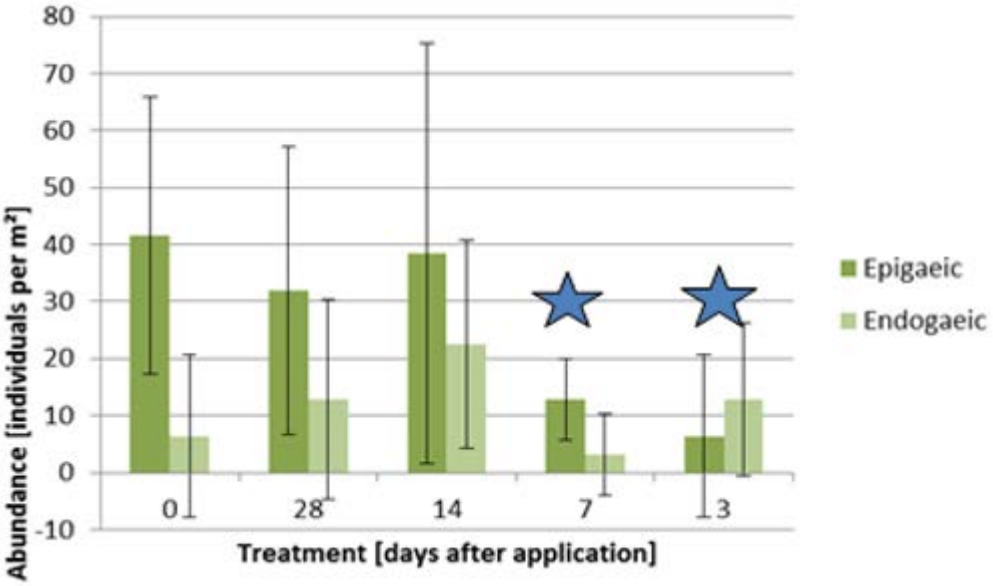
Asterisks = significant different at $p < 0.05$

Figure 76: Number of adult and juvenile earthworms per square meter at the different concentrations one month after the start of the study in Wageningen



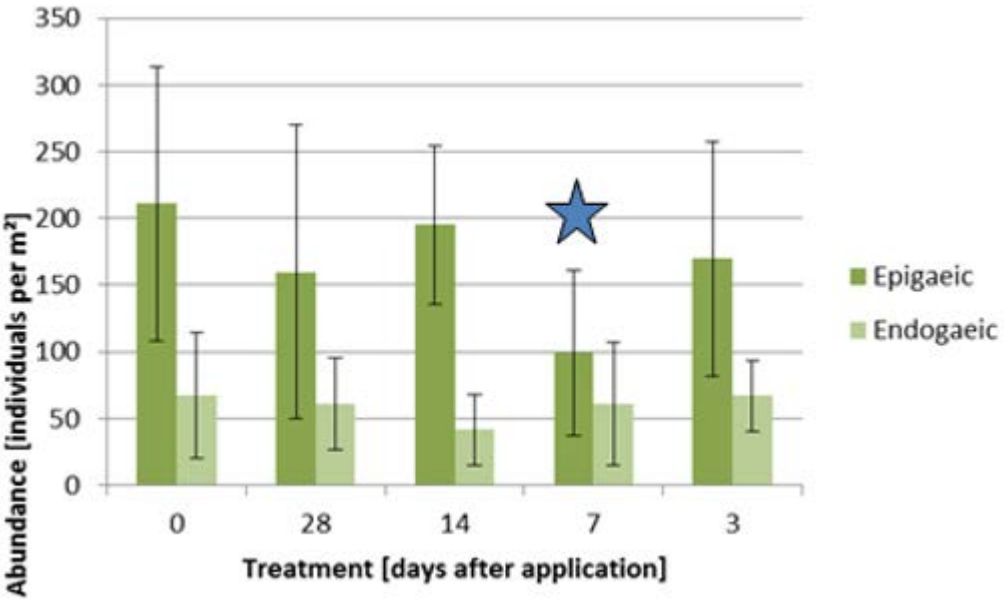
Asterisks = significant different at $p < 0.05$

Figure 77: Number of endogeic and epigeic earthworms per square meter at the different concentrations one month after the start of the study in Wageningen



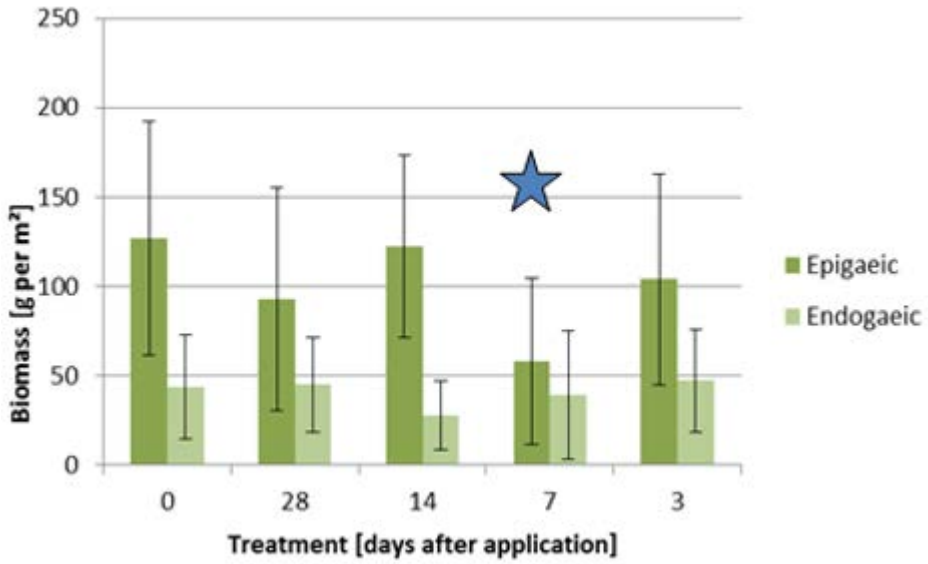
Asterisks = significant different at $p < 0.05$

Figure 78: Number of endogeic and epigeic earthworms per square meter at the different concentrations three months after the start of the study in Wageningen



Asterisks = significant different at $p < 0.05$

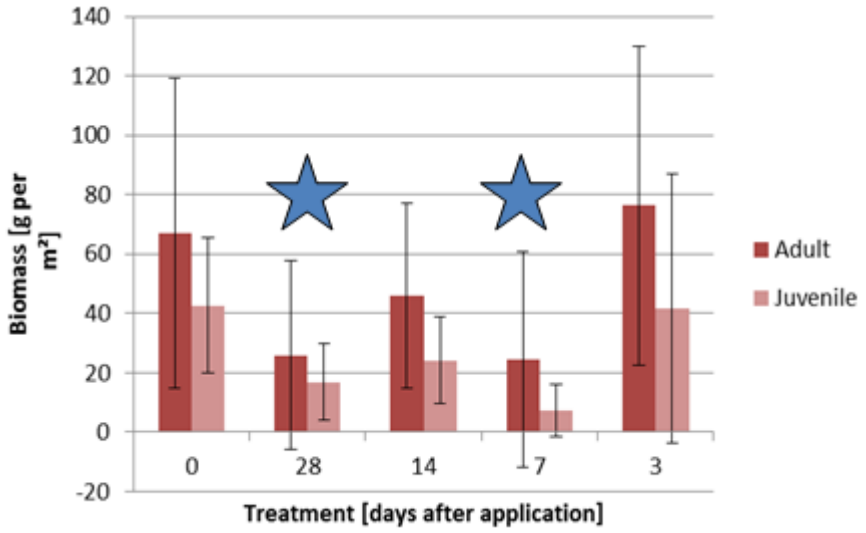
Figure 79: Biomass of endogeic and epigeic earthworms per square meter at the different concentrations three months after the start of the study in Wageningen



Asterisks = significant different at p < 0.05

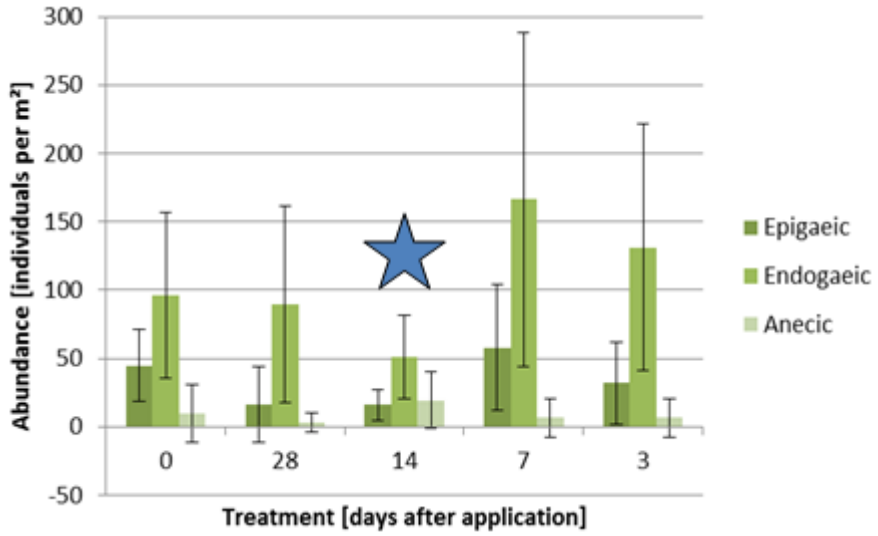
In Zurich, almost no differences between individual treatments and the control were found on the level of the whole lumbricid community. An exception was the total biomass one month after the start of the study which was significantly lower in the treatments D7 and D28 compared to the control (Figure 80). In addition, the number of epigeic worms was significantly lower in the treatment D 14 compared to the control five months after the start of the study (Figure 81). In both cases it seems that there is no steady relationship between treatments (and thus concentrations of ivermectin in soil) and the reaction of the earthworm community. No further significant differences between control and individual treatments were found.

Figure 80: Biomass of all earthworms per square meter at the different concentrations three months after the start of the study in Zurich



Asterisks = significant different at p < 0.05

Figure 81: Abundance of all anecic, endogeic and epigeic earthworms per square meter at the different concentrations five months after the start of the study in Zurich



Asterisks = significant different at $p < 0.05$

Assessment based on Regression Analysis

This kind of analysis did only make sense for the data sets from Wageningen and Zurich (Figure 82 and Figure 83). According to this evaluation no adverse effect of ivermectin in the soil on the total number of earthworms could be identified. Due to the sometimes low absolute numbers of earthworms in combination with small differences in concentrations, especially at the later dates, some results are not reliable: for example, the huge increase in Wageningen six months after application is considered to be an artifact (Figure 82). The same is true for the results five months after application in Zurich (Figure 83). In summary, using this way of analysis, no significant negative impact of ivermection on the total number of earthworms could be identified. Due to this clear outcome, no other endpoints were assessed this way. When assessing the influence of ivermectin in dung on earthworms, again no adverse effect was found (data not shown).

Figure 82: Total number of earthworms per square meter at the different concentrations one, three and six months after the start of the study in Wageningen

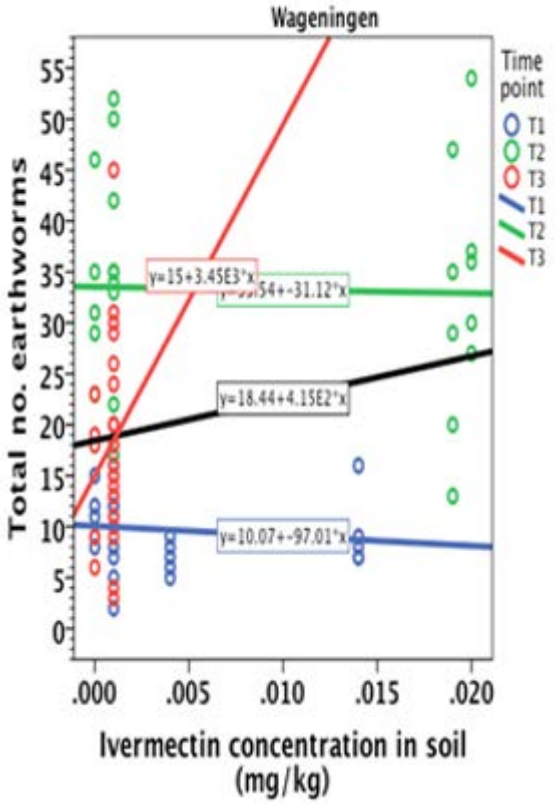
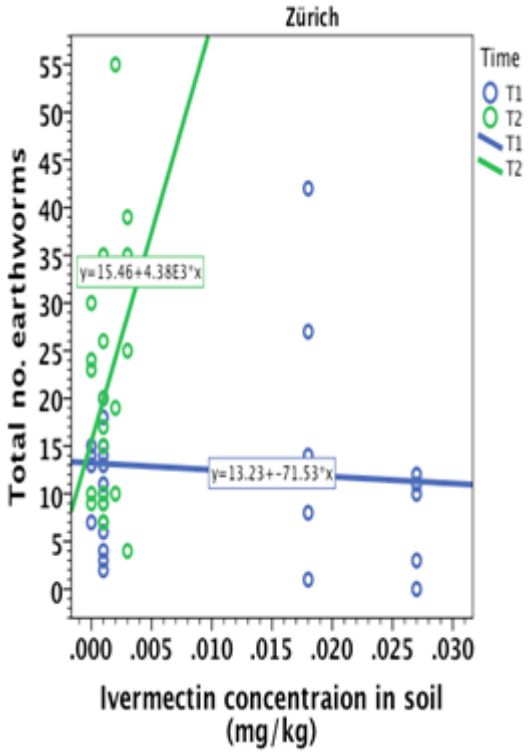


Figure 83: Total number of earthworms per square meter at the different concentrations one and five months after the start of the study in Zurich



15.4.3 Species composition, abundance, and biomass of springtails

Overview on the springtail communities at the four sites

While samples were taken three times in Montpellier (at two other occasions sampling failed due to the dryness of the soil), five times samples were taken in Lethbridge, Wageningen and Zurich, but not always at the same point in time.

Springtail abundance in the controls was rarely high and always varied considerably in time: in Montpellier mean abundance differed between 1000 and 10.000 ind/m2, in Wageningen between 2000 and 40.000 ind/m2, in Zurich between 2000 and 14.000 ind/m2 and in Lethbridge between 500 and 8.000 ind/m2.

The diversity of the springtail communities at the four study sites differed considerably (Table 24): 10 species were found at Lethbridge and Montpellier, but 16 species occurred at Wageningen and Zurich. At all four sites mainly juvenile individuals could not be classified to a certain species but to a genus or even family. Therefore, the number of taxa increases by four for Lethbridge, eight for Montpellier, seven for Wageningen and even 11 for Zurich. No species has been found at all four sites, which is, considering the locations of the sites on different continents and in different biogeographic regions, not a big surprise. Interestingly, five out of 10 species found in Lethbridge did also occur in at least one European site. Only two species were found at all three European sites. The number of species found only at one site is as follows: five at Lethbridge, four at Montpellier, eight at Wageningen, and six at Zurich. Thus, with the exception of Zurich, about 50% of all named species were endemic to one of the study sites. At all four sites, representatives of the three main ecological groups did occur. Therefore, the diversity of the springtail communities, measured as species composition and/or species number, should be sufficient as an assessment endpoint. However, as in the case of earthworms the evaluation will start by using total abundance, number of juveniles or adults, and number of springtails belonging to one of the ecological groups. It should be noted that the species used in standard laboratory tests (Folsomia candida), was only found at one site (Wageningen).

Table 24: List of springtail species (Collembola) found at the four study sites

Species	Ecol. Group	Leth.	Montp.	Wagen.	Zurich
Brachystomella parvula	Hemiedaphic			X	
Ceratophysella denticulata	Hemiedaphic	X	X	X	
Cryptopygus ponticus	Hemiedaphic		X		
Cryptopygus thermophilus	Epigeic	X	X	X	
Cyphoderus albinus	Hemiedaphic				X
Desoria tolya	Epigeic			X	X
Folsomia candida	Hemiedaphic			X	
Folsomia quadrioculata	Hemiedaphic				X
Folsomides parvulus	Hemiedaphic				X
Frisea mirabilis	Hemiedaphic	X		X	
Hypogastrura assimilis	Epigeic			X	X
Hypogastrura manubrialis	Epigeic		X		

Species	Ecol. Group	Leth.	Montp.	Wagen.	Zurich
Hypogastrura perplexa	Epigeic	X			
Isotoma viridis	Epigeic	X			
Isotomiella minor	Hemiedaphic	X		X	X
Lepidocyrtus cyaneus	Epigeic		X	X	X
Lepidocyrtus lignorum	Epigeic			X	X
Lepidocyrtus violaceus	Epigeic	X			
Mesaphorura critica	Euedaphic			X	
Mesaphorura italica	Euedaphic			X	
Mesaphorura macrochaeta	Euedaphic			X	X
Metaphorura affinis	Euedaphic		X	X	X
Neanura muscorum	Hemiedaphic			X	
Neotullbergia crassicuspis	Euedaphic				X
Neotullbergia ramicuspis	Euedaphic		X		
Parisotoma notabilis	Epigeic			X	X
Pogonognathellus falvescens	Epigeic	X			
Protaphorura armata	Euedaphic		X		X
Pseudosinella alba	Hemiedaphic	X	X		X
Pseudosinella petterseni	Hemiedaphic	X			
Sminthurides aquaticus	Epigeic		X		
Sminthurinus aureus	Epigeic				X
Tomocerus vulgaris	Epigeic				X
Species number		10	10	16	16

Effects of the VMP on Collembola

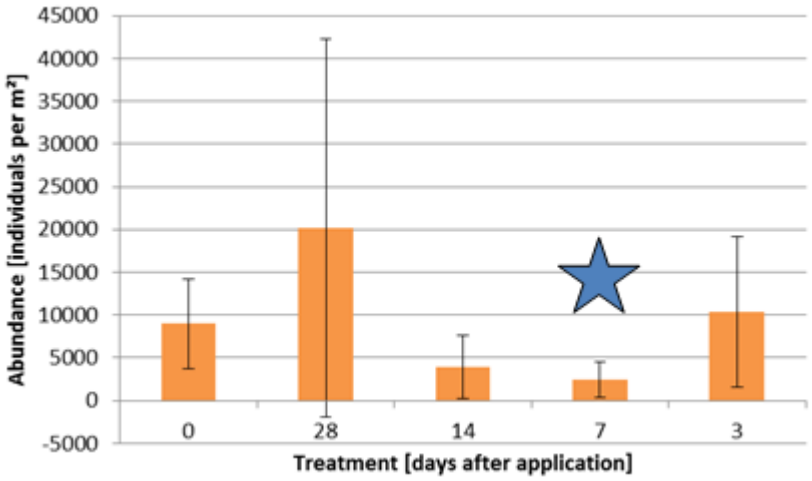
Assessment based an ANOVA:

In Montpellier, no significant differences were found between individual treatments. At T1, the average abundance was about 10.000 ind/m2, but at the later sampling dates this value dropped to 100 – 1100 ind/m2, without any relationship to the respective treatment. Variability at all dates and treatments was high.

In Wageningen, there was almost always no significant difference in total springtail numbers between the five treatments. The one exception occurred at the D7 treatment at T1 (Figure 84). The overall numbers differed considerably between the five dates and between treatments at each date, but variability was again very high. Abundance was highest at T1 and T5 (up to 40.000 ind/m2), but as low as 1000 ind/m2 per treatment at the other dates. No tendency between total springtail numbers and the

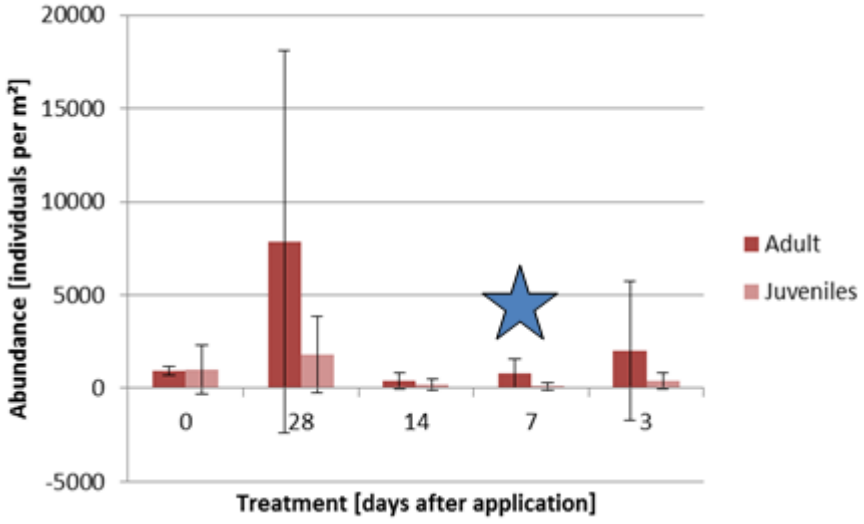
VMP treatment is identifiable; in fact, numbers in different treatments were often higher than in the control. However, when looking at the total number of juvenile and adult springtails separately, a significant decrease was found for adults for treatment D7 at date T1 and for treatment D28 at T4 (Figure 85 and Figure 86). However, absolute numbers are quite low while variability is high. No effects were observed when looking at the standard test species F. candida individually.

Figure 84: Total number of springtails per square meter at the different concentrations one month after the start of the study in Wageningen



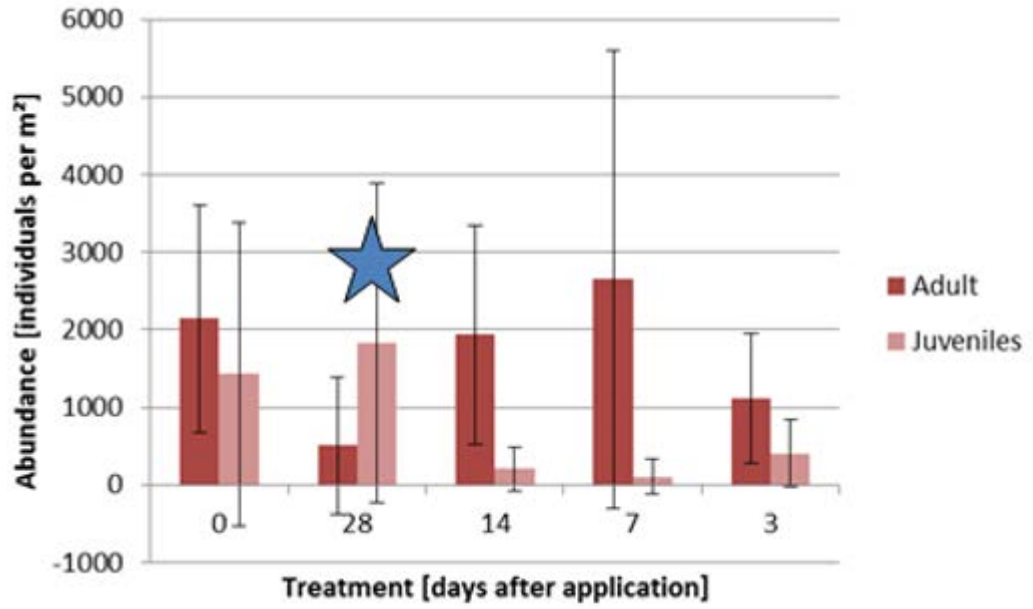
Asterisks = significant different at $p < 0.05$

Figure 85: Number of adult and juvenile springtails per square meter at the different concentrations one month after the start of the study in Wageningen



Asterisks = significant different at $p < 0.05$

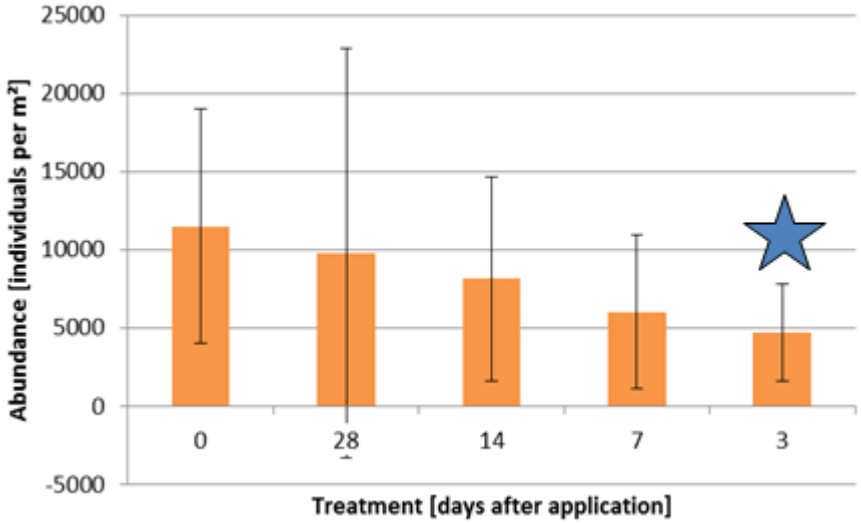
Figure 86: Number of adult and juvenile springtails per square meter at the different concentrations four months after the start of the study in Wageningen



Asterisks = significant different at $p < 0.05$

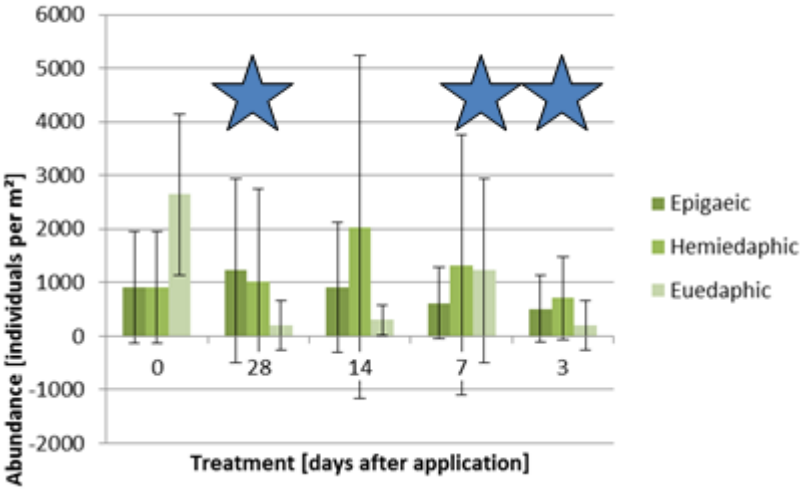
In Zurich, the total number of springtails in the control and the treatment differed only once: at T4 in the treatment D3 (Figure 87). Again, no tendency was found when looking at the numbers at all five dates. Absolute numbers varied between 1000 and 10.000 ind/m2, with almost no springtails at some treatments (including the control) at T1 and at almost all treatments (except D28) at T4. At the same date and treatment, the number of adults was significantly lower than in the control (data not shown). Interestingly, still at T4 several significant differences between the control and three treatments (D3, D7 and D14) did occur when looking at the ratio between the three ecological groups of Collembola. In fact, the high variability at D14 hid the fact that also at this treatment the number of euedaphic springtails was lower than in the control (Figure 88).

Figure 87: Total number of springtails per square meter at the different concentrations four months after the start of the study in Zurich



Asterisks = significant different at $p < 0.05$

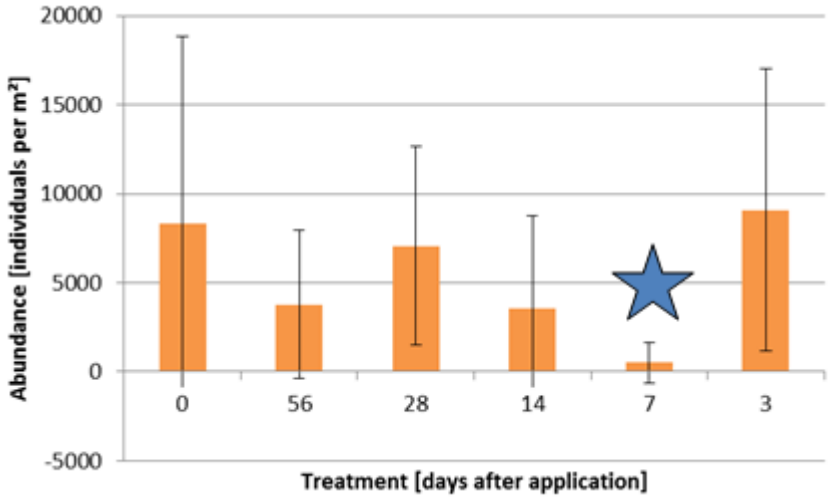
Figure 88: Abundance of all hemiedaphic, euedaphic and epigeic springtails per square meter at the different concentrations five months after the start of the study in Zurich



Asterisks = significant different at $p < 0.05$

Finally, in Lethbridge the total numbers of springtail were almost always not significantly different in any treatment from the control. The only exception occurred at treatment D7 at sampling date T2 (Figure 89). In general, the springtail numbers were relatively low at all dates (in particular T4), i.e. between a few hundred an about 20.000 ind/m2). Again there is no general tendency regarding potential effects of the VMP on these organisms.

Figure 89: Total number of springtails per square meter at the different concentrations two months after the start of the study in Lethbridge



Asterisks = significant different at $p < 0.05$

Assessment based on Regression Analysis

Despite the fact that the number of data was much higher for Collembola than for earthworms, similar problems were observed, mainly due to the fact that in many samples the concentration of ivermectin was very low, i.e. at the detection limit (Figure 90 and Figure 91). Independently from the endpoint evaluated (total richness, i.e. number of taxa, and number of individuals), there was no effect of ivermectin in soil on Collembola in Montpelllier and Zurich. In Lethbridge, a significant increase of both endpoints was observed, but this is considered to be an artifact, caused by the lack of samples with

higher concentrations of ivermectin. Only in Wageningen, there was a significant decrease in the number of taxa and the number of individuals. No effect at all was found when looking at the influence of ivermectin in dung on springtails (data not shown). This difference can be explained by the fact that the springtails were mainly staying in the soil, being exposed to ivermectin mainly when feeding on dung particles.

Figure 90: Taxon richness (number of taxa) of Collembola at all four sites depending on the concentration of ivermectin in soil

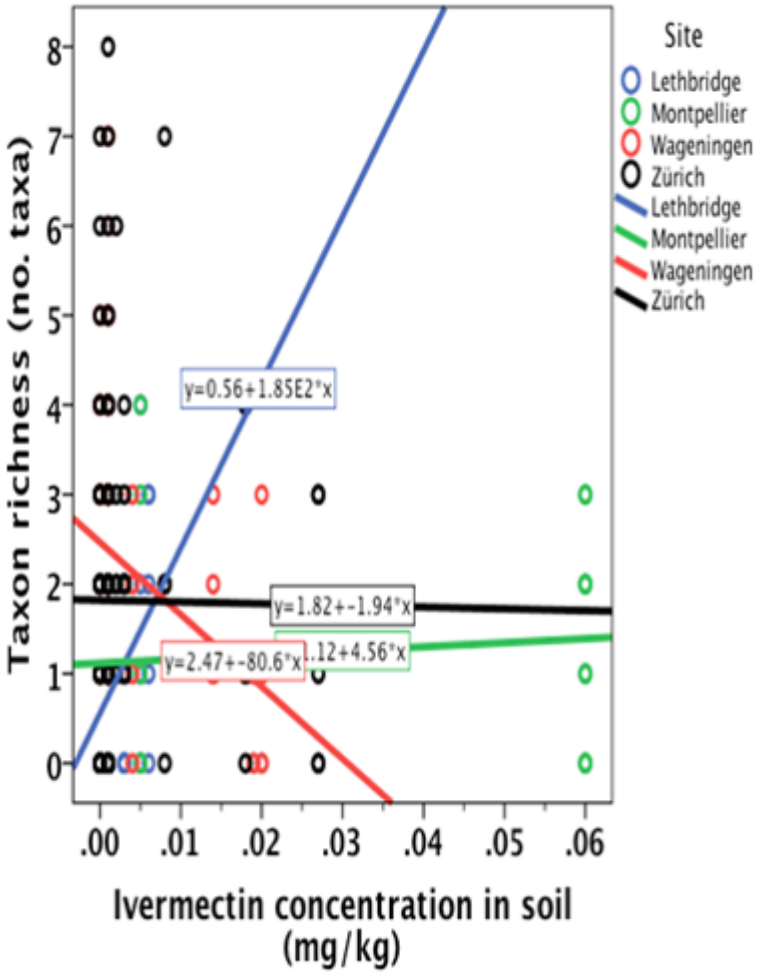
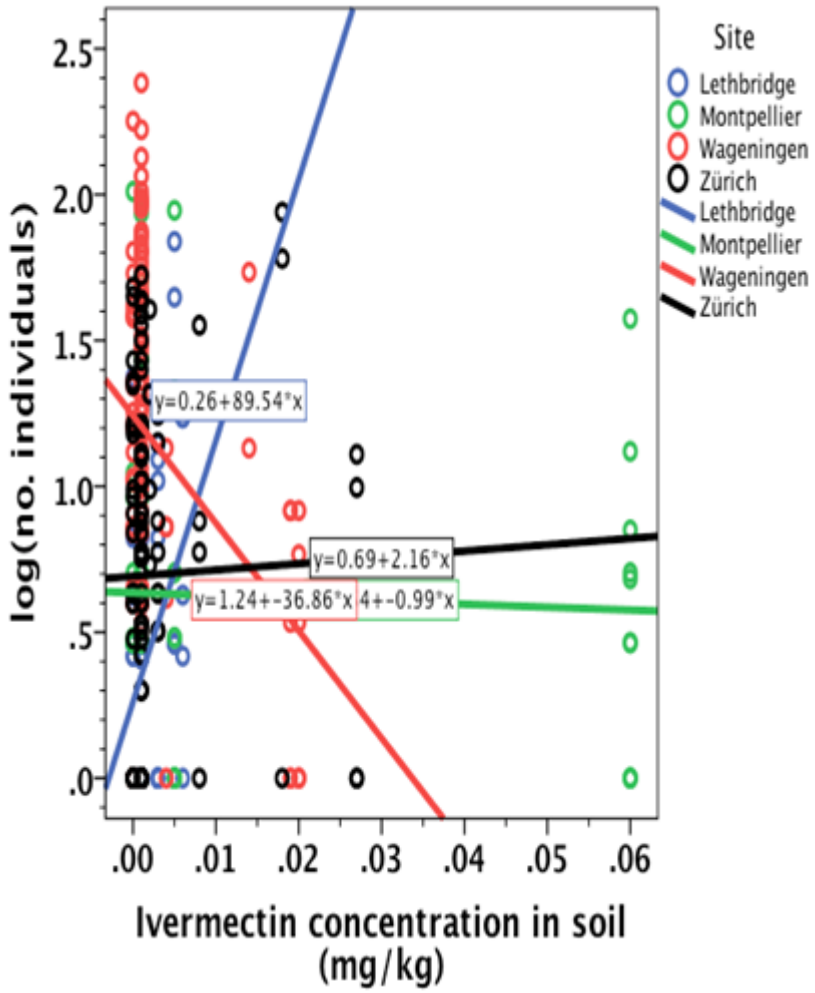


Figure 91: Number of individuals of Collembola per square meter at all four sites depending on the concentration of ivermectin in soil



15.5 Discussion

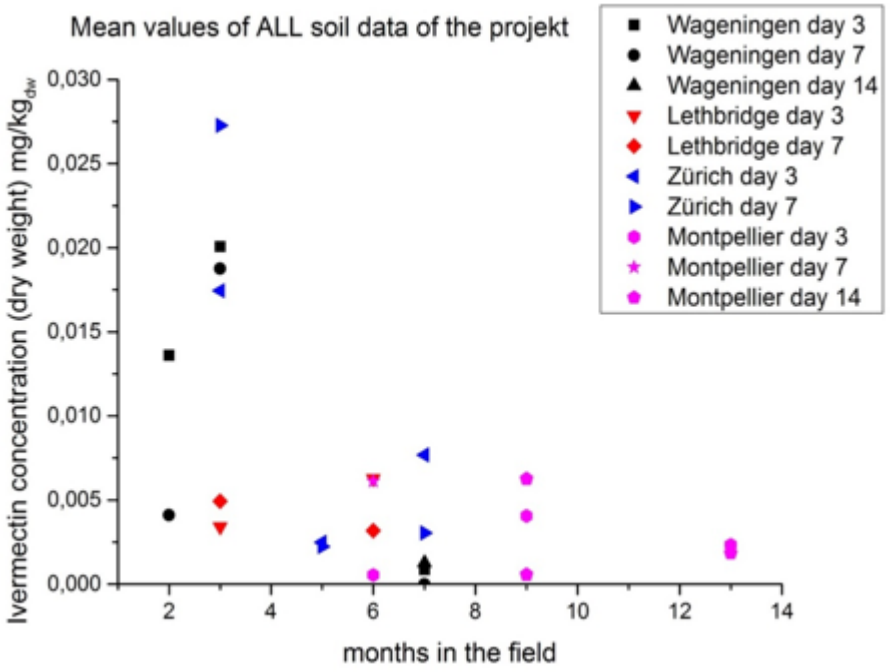
15.5.1 Methodological aspects

Technically, the sampling of earthworms and springtails below the dung pats was not a problem, following the respective guidelines (ISO 200a, b). Extraction of Collembola from soil samples depend much on the technology used, meaning that some kind of validity control should be included in respective guideline. However, sampling strategy and design could be optimized. Firstly, despite its high diversity on a regional scale the earthworm community is not an ideal endpoint in Mediterranean countries due to the low numbers usually encountered during most times of the year. Secondly, the high variability in springtail abundance could be addressed by increasing the number of samples taken at a given point in time (e.g. two samples per pat). However, when doing so, no other samples (e.g. for earthworms) can be taken in such a case. Therefore, and in addition to the recommendations given by Jochmann et al. (2011) the design of sampling soil organisms has to be fixed on a case-by-case basis, meaning that in particular site properties (such as climate), use pattern (when is the VMP used?) and the results of lower tier tests have to be taken into account. Regarding species determination, no problems occurred due to the fact that all study sites are located at sites with a well-known earthworm or springtail community, i.e. keys are available. However, in the case of Montpellier, located in a region with a very diverse earthworm community, this good knowledge happened by chance (a monography published in 1972). Therefore, it is recommended to use barcoding methods in order to facilitate addressing the species level.

15.5.2 Residue analysis

The results of chemical analyses confirmed the presence of IVM residues in soil (i.e. in the uppermost 5 cm) (Figure 92). The highest concentrations found were in a range of about 0.02 to 0.03 mg IVM/kg dw soil (Wageningen, Zurich) three months after starting the study, while at the same time the IVM concentration in Lethbridge was much lower (0.005 mg IVM/kg dw soil). At all later dates (five to 13 months after starting the study) concentrations were almost always lower than 0.006 mg IVM/kg dw soil. These concentrations were mostly found below pats containing the highest IVM concentration (D3, D7). Measurable concentrations of the D14 treatment were found once in Wageningen (after nine months) and twice in Montpellier (after nine and 13 months). At other sites and for all treatments D28 the measured concentrations below other pats were below the limit of quantification. In the future, one should consider sampling only the uppermost 2.5 cm of soil in order to improve the determination of IVM. The depth of 5 cm was used here, because this is the depth most often sampled when looking at micro-arthropods such as springtails. However, recent recommendations for the risk assessment of pesticides do include the sampling for residues as well as organisms in a soil depth of 2.5 cm, among others (EFSA 2010a, b).

Figure 92: All measured concentrations of ivermectin for all treatments at all four study sites



The concentration of IVM in soil has rarely been measured in field studies. For example, at a dry meadow site near Madrid (Spain), cattle was treated with a single application of 200 µg IVM/ kg body weight cm (Römbke et al. 2010a). In soil below pats containing 0.3. – 0.8 mg IVM/kg dung d.w., IVM was found at concentrations between 0.001 and 0.005 mg/kg soil d.w. in a depth of 0 – 2 cm and between 0.0002 and 0.001 mg/kg soil d.w. in a depth of 2 – 5. Comparable concentrations, but almost only in the uppermost centimeter of soil, have been found in a similar field study performed near York, England (Pope, 2010). These values are in the same order of magnitude as those found in this study. Obviously, the mobility of ivermectin from dung to soil – while generally slow and limited – can be influenced by environmental factors, mainly precipitation. Using soil from the Madrid site, Krogh et al. (2009) determined dissipation time (DT) values for ivermectin as 10–16 days (DT50) and 54–89 days (DT90) in the laboratory. Our own observations are probably in the same range.

15.5.3 Earthworms

Representativity of the earthworm communities at the three study sites

The diversity of the earthworm communities differed considerably (2 – 11 species), this difference was expectable as far as can be said in the light of the very different information regarding these organisms in the three study sites (Lethbridge was not sampled for earthworms due to the fact that in prairies lumbricids do not occur for natural regions). Most interesting is the fact that in Montpellier an individuum of the very rare species Vignysa teres (Hormogastridae) was sampled. So far it has only been found twice, in both cases close to Montpellier (Bouche´1972). While lumbricid earthworms (including the species-rich genus Aporrectodea) are found all over Europe hormogastrid worms are restricted to the regions around the Western Mediterranean basin. No abundance or biomass earthworm data from literature are known from the region around Montpellier.

In Wageningen the observed and expected earthworm community in terms of species composition is comparable. For example, Rutgers et al. (2008) list mean values of 4.6 (3 7) species for such grasslands on sandy soils. With the exception of *P. antipai* all species found belong to the "normal" earthworm fauna of central European grasslands (Römbke et al. 2012). The occurrence of *A. chlorotica, Octolasion sp.* and *P. antipae* can be seen as an indication of (at least sometimes) high moisture levels in the Wageningen soil. This observation is in line with the fact that the study site is surrounded by small ditches, some of them less than 100 m away. The abundance (on average about 350 ind/m2) on the control plots seems to be at the higher end of the typical range for Dutch grasslands on sandy soils. For example, Rutgers et al. (2008) lists 163 (24 – 388) Ind/m2 for this land use form.

In Zurich, the species composition is heterogeneous: most of them are typical inhabitants of Central European grasslands (e.g. *A. caliginosa, A. longa* or *L. terrestris*), while others, especially A. chlorotica are indicators for moist soils. However, there are no surface waters close to the sampling area. Even more difficult to explain is the occurrence of two species usually found in acid soils (*D. rubidus, D. attemsi*), especially at coniferous forest sites. Probably such a forest did originally grow here, but the study site belongs to the university ground and the next forest stand is several hundred meters away. More importantly, the pH (7.4) of the soil is clearly higher than that usually preferred by these two species (i.e. < 4.5) (Sims & Gerard 1999). Despite the fact that no earthworm data from comparable sites in the Zurich region are available it seems that all species found at our study can be considered to be a normal part of the lumbricid fauna in this part of Switzerland (e.g. most of them were also found in a nearby forest sites (Daniel 1991)). No data on earthworm abundance in grasslands sites of the Zurich region are available. However, using information from South German meadows the study site seems to be well inhabited (Römbke et al. 2012).

Effects of ivermectin on earthworms

In the literature, no quantitative data on the toxicity of IVM on earthworms under field conditions were found. However, the chronic toxicity of this VMP on the compost worm Eisenia fetida (Lumbricidae) was studied several times in the laboratory, usually using artificial soil (e.g. Halley et al. 1989; Gunn & Sadd 1994). The lowest effect values were determined in a reproduction test with a duration of 56 days (Römbke et al. 2010a): NOECReproduktion = 2.5 mg IVM kg-1 soil d.w.; EC50_Reproduktion = 5.3 mg IVM kg-1 soil d.w. When assessing the risk of IVM to earthworms, usually the exposure, i.e. the highest soil concentrations (worst case: 4.8 µg/ IVM kg-1 soil d.w.) is compared with the lowest effect values (i.e. here: 2.5 mg IVM/kg soil d.w.). The ratio between these two values is clearly <1, meaning that laboratory tests do not indicate any risk of ivermectin to earthworms (Liebig et al. 2010). However, this statement seems to be contradicted when looking at the results of this study: At Wageningen and Zurich, significant differences between the control and individual IVM treatments on total earthworm abundance, number of adults or the number of individual ecological groups were found in some cases. In most of them, this difference was observed at the two highest treatment groups (and

thus ivermectin concentrations). However, looking at the standard deviation at the individual treatments and dates, the absolute differences in numbers and biomass are often quite small. In the light of the results of the regression analysis performed at two sites (in Lethbridge and Montpellier there were either no or not enough earthworms for analysis) it seems that there is no consistent effect of ivermectin on these invertebrates under field conditions.

15.5.4 Collembola

Representativity of the springtail communities at the four study sites

Right now, information on the "normal" springtail community at the four study sites is very limited. Therefore, we can only assume in analogy to what has been said about the respective earthworm communities at these sites: there is no indication that the study sites were strongly impacted by some unknown stress factor. For example, the species number for springtails in German grasslands is 13 – which is the mean of the numbers found at the three study sites. However, some interesting observations have been made (J. Salamon, pers. Comm.): In Montpellier, the species *Sminthurides aquaticus* was found. Usually, it occurs close to eutrophic lakes. Maybe this species was attracted by the microclimatic conditions of fresh dung pats. At the Zurich site *Neotullbergia crassicuspis* was determined regularly, but this species is usually classified as being rare. At the same site *Cyphoderus albinus* was found several times, which is associated with ants – i.e., it is not considered to be a dung species.

Effects of ivermectin on springtails

In the literature, almost no quantitative data on the toxicity of IVM on springtails under field conditions were found. However, the chronic toxicity of this VMP on the standard test species *Folsomia candida* and *Folsomia fimetaria* was studied several times in the laboratory, usually using artificial soil. The lowest effect values were determined in a reproduction test (duration: 28 days) with F. candida: NOECRepro. = 0.3 mg IVM kg-1 soil d.w.; EC50_Repro. = 1.7 mg IVM kg-1 soil d.w. (Römbke et al. 2010a). The same effect values were found when testing the closely related species *F. fimetaria* (Jensen et al. 2003). Finally, even lower values were measured when testing again *F. fimetaria* using the same method but a test duration of 21d: NOECRepro. = <0.2 mg IVM kg-1 soil d.w.; EC50_Repro. = 0.11 mg IVM kg-1 soil d.w. According to Liebig et al. (2010), using data from a two-species laboratory test (Jensen et al. 2009), a risk to springtails cannot be excluded. Thus, highest exposure and lowest effect values differ only by a factor of about 17. The relevant exposure concentration is probably higher when looking only at the uppermost 2.5 cm of the soil profile. In addition, the springtail community consists of at least 10 species with, probably, different sensitivities, meaning that this difference is not very large. Based on the results of the ANOVA analysis, in eight cases a significant difference between springtail numbers under control and treatment pats was observed. These differences did occur most often at D3 (two times) and D7 (four times), but – difficult to explain - also twice at D28 (four and five months after the start of the studies in Wageningen and Zurich). High standard deviations and a lack of clear concentration-effect relationships were regularly found, which might be caused by the fact that in many soil samples only low concentrations of ivermectin were detected. However, a significant decrease of both taxon number and number of individuals in Wageningen proofs that springtail communities could be affected by ivermectin under field conditions.

15.5.5 Summary and outlook

In this study, it could be shown that IVM is found in quantifiable amounts below dung pats from treated cattle. At the study sites, earthworm and springtail communities did occur as expected, taking climatic and biogeographical factors into consideration (i.e. (almost) no earthworms at Montpellier and Lethbridge). At all sites (but in different intensity) significant differences in the number of earthworms or springtails between the soil below control and treated pats, respectively, could be found at several dates. Despite indications that IVM was responsible for these differences (they occurred most often at the two highest concentrations, D3 and D7), a clear concentration-effect relationship is – with the exception of

the effects of ivermectin on the springtails in Wageningen - often lacking. These effects are probably being caused by additional factors than just the IVM concentration: different sensitivity and/or behavior of the individual species, small-scale differences in soil conditions or dung organism activities, influencing the transport of IVM from the dung pat to the soil might be listed here. Referring to the results of laboratory tests, effects on earthworms were not expectable but could not be excluded for springtails. This difference is confirmed by the results of this study. Surely further research is needed in order to improve our understanding of the cause-effect-relationships of ivermectin and the soil organism community.

Summarizing the experiences made in this study it is recommended to include the study of soil organism in field studies assessing side-effects of veterinary pharmaceuticals on a case-by-case basis (i.e. mainly when already effects in lower tiers were observed). Further guidance concerning the performance of such work has to be prepared, using recommendations provided by Jochmann et al. (2012; see also Adler et al. 2013) as a starting point. In the long run, a detailed guidance document according to OECD rules should be prepared. Finally, further efforts are needed to facilitate the evaluation of the results of such complex field studies, aiming to improve the environmental risk assessment of VMPs.

Acknowledgement

The work described here was sponsored by the German Federal Environmental Agency (Project-No. FKZ 371063412). Many thanks go to Dr. Jörg Salamon (Hannover, Germany), who determined the collembolans. Finally, we thank all colleagues who helped during the sampling campaigns at the four study sites.

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16 Annex 1: Standard Operation Procedure (SOP) and checklist for the performance of field studies with VMPs: a result of this project

16.1 Standard Operation Procedure (SOP) for the performance of field studies using structural (diversity and abundance of dung and soil organisms) and functional (dung degradation) endpoints in the context of the ERA of VMPs

This SOP is based on the recommendations given in Jochmann et al. (2011) and on the discussions of the first workshop of the UBA project on dung biodiversity (Montpellier, France; April 01, 2011). The text has been compiled by Bernhard Förster.

Keeping of Animals (Livestock)

The number of animals that are needed to produce dung for the experimental dung pats depends on the test design, i.e. on the

- Number of concentrations
- Size of the experimental dung pats
- Replication
- Number of dung pat retrievals from the field (sampling dates)

As a rough estimate for cattle, approximately 22 kg fresh weight of dung is produced per animal within 24 hours.

Animals for dung production, e.g. cattle, should be held separately from other animals, especially if these animals have been treated with VMPs, to avoid any kind of cross contamination. Animals should preferably be of the same gender, age and breed. They should be fed on the same diet for at least 4 weeks before dung sampling and must not receive any VMPs for at least 6 months before dung sampling.

Application of the Test Substance

Prior to handling of the test substance the instructions given by the producer should be read carefully. The test item should be applied according to the method described in the instructions, i.e. application should be a single application at a field relevant rate. Pour-on substances should not be applied to parts of the skin that are either injured or dirty.

Sampling and Storage of Dung

Dung should be sampled before treatment of the animals (= untreated control dung) and at various time points post treatment of the animals with the test substance (= dung with different test substance concentrations). Suggested time points are 3, 7, 14 and 28 days post treatment, assuming peak excretion of the test substance in dung on day 3. In case of a known different excretion profile the sampling schedule should be adapted accordingly. The dung sampled from treated animals during a particular sampling day is combined to one composite dung sample (bulk sample). Each sampling day represents a certain test substance concentration in the dung. The concentration of the test substance needs to be verified analytically (see chemical verification of VMP concentration in dung).

Dung can either be sampled after excretion (Option 1) or actively from the rectum of the animal (Option 2).

Option (1): If dung is to be sampled after excretion the cattle should be placed on concrete floor (without straw), e.g. in a pen. Dung should be collected from the floor immediately after excretion. This can be done by the help of a dustpan and a brush. Dung contaminated with urine should be avoided. Alternatively, dung can be collected in special bags tied around the animal's rump.

Option (2): Active dung sampling from the rectum requires experienced staff (veterinary) and is therefore not recommended as the standard procedure.

In any case dung should be collected into a sealed vessel to avoid/reduce immediate colonization by dung organisms. If not used the same day, dung should be stored at ≤-18°C. After thawing, the dung should be thoroughly mixed (by the use of an electric stirrer) to ensure uniform constituency and texture.

Note: Be aware that thawing of a large amount of dung, e.g. 20 kg fresh weight, will be time consuming. Therefore, dung should be frozen in smaller units (up to 5 L) and mixed after thawing; overnight thawing should be considered.

Characterization of Dung

To determine the physico-chemical properties of the dung used for the test, sub-sampling of dung for characterization should be performed once prior to placing the experimental pats out in the field.

A minimum set of data would comprise water content (% dry mass), coarse fiber content, ash content and pH.

To determine the water content, a sub sample of at least 20 g fresh weight is weighed into appropriate glassware and dried at 105°C for at least 12h.

The coarse fiber content is measured by washing a defined amount of dung, (e.g. 100 g wet weight) through a fine mesh (0.5 mm) followed by drying and reweighing the coarse fraction.

To assess the ash content of the dung, a 50 g sample of the fresh dung is collected and the water content is determined after oven-drying for 24 h at 105°C. The dry dung sample is then finely ground and approximately 1 g is placed into a weighed silica crucible, weighed, heated in a muffle furnace to 500 °C for 4 h, cooled and reweighed to determine the ash content.

The pH should be measured in fresh dung/water slurry of 2.5 parts fresh dung mixed with one part water (ratio 2.5:1 w/w) in triplicate.

If dung was stored, sub sampling for characterization should take place on day 0 (i.e. the day when the pats are placed in the field) to describe the properties of the dung at the starting point of the field experiment. If the dung was stored frozen samples should be taken after thawing and homogenizing.

Preparation of Experimental Dung Pats

The bulk dung sample is homogenized (after thawing, if stored frozen), e.g. by the use of an electric stirrer. Cross contamination between dung batches with different VMP concentrations due to the use of the same equipment must be ruled out (e.g. by starting homogenization with the lowest concentration).

Depending on the desired size of the dung pat a defined amount of dung (500-800 g fresh weight) is weighed into a labeled plastic bag or box and sealed. All pats should have the same weight (± 5%) and shape, as far as reasonably possible. The bags/boxes are brought to the field at ambient temperature and protected from direct sunlight (to prevent from heating-up). Alternatively, a container ('mould') that holds a defined amount of fresh dung by volume (e.g. 500 g) can be used. In this case a pail of mixed manure is taken into the field, and then the mould is used to place a '500 g' mound of manure on the plate with the layer of sand.

Chemical Verification of VMP Concentration in Dung

Chemical analysis is needed to determine the concentration of the VMP in the dung used for the experiment. Following the homogenization step on day 0 at least two analytical sub-samples of at least 100 g fresh weight, each, are taken from the bulk dung of each concentration (dung sampling date). One of the duplicate samples is stored as a reserve. If chemical analysis cannot be performed immediately, the

analytical samples are stored in appropriate and sealed vessels (e.g. glass, depending on the adsorption properties of the substance) at ≤ -18°C.

Description of the Experimental Field

The field/pasture on which the experiment takes place should be described with at least the following data: location (coordinates) vegetation, use history, e.g. former use of plant protection products and fertilizers and daily precipitation and air temperature during the trial. Also, the top soil (0-10 cm depth) should be characterized. Data should include texture, pH, organic matter content, maximum water holding capacity and C/N ratio.

Placing of Experimental Dung Pats in the Field.

If the experiment is to be performed on grassland pasture, vegetation within the experimental area should be cut, if necessary, and the area should be fenced or otherwise protected from animal trampling.

The experimental dung pats are placed out in a grid. Distance between the pats within a row should be approximately 5 m, and the distance between rows should also be 5 m. If the space is limited, the distance can be smaller but should not fall below 2 m. All pats should be numbered and each pat should be clearly marked, e.g. by a stake equipped with a label, indicating the number, the concentration (or dung sampling date), the set of pats to which the pat belongs to (structural or functional) and the retrieval date.

For the structural part (dung organisms), a 1-2 cm thick layer of sand is placed on styrene plates. An experimental pat is then placed on the sand. Styrene plates of 23 cm diameter work well for pats of 500 g fresh weight. Small holes (e.g., 3 each of 2-3 mm diameter) are made in the bottom of the plate before adding the sand, to prevent rain water from pooling in the plate.

Experimental pats can be weighed and held in individual containers prior to placement in plates. Alternatively, the weight of fresh pats can be estimated by volume; e.g., a 500 ml container will hold 500 g of fresh dung. For this latter method, fresh dung is gently packed in a polythene container to remove air pockets, and then the container is held upside-down to deposit the pat on the sand. Regardless of the method used, all pats should be of similar shape (e.g., height, diameter) and of the same weight.

In areas where tunnelers are dominant, mainly in Mediterranean areas, plastic containers of twenty centimeters deep are completely buried in the ground up to their rim and filled with sieved soil. Dung placed on the surface of the sieved soil is colonized by dung beetles which dig galleries beneath the pat. In areas where tunnelers are dominant, mainly in Mediterranean areas, plastic containers of twenty centimeters deep are completely buried in the ground up to their rim and filled with sieved soil. Dung placed on the surface of the sieved soil is colonized by dung beetles which dig galleries beneath the pat

To determine which species of dung insects are active during the exposure of structural pats, dung-baited pitfall traps (e.g., 3-5) can be operated at the site during the exposure period. Best results will be obtained by emptying traps and renewing baits every 3-4 days. The collection chamber of each trap should contain a preservative replaced as needed. The preservative can be a strong saltwater solution (e.g., 250 ml) with 2-3 drops of dish detergent to reduce surface tension. Non-toxic propylene glycol also can be used for this purpose.

For the functional part (dung decomposition) the dung is placed on a piece of netting (size about 25 by 25 cm, mesh width 4 to 8 mm) to allow free access of soil organisms from dung to soil and vice versa.

To prevent pats from being disturbed by birds, such as e.g. corbies, each pat has to be covered by a pyramid-like protective hood made of chicken wire or other appropriate netting. If soft netting is used, a stake may be put into the soil beneath the pat to hold the net (like a tent pole).

Experimental Phase - Dung Organisms (Structural Part)

To examine the number and species diversity of dung organisms, pats with their plates are placed out on pastures preferably in spring when dung insects are normally most abundant. Pats are placed at least 2 m apart and 5 m from the boundary of a field used for grazing the herd.

Seven days after exposing pats in the field, all pats of the structural set including their styrene plates are transferred to the laboratory where each pat and its corresponding plate is placed in a cage to catch emerging adults. In case the 7-day exposure is unusually cool pats could remain out a little longer; e.g., 10 days.

Experimental Phase - Dung Degradation (Functional Part)

To examine the natural rates of dung decomposition, pats are placed out on pastures preferably in spring. Pats are placed at least 2 m apart and 5 m from the boundary of the pasture used for grazing the herd. Each pat is placed on a plastic netting (size about 25 by 25 cm, mesh width 8 to 10 mm).

At defined time points, e.g. 14, 28, 56, 112 after placing out the pats a number of five pats per test concentration and control is selected at random and placed directly into polythene bags. The bags are brought back to the laboratory and the dung is weighed to determine the actual fresh weight. Thereafter, water and ash-free organic matter contents of the remaining dung are determined as described above and the weight loss (based on ash-free dry weight) of the dung pat is calculated as the difference between the initial ash-free dry weight of the pat and the ash-free dry weight after exposure in the field.

Soil Organisms in Below-Pat Soil

To assess potential effects of the test substance on soil organisms, the soil from underneath each pat of the functional set is investigated for microarthropods, nematodes (possibly) and earthworms immediately after removal of the pat.

For microarthropods, one soil core of 5 to 6 cm in diameter and 5 cm in depth is taken by the help of a split corer. Extraction of microarthropods from the soil sample is done via Kempson according to ISO Standard 23611-2 (2006b).

► ISO (International Organization for Standardization) (2006b): Soil quality - Sampling of soil invertebrates Part 2: Sampling and extraction of microarthropods (Collembola and Acarina). ISO 23611-2. Geneva, Switzerland.

For nematodes one soil core of 1.5 to 2 cm in diameter and 5 cm in depth is taken. Extraction of nematodes from the soil sample is done via wet extraction according to ISO Standard 23611-4 (2007).

► ISO (International Organization for Standardization) (2007): Soil quality - Sampling of soil invertebrates Part 4: Sampling, extraction and identification of free-living stages of nematodes. ISO 23611-4. Geneva, Switzerland.

After taking these two samples, the remaining soil within an area of 25 by 25 cm and 10 cm depth is excavated and filled into a tub (or on a foil) for hand sorting to determine abundance and species diversity of earthworms. About 5 L of a 4% formalin solution is then poured into the hole to make deep borrowers escape from their holes; all earthworms caught by hand sorting or appearing on the soil surface within the 25 by 25 cm area are stored in 70% alcohol. This step is done according to ISO Standard 23611-1 (2006a).

► ISO (International Organization for Standardization) (2006a): Soil quality - Sampling of soil invertebrates Part 1: Hand-sorting and formalin extraction of earthworms. ISO 23611-1. Geneva, Switzerland.

Health and Safety

All employment protection conditions should be considered. During test substance application protective clothing, goggles, rubber gloves and rubber boots should be worn.

Pour-on substances should always be applied under well ventilated conditions.

During application, eating, drinking or smoking is not allowed. After termination of work, hands should be washed.

16.2 Checklist for the performance of field studies using structural (diversity and abundance of dung and soil organisms) and functional (dung degradation) endpoints in the context of the ERA of VMPs

Table 25: Checklist for the performance of field studies using structural (diversity and abundance of dung and soil organisms) and functional (dung degradation) endpoints in the context of the ERA of VMPs

Issue	Description of the UBA study (Draft guideline may be more flexible)	Remarks
Aims of the study		
Protection goals	Diversity of dung and soil organisms, Ecological functions of these organisms	
Study design	Effects of a single application of a model VMP to cattle at a field relevant rate on the species diversity and abundance of dung and soil organisms (structure) and the decomposition of dung (function) within one season (i.e. two sets of dung pats are needed).	Duplication of the design without change possible, e.g. by exchanging dung pats from treated cattle with dung pats spiked with the test VMP
Product	Test report of a higher-tier field study with VMPs (pasture scenario) and/or a scientific publication.	Such tests will be performed using an OECD Guidance Paper (to be prepared using the UBA study).
Methods		
Livestock	Cattle: Minimum: same gender and age; if possible, same breed. Not necessary to keep cattle always in the stable or in the field	
Livestock diet	Grass / hay (in any case: constant diet within the study: i.e. 4 weeks before and after treatment.	
Restrictions	Do not use animals previously treated with a parasiticide within 6 months before application. Any other drug use has also to be documented.	
Test item	Pour-on formulation of Ivermectin (both injection and pour-on are regularly used in EU and NA, but the latter is easier to use)	

Issue	Description of the UBA study (Draft guideline may be more flexible)	Remarks
Housing	Separate treated cattle from other cattle	
Test item amount	Ivomec pour-on bovins = ivermectin (IVM) 0.5g/excipient q.s.p. 100 mL. That is 0.5 mg IVM/kg body weight (=1 mL Ivomec/10 kg b.w.).	Example. Values can be adapted due to local conditions
Method	Fresh dung from one group of animals (5 – 10 cows) collected before and at various times post-treatment. The dung is frozen (-18°C) until collections are completed.	
Positive control	Pats spiked with a high concentration of ivermectin. Initial ivermectin concentrations have to be measured in all treatments.	Spiking may cause attraction behavior of dung beetles
Negative control	Dung from animals prior to application (day 0)	
Concentrations	Determined by the sampling schedule of dung: e.g. just before treatment and then 3, 7, 14 and 28 days post treatment (each sampling date represents a different concentration)	Additional dung sampling at Day 56 after treatment recommended for ivermectin
Replication	Structural set: 10 pats x 5 concentrations (including control) x 1 sampling date = 50 pats Functional set: 5 pats x 5 concentrations x 5 sampling dates = 125 pats	Example, but also minimum requirment
Dung pat preparation	Estimation of minimum amount of dung (without reserve). Structure set: 10 repl. á 500 g of pats ➔ 5 kg dung FW à 5 concentrations is 25 kg in total Functional set 5 repl. á 500 g of pats á 5 sampling dates➔ 12.5 kg dung á 5 concentrations = 62.5 kg dung FW in total ➔ both sets together = 25 + 62,5 = 87,5 kg dung FW (+ analytics)	
Characteri-za-tion of dung	Dry mass (or Corg?), fibre content (?), pH, (after thawing the dung to prepare the pats day 0)	
Dung pat placement	Pats be placed in a grid (e.g., 5 m x 5 m) with treatments randomized within rows	
Dung pat exposure and sampling	Using the “Floate-Method”, pats are placed on Styrofoam plats plus a sand layer. Functional pats are placed on a net on the ground	In order to get tunne-lers (more relevant in Mediterranean areas), plastic vessels are buried directly below the pats in the field
Study sites and field work		

Issue	Description of the UBA study (Draft guideline may be more flexible)	Remarks
Test sites	Sites should be located in a grazing area. Exact site is identified by GPS.	
Test start date	May (slightly variable due to regional climate). Most often in Europe: May to June	
Test duration (field)	Structural set: sampling day: 7 (= 1 week) => 50 pats in total Functional set: sampling days: 28, 56, 112, 224 and 365 days (= 125 pats in total)	Functional series: Individual dates could differ due to regionally different climatic conditions; but 5 sampling dates are the minimum.
Characterization site	Coordinates, vegetation, precipitation, daily temperature, use history, anthropogenic stress	
Characterization soil	Texture, pH, organic matter content, maximum water holding capacity, C/N ratio	
Field measures	Protection measures against birds etc. can differ technically as long as they are efficient (e.g. nets, cages, etc.)	
Measurement endpoints		
Endpoints	Dung organisms, especially flies and beetles, parasitic wasps, soil micro-arthropods, earthworms, dung mass loss (organic matter dry weight)	Set-up of 3-4 baited pitfall traps by all partners to assess overall diversity (control dung could be used as bait; exposure dung exposed for 1 week; roof against rain).
Sampling method: dung	Dung: pats from the structure part are taken into the lab in order to get hatching adults (emergence cages).	In case of high importance of tunnelers, the method has to be adapted
Sampling method: soil	Soil taken below the pats from the functional part: hand-sorting; dry extraction of soil-core samples; possibly: wet extraction (nematodes)	
Taxonomic resolution	Species level: dung beetles, flies, parasitoid wasps (?), earthworms, springtails; Genus level: soil mites, staphylinid beetles; Family / trophic group level: nematodes; General: Identification of the smallest taxonomic unit that can be distinguished with confidence	Note: Check possibility of genetical determination (e.g. bar-coding)

Issue	Description of the UBA study (Draft guideline may be more flexible)	Remarks
Endpoints: Fate	Ivermectin concentrations for all treatments in dung (Day 0 = initial values) and soil (Day 28) Amount needed: 500 g dung FW; 500 g soil FW	Note: Initial dung values needed for risk assess-ment
Residue analysis		
Dung: Method	Method set-up by M. Alvinerie (INRA, France) or by AL-TERRA (Wageningen)	
Soil: Method	Method set-up by M. Alvinerie (INRA, France) or by AL-TERRA (Wageningen)	
LoD / LoQ	Depending on the method chosen	
Data assessment (to be fixed later)		
	Not clear	To be decided later