OECD Environmental Health and Safety Publications

Series on Testing and Assessment

No. XX

GUIDANCE DOCUMENT ON THE DETERMINATION OF THE TOXICITY OF A TEST CHEMICAL TO THE DUNG BEETLE *APHODIUS CONSTANS*

Environment Directorate

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

Paris

May 2009

INTRODUCTION

1. For more than two decades the environmental risk of chemicals in general and pesticides in particular are assessed before these products can be marketed in the European Union. At about the same time when this assessment was codified in guidelines [1], the discussion about environmental impacts of pharmaceuticals just began within the scientific community [2]. Due to increasing evidence of potential side effects of certain pharmaceuticals in the environment, the European Union developed respective guidelines in the mid-nineties [3]. The focus on veterinary pharmaceuticals and especially parasiticides is caused by their direct entry into the environment, and their biocidal mode-of-action [4]. Cattle, sheep, pigs, and horses are treated regularly (metaphylactically as well as therapeutically) with veterinary pharmaceuticals used against endo- and ectoparasites, which often have a nematicidal or insecticidal mode-of-action [5]. In addition, these parasiticides can also impact ecosystem functions in the field, in particular the decomposition of dung [6, 7]. However, such side effects are not always detected [8, 9], which at least partly may be the result of using different and non-standardized methods.

2. Dung beetles (Family Scarabaeidae) are among the most abundant and species-rich organisms associated with fresh dung [2]. In close interaction with micro-organisms and other fauna (e.g., fly maggots, nematodes, oligochaetes) feeding, shredding, and burying of the pat by dung beetles accelerates its decomposition [10]. This facilitates the return of nutrients contained in the dung back into the soil to promote growth of the plants. It also limits the area of the pasture with undecomposed dung, near which cattle will avoid grazing (an effect known as “pasture fouling”; e.g., [11]). Further information on the biology and ecology of dung beetles is presented in ANNEX 5. To identify potential adverse effects of veterinary parasiticides on the dung organism community, data on the effects of these substances on dung flies and dung beetles are required by the responsible agencies in the European Union, North America and Japan (International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) Guidance Paper [12]).

3. A guideline to test for adverse effects of pharmaceuticals on dung flies recently was submitted to OECD. Validated with an international ring test [13] organised by the Society for

Environmental Toxicity and Chemistry (SETAC) advisory group DOTTS (Dung Organism Toxicity Test Standardization), this guideline was finalised in 2008 [14]. However, no comparable guideline exists for dung beetles. The DOTTS group currently is working to develop standardised test methods for two species of dung beetles representing different geographic regions and life histories. *Onthophagus taurus* is a Mediterranean/temperate species that develops in dung buried beneath the pat. *Aphodius constans* is a temperate species that develops within the dung pat (http://www.dottsgroup.org). Methods for *O. taurus* derive mainly from research in Australia [15, 16], while methods for *A. constans* were developed in research supported by the German Federal Environment Agency (UBA, Dessau, Germany) [17, 18, 19]. Further information on the ecotoxicology of dung beetles is compiled in ANNEX 6.

4. Preparation of test guidelines for dung beetles for formal submission to OECD currently is not possible. Partly this is due to unanswered questions concerning methods for *O. taurus* and *A. constans*, and partly because methods developed thus far have not been validated with a ring test. Nevertheless, guidance is needed in cases for which tests on dung beetles are required for environmental risk assessments of parasiticides.

5. The approach and test methods described in this document mainly derive from studies in Europe, Australia and Canada. The tests are required in Phase II, Tier A of the VICH regulations [12, 20] as adopted by the USA, the European Union and Japan. It is recognized that other OECD member countries may have different regulatory requirements for veterinary pharmaceuticals, in particular parasiticides. However, the methods identified in this document add important tools to a battery of existing standardized protocols for assessing chemical impacts on the dung organism community.

6. This test method is designed to assess the effects of a test chemical, e.g. veterinary pharmaceuticals, to dung dwelling life stages of dung beetles. In this test, insects are exposed under controlled conditions to the test chemical spiked into the dung. An extended test, in which the beetles are exposed to dung originating from livestock treated with the test substance is described in ANNEX 4.

7. Besides Diptera, beetles of the family Scarabaeidae are the most ecologically important dung organisms [21]. In close interaction with micro-organisms and other fauna like nematodes and oligochaetes, they promote the decomposition of the dung pat [10]. This, in turn, allows the release of nutrients contained in the dung which are necessary for the growth of the plants. In addition to their role in removal and degradation of dung in pastures, they are also an important food source for insectivorous birds and mammals. Lack of dung insects has been shown to adversely affect dung degradation in climates where these are the key dung degraders, e.g. Australia [2, 22, 23].

8. *Aphodius (Agrilinus) constans* Duftschmidt (1805) is considered to be a suitable indicator species for estimating the toxicity of veterinary parasiticides in dung for the following main reasons: This species covers a wide geographic range in Europe, [24], has a long activity period, a short larval development time [25] and it plays an important role for the decomposition of dung, since it prefers fresh cattle dung for its nutrition and reproduction [7]. It is also well-known that *A. constans* reacts sensitively to veterinary drugs [26, 27]. In addition, a lot of experience in handling and testing this species is available [17, 18, 19].

PRINCIPLE OF THE TEST

9. This test method is designed to assess the effects of a test chemical, e.g. veterinary pharmaceuticals, to dung dwelling life stages of dung beetles. The possible impact of the test chemical spiked into the dung on the development of the beetle first instar larvae (up to 7 d old) is compared to the negative control(s) (an extended test using dung from drug-treated livestock as test substrate is described in ANNEX 4). A positive control should be tested (see § 12). The test chemical is mixed with dung, to which the larvae are added. Then the effects of the test chemical on the following measurement endpoints are assessed under controlled conditions after exposure of the larvae to the test substance (always in comparison to the control):

- Number of surviving larvae after exposure;
- Morphological change, i.e. any visual abnormalities, including body size, biomass etc.

Depending on the experimental design, the ECx (Effect concentration for x% effect e.g. EC50) or the No Observed Effect Concentration (NOEC) can be determined (see ANNEX 1 for definitions).

INFORMATION ON THE TEST SUBSTANCE

10. The water solubility, the log Kow, and the vapor pressure of the test substance should be known to assist the test design. Additional information on the fate of the test substance in dung, such as degradation times, is desirable. Details of the source, batch or lot number and purity of the test and reference chemicals also need to be provided.

11. This Guidance Document can be used for water soluble or insoluble substances. However, the mode of application of the test substance will differ accordingly. The Guidance Document is not applicable to volatile substances, i.e. substances for which the Henry's constant or the air/water partition coefficient is greater than one, or substances for which the vapour pressure exceeds 0.0133 Pa at 25 °C.

REFERENCE SUBSTANCE

12. Ivermectin (tech.) is a suitable reference substance that has been shown to affect beetle larval development [17, 18, 19, 27]. The reference substance should be tested regularly, but two options are possible:

- The ECx of a reference substance can be determined 1 - 2 times per year to provide assurance that the laboratory test conditions are adequate and to verify that the response of the test organisms does not change significantly over time. The EC50 for the endpoint survival should be between 0.5 and 1.5 mg active ingredient (a.i.)/kg dung dry weight (d.w.).
- However, it is more advisable to test a reference substance in parallel to the determination of the toxicity of a test substance. In this case, one concentration is used and the number of replicates should be the same as that in the solvent only control (20). Significant effects on larval survival should be observed at a concentration of 1.0 mg a.i./kg d.w. dung.

The performance of a reference test is always required when a new batch of beetles is tested for the first time, independently whether they were bought from an existing culture or whether they were collected in the field. The period between reference test and definitive test should be less than plus/minus three months.

VALIDITY OF THE TEST

13. The definitive/limit test is valid if in the solvent only control the mortality is lower than 20% (formulated dung) or 30% (fresh dung), see § 18. When a test fails to meet the above validity criteria the test should be terminated unless a justification for proceeding with the test can be provided. The justification should be included in the report.

DESCRIPTION OF THE TEST

Equipment

14. Test vessels must be of an appropriate size, e.g. clear plastic cell counter tubes (20 ml volume made of PS/LD-PE plastic with a diameter of ca. 2.4 and a height of 4.5 cm). Micro well plates (six wells with a diameter of ca. 3.5 cm, a height of 1.5 cm and a volume of 15 mL are also possible). For identification purposes, each tube or plate will be labelled with treatment number, replicate number, test or reference chemical concentration and study initiation date. Plastic test vessels will be discarded at the end of each assay.

15. Standard laboratory equipment is required, specifically the following:

- drying cabinet;
- stereomicroscope;
- brushes for transferring larvae
- pH-meter;
- suitable accurate balances;
- adequate equipment for temperature control;
- adequate equipment for humidity control (not essential if exposure vessels are covered by lids).

Selection and collection of the dung

16. Non-contaminated bovine dung will be obtained from cattle of documented veterinary history. At the time of collection the animals must not have been treated with any veterinary pharmaceuticals for at least 2 months or even longer in case the compound is still excreted after that time. No contaminants should be expected in the dung that might interfere with the conduct of the study.

17. The dung may be collected directly from cattle (internal or bag collection) or ground collected. If dung is ground collected, care should be taken to avoid urine contamination. Ground collected dung should be less than 2 hours old at the time of collection to minimise dung fauna colonisation and should be frozen at ca –20°C for at least 1 week before use in order to avoid mite contamination. In the case of directly collected dung mite infection is very unlikely. Therefore, this dung must not be frozen, but it could be frozen if not needed immediately. The husbandry, in particular the diet, of the cattle providing the dung should be recorded. Samples of the dung should be taken to determine moisture content and pH (see ANNEX 2).

18. The collected dung can be used in the tests in two different ways: Either fresh, with or without being frozen beforehand (= fresh dung) or after being dried, grounded and re-wetted (= formulated dung). In the latter case, handling and mixing-in of the test substance is easier and the homogenised distribution of the test substance is better compared to use of fresh dung.

Selection and preparation of test animals

19. The species to be used in this test is *Aphodius (Agrilinus) constans* Duftschmidt (1805). First instar larvae or very early 2nd instar larvae (age: ≤ 7 days after hatching) are used for testing the effects of the test substance, after larvae began to feed from their substratum of origin, what guarantees their good physiological state (black digestive tract visible). Beetles can be obtained from an established laboratory culture, but continuous breeding of this species is not easy due to its summer diapause. ANNEX 3 contains more information on how to culture the test species..

20. Alternatively, adult beetles and cow pats can be taken from the field. After transfer to the laboratory the larvae hatching from eggs laid by the adults or by the adults which developed from eggs already in the manure could be used for testing purposes. However, this way is only possible in the period between December and end of April (times valid for Southern France and other parts of Europe) since in this time the beetles are active [24, 25]. Where field-collection of beetles to initiate a culture is conducted, the species identity must be verified using an appropriate key [24]. In fact, in areas like in the hilly region north of Montpellier (France) this species is the dominant dung beetle in winter and early spring (>95% of all individuals found in cattle dung). Colonies initiated from field-collected organisms should be cultured for a minimum of one generation prior

to test initiation. The species confirmation, source and history of the organisms should be documented.

Adult individual of *A. constans*

Three larvae of *A. constans*

Test conditions

21. The rearing vessels for laboratory culturing of beetles and test vessels will be maintained within the laboratory at a temperature of 20°C ± 2°C. The tests are conducted in permanent dark.

22. The water content of the dung substrate in the test vessels is not maintained throughout the test because a slight drying of the dung surface is allowed in order to drive the larvae through the test substrate.

TEST PROCEDURE

Dung Preparation

23. Dung should be removed from the freezer in time to ensure that it is completely thawed before use (directly collected dung could be used immediately; see § 18). The dung should be homogenised for ca 10 minutes, for example in a large-scale laboratory mixer, prior to preparation of the separate treatment groups. No change of the moisture content is usually required.

Experience has shown that a moisture content of 60 - 65% fresh weight (fw) is suitable for oviposition by adults and the development of larvae.

24. Moisture content and pH of a sample of dung from cattle has to be determined at the start of each test. The dung should be wet enough to be easily moulded into a ca 7 cm diameter ball, but dry enough that the ball will retain its shape. Nitrogen and carbon content (incl. C/N ratio) should be determined. The methods used for measuring these parameters will be recorded. Possible methods for parameter determination are included in ANNEX 2.

Cell counter tubes

Micro well plates

Application of Test Chemicals

25. All test concentrations must be given on a dry weight basis in order to ensure comparability of the results from different studies.

26. A known amount of fresh dung will be placed into a large-scale laboratory mixer. Test and reference chemicals will be introduced in a known amount of deionized water. If chemicals are poorly soluble in water, they will be introduced in a known amount (depending on the solubility of the test substance 1 – 10 mL/120 g dw of dung have been proved to be suitable) of an organic volatile solvent (e.g. acetone, ethanol or DMSO) and mixed thoroughly for ca 10 minutes. Control dung will be inoculated either with a known amount of solvent only (solvent control) or with an appropriate amount of water only (untreated control). Afterwards, the dung and the respective

addition will be mixed thoroughly. Where a solvent carrier is used, the solvent must be allowed to fully evaporate using an extraction hood for at least 4 hours at room temperature before the test organisms are added.

27. The concentrations of application must be confirmed by an appropriate analytical verification. For soluble substances, verification of all test concentrations can be confirmed by analysis of the highest test solution used for the test with documentation on subsequent dilution and use of calibrated application equipment (e.g., calibrated analytical glassware, calibration of sprayer application equipment). Nominal values can be used for ECx and NOEC calculations in case the concentrations within test duration will vary less than 20% of nominal. Otherwise real mean measured concentrations should be used for the calculation of ECx or NOEC.

Preparation of Test Vessels and Addition of Organisms

28. Five to seven g f.w. of dung will be added to each test vessel. The larval phase is used as the starting point of the test.

29. Harvested larvae should be divided into separate groups corresponding to the number of treatments prior to addition. This ensures that the transfer of organisms to a particular dung type does not result in any chemical cross-contamination. Allocation of larvae to treatment groups should be done progressively, in small batches, so as to further randomise larval distribution. Each group of larvae should be kept on moist filter paper in a closed container until ready for use in the test.

30. One larva will be placed in a small hole on the dung surface of each test vessel. The total number of vessels containing one larva each (= replicates) depends on the chosen test design (see § 33 – 36).

Observations

31. Main endpoint is larval survival. The mortality is assessed by emptying the dung from the individual test vessel on a glass tray or plate, sorting the larva from the dung and testing its reaction to a mechanical stimulus: if there is no reaction, the larva is considered to be dead.

Facultative assessments of this endpoint are done one and two weeks after starting the test. A mandatory is performed three weeks after starting the tests. Any visual morphological abnormalities will also be recorded during these assessment(s).

32. The test will be terminated three weeks after application of the test substance.

Test design

33. Range Finding Test: If the toxicity of the test chemical is unknown, five nominal test concentrations of 0.1, 1.0, 10, 100, and 1000 mg/kg (dry weight of dung) plus an untreated control and a solvent only control (if solvent is not water) should be conducted. If information about the toxicity is available, the test concentrations can be adapted accordingly (see § 34). All test concentrations have also to be given on a dry weight basis. At least seven replicates should be used.

34. Limit Test: If the range finding test indicates that the no-observed effect concentration (NOEC) of the test chemical is greater than the tested concentrations (e.g. 1000 mg/kg dung d.w.), a limit test at an appropriate concentration (usually 1000 mg/kg dung d.w.) may be carried out instead of a definitive test. The limit test will be conducted with twelve test chemical vessels and twelve untreated vessels. A reference substance and a solvent only control (if solvent is not water) will also be included (twelve replicates each). This design was selected in accordance with OECD Guidance Document No. 54 [28].

35. If effects of the test chemical are observed within the range tested in the range-finding study (corrected for control mortality using Abbott's (1925) formula [29]), a definitive test will be conducted. At least five concentrations in a geometric series should be tested. Twenty replicates for each test concentration treatment plus twenty controls are recommended. The spacing factor between concentrations should not exceed 3.16. In addition to an untreated control and a solvent only control (if solvent is not water) a reference substance (not always, see § 12) can also be tested.

36. Positional bias will be eliminated by using a randomised complete block design for all studies carried out (range test, limit test or definitive test).

STATISTICAL EVALUATION

37. No definitive statistical guidance for analysing test results is given in this Guidance Document. However, based on recent recommendations in other OECD guidelines (mainly the Guidance Document on statistics [28] but also other recently published guidelines [30]) some proposals can be made. This Guidance Document primarily focuses on the determination of the ECx. According to the recent VICH guideline [12] the EC50 is required by many regulatory authorities (e.g. in the European Union), mainly resulting from statistical and ecological considerations. However, for reasons of flexibility guidance is also given for the determination of the NOEC [30].

38. The number of surviving larvae will be tabulated along with each concentration of test chemical. In addition, all other observations of morphological changes, always compared to the control, will be provided in a tabular format.

TEST REPORT

39. On completion of the study a final report will be prepared. The report must include the following information (but not be limited to):

Test substance:

- Test chemical (CAS no., name, common name, chemical name, Batch no., purity etc.)
- Reference chemical (CAS no., name, common name, Batch no., purity etc.)
- Properties of the test substance (e.g. log Kow, water solubility, vapour pressure and information on fate and behaviour), if possible

Test species:

- Test species used (confirmation of species, source of organism, breeding conditions)
- Handling of organisms
- Age of organisms when added to test vessels

Test conditions:

- Source of dung and recent veterinary history of livestock used
- pH and moisture content of the dung
- Depth of dung in the test vessels
- Test vessels (material, dimensions and size)
- Test concentrations and number of replicates
- Description of the preparation of test and reference chemical dosing solutions
- Environmental conditions (temperature, humidity)
- Test duration and number as well as timing of assessment dates

Test results:

- Number of surviving larvae at the end of the test
- Morphological abnormalities (e.g. body size) per replicate
- Results of the tests with the reference substance
- Results presented in tabular and/or graphical form
- Estimates of toxic endpoints (e.g. ECx, NOEC), and the statistical methods used for their determination including, in case of NOEC determination, the minimum detectable difference (MDD) between control and treatments
- Parameters of the fitted statistical models (usually slope and EC50), including their 95% confidence intervals.

Evaluation of the test results:

- Fulfilment of validity criteria
- Review/discussion of results obtained
- Conclusion reached

ANNEX 1

DEFINITIONS

The following definitions are applicable to this Guidance Document:

NOEC (No Observed Effect Concentration) is the highest test substance concentration at which no effect is observed. In this test, the concentration corresponding to the NOEC, has no statistically significant effect ($p < 0.05$) within a given exposure period when compared with the control.

ECx (Effect concentration for x% effect) is the concentration that causes x% of an effect on test organisms within a given exposure period when compared with a control. For example, an EC50 is a concentration estimated to cause an effect of 50% on a test endpoint in an exposed population over a defined exposure period. In this test the effect concentrations are expressed as a mass of test substance per dry mass of the test dung.

ANNEX 2

DETERMINATION OF DUNG PROPERTIES

Dung pH can be determined by adding a weighed amount of dung (3 - 5 grams) to a 1.0 M potassium chloride solution or 0.01 M calcium chloride in a vial [31]. The ratio between dung and aqueous phase should be 1 : 5 v/v. The suspension is then shaken thoroughly for five minutes and then left to settle for at least 2 hours but not for longer than 24 hours. The pH of the liquid phase is then measured using a pH-meter that has been calibrated before each measurement using an appropriate series of buffer solutions (e.g. pH 4.0 and 7.0).

Moisture content can be determined by weighing three replicate dung samples (ca 20 g) into vessels and drying overnight in an oven at ca 105°C [32]. The samples are then removed, cooled

at room temperature in a desiccator and reweighed, the moisture content is calculated and expressed on an oven dry basis.

Nitrogen content can be determined using the method of Tilman and Wedin [33] or the micro-Kjeldahl procedure as described by Hesse [34]. Again, ISO methods should be preferred [35, 36, 37]. Accordingly, the carbon content in dung should be determined by using modified ISO guidelines [e.g. 38].

ANNEX 3

CULTURING OF THE DUNG BEETLES

Continuous breeding of the species *Aphodius constans* which needs fresh dung for feeding and reproduction is difficult since this spring/winter-active beetle has usually a summer diapause. However, it is possible to keep some generations of beetles in the laboratory starting with field-collected animals, meaning that in Europe larvae can be gained for several months between December and May. In the following, experiences made at the University of Montpellier (France) and in the laboratory of ECT Oekotoxikologie GmbH Flörsheim (Germany) are summarised.

Adult and larval beetles are kept in transparent plastic boxes with a size of about 42 x 26 x 15 cm. The boxes are covered with a gauze (mesh size: 200 µm) in order to provide a permanent air exchange. As breeding substrate an agricultural sandy to loamy soil (e.g. the German standard soil LUFA 2.2) at the bottom of the boxes (200 – 300 g dry weight) is used. The pH value of this soil should be about 7 (measured according to ISO 10390) [31]) and its moisture content should be about 25 – 30% (determined according to ISO 11461 [32]). A ratio of ca. 750 g of cattle dung (4-5 cm in thickness) and 20 adult beetles per culture box are giving good results in terms of eggs and larvae. Beforehand, the dung should by air-dried for about 4-5 hours in order to obtain a thin crust. After putting the dung into the boxes, the surface of the dung pats are slightly cut (several notches; 5-6 mm deep). In ach of these notches 3-4 adults are arranged together which bury themselves very quickly into the dung.

Suitable dung has to be collected as fresh as possible, since otherwise contamination with dung flies may occur. After transport to the laboratory, the dung should be frozen at -20°C for at least one week before its use in the cultures. Since thawing takes 2 – 3 d, the general appearance of the pats does not change compared to their physical appearance before freezing. Larval and adult boxes are kept in an air-conditioned room (20 ± 2 °C) at a 16 / 8 h day/night schedule. Eggs and larvae remain in the dung until the latter pupate in the soil below the dung pats.

Three stages of larval development can be distinguished in the species *A. constans*: under laboratory conditions (e.g. 20°C) the first and second stage last for about one week each, while the third stage differs in duration between one and two weeks. First-instar larvae are characterized by a reddish head capsule, while those of the second and third stage have brownish to black capsules (the difference between these two stages is mainly size). Pupation requires about three to four additional weeks before adults will appear. Freshly hatched beetles have a red colour but after some days they are overall black.

ANNEX 4

Testing of dung collected from livestock treated with veterinary pharmaceuticals

In contrast to use dung spiked with a test substance the dung beetles can also be exposed to dung which was collected from livestock (often cattle) treated with the test substance. This test design is considered to be more realistically since it includes all metabolism occurring during the passage of the drug through the body of the treated animal. In addition, the exposure situation reflects the real availability of the test substance in the dung which may differ from the one reached after spiking and homogenisation. For these reasons, such an extended laboratory test may be required at higher tiers when assessing the potential risk of veterinary pharmaceuticals for dung organisms.

Basically the test is performed as described in the main body of this Guidance Document. Therefore, in the following only those issues which need to be modified are listed (for example, no changes are necessary concerning reference testing, validity criteria or the culturing of the two test species).

Information on the test substance:

5,6. In addition to the physico-chemical properties of the test substance the formulation used in the test with treated dung has to be described.

DESCRIPTION OF THE TEST

10. To be added: Equipment to treat livestock with the test substance (depending on the formulation used, e.g. a syringe).

New § after 10.

In addition, the treated animals (e.g. race, age, weight of cattle; husbandry, feeding) and their treatments (e.g. how often and in which frequency the livestock was treated etc.) have to be described in detail.

20. Since it is not known how much of the test substance will occur in the dung it is necessary to analyse the dung for the test substance and its main metabolites. Residue analysis has to be performed as long as test substance is appearing in the faeces of the treated livestock.

21 ff. Dung from treated cattle is collected at different dates after treatment, depending on the excretion profile of the test substance (e.g. for a pour-on formulation containing ivermectin used on cattle, samples were taken up to 12 days after treatment [18]). Dung samples from one animal and from the same day are combined and mixed in order to get a homogenized batch. From each batch, 5 - 7 g (f.w.) are taken for each replicate (= vessel).

26 ff. Depending on the aim of the study, the same test designs could be used as for the tests with spiked dung, since each dung sample from treated livestock contains a different concentration of test substance depending on the excretion profile. Therefore, both limit tests (just one sampling date) or dose-response designs (ECx, NOEC) are possible. For the same reasons, there is also no difference concerning statistical assessment.

32. In the test report, the additional information referring to the test modifications described in this ANNEX No. 4 have to presented.

ANNEX 5

Biology and Ecology of Dung Beetles

Livestock dung is colonized by diverse organisms that form a very complex food web [21, 39, 40] (Figure 1). Most of these organisms are insects, of which dung beetles are among the most abundant and species-rich. Other members of the community include mites, nematodes and annelids.

Together, the community of dung organisms fulfil the following ecological services [21, 41, 42, 43]:

- remove dung from the pasture surface that would otherwise remove area from grazing;
- recycle organic matter, nitrogen and other nutrients by incorporating dung back into soil (the nitrogen would otherwise be lost to the atmosphere);
- reduce the suitability of dung pats as breeding sites for parasites (e.g. helminths) and pest flies (e.g., bush fly – *Musca vetustissima*; horn fly – *Haematobia irritans*) affecting livestock and humans;
- improve soil aeration and water retention by tunnelling in the soil to bury dung in which to rear offspring.

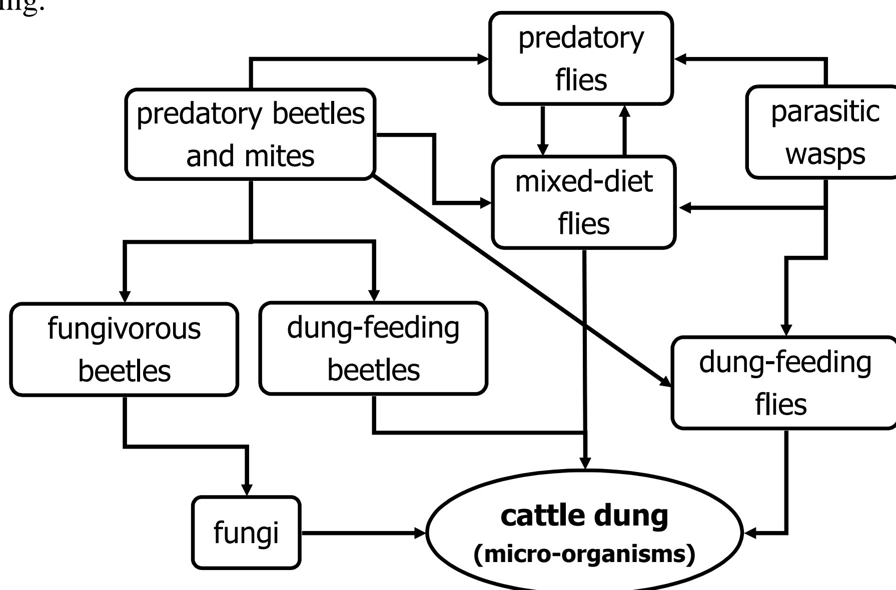

Fig. 1: Schematic food web of a cattle dung pat, showing the most important groups of the dung community (from [76]).

The value of these activities has been estimated to exceed $2 billion / yr in North America [44]. In Northern Australia, the benefits of dung beetles (in particular the reduction of pests like bush flies) are considered to be worth $ 13 million per annum (John Feehan, Hackett, ACT, Australia; pers. comm.). Comparable numbers have been reported from the Netherlands [39] and the USA [41]. It is emphasized that very few species of insects breeding in dung are considered pests; e.g., bush fly, horn fly. Actually, several dung beetle species, originally from South Africa or Mediterranean Europe, were introduced deliberately in Australia since the native dung beetle species did not use cattle dung as food, meaning that vast areas of Australian grassland became devastated [45, 46].

Species of organisms associated with dung typically differ in their periods of seasonal activity (e.g., occurring only in spring or autumn), number of generations per year, and time of arrival at fresh dung. Under temperate conditions, fresh dung is colonized almost immediate by adult flies (e.g., Muscidae, Scatopsidae, Sepsidae, Sphaeroceridae) [21]. Dung beetles (Scarabaeidae) arrive shortly after to feed and oviposit, with peak colonization finished by the end of the first week after deposition. Parasitic wasps (e.g., Braconidae, Ichneumonidae, Pteromalidae) and predaceous beetles (e.g., Histeridae, Staphylinidae) arrive concurrently with the flies and dung-feeding beetles to feed on immature insects developing in the dung pat or to oviposit [47]. In particular the diversity of staphylinids can be very high (10 – 120 species depending on the region [21]). There is very little additional colonization of dung by coprophilous insects two to three weeks after deposition. In the latter stages of decomposition, pats may be colonized by saprophagous species including beetles in families Lathridiidae, Ptiliidae and Rhizophagidae, and earthworms). In fact, the colonization of dung pats is not only a complex but also fragile process. In England, the exclusion of mainly dung beetle larvae (*Aphodius* spp.) for as little as two days following pat deposition significantly reduced both the insect population and the rate of dung pat degradation [48]. Similar results were obtained under Mediterranean conditions when insects were excluded from colonisation [49].

The “true” dung beetles (Scarabaeidae) are of primary interest in this document. These beetles comprise at least ten genera and about 7000 species worldwide [50]. High diversities of

scarabaeid beetles were reported from many temperate regions, e.g. from a grassland in South-Western Germany where 38 species (including 22 *Aphodius* spp. and 12 *Onthophagus* spp.) were sampled within one year [51]. Depending on factors including climate, soil type, vegetation, and the diversity of animal dung, about ten to 50 dung beetle species can occur in one region [21], e.g. 15 *Onthophagus* spp., 35 *Aphodius* spp. and 6 Geotrupidae species in the Southern Alps [52]. Within Europe, the Mediterranean region probably has the highest diversity of scarabaeid dung beetles in Europe [53, 54, 55]. The abundance of individual species can vary considerably. Captures of 39 631 dung beetles in an Irish grassland comprised 24 species of which eight species accounted for 94% of all individuals [56]. Fewer than 12 dung beetle species might normally occur within the same dung pat.

Different species of dung beetles can be classified by their feeding and reproductive behaviours as ‘dwellers’, ‘tunnelers’, or ‘rollers’ (Fig. 2) [57, 58]. Adult ‘dwellers’ lay eggs in the dung pat, wherein the immature beetles develop from egg-to-adult. Adult ‘tunnelers’ bury fragments of dung in tunnels that extend down from beneath the dung pat. This dung provides food for immature beetles that hatch from eggs laid in the tunnels. Adult ‘rollers’ remove balls of dung that are rolled away from the pat, before being buried in tunnels. As with the tunnelers, the buried dung provides food for immature beetles developing from eggs laid in the tunnels. The degradation of dung pats by species of tunnellers and rollers can occur within a span of hours or days. In contrast, the degradation of dung pats by dwellers may take weeks or months. Knowledge of these different functional groups (dwellers, tunnelers, rollers) and how their representation may vary with region and season, is needed to best assess the potential for veterinary pharmaceuticals to adversely affect the community of dung organisms and dung degradation.

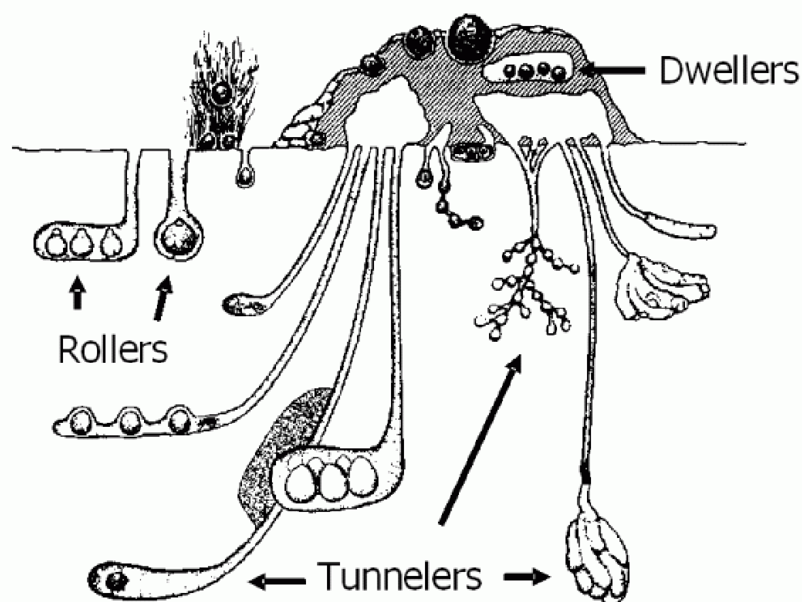

Fig. 2: Schematic view of the three different life and reproduction strategies of dung beetles (after [58], modified according to [57])

The most common method to catch dung beetles in the field is via dung-baited pitfall traps for which various versions exist [57, 59, 60]. Although most of the information provided in this document was gained from studies using cattle dung, the same diversity and type of ecological services are also provided by insects in dung of other livestock; e.g., sheep [61, 62].

ANNEX 6

Ecotoxicology of Dung Beetles

Numerous field studies have reported on the effects of veterinary pharmaceuticals, in particular the avermectins, on dung beetles [27, 63, 64, 65, 66, 67, 68, 69, 70]. This focus is explained partly by the high persistence of these compounds, partly by their toxicity at extremely low concentrations, and partly by their mode of action; e.g., impacting the nervous system of both adult and larval insects [5]. Ingestion is not necessary – contact is often sufficient for mortality. Almost all studies confirm that:

- dung beetle communities (i.e. a wide range of species) can be affected by the residues of parasiticides in dung after treatment of livestock under realistic conditions;
- genera most often affected are *Aphodius* spp. and *Onthophagus* spp. but this might be an artefact of the species most often tested;
- even species introduced into a certain region like *Onthophagus binodis*, a beneficial insect introduced to Australia to increase the rate of breakdown of cattle dung dispersal on pastures , were strongly affected [71].
- these effects usually occur during a period of two weeks after application, but depending on the treatment (active ingredient, concentration and frequency) and the excretion pattern these numbers may vary;

- effects were reported as starting at concentrations of 0.5 - 4.0 mg/kg dung [72], but these numbers are difficult to verify since details of the application, residue analysis or the reference (dry or wet weight?) were often not presented;
- usually, the larvae are clearly more sensitive than the adults. However, the dispersal activity of the adults could be severely affected [73, 74];
- avermectins in dung pats can attract as well as repel dung beetles [70]; thus, effects can increase or decrease, making predictions on the overall impact difficult;
- however, in a few cases contradictory results were found (e.g. no effects of ivermectin on the mortality of dung beetles or on the degradation), which may be caused by the use of non-standardised methods [8, 59].

When compiling the effects of veterinary pharmaceuticals on dung beetles in the field it should not be forgotten that these communities are at the same time also affected by other forms of intensive agricultural management like removal of herbaceous field boundaries [56]. However, in reviews of various laboratory studies it has been confirmed that veterinary pharmaceuticals and in particular avermectins as well as synthetic pyrethroids have detrimental effects on dung beetles at environmentally relevant concentrations [7, 23, 75, 76].

In the few cases where dung flies and dung beetles have been tested in the laboratory under comparable conditions (mainly with ivermectin) it seems that on average the beetles were less sensitive than the fly larvae [13, 17, 18]. However, the experience gained so far is much too low in order to draw any final conclusion on this matter.

During the inaugural meeting of the SETAC advisory group DOTTS, held at Huntingdon, England, in February 2002, 17 participants from eight countries represented governmental agencies, industry, contract laboratories and universities. Discussion centred on suitable dung beetle test species and test methods for the assessment of effects of parasiticides. Proposed species included: *Anoplotrupes stercorosus, Aphodius constans, A. haemorrhoidalis, Bubas bubalus, Copris hispanus, Diastellopalpus quinquedens, Euoniticellus fulvus, E. intermedius, Geotrupes spiniger, Onitis alexis, O. belial, Onthophagus binodus, O. gazella, O. taurus, Sisyphus rubrus*. Consideration was given to ecological role, geographic distribution, sensitivity,

representativeness, ease of rearing, and available experience. General agreement ultimately was reached on the use in laboratory tests, of *O. taurus* and *A. constans*. Colleagues from South Africa subsequently also proposed use of *Euoniticellus intermedius* [77, 78].

General agreement was not reached on specific test methods, mainly because participants had experience with different techniques. The situation has since improved with development of a standard method for use with dung flies [14], and because of experience gained in recent studies on *O. taurus* and *A. constans* [16, 19]. Obviously, it is advantageous to perform fly and beetle tests as similarly as possible (e.g. in terms of using the same reference compound or test design). Pending clarification of further research, the methodology presented in Part B of this Guidance Document is considered to be sufficiently well developed for application in regulatory ecotoxicology.

Open issues for research are mainly related to the breeding process. For example, the larvae of *Aphodius* species are known for almost 70 years [25, 79]. However, trials to culture started only quite recently and despite some progress mass cultures are difficult to be achieved. Laboratory culture of *Onthophagus taurus* has been achieved [15], but is not easy. In addition, the long time needed by this species is a disadvantage, as well as the mode of nesting which allows with difficulty to have an easy access to larvae (larvae included inside individual pellets). Preliminary results for *Euoniticellus intermedius* are promising, but work with this species in different laboratories has just been started. Current research needs can be summarised as follows:

- Improvement of the breeding and culturing methods with the three species mentioned above;
- Performance of laboratory tests with various parasiticides in order to evaluate their sensitivity.

REFERENCES

[1] European Union (1991): Council Directive concerning the placing of plant protection products on the market. 91/414/EEC. Brussels, Belgium.

[2] Madsen M, Overgaard Nielsen B, Holter P, Pedersen OC, Brochner Jespersen J, Vagn Jensen KM, Nansen P, Gronvold J. (1990): Treating cattle with ivermectin: Effects on the fauna and decomposition of dung pats. J Appl Ecol 27:1-15.

[3] European Medicines Agency (1998): Note for guidance: Environmental risk assessment for veterinary medicinal products other than GMO-containing and immunological products. EMEA/CVMP/055/96. London, UK.

[4] Römbke J, Knacker Th, Teichmann H. (2000): Ecotoxicological evaluation of pharmaceuticals. In Kümmerer K (ed). Pharmaceuticals in the Environment. Springer-Verlag, Berlin, Germany, pp 123-141.

[5] Campbell WC. (1989): Ivermectin and Abamectin. Springer Verlag, New York, NY, USA.

[6] Edwards CA, Atiyeh RM, Römbke J. (2001): Environmental impact of avermectins. Rev Environ Contam Toxicol 171:111-137.

[7] Lumaret J-P, Errouissi F. (2002): Use of anthelminthics in herbivores and evaluation of risks for the non target fauna of pastures. Vet Res 33:547-562.

[8] Barth D, Heinze-Mutz EM, Roncalli RA, Schluter D, Gross SJ. (1993): The degradation of dung produced by cattle treated with an ivermectin slow-release bolus. Vet Parasitol 48:215-227.

[9] Herd R, Strong L, Wardhaugh K. (1993): Environmental impact of avermectin usage in livestock. Vet Parasitol 48:1-340.

[10] Swift MJ, Heal OW, Anderson JM. (1979): Decomposition in Terrestrial Ecosystems– Studies in Ecology, Vol 5. Blackwell Scientific, Oxford, UK.

[11] Herd R. (1995): Endectocidal drugs: Ecological risks and counter-measures. International Journal for Parasitology 25:875-885.

[12] VICH (International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products) (2004): Environmental Impact Assessment for Veterinary Medicinal Products – Phase II. Guidance. VICH GL 38, 38 pp.

[13] Römbke J, Floate KD, Jochmann R, Schaefer MA, Blanckenhorn W, Puniamoorthy N, Knäbe S, Lehmhus L, Rosenkranz B, Scheffczyk A, Schmidt T, Sharples A. (2009): Lethal

and sublethal toxic effects of a test chemical (ivemectin) on the Yellow Dung Fly *Scathophaga stercoraria* based on a standardized international ring test. Envir. Toxicol. Chem. (in press)

[14] OECD (Organisation for Economic Co-operation and Development) (2008): OECD Test Guideline for the testing of chemicals: Determination of Developmental Toxicity of a Test Chemical to Dipteran Dung Flies (*Scathophaga stercoraria* L. (Scathophagidae), *Musca autumnalis* De Geer (Muscidae)). No. 228. Paris, France.

[15] Wardhaugh KG. (2002): Working paper on protocol for rearing and testing *Onthophagus taurus* and/or *Euonicellus fulvus* – for comment, discussion and revision. Internal paper distributed with the DOTTS group, 7 pp.

[16] Chapman A, Gray J, Taylor K, Barrett KL. (2003): Development of a toxicity test method using the dung beetle *Onthophagus taurus*. Abstracts, ENVIRPHARMA Conference, Lyon, France, April 14-16, p 60.

[17] Hempel H, Scheffczyk A, Schallnaß H.-J, Lumaret J-P, Alvinerie M, Römbke J. (2007): Toxicity of four veterinary parasiticides on larvae of the dung beetle *Aphodius constans* in the laboratory. Environ. Toxicol. Chem. 25 :3155-3163.

[18] Lumaret J-P, Alvinerie M, Hempel H, Schallnaß H-J, Claret D, Römbke J. (2007): New screening test to predict the potential impact of ivermectin-contaminated cattle dung on dung beetles. Vet. Res. 38:15-24.

[19] Römbke J, Hempel H, Scheffczyk A, Schallnass H-J, Alvinerie M, Lumaret J-P. (2007): Environmental risk assessment of veterinary pharmaceuticals: development of a standard laboratory test with the dung beetle *Aphodius constans*. Chemosphere 70:57-64.

[20] EMEA/CVMP (2007): Guideline on environmental impact assessment for veterinary medicinal products in support of the VICH guidelines GL6 and GL38. EMEA/CVMP/ERA/418282/2005. EMEA, London (U.K.), May 2007.

[21] Hanski, I. & Cambefort, Y. (1991): Dung beetle ecology. Princeton University Press, Princeton. 481 pp.

[22] Ridsdill-Smith T.J. (1993): Effects of avermectin residues in cattle dung on dung beetle (Coleoptera: Scarabaeidae) reproduction and survival. Vet. Parasitol. 48: 127-137.

[23] Floate KD, Wardhaugh KG, Boxall ABA, Sherratt TN. (2004): Fecal residues of veterinary parasiticides: Nontarget effects in the pasture environment. Annu Rev Entomol 50:153-179.

[24] Lumaret (1990) : Atlas des Coléoptères Scarabéides Laparosticti de France. Ministère de l'Environnement, Secrétariat Faune Flore (édit.), Paris. ISBN 2-86515-057-7. 420 p.

[25] Lumaret, J.-P. (1975): Etude des conditions de ponte et de développement larvaire d´*Aphodius (Agrilinus) constans* Duft. (Coléoptère Scarabaeidae) dans la nature et au laboratoire. Vie Milieu 25: 267-282.

[26] Lumaret, J-P. (1986): Toxicité de certains helminthicides vis-à-vis des insectes coprophages et conséquences sur la disparition des excréments de la surface du sol. Acta Oecol. Oecol. Appl. 7: 313-324.

[27] Errousissi, F., Alvinerie, M., Galtier, P., Kerboeuf, D. & Lumaret, J.P. (2001): The negative effects of the residues of Ivermectin in cattle dung using a sustained-release bolus on *Aphodius constans* (Duft.) (Coleoptera: Aphodiidae). Vet Res 32: 421-427.

[28] OECD (Organisation for Economic Co-operation and Development) (2006): Current Approaches in the Statistical Analysis of Ecotoxicity Data: a Guidance to Application. OECD Series on Testing and Assessment. Guidance Document No. 54, 146 pp. Paris

[29] Abbot, W.S. (1925): A method of computing the effectiveness of an insecticide. Journal of Economic Entomology. 18: 265-267.

[30] OECD No. 220 (2004): OECD Guidelines for the Testing of Chemicals. Enchytraeid Reproduction Test. Paris, France.

[31] ISO (International Organisation for Standardisation) (1994): Soil Quality — Determination of pH. ISO 10390. Geneve, Switzerland.

[32] ISO (International Organisation for Standardisation) (1992): Soil Quality — Determination of soil water content on a volume basis — Gravimetric method. ISO 11461. Geneve, Switzerland.

[33] Tilman, D., Wedin, D. (1991): Plant traits and resource reduction for five grasses growing on a nitrogen gradient. Ecology 72: 685-700.

[34] Hesse, P.R. (1971): A textbook on soil chemical analysis. John Murray, London.

[35] ISO (International Organisation for Standardisation) (1995a): Soil quality – Determination of organic carbon and total carbon after dry combustion. ISO 10694. Geneve, Switzerland.

[36] ISO (International Organisation for Standardisation) (1995b): Soil Quality — Determination of total nitrogen – Modified Kjeldahl method using titanium dioxide as catalyst. ISO 11261. Geneve, Switzerland.

[37] ISO (International Organisation for Standardisation) (1997): Soil Quality — Determination of total nitrogen content after dry combustion (element analysis). ISO 13878. Geneve, Switzerland.

[38] ISO (International Organisation for Standardisation) (1995): Soil Quality — Determination of organic carbon and total carbon after dry combustion. ISO 10694. Geneve, Switzerland.

[39] Lahr J, Van der Pol JJC (2007): Mestfauna en duurzame landbouw. Belangrijkste groepen dieren, levenswijze and ecologische diensten. Alterra Report 1473, 58 pp. Wageningen, The Netherlands.

[40] Skidmore P. (1991): Insects of the British cow-dung community. Field Studies Council, Shrewsbury, England. Occasional Publication No. 21, 166 pp.

[41] Losey JE, Vaughan M. (2006): The economic value of ecological services provided by insects. BioScience 56:311-323.

[42] Cuddeford V. (1999): Beneficial bugs at risk from pesticides. World Wildlife Fund, Toronto, Canada. 68 pp.

[43] Nichols E, Spector S, Louzada J, Larsen T, Amezquita S, Favila ME. (2008): Ecological functions and ecosystem services provided by Scarabaeinae dung beetles. Biological Conservation 141:1461-1474.

[44] Fincher GT. (1981): The potential value of dung beetles in pasture ecosystems. J. Georgia Entomol. Soc. 16:301-316.

[45] Bornemisszza GF. (1976): The Australian dung beetle project – 1965 – 1975. Australian Meat Research Committee Review 30:1-30.

[46] Edwards P. (2007): Introduced dung beetles in Australia 1967 – 2007. Current status and future directions. A Landcare Australia project funded by The Orica Community Foundation. 66 pp.

[47] Lee C, Wall R. (2006b): Distribution and abundance of insects colonizing cattle dung in South West England. J. Natural Hist. 40:1167-1177.

[48] Lee C, Wall R. (2006a): Cow-dung colonization and decomposition following insect exclusion. Bull. Entomological Res. 96:315-322.

[49] Lumaret J-P, Kadiri N. (1995): The influence of the first wave of colonising insects in cattle dung dispersal. Pedobiologia 39:506-517.

[50] Blume RR. (1985): A checklist, distributional record, and annotated bibliography of the insects associated with bovine droppings on pastures in America north of Mexico. Southwestern Entomologist, Supplement 9:1-55.

[51] Wassmer Th. (1995): Mistkäfer (Scarabaeoidea et Hydrophilidae) als Bioindikatoren für die naturschützerische Bewertung von Weidebiotopen. Z. Ökologie u. Naturschutz 4:135-142.

[52] Jay-Robert P, Lumaret J-P, Lebreton J-D. (2008): Spatial and temporal variation in mountain dung beetle assemblages and their relationships with environmental factors (Aphodiinae: Geotrupinae: Scarabaeinae). Ann Entomol Soc Am 101:58-69.

[53] Hortal J, Lobo JM, del Rey L. (2006): Distribucion y patrones de diversidad de los Afididos en la Communidad de Madrid (Coleoptera, Scarabaeoidea, Aphodiidae: Aphodiinae y Psammodiinae). Graellsia 62 :441-462.

[54] Lobo JM, Hortal J. (2006): Los escarabaeidos y Geotrupidos de la Communidad de Madrid: lista de especios, distribucion geografica, y patrones de diversidad (Coleoptera, Scarabaeoidea: Scarabaeidae y Geotrupidae. Graellsia 62:421-440.

[55] Lobo JM, Lumaret J-P, Jay-Robert P. (2001): Diversity, distinctiveness and conservation status of the Mediterranean coastal dung beetle assemblage in the Regional Natural Park of the Camargue (France). Diversity and Distributions 7:257-270.

[56] Hutton SA, Giller PS. (2003): The effects of the intensification of agriculture on Northern temperate dung beetle communities. J. Appl. Ecol. 40:994-1007.

[57] N.N. (2005): A guide to dung beetles. Brochure, Fort Dodge, 8 pp.

[58] Doube BM. (1990): A functional classification for analysis of the structure of dung beetles assemblages. Ecological Entomology 15:371-383.

[59] Fincher GT, Stewart TB, Davis R. (1970): Attraction of coprophagous beetles to faeces of various animals. J. Parasitology 56:378-383.

[60] Floate KD, Gill BD. (1998): Seasonal activity of dung beetles (Coleoptera: Scarabaeidae) associated with cattle dung in southern Alberta and their geographic distribution in Canada. Canadian Entomologist 130:131-151.

[61] Sowig P, Wassmer Th. (1994): Ressource partitioning in coprophagous beetles from sheep dung: phenology and microhabitat preferences. Zool. Jahrbuch Syst. 121:171-192.

[62] Lumaret J-P, Kadiri N, Bertrand M. (1992): Changes in resources: consequences for the dynamics of dung beetle communities. J Appl Ecol 29:349-356.

[63] Strong L, Wall R. (1988): Ivermectin in cattle treatment: Non-specific effects on pastureland ecology. Aspects of Applied Biology 17:231-238.

[64] Strong L. (1992): Avermectins: a review of their impact on insects of cattle dung. Bulletin of Entomological Research 82:265-274.

[65] Strong L. (1993): Overview: the impact of avermectins on pastureland ecology. Veterinary Parasitology 48:3-17.

[66] McCracken DI. (1993): The potential for avermectins to affect wildlife. Veterinary Parasitology 48:273-280.

[67] Strong L, Wall R. (1994): Effects of Ivermectin and Moxidectin on the insects of cattle dung. Bulletin of Entomological Research 84:403-409.

[68] Floate KD. (1998): Off-target effects of ivermectin on insects and on dung degradation in southern Alberta, Canada. Bull Entomol Res 88:25-35.

[69] Floate KD. (2006): Endectocide use in cattle and fecal residues: Environmental effects in Canada. The Canadian J. Veterinary Research 70:1-10.

[70] Floate KD. (2007): Endectocides residues affect insect colonization of dung from treated cattle: implications for toxicity tests. Medical and Veterinary Entomology 21:312-322.

[71] Ridsdill-Smith TJ. (1988): Survival and reproduction of *Musca vetustissima* Walker (Diptera: Muscidae) and a scarabeine dung beetle in dung of cattle treated with avermectin B1. Journal of the Australian Entomological Society 27:175-178.

[72] Schaper R, Liebisch, A. (1991): Einfluß eines systemisch wirkenden Antiparasitikums (Ivermectin) auf die Dungfauna und den Dungabbau der Rinder bei Weidehaltung. Tierärztliche Umschau 46:12-18.

[73] Dadour IR, Cook DF, Neesam C. (1999): Dispersal of dung containing ivermectin in the field by *Onthophagus taurus* (Coleoptera: Scarabaeidae). Bulletin of Entomological Research 89:119-123.

[74] Krüger K, Scholtz CH. (1997): Lethal and sublethal effects of ivermectin on the dung-breeding beetles *Euoniticellus intermedius* (Reiche) and *Onitis alexis* Klug (Coleoptera, Scarabaeidae). Agriculture Ecosystems & Environment 61:123-131.

[75] National Registration Authority, Chemical Review Section. (1998): NRA Report on the special review of macrocyclic lactones. NRA Special Review Series 98.3. Canberra, Australia.

[76] Boxall ABA, Fogg LA, Blackwell PA, Kay P, Pemberton PJ, Croxford A. (2004): Veterinary Medicines in the Environment. Rev. Environ. Contam. Toxicol. 180:1-91.

[77] Kryger K, Lukhele OM, Scholtz CH. (1999): Survival and reproduction of *Euoniticellus intermedius* (Coleoptera: Scarabaeidae) in dung following application of cypermethrin and flumethrin pour-ons to cattle. Bull. Entomological Res. 89 :543-548.

[78] Kryger U, Deschodt C, Davis AL, Scholtz CH. (2006): Effects of cattle treatment with a cypermethrin/cymiazol spray on survival and reproduction of the dung beetle species *Euoniticellus intermedius* (Coleoptera:Scarabaeidae). Bull. Entomol. Res. 96 :597-603.

[79] Madle H. (1935): Die Larven der Gattung *Aphodius* I. Arb. Phys. Angew. Entomologie 2:289-304.