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4 Guideline on the higher tier testing of veterinary 5 medicinal products to dung fauna

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Guideline on the higher tier testing of veterinary medicinal products to dung fauna

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1. Introduction

Dung, especially from larger mammals, makes up a complex and highly dynamic ecosystem within a small environmental scale. Odour from excreted dung almost instantly attracts flies, which feed, mate, and lay eggs on the dung, leading to a new generation of flies within a few weeks. Fly numbers on the dung rapidly decline after a few hours when crust formation on the dung pat reduces the scent. After the flies, dung-feeding beetles arrive at the pat, with the colonisation peak typically finishing by the end of the first week. In contrast to flies, the development time of beetles may take weeks to months. Parasitic wasps and predatory beetles arrive concurrently with their prey (i.e., flies and beetles), and may either lay eggs or feed on the immature insects developing in the dung pat. In less than three weeks after the pat is dropped, the colonisation of dung by dung-loving insects is almost finished. After this time, tunnelling and feeding activities by other insects and the penetration of vegetation accelerate dung pat degradation. With this, access is provided to soil-dwelling organisms like earthworms and bacteria which complete the breakdown of dung into parts that are finally incorporated into the soil matrix. From time of deposition to total degradation, a dung pat may contain several dozen species of dung-loving insects exceeding thousands of individuals.

This complex and dynamic ecosystem may be put under threat from natural stressors as well as a number of agricultural practices, and the use of antiparasitic substances (a group of veterinary medicinal products that provide internal and external parasite control in husbandry by oral or topical application) is one of them.

The high effectiveness against invertebrate parasites in pasture animals has, however, also been the reason for their documented high toxicity to non-target invertebrates, like dung insects.

2. Scope

The VICH guideline on the environmental impact assessment for veterinary medicinal products Phase II (CVMP/VICH/790/03-FINAL, 2005) requires effect studies (a Tier A assessment) on dung fly and dung larvae for endo/ectoparasiticides used for pasture treatments. Yet, no specific guidelines on dung fly or dung larvae studies are listed, as no harmonised OECD documents were available at the time when the VICH Phase II was published[1]. Since the publication of the VICH GL 38 in 2005, the OECD has published two relevant guidelines (OECD 122 and 228) for Tier A ecotoxicity testing of substances to dung fauna.

The CVMP guideline 'Environmental Impact Assessment for Veterinary Medicinal Products in support of the VICH guidelines GL6 and GL38' (EMEA/CVMP/418282/2005-Rev.1) (CVMP TGD), was developed to give further technical support to the implementation of the VICH guidelines GL6 and GL38 on the environmental risk assessment (ERA) of VMPs, where additional regulatory guidance was deemed necessary for the ERA of VMPs. However, in this particular case the CVMP TGD does not include any reference on how to proceed if the initial Tier A risk assessment indicates a risk to dung flies or beetles.

This guideline is intended to provide guidance on how to investigate the environmental effects of VMPs containing antiparasitic substances in higher tier laboratory tests and field studies, in situations where

[1] VICH GL 36 notes that at the time of the publication no internationally accepted guidelines or processed drafts were available for these studies, but acknowledges ongoing work in developing standardised studies for dung fly and dung beetle larvae and their inclusion into the OECD Test Guidelines Program.

the initial Tier A risk assessment indicates a risk to dung flies or beetles. The guideline aims to provide harmonisation of the study design for an easier interpretation and comparison of the results.

3. Scientific considerations

It is generally accepted that veterinary parasiticides are toxic to insects like dung flies and beetles. The question of fundamental concern is, however, whether the often very strong impacts of antiparasitics seen in experimental laboratory studies at realistic exposure concentrations are likely to have impact on insect populations, community interactions and the economically important process of dung decomposition under realistic large scale field conditions. Wall and Beynon (2012) reviewed the current information on large scale studies on the ecological impact of parasiticides, and concluded (citation): *"The extent to which chemical residues may have any sustained ecological impact will depend on both a range of farm management factors, such as the temporal and spatial patterns of chemical use, the number of animals treated and the choice of active ingredient, and a range of insect-related factors, such as abundance, population dynamics and dispersal rates. However, they also demonstrate that considerable uncertainty remains about the likely extent of such effects and that current data are insufficient to support firm conclusions regarding sustained pasture-level effects. More large-scale, long term field experiments are required, particularly in relation to insect dispersal and functional interactions within the dung insect community".* Furthermore, other spatial and temporal factors like the local weather conditions, period and number of treatments throughout the year, and species life cycles may have confounding influences on the toxicity of the VMP.

Therefore more information may be needed in order to evaluate the potential long term and large scale effects of antiparasitics. Performing field studies may be challenging, as the natural variation and temporal and spatial fluctuations caused by a large set of confounding and co-existing (side) effects is likely to hamper the interpretation of results. The task is hence to design field studies that are on the one hand as realistic as possible and on the other hand so robust, standardised and reproducible that the results can be used universally and interpreted in a straightforward and transparent fashion.

Scientific works by for example Römbke et al. (2010), Jochmann (2011) and Adler et al. (2016) may also provide relevant information in this context.

The guideline is focusing on assessing the impact of antiparasiticides on the dung fauna typically associated with cattle dung. Acknowledging the fact that non-target dung communities to a certain degree deviate according to the target species, it is anticipated that VMPs tested safe for use in cattle also are safe for use in other target species like sheep and horse.

3.1. Selection of protection goals

The protection goals for the studies included in this guideline have been identified as being:

- The populations of dung dwelling beetles species
- The populations of dung dwelling flies at family level
- The populations of endangered dung fauna species[2]
- The degradation of dung pats
- The populations of soil dwelling fauna associated to dung pats

All protection goals may, however, not be equally important for all scenarios as outlined in more details in the following sections.

[2] See Annex III for information on the inclusion of endangered species in the list of protection goals

4. Decision tree for higher tier testing of VMPs on dung fauna

When designing a higher tier testing strategy, the results from the Tier A testing should be available (Table 1, VICH GL 38 (2005)).

Table 1. Toxicity studies and associated assessment factors in Tier A of the risk assessment procedure for antiparasitics (VICH, 2005).

Study	Toxicity endpoint	Assessment factor
Dung fly larvae (OECD 228)[1]	EC50	100
Dung Beetle larvae (OECD 122)[2]	EC50	100

1. OECD 228. Determination of Developmental Toxicity of a Test Chemical to Dipteran Dung Flies (*Scathophaga stercoraria* L. (Scathophagidae), *Musca autumnalis* De Geer (Muscidae))
2.OCED 122 Guidance Document on the Determination of the Toxicity of a Test Chemical to the Dung Beetle *Aphodius constans.*

Based on the outcome of the tier A assessment, the PNEC is calculated and compared to the predicted environmental concentration in dung (PEC) in order to derive the risk quotient (RQ)[3]. Typically the PEC is established as the maximum measured concentration in dung observed in the ADME study.

For antiparasitics this comparison often results in a RQ ≥ 1000 in Tier A, and as noted in Section 2 no recommendations are given in the CVMP TGD on how to proceed in these situations (particularly, when the PEC has already been refined with metabolisms data and the RQ is still considerably high).

When RQ values are that high (>1000), it is likely that any additional laboratory testing (i.e., Tier B testing) will not result in RQ< 1. Therefore, only in cases where the Tier A results in a RQ < 50 it is **recommended** to continue with (extended) laboratory Tier B testing. In cases when RQ values are above 50, it is instead **recommended** to direct the effort to field testing (Tier C testing) as a field study will elucidate the environmental risk under realistic conditions and create the scientific foundation for potential risk mitigation measures. This can be summarised as below.

- If RQ in Tier A < 1 ⇒ stop the assessment.
- If RQ in Tier A ≥ 1 ⇒ further assessment is needed.
  - If RQ in Tier A ≥ 1 and < 50 ⇒ go to Tier B
  - If RQ in Tier A > 50 ⇒ go to Tier C

It is, however, up to the investigator to decide which approach to take, as an RQ>1 in Tier A does not automatically requires additional tier B testing studies, and Tier C studies can be considered as well at this stage.

5. Tier B – Extended laboratory studies

No international guidelines for dung fauna laboratory testing exist, which can be used for Tier B guidance. However, recommendations can be found in scientific publications e.g. Adler et al (2013).

Indeed, two methodologies using the dung beetle *Aphodius constans* are currently under development: the elongated larvae test and the reproduction test.

- In the elongated larvae test (70 days) larvae (first larval stage of the beetle) is incubated in dung spiked with the test substance for the first 21 days of the development. At day 21 beetle larvae are transferred from the spiked dung to uncontaminated soil, e.g. LUFA2.2 soil, in order to guarantee

[3] Risk quotient (RQ) = PEC/PNEC

good conditions for the pupation of the larvae. Endpoints include mortality, development and rate of hatched adult beetles.

- The reproduction test (21 days) uses dung spiked with test substance. Twenty to 30 adult beetles are used per test vessel. Endpoints include adult mortality, and the number and age stage of larvae. This test is appropriate for substances indicated to have a repellent effect on dung organisms.

Preliminary results have shown that the two tests listed above are more sensitive than the current Tier A studies, with the elongated test being the most sensitive (Adler et al 2013).

5.1. PNEC derivation in Tier B

Based upon the results of the Tier B testing, the PNEC for dung organisms is derived according to the principles listed in Table 2.

Table 2. Recommended Tier B tests for dung fauna and associated toxicity endpoints and assessment factors.

Study	Toxicity Endpoint	AF
Elongated larvae test	NOEC/EC10	10
Reproduction test	NOEC/EC10	10

6. Tier C - Field Testing

6.1. Overall principles

The overall principles when designing a field test for evaluating risk of VMP to populations of dung associated fauna are stated below. Details on the recommended Standard Testing Procedures are presented in Annex I.

1. Over time, up to three endpoints should be monitored: 1) abundance of dung dwelling species; 2) degradation rate of dung pats; and 3) abundance of soil dwelling fauna associated with dung pats. The two first endpoints are mandatory whereas the third endpoint depends on the properties, toxicity and use of the VMP in question (see below).

2. Field studies should be conducted in sufficient and representative EU regions to cover all concerned Member States, i.e. at least a study under temperate or Atlantic as well as Mediterranean conditions. The studies should be performed at the time of year where the most relevant dung species are active in the region hosting the study (typically spring).

3. The study is design as a simple relative comparison to a control (no VMP) situation. The study should have significant statistical power to be able to distinguish between separate groups with a 25 % difference at the relevant taxonomic level (see Annex II).

4. The study should use control dung collected from non-treated animals the day before medication, and dung from medicated animals collected post-medication, including the dung with the maximum VMP concentrations (based on absorption, distribution, metabolism, and excretion (ADME) study results).

5. Samples should be collected at least up to 28 days after medication even if this includes sampling dung with concentration of VMP below the limit of quantification (LOQ). A minimum of two dung

sampling points post medication is recommended, i.e. date with maximum excretion and 28 days (See Annex I). Furthermore a positive control made up by control manure spiked with VMP to a level corresponding to the highest EC50 value in Tier A has to be included.

6. All dung pats are collected simultaneously for fauna extraction one week after placing in the field – or are simultaneously covered by in-situ traps in the field one week post placing (Procedures for sampling is found in Annex I).

7. The degradation of dung pats is monitored as loss of mass over a time span reliant on the degradation rate (See Annex I).

8. Soil taken from below the removed dung pats may – if required according to the sampling plan – be analysed for species composition and species number of the major taxonomic groups, e.g. collembolans, mites and earthworms.

9. The concentration of VMP needs to be verified by analytical means in all dung and soil samples. Appropriate extraction techniques should be used since a non-suitable extraction technique may not extract all residues, with erroneous concentrations as a result. The investigator is advised to follow the same principles for extraction as outlined in the reflection paper on poorly extractable substances (EMA, 2016)

10. The choice of endpoints to monitor depends to some extent on the outcome of Tier A and, if applicable, Tier B. Structural and functional endpoints related to dung organisms must be assessed in Tier C unless the investigator has scientifically justified otherwise.

Soil fauna need to be included in the monitoring program only if:

- Ecotoxicity data from Tier A shows a similar or higher toxicity for soil fauna compared to dung fauna;

or

- Effects on earthworms are likely at the predicted exposure concentration[4] in soil.

and

- The field study is conducted in a region where earthworms play a major role in the degradation of dung pats (typically not a situation in e.g. the Mediterranean region).

Specific recommendations regarding monitoring endpoints and taxonomic resolution of soil species can be found in Annex III.

6.2. Decision tree in Tier C

The intrinsic properties of parasiticides will most likely make them toxic to dung fauna for a certain period of time, also in field situations. This has been demonstrated and published on several occasions (e.g. Römbke et al 2010). Thus, the field study should focus on setting up boundaries for when the use of VMPs are considered sufficiently safe for the dung fauna communities, and thereby identifying scenarios where alternative risk mitigating measures must be taken into account.

[4] Potential effects is indicated by LOEC>PEC_{soil}. No specific guidance for how to calculate the PEC_{soil} below dung pats is given in the VICH- and CVMP Guidance documents. Furthermore very few studies have measured this under realistic conditions. Römbke et al (2010), did, however, measure the soil concentration below dung pats from ivermectin-medicated cattle in a field study. Here they found soil concentrations above the limit of detection in soil below dung pats from medicated animals at the two highest dung concentrations and below dung pats artificially spiked to high concentrations. The ratio of the concentrations in dung-to-soil ranged from 107 to 405 in the upper 2 cm, and markedly higher in lower parts of the soil. It is therefore recommended to calculate an indicative and conservative PEC_{soil} as: $PEC_{dung\ (max)}$ x 0.01. Both PEC-values in dry weights.

The VMP can be considered environmentally safe for dung fauna if all of the following requisitions are fulfilled in all[5] of the individual studies:

- The field study/studies has/have met the requirements specified in the procedures found in the Annexes of this guideline, and
- the numbers of individual dung fauna species[6] in dung pats collected 28 days post medication are at least 75% of the numbers found in control pats collected prior to medication, i.e. the ET25[7] <28 days.
- The degradation of dung pats (measured as loss of mass) collected 28 days post medication is minimum 75% of the controls at the last sampling date.

In cases where one or more of the criteria above is not met, the VMP is considered to have the potential to pose a long term risk for dung fauna. When this is the case, risk mitigation measures should be considered. If no measures can be found that mitigate the risk, this should be taken into account in the benefit/risk analysis of the marketing authorisation processes.

[5] The listed minimum requirements need to be fulfilled in both climatic regions if the MA is intended for the whole of EU.
[6] Dung fauna species must be monitored at the family level for dung flies and species level in the case of dung beetles.
[7] ET25 is the time (days) post medication where collected dung affects dung fauna species (at the family level) with 25% compared to the controls.

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ANNEX I. Standard testing procedure for assessing the impact of VMP on dung fauna in the field.

The standard testing procedures recommend in this guideline is largely based upon the recommendations laid down in the UBA report: “Comparison of dung and soil fauna from pastures treated with and without ivermectin as an example of the effects of a veterinary pharmaceutical” (Römbke et al, 2013).

Planning, scaling, timing and location of the field study

1. The field study needs to be conducted within realistic season(s) of treating herds with parasiticides, as well as when the majority of dung fauna species are expected. Spring would, in most cases, be considered most appropriate (See Annex III).

2. The number of treatment groups and dung pats replicates will have to reflect the natural variation expected to be found in the field, and needs to be sufficient in numbers to statistically detect a difference of 25% ($p < 0.05$, see Annex II)

3. The field study should be located within grazing areas covering the climatic zones for which the marketing authorization is requested. This would normally as a minimum require one location in an arid or semi-arid Mediterranean location and a temperate location in the northern or Atlantic zone of the EU.

4. The site and study characteristics defined in the Table 3 below need, as a minimum, to be documented and listed.

Table 3. Recommended site and study characteristics

Issue	Documentation	Recommendation
Livestock	Age, gender and breed.	Highest possible similarities in age, gender and breed. Do not use animals medicated within the last six months. Always keep treated and non-treated animals separated.
Livestock diet	Grass or hay	Constant diet throughout the study, starting at least four weeks before medication.
Medication	Application form and dose	Use the relevant form and specified dose for the specific MA. 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.
Dung	Water content, organic carbon, ash content, pH at day 0	
Dung pats	Individual wet weight for each of the constructed dung pats (See section 2)	A variation of less than 10% in wet weight between pats is required.
Study area	GPS coordinates, vegetation, precipitation and weather conditions, daily temperature, land management history	Potential drift of insecticides needs to be evaluated by documented distance to crop fields and/or time since spraying. Weather conditions, e.g. clouds, wind intensity or precipitation can influence the behaviour of

Issue	Documentation	Recommendation
		insects.
Soil	Texture, pH, organic matter content, maximum water holding capacity, C/N ratio	Extreme soil types in the context of e.g. texture and pH should be avoided.
Analytical method	A full description of the analytical method is required including LoD and LoQ	LoQ should as a minimum requirement be able to match the observed NOEC values observed in the laboratory during Tier A.

Preparation of dung pats

1. Dung pats for control/VMP spiked groups are collected from untreated cattle (Day 0). Collection is to be done as close as possible to the time the animals will be medicated.
2. Cattle is treated with the VMP at the recommended dose, formulation and application form, e.g. oral or pour-on.
3. Dung (for treatment groups) is subsequently collected from the medicated animals at a temporal interval covering as a minimum the expected peak of excretion according to the ADME study and one subsequent sample from day 28[8].
4. For each collection date, fresh dung (less than 3h old) from multiple pats is placed in large sealed plastic bags and stored at -20°C until use.
5. Dung can either be sampled after excretion or from the rectum of the animal (not recommended). The cattle should be placed on an area without straw, e.g. in a pen, and dung is collected from the floor immediately after excretion. This can be done by the help of a dustpan and a brush. Alternatively, dung can be collected in special bags tied around the animal's rear. Sampling dung contaminated with urine should be avoided.
6. Frozen dung from each sampling date is thawed overnight and mixed, and standardized dung pats with uniform shape are prepared to be placed in the field the following day. The selected wet weight of the dung pats must be within the range of 500-1000 g. The variation among individual dung pats in wet weight should not exceed 10%. Dung pats may be stored at temperature +5°C or lower for no more than 24 hours prior to the start of the field study.
7. Dung from untreated animals is spiked with the VMP to a dry weight concentration corresponding to the lowest EC50 value observed in Tier A. If a solvent is used in spiking procedure in order to make the VMP soluble, the dung is left overnight in a fume heads in order to eliminate the solvent prior to field study. The positive control pats must resemble the pats from the other part of the study in age, size, form and shape.

On-site procedures

A. Structural Assessment of dung fauna

The main target for the structural assessment is the potential effect of VMP on dung fauna at the family level (See Annex II). It is recommended to identify the collected species to the highest possible

[8] Sampling dates should as a minimum be targeted to enable the determination of the environmental impact 28 days post medication. This may include dung collected at stages post medication where the VMP is below the limit of detection or quantification.

taxonomic level if possible, e.g. genus or species. Although the evaluation and subsequent decision making should be based on the effects at family level in the case of dung flies.

1. In order to collect potential burying species, a container e.g. with a capacity of 7 L (25 cm high, Ø 15 cm), is buried to their rim in the soil and filled with soil collected in the field. Dung pats (see above) are hence deposited at the surface of each container.

2. Wire mesh cages are placed over pats to exclude interference from birds, but still allowing the pats to be colonization by insects. Dung pats are placed at least 2 m apart and 5 m from the edge of the field.

3. Preferably the study site has previously been used for grazing herds of the husbandry targeted in the Application, typically being cattle.

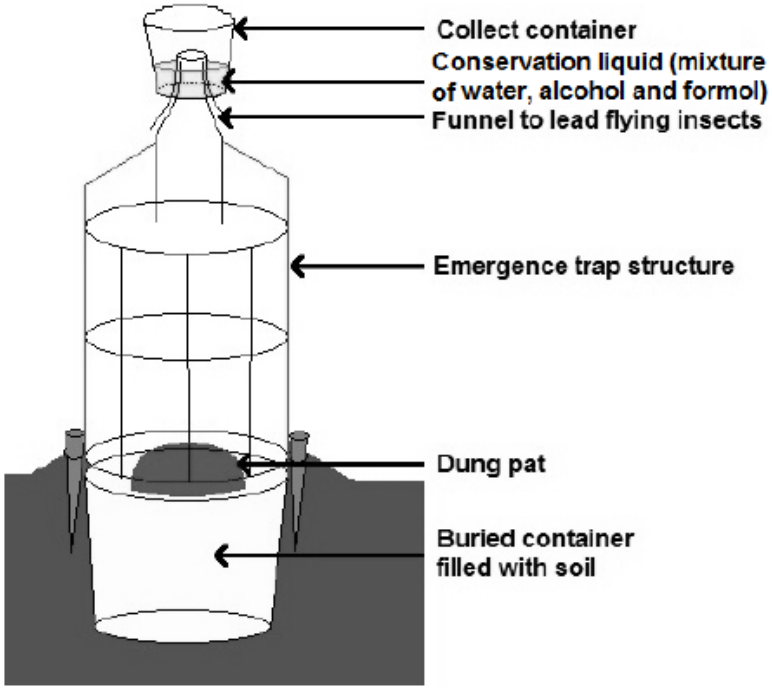
Two different sampling methods for dung fauna may be applied as described in 4(a) and 4(b). In both cases, the soil in the underlying container needs to be examined for presence of dung related organisms after removal of the dung pat.

4. (a). All dung pats are left one week in the field to be colonized by insects. After 7 days in the field, the pats are collected and transported to the laboratory. The underlying soil is carefully examined. In the laboratory each replicate dung pat is placed separately in an emergence trap that captures any flying and crawling insects emerging from the dung. Emergent insects are collected at regular intervals over a period of three months and preserved in 70% or 95% ethanol for later identification quantification. When emergence of insects stops, the remaining dung is carefully examined and any insects in the dung are collected.

   (b). All dung pats are left one week in the field to be colonized by insects. Then emergence traps (see figure 1 below) are set up directly in the field to collect insects as they emerge. Regularly, e.g. weekly, collections are made during the first 3 months and subsequently less frequently. When emergence of insects stops, the remaining dung and the underlying soil are carefully examined.

5. In addition to the pat-specific collection of dung fauna described in point 4. (a) or (b) above, a minimum of five pitfall traps, using manure from control animals as bait, need to be established within the study area. These are used to elucidate the overall presence of insects active at the study site before, during and after the time that pats are exposed in the field. The 5 pitfall traps must be monitored once a week from one month before the study until one month after terminating the study. The traps must be emptied and bait renewed every week. The collection chamber of each trap should contain a preservative replaced as needed. The preservative can be a strong saltwater solution with 2-3 drops of dish detergent to reduce surface tension or non-toxic propylene glycol. The collected organisms are stored in 70-95% ethanol and later sorted, counted and identified.

6. The endpoints to be monitored need to reflect the dung fauna communities in the specific region of the study. Furthermore, the endpoints need, as a minimum, to be sufficient detailed to monitor effects on family level for dung flies and species level for dung beetles See also Annex III for the evaluation of endangered species. It is imperative that the taxonomic determination of dung insects is reliable. It is therefore important that the taxonomic work on dung and soil fauna is performed by specialists with documented expertise within dung fauna and soil fauna taxonomy, respectively.



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359 **Figure 1. Example on an on-site emergence trap (Figure reproduced from Tixier 2014).**

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B. Functional assessment of dung fauna

Dung pats from Section A.2 above are used for the assessment of dung degradation as listed below.

1. Each dung pat is placed on a plastic net (e.g. 25 x 25 cm, mesh width 8 to 10 mm), being in direct contact with the soil. The use of a net should facilitate recovery of pats from the field, without impeding biological activity at the dung-soil interface.
2. Five dung pats from each treatment, i.e. VMP concentration[9], are removed from the field at differing dates after the start of the study covering a period until the control pats is fully degraded or if climatic or other conditions prevent degradation of control pats, at least six months.
3. The individual replicate dung pats are collected into plastic bags; ground with a blender and weighed. Sub-samples are then oven-dried for at least 48h at 100°C to determine water content. Approximately 50 g of the sample is heated in a muffle furnace at 500°C for 12 h to determine the ash content.
4. Main measurement endpoints are dung mass loss, determined either as total dry weight or as ash-free dry weight, i.e. organic matter[10].

C. Structural assessment of soil fauna

It is not mandatory to assess the potential impact to soil dwelling species in all cases, as it depends on the outcome of the studies performed in Tier A (see Chapter 6 above). If required, the study follows the principles described below.

1. From just below each of the removed dung pats in Section B (Functional Assessment), soil samples are taken for analyses of earthworms and micro-arthropods following the respective ISO guidelines (ISO 2006 a, b).
2. Below each dung pat, a homogenous mix of two sub-samples taken from the upper 0-5 cm of the soil surface. The concentration of the VMP is measured following the best available analytical practice. Appropriate extraction techniques should be used, since a non-suitable extraction technique may not extract all residues, with erroneous concentration as a result. The investigator is advised to follow the same principles for extraction as outlined in the CVMP reflection paper on poorly extractable substances (EMA 2016).

See also for example Scheffczyk et al. (2016) for additional information and a published example of a field study with antiparasitics looking at the effects on soil fauna.

Reporting results

The report should, as a minimum, contain the following aspects and data:

- A detailed description of the technical and practical aspects of the study including a specification of any deviations from the recommendations found in this guideline document.
- A list of all identified species and taxa, including dates and numbers.

[9] A minimum of two dung sampling periods post medication (VMP concentration) including a control sampled prior to medication is recommended. Dung pats should cover the excretion profile ranging from peak concentration according to the ADME study to at least 28 days post treatment. Furthermore, a set of dung pats spiked to the highest EC50 value observed in Tier A, should be used as a positive control.

[10] Ash content can be used as a proxy of soil invertebrate activity as higher burying activity increases the amount of soil incorporated into the pats leading to higher ash content of the dung pats.

- Documentation of the analytical methods including extraction method used and limit of detection (LoD) and limit of quantification (LoQ).
- Determination of the following endpoints:
  - Quantification of dung fauna (mean and standard deviation) identified in the following samples: Dung pats sampled prior to medication (T_0, control); Dung pats sampled at the date with maximum excretion (T_{max}); Dung pats sampled 28 days post medication (T_{28}); Dung pats spiked to the lowest EC50 value in Tier A (positive control)
  - Degradation rate (loss of mass) of dung pats after 3 months in the field for the T_0, T_{max}, T_{28} and Positive Control groups.
  - The effects on soil fauna **only** in cases where this is considered relevant according to the criteria listed in Chapter 4 (Tier C – Field study) above.

References

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ANNEX II. Selecting the number of replicates

To obtain reliable results, it is very important to apply the right statistics. This already starts at the design of the test, when deciding on the number of replicates to use in order to gain sufficiently high statistical power.

The power of the test is defined as 1-β. Power increases with increasing sample size and with decreasing variability and also depending on the value of α, i.e. the probability of making a Type I error. The statistical power of a given study is inversely related to the probability of making a Type II error, i.e. to conclude that there is no effect, even though an effect is present. That is, an effect has not been detected because of missing statistical significance.

Ecological experiments can be improved to increase the statistical power by selecting the sample sizes necessary to detect a given difference between treatments. The statistical power should be equal to at least 0.8, i.e. β should not exceed 0.2.

Using statistical methods and information on the natural variation typically observed for the sampling endpoints, it is possible upfront to predict the theoretical minimum detectable (significant) difference (MDD) between a control and exposed group with a given number of replicates. Similar, it can be predicted what the theoretically minimum number of replicates (MNR) would be in order to statistically demonstrate a given significant difference between a control and exposed group (e.g. Kraufvelin (1998).

As indicated in the Guideline text above, it should be the aim of the Higher Tier study to identify statistical effects on dung beetle species and family level of dung flies at the magnitude of 25%.

Unpublished screening analysis of field data has indicated that a 25% difference between exposure groups can be differentiated statistically when designing a field study having a total of 60 replicates, i.e. 30 replicates in control group and 30 replicates in the exposure group.

It is therefore recommended to design the field study in order to have a minimum of 30 replicates in each treatment group, e.g. T_0 (control), T_{max} (dung with the highest concentration according to the ADME study), T_{28} (Dung collected 28 days post medication), as well as a positive control spiked with the concentration of the VMP corresponding to the highest EC50 value found in Tier A. In total this would be 120 replicates per field study.

The recommended minimum number of replicates can be deviated, provided it is possible, by scientific means, to demonstrate that a sufficient statistical power (0.8) of the study can be obtained with less replicates having in mind that the study should be able to demonstrate statistical significance between exposure groups having a 25% difference in the number of individuals measured at the species level for dung beetles and family level for dung flies.

References

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ANNEX III. Endangered dung species

As identified in section 3.1 Selection of protection goals, it may be necessary to consider the potential effects of antiparasitics on dung fauna, typically dung beetles, classified as endangered species in member states and/or international bodies.

The European Food Safety Agency (EFSA) recently published a draft opinion (EFSA, 2016) exploring to what extent endangered species are covered in the current ERA schemes of EFSA. Some of their conclusions are summarised below. Due to their legal status as endangered typically no effect- and exposure data are available. A major open question is whether or not it is reliable to use data from other species, using the same assessment factors and level of protection. For instance, it could be hypothesised that endangered species could be more vulnerable than other species because of their their decreased potential for recovery, their lower genetic diversity, their small population sizes and the fact that they typically inhabit limited, marginal or fragmented habitats.

With respect to sensitivity against toxicological stressors, EFSA concluded there is no evidence that endangered species are *per se* more sensitive towards these chemicals. However, since many of the endangered species are highly specialised, e.g. in their food or choice of habitats, they may only have been exposed to a restricted range of natural occurring hazardous chemicals, which could have resulted in a phylogenetic loss of certain detoxifying pathways relevant for anthropogenic chemicals. Furthermore, some endangered species appear to suffer more from indirect effects than many non-endangered species. Hence, endangered species can indeed be more vulnerable than the species currently considered in the ERAs of Plant Protection Products and VMP.

The IUCN (International Union for Conservation of Nature) and species on national lists of endangered species frequently include species that are associated with dung. In the UK for example the dung beetle *Aphodius niger* is listed as highly endangered. In Germany the species listed below are all associated to dung and endangered (Binot et al. 1998).

Table 5. Examples of red list status of dung beetle and fly species (Germany)

IUCN category German Red list category	Beetle species name
0 "already extinct"	*Aphodius coniugatus*
	Onthophagus gibbulus
1 "endangered or critically endangered"	*Euoniticellus fulvus*
	Aphodiua quadriguttatus
	Aphodius hydrochaeris
2 "seriously threatened"	*Aphodius arenarius*
	Aphodius brevis
	Aphodius consputus
	Aphodius constans
	Onthophagus lemur
	Onthophagus semicornis
3 "threatened or vulnerable"	*Aphodius niger*
	Aphodius varians
	Onthophagus taurus

A more recent German report states that about 60% of the Scarabaeidea species proved in the federal state Saxony-Anhalt are threatened or already extinct, i.e.

10.9% being already extinct (IUCN 0), e.g. *Aphodius foetidus, A. quadriguttatus;*

15.1% being endangered or critically endangered (1), e.g. *Aphodius hydrochaeris, Onthophagus lemur*;

18.5% being seriously threatened (2), e.g. *Aphodius foetens, A. plagiatus;*

16.8% being threatened or vulnerable (3) e.g. *Aphodius fasciatus, Onthophagus similis*.

The decline and threats qualitatively addressed above, is not solely a result of the use of antiparasitics, but rather a result of a complex combination of changed agricultural practices with a higher degree of intensive husbandry, leading to fewer non-stabled and free range animals in combination with the widely use of antiparasitics since the 1980's. In the light of this and challenges highlighted by EFSA, it is not possible to come up with a general approach on how to address the specific concern of antiparasitics associated to endangered dung species. Instead it is recommended to consider this in the planning of the field study, so that endangered species are monitored and reported **at species level** if they are occurring in the region hosting the field study, whereas non-endangered fly species for example can be assessed at family level.

References

Binot M, Bless R, Boye P, Gruttke H und Pretscher P. 1998. Rote Liste gefährdeter Tiere Deutschlands (Red list of the endangered species in Germany; in German). Schriftenreihe für Landschaftspflege und Naturschutz vol 55, 434pp, Bundesamt für Naturschutz.

EFSA. 2016. Draft Scientific Opinion. Coverage of endangered species in environmental risk assessments at EFSA. European Food Safety Authority (EFSA), Parma, Italy.