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Guideline on the plant testing strategy for veterinary medicinal products

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Guideline on the plant testing strategy for veterinary medicinal products

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1. Introduction

1.1. Background

Plant toxicity tests are used in the terrestrial environmental risk assessment (ERA) of veterinary medicinal products (VMPs) as described in VICH guideline (GL) 38 on the environmental impact assessment for veterinary medicinal products — Phase II (EMA/VICH, 2005).

OECD Test Guideline (TG) 208 for plant testing (OECD, 2006) has been updated since the publication of VICH GL38. In the updated OECD TG 208, guidance on how many plant species are needed for testing of VMPs is no longer provided.

If a risk for plants is still identified in Tier B of a Phase II ERA for VMPs, the following three options for a further risk assessment exist, by refinement of the predicted no effect concentration in soil ($PNEC_{soil}$):

- A statistical extrapolation technique, the so-called species sensitivity distribution (SSD).
- Testing of metabolites/transformation products, as described in the OECD TG 208.
- An extended plant test for substances which form non-extractable residues and/or transformation products in manure.

1.2. General considerations

Guidance on how to perform Tier A and Tier B plant testing, including an explanation of the SSD approach for higher tier assessment, was provided in the reflection paper on testing strategy and risk assessment for plants (EMA, 2011). The current guideline replaces this reflection paper and provides additional options for a higher tier assessment for plants. The SSD approach presented in the reflection paper as the sole option for $PNEC_{soil}$ refinement has now been complemented with two additional options: testing of metabolites or transformation products, and a plant toxicity test using manure-mediated exposure. Chronic plant tests are also considered, but currently not recommended.

The extended toxicity test using manure-mediated exposure on plants for VMPs is suitable for those substances which form a high amount of non-extractable residues or transformation products. For example, studies on the determination of the fate of VMPs in manure have shown that some antibiotics with high plant toxicity may form a high amount of non-extractable residues. However, it is not known whether these fractions of non-extractable residues are bioavailable in the manure, since non-extractability also depends on the extraction method. Besides this, the manure matrix consists of a high amount of organic matter and undergoes decomposition after spreading onto soil. During this process, non-extractable residues might be released and become bioavailable again. Consequently, the risk of VMPs that bind strongly to manure is unknown, and adapted ecotoxicological tests may need to be considered for the refinement of the risk assessment following Tier B.

In some cases, the active substance may be metabolised in the animal or transformed to transformation products in manure or soil. In case these major metabolites or transformation products (≥ 10% of the applied amount) are identified and available for testing, it may also be an option to test those metabolites/transformation products in a standard plant test according to OECD TG 208.

2. Plant testing in Tier A and B

2.1. Tier A

Six plant species from six different families should be tested in Tier A. It is highly recommended to use species belonging to six different families of four dicotyledonous (including a *Brassica* species) and two monocotyledonous species, which represent the types of plants grown on agricultural land which would receive a manure application. This is to better reflect the variety in the plant kingdom. Acceptable plant species for use in the test are presented in annex 2 of OECD TG 208. The lowest EC_{50} value for the most sensitive endpoint is used, in combination with an assessment factor of 100, to derive the predicted no effect concentration (PNEC). The PNEC is compared to the predicted environmental concentration in soil ($PEC_{soil\_initial}$) (EMA/VICH, 2005). If the PEC/PNEC ratio (risk quotient [RQ]) using the $PEC_{soil\_initial}$ is higher than 1, the $PEC_{soil\_initial}$ can be refined as explained in the 'Guideline on environmental impact assessment for veterinary medicinal products in support of the VICH guidelines GL6 and GL38' (EMEA/CVMP/ERA/418282/2005-Rev.1-Corr.) (EMA, 2016). If the resultant RQ calculated with the $PEC_{soil\_refined}$ is below 1, the assessment can stop. If the RQ is ≥ 1, it is necessary to proceed to Tier B.
Studies with three plant species that were performed before the reflection paper came into force in 2012 (EMA, 2011) could still be accepted in Tier A, provided that the PEC/PNEC is < 0.1.

2.2. Tier B

From the same plants species tested in Tier A, the lowest 'No Observed Effect Concentration' (NOEC) or EC_{10} value is used in combination with an assessment factor of 10. If the resultant RQ is below 1, the assessment can stop. If the RQ is ≥ 1, it is necessary to proceed to the higher tier assessment.

It should be noted that NOEC values often depend on the experimental design, variation within the treatments and the power of the statistical test. Thus, the design of the test (including number of replicates) should be optimised in order to obtain reliable and statistically significant results. Experience has shown that statistically derived NOEC values obtained from plant studies sometimes are associated with effects significantly above 10%. In such cases, it is recommended to use EC_{10} values. It should be noted that EC_{10} values can only be derived if a reliable dose-response relationship is generated and the EC_{10} is within the range of the tested concentrations (including the controls).

No further refinement options for PEC_{soil} are available in Tier B, therefore the PNEC is compared to the refined PEC_{soil} as determined at the end of Tier A.

3. Higher Tier Assessment

3.1. Species Sensitivity Distributions (SSD)

The species sensitivity distribution (SSD), a statistical extrapolation technique, can be used to derive a PNEC if a potential risk for plants is still identified in Tier B. Using the SSD method, the concentration at which 95% of the species are theoretically protected (HC_5) can be estimated. More information about the SSD method can be found in Posthuma *et al*. (2001).

To better reflect the variety of plant species and to improve the statistical power of the SSD, two additional new species, preferably from two additional plant families, should be tested and used in combination with the six species/families tested in Tier B. Only one data point for each species should be included in the SSD.

In other legal frameworks such as the EU regulation on chemicals[1], i.e. REACH, the HC_5 of the SSD is used as the basis for deriving a PNEC in combination with an additional assessment factor ranging typically between 1 and 5. However, no specific and generic criteria for selecting the assessment factor is outlined in any of the legal frameworks, as it should be determined on a case-by-case basis. To move away from case-by-case decisions on the magnitude of this assessment factor, the CVMP recommends using the lower confidence level of the HC_5 (LL HC_5) directly as the PNEC.

An improved dataset in the SSD assessment, i.e. increased number of tested species covering the same endpoint (e.g. growth), will result in a narrower difference between the median (HC_5) and the lower confidence level (LL HC_5) of the HC_5, and consequently in an enhanced confidence in the assessment.

All data used in the SSD assessment have to meet the general requirements for quality as applicable already in the lower tier risk assessment of VMPs. The additional tests should be performed and reported according to the OECD TG 208, including a report on the fulfilment of validity criteria.

In order to use the SSD, the following additional criteria have to be fulfilled in addition to the general quality criteria:

- A minimum of eight plant species from at least six different families have to be tested.
- A minimum number of two monocotyledonous and six dicotyledonous plant species should be included.
- When reliable EC_{10} values are available, it is highly recommended using these for the SSD. When this is not the case, it can be acceptable to use a combination of NOEC and EC_{10} values. Only definitive EC_{10} or NOEC values (excluding '>' and '<' values) can be used in the SSD calculation to ensure the SSD is statistically correctly fitted. In case no reliable EC_{10} value or NOEC can be calculated because significant effects are found at the lowest test concentration, these species should then be retested. The only exemption is that '<' or '>' values can be included (without '<' or '>' sign) in the SSD if it is clearly outside the range of all other available EC_{10}/NOEC values for the tested plant species. If '<' or '>' values are included in the SSD, this should always be clearly indicated and justified. The SSD needs to contain a minimum of 6 definitive (no '<' or '>') values, and at least 8 species have to be tested.
- The NOEC or EC_{10} values should all reflect the same most sensitive endpoint. If a plant species has been tested more than once, the geometric mean of the NOEC and/or EC_{10} values of the same endpoint should be used in the SSD assessment. It is not possible to mix NOECs and EC_{10} values determined in standard tests with those determined in tests with manure.
- The HC_5 and LL HC_5 are calculated based on a log-normal distribution. The data should be tested by 'goodness-of-fit' methods to confirm the likelihood of the data coming from a normal distribution. The Anderson-Darling test on normal distribution is recommended for datasets with less than 20 numbers. If the Anderson-Darling statistic is above the 5% critical value, normality must be rejected and data cannot be used for the SSD.

If it is known that plants are sensitive to the substance under evaluation, the stepwise approach of Tier A and Tier B could be waived, eight or more plants species could be tested in the first instance, and the data could be used in the SSD method, provided the criteria mentioned above are met.

[1] Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. OJ L 396, 30.12.2006, p. 1–849.

Different software programmes are available to calculate the HC_5 and LL HC_5 and to assess whether the data follow a normal distribution, e.g. 'ETX' developed by RIVM (2023) and the 'SSD Generator' developed by the US EPA (2005). The choice of software program is optional.

The PNEC determined with the SSD is compared to the PEC_{soil} as refined at the end of Tier A to determine the risk quotient for plants.

3.2. Testing of transformation products

If the active substance is metabolised in the target animal or transformed in manure to relevant transformation products (≥ 10 %), the standard OECD TG 208 test may also be performed with the relevant metabolites and transformation products. The criteria for Tier A and Tier B testing as described above apply.

The results of the OECD TG 208 study feed into the risk assessment, where the PEC is calculated for the parent and all metabolites or transformation products ≥ 10%. To assess the overall risk of the mixture of parent and metabolites/transformation products, the resulting risk quotients have to be summed up.

3.3. Plant test using manure-mediated exposure

The aim of the extended plant test is to assess the effects of VMPs on terrestrial plants considering a more realistic exposure scenario through the application of pig or cattle manure spiked with the substance of concern into the soil, by performing an extended study in accordance with OECD TG 208. All requirements and recommendations of OECD TG 208 still apply to this extended approach. As in Tier A, six plant species from six different families should be tested.

VMPs administered to the target animal orally or by injection enter the environment via manure. The modified exposure scenario used in this approach takes into account the degradation of the parent compound into transformation products and/or formation of non-extractable residues. More information on non-extractable residues is available in the CVMP 'Reflection paper on poorly extractable and/or non-radiolabelled substances' (EMA, 2024a). For manure, it is assumed that chemicals are potentially released when manure is mixed into soil or undergoes decomposition.

In this extended OECD TG 208 study, manure is spiked with the test substance and incubated under anaerobic conditions. The scenario of spiking manure is intended to simulate the fate and behaviour of VMPs in manure which is usually stored in tanks before spreading onto agricultural soil. The relevant type of manure should be used for this test. For instance, cattle manure should be used if the product is intended for use in cattle and pig manure should be used if the product is intended for use in pigs. The test design has been successfully verified with pig and cattle manure (Simon *et al*., 2015). The technique for manure storage and acclimation generally follows the CVMP 'Guideline on determining the fate of veterinary medicinal products in manure' (EMA, 2024b).

To determine the PNEC of the extended plant test, the same assessment factors apply as in Tier A or B. The PNEC is compared to the $PEC_{soil\_refined}$ determined at the end of Tier A. It is not possible to further refine the PEC for degradation in manure, because this process is already taken into account in the determination of the PNEC.

Details on the test design, performance, evaluation and reporting for the extended plant test are given in Annex I to the present guideline.

3.4. Chronic toxicity in higher plants

The 'International Organisation for Standardisation' (ISO) has developed a chronic toxicity test for higher plants, i.e. ISO 22030:2005 (ISO, 2005) mainly for the testing of contaminated soils. In this test, not only emergence and growth, but also reproduction parameters such as number of flowers or seed pods are measured. The European Food Safety Authority (EFSA) evaluated this test method and concluded that its usefulness for testing herbicide effects on non-target terrestrial plants is very limited, as only two crop species with a very short life cycle are recommended for use in testing, and the artificial soil recommended is a very poor soil in which plants do not grow well (10% *Sphagnum* peat, 20% kaolin clay, 69% sand) (EFSA, 2014). Furthermore, experience has shown that the test may be difficult to perform and that the variability in the measured reproductive endpoints is often very high. Therefore, the test is currently not recommended for higher tier testing of VMPs.

4. References

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Annex I

Guidance on test design, performance, evaluation and reporting for the extended plant test

I.1 Definitions

- Manure means liquid manure from a tank (mixture of urine and faeces) for the purpose of this guideline.
- Manure storage or pre-storage tank is the basin where the manure is stored at the farm.
- Storage means storing of manure after sampling under unaltered conditions (unprocessed, anaerobic conditions, 4–20 °C, in the dark), comparable with those of storage or pre-storage tanks at farms until use.
- Acclimation means storing of manure after homogenisation and adjustment to standardised dry matter content, at conditions to acclimate microorganisms before incubation. An acclimation period of 21 days is recommended to ensure comparable conditions between experiments (Hennecke *et al*., 2015).
- Half-maximum storage duration is the half of the mean maximum storage time of manure in storage tanks at farms according to table 6 in the 'Guideline on environmental impact assessment for veterinary medicinal products in support of the VICH guidelines GL6 and GL38' (EMEA/CVMP/ERA/418282/2005-Rev.1 Corr.1).
- Incubation means storage of manure after acclimation and application of the test substance, at conditions mimicking abidance of manure in storage tanks at farms under standardised conditions.

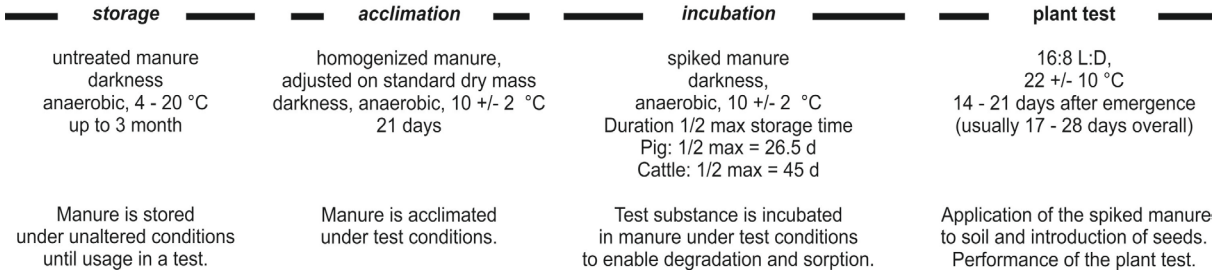

Figure 1. Schedule and definition of main phases in the extended test design

I.2 Manure

- The manure applied should originate from animals reared under well controlled conditions. The use of manure contaminated with VMPs, biocides and other material that might impair plant growth or survival should be avoided. The type of animal feed, the feeding regime and the veterinary history of the animals from which the manure will be collected should be recorded and reported.
- The manure used should reflect the target animals for the intended use of the product. For example, pig manure when the product is intended to be used in pigs and cattle manure if it is intended to be used in cattle.
- Manure should be sampled from manure storage or pre-storage tanks which are above or below ground. Prior to collection, the liquid manure should be thoroughly mixed in the respective manure tank. Pig manure should be stirred immediately before sampling, as separation into liquid and solid phase easily occurs. Duration of mixing depends on the kind of storage tank. However, it should be ensured that the sample of liquid manure is a representative mixture of liquid and solid phase. The

sampling site, procedure and the type and size of manure tank (above/below ground, covered/open) should be recorded.

- Prior to further processing, the manure should be stored preferably at acclimation and incubation temperature for a maximum of three months (EMA, 2024a) and under anaerobic conditions.
- For acclimation, the dry matter content of the manure has to be adjusted to standardised values. The recommended dry matter content in pig manure is 5 ± 1% and 10 ± 1% in cattle manure (EMA, 2024a; Weinfurtner, 2011). Manure should be processed using a mixer (e.g. a food processor or similar apparatus), in order to obtain a homogenised phase and to reduce the variability of the test result. All operations should be carried out under anaerobic conditions; exposure to oxygen has to be kept to an absolute minimum if it cannot be avoided. The period of anaerobic acclimation should be 21 days at 10 ± 2 °C in the dark.
- Key parameters of the manure as mentioned in the EMA 'Guideline on determining the fate of veterinary medicinal products in manure' (EMA, 2024a) and listed in **Table 1** should be measured and reported.

Table 1: Key parameter measurements in manure

Parameter	**Stage of test procedure**	
	Start of storage	During acclimation
pH	X	X
Microbial activity		X
Organic carbon content [C_{org}; mg/kg]		X
Total nitrogen content [N_{total}; mg/kg]		X
Ammonium content [NH_4-N; mg/kg]		X
Phosphate content [P; mg/kg]		X
Copper content (for pig manure only) [Cu; mg/kg] optional	X	
Redox potential [mV]	X	X*
Dry matter content [%]		X*
Temperature [°C]	X	X

* Should be measured at the start and end of acclimation.

- Anaerobic conditions in manure should be ensured and demonstrated by measuring and reporting the redox potential at the end of the acclimation and incubation period where -100 mV should never be exceeded. Typical redox potentials measured in pig and cattle manure have been found to range from -230 to -400 mV (Weinfurtner, 2011).

I.3 Application of the test substance

- Untreated manure by itself can also impair seedling emergence (Simon *et al*., 2015). Therefore, it is advised to check in a pre-test without test substance whether the intended manure concentration in soil has adverse effects on the test plants.
- Based on nitrogen content, the maximum amount of manure must not exceed 227 mg N_{total} /kg dry soil (170 kg N_{total} /ha per year, assuming an incorporation depth of 5 cm and a soil density of 1.5 g/cm^3). An amount of 20 g fresh manure per kg dry soil, corresponding to approximately 45–55 kg N_{total} /ha, was shown to be a suitable amount regarding seedling tolerance (Simon *et al*., 2015).

- The quantity of test substance required to obtain the theoretical test concentrations in soil, assuming no transformation during incubation, is mixed with a portion of manure (dry mass content of the manure: 5 ± 1 % for pig manure, 10 ± 1 % for cattle manure), e.g. in glass beakers.

  Example: If 20 g fresh manure should be applied to 1 kg dry soil and a theoretical test concentration in soil assuming no transformation during incubation should be 100 mg/kg, 100 mg test substance have to be applied to 20 g fresh manure.

- Water-soluble substances or those suspended in water can be added directly to the manure and mixed, for instance, with a pipette tip. The volume of water added should be the same for each test concentration and should not result in a difference to the desired dry mass content of the manure. The water additionally provided by the stock solution has to be taken into account when adjusting the manure for acclimation (i.e. the manure should be adjusted to an appropriate higher dry mass content for acclimation).

- Substances of poor solubility in water should be dissolved in a suitable volatile solvent and mixed either directly with the manure or via quartz sand. For direct application, the solvent concentration should not be greater than 0.1 ml/l manure and should be the same concentration in all test vessels. The solvent should be removed from the manure, for instance by using low pressure followed by pressure compensation using oxygen free air or nitrogen. If the test substance is applied in a solvent, a respective solvent control has to be included. For direct application, this should be a solvent control containing manure and solvent, for application via spiked quartz sand (as little as possible), a solvent control containing manure and evaporated spiked quartz sand. The quartz sand added is not considered for dry mass content of the manure. However, every effort should be made to keep the solvent concentration to a minimum.

- Solid, insoluble test substances can be applied either directly to manure or via quartz sand. For the latter, the test substance and (as little as possible) finely ground industrial quartz sand is mixed in a suitable mixing device. Hereafter, the mixture is added to the manure and mixed thoroughly. The quartz sand added is not considered for dry mass content of the manure.

- It should be kept in mind that all spiking and mixing operations should be carried out in a way that the manure has minimal contact with oxygen.

- To reflect representative influences of storage on manure, the spiked manure is incubated under anaerobic conditions in the dark for a period representing the half-maximum storage duration of the respective manure type (26.5 days for pig manure, 45 days for cattle manure) (EMA, 2024a). To reflect a realistic scenario, incubation temperature should be 10 ± 2 °C.

- It is recommended to mix the spiked manure with soil in a two-step approach to ensure a homogenous distribution. The spiked manure is added to a sub-portion of test soil and mixed thoroughly. Subsequently, the pre-mixture is added to the rest of test soil and mixed thoroughly.

I.4 Verification of test substance concentration

- The concentrations/rates of application into the fresh manure must be confirmed by an appropriate chemical analysis, comparable to the requirements of the standard OECD TG 208 (OECD, 2006).

- It is strongly recommended to measure the test substance concentration in the incubated manure prior to the start of the plant test at the time of incorporation of manure into soil. As a minimum, samples of manure with the highest concentration and one lower concentration should be considered for analysis. These determinations of test substance concentration provide information about the degradation/adsorption of the test substance in the manure. Depending on the question to be addressed, determination of transformation products and non-extractable residues might be

required. As mentioned in the 'Reflection paper on poorly extractable and/or non-radiolabelled substances (EMA, 2024b), the best available extraction technique should be used. This means that determination of the extractable fraction may have to be pursued by various extraction methods with increasing strength. The evaluation of the feasibility of various extraction techniques should be reported in the final study report.

I.5 Plant test

- In general, the extended test approach follows the standard test in accordance with OECD TG 208 (OECD, 2006) with all respective requirements and recommendations. Additionally, six plant species from six different families should be tested as well as in Tier A. Any potential acceptable modifications are listed below.
- The planting of seeds has to be done for all replicates on the same day when the test substance/manure mixture is incorporated into soil to prevent aerobic transformation of the test substance before contact with the seeds.
- Control groups with non-spiked manure only are established to assure that effects observed are associated with or attributed only to the test substance exposure. The manure controls or solvent/manure controls are used for evaluation of the effects caused by the test substance. The number of replicates and seeds depend on the chosen test design.
- A standard control without manure has to be established to detect possible adverse effects on seedling emergence or growth caused by manure by comparing with the non-spiked manure control. The standard control should consist of at least four replicates with at least 20 seeds in total, independent from the chosen test design. The standard control should not be used for test substance effect evaluation.
- The start of the 14–21 day growth period is defined by 50% emergence in the manure control and not in the standard control.
- Endpoints: The purpose of this approach is to obtain NOEC and/or EC_x values.
- For establishment of the number and spacing of concentrations, the following should be considered:
  - Prior knowledge of the toxicity of the test substance to plants, e.g. derived using a standard test according to OECD TG 208 (OECD, 2006), could help selecting appropriate test concentrations. However, it is strongly recommended to perform a range-finding test following the extended test design, as the magnitude of effects caused by the test substance together with manure is often not predictable.
  - A combined approach allowing for the determination of both NOEC and EC_x values is highly recommended. Eight treatment concentrations in a geometric series should be used with four replicates each, together with eight manure control replicates. The concentrations should be spaced by a factor not exceeding 2.5.
  - For determination of the NOEC, at least five concentrations in a geometric series should be tested. Eight replicates for each test concentration plus eight manure control replicates are recommended. The concentrations should be spaced by a factor not exceeding 3.
- Effect concentrations should be related to soil dry mass and calculated on the basis of either the measured concentrations in the applied stock solution (in case of water-soluble substances) or the applied weights (in case of insoluble test substances).

I.6 Validity of the test

- All requirements as stated in OECD TG 208 (OECD, 2006) apply to this extended approach as well. Especially the performance criteria in terms of seedling emergence rate (70%) and post-emergence survival rate (90%) have to be fulfilled in all controls.

I.7 Test report

- All requirements as stated in the OECD TG 208 (OECD, 2006; test substance, test species, test conditions, results) apply to this extended approach as well. However, additional issues regarding the manure and its preparation, acclimation, incubation and application are listed below and should be reported too.
- Type of manure (pig or cattle).
- Name and location of the farm the manure originates from.
- Feed type, feeding regime and the veterinary history of the animals from which the manure originates (if data are available).
- Type of manure tank from which the manure originates (e.g. above/below ground, open/covered, size; if data are available). Sampling procedure and how the manure was mixed before sampling.
- Key parameters of the manure at the respective time as outlined in **Table 1** (e.g. temperature, pH, redox potential, dry matter content, C_{org}, N_{total}, P).
- Techniques and conditions (duration, temperature) for manure storage, preparation, acclimation, and incubation (e.g. cooling and/or incubation chamber, mixing device for manure homogenisation).
- Details on preparation of the spiked manure and verification of the test concentrations.

I.8 References

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