Proposal for a Technical Protocol (Draft Version)

Transformation of Veterinary Medicinal Products and Biocides in Liquid Bovine and Pig Manures

INTRODUCTION

1. This technical protocol is designed for evaluating the transformation of veterinary medicinal products (VMP) and biocides in liquid bovine and pig manures. Special focus is put on the environmentally relevant entry routes of VMP and biocides into liquid manures, i.e., after excretion of cattle and pigs via urine and feces of VMP as parent compounds or metabolites, and after the use of disinfection agents in animal houses, respectively. In loose housing stables with slatted floors, the excrements are discharged into manure aboveground silos or underground pits. After the storage of liquid manures up to several months, they are applied to arable and grassland soils as organic fertilizers. Via this route, VMP may enter soil environments. Thus, the persistence of VMP during manure storage under anaerobic conditions decides on the environmental relevance of this entry route.
Further entry routes, i.e., solid dung application and direct dung pat deposition by production animals on pasture, are not considered by this technical protocol. Solid dung of poultry is also not in the scope of this technical protocol due to its aerobic storage conditions and then used for laboratory testing.

2. Taking into special consideration that liquid manures are heterogeneous matrices of high complexity and variability, the representative and reproducible sampling in manure tanks is considered impossible. Therefore, this technical protocol focused on the sampling of excrements from cattle and pigs kept in an experimental stable and fed under standard nutrition conditions. This approach additionally ensures that excrement samples are free of any contamination by VMP and disinfection agents [1]. After a sophisticated matrix characterization, reference-manure samples are prepared from the excrement samples by the adjustment of defined dry substance contents typical for bovine or pig manures.

3. This technical protocol comprehends a tiered experimental design in two parts:

I. Sampling of excrements and preparation of reference bovine and pig manures.

II. Anaerobic transformation tests of VMP in reference manures.

PRINCIPLE OF THE TESTS

4. The principle of the transformation tests in liquid manures is based on the tiered experimental design starting with the reproducible sampling of excrements. After a sophisticated matrix characterization, tap water is added to the excrement samples to prepare the reference-manure samples for these transformation tests under controlled experimental conditions. With respect to the high complexity of the manure samples, test substances should be preferably fortified as ^{14}C-labeled radiotracers, if available. Mass balances are set up considering the mineralization and the formation of extractable and non-extractable residues. Extracts were additionally analyzed for the parent compound initially applied and metabolites formed during appropriate incubation intervals up to 100 d.

APPLICABILITY OF THE TESTS

5. These tests are principally applicable to every veterinary medicinal product and every biocide applied in animal houses (^{14}C-labeled or non-labeled) for which an analytical method with sufficient accuracy and sensitivity is available. With respect to the high complexity of the manure samples, the use of ^{14}C-labeled test substances is strongly recommended. Otherwise, mass balances including the release of ^{14}C-carbon dioxide and the formation of non-extractable residues cannot be set up and the metabolic fate of the test substance can only be assessed by the determination of the disappearance time (DT_{50}, DT_{90}) of the parent compound under study.

INFORMATION ON THE TEST SUBSTANCE

6. The ^{14}C-labeled test substance is to be specified by the position of the labeling (most stable moiety in the molecule is to be preferred), the radiochemical purity ($\geq$ 95 %) and the specific radioactivity (MBq mg^{-1} or MBq mmol^{-1}).

7. Before carrying out transformation tests in manure, the following information on the test substance should be available:

– Solubility in water according to OECD Guideline 105 [2].
– Solubility in organic solvents.
– Vapour pressure according to OECD Guideline 104 [3] and Henry´s law constant.
– n-Octanol/water partition coefficient according to OECD Guidelines 107 and 117 [4,5].

– Chemical stability in dark (hydrolysis) according to OECD Guideline 111 [6,7].
– pK_a if a molecule is liable to protonation or deprotonation according to OECD Guideline 112 [8].

8. Other useful information may include data on toxicity of the test substance to manure and soil microorganisms according to OECD Guidelines 216 and 217 [9,10].

9. Analytical methods (including extraction and clean-up methods) for identification and quantification of the test substance and its main metabolites (> 10 % of the parent compound initially applied) should be available or have to be elaborated. Furthermore, reference substances should be used for the identification of transformation products by spectroscopic and chromatographic methods.

DEFINITIONS

10. See Annex A.

QUALITY CRITERIA

Recovery

11. Data for the quality assurance of the matrix characterization are given in the ISO and DIN EN Guidelines listed in Annex B.

12. The analyses of at least duplicate manure samples directly after the addition of the test substance give a first indication of the repeatability of the analytical methods and of the uniformity of the application procedure for the test substance. Recoveries for the experiments are given by the respective mass balances that should range from 90 % to 110 %, if possible. When residue analytical methods are applied, recoveries are considered acceptable at 70-110 %.

Repeatability

13. Differences of independently analyzed duplicates should not exceed 10 %.

14. Repeatability of the analytical method to quantify test substance and metabolites can be checked by duplicate analysis of the same extract of the manure incubated long enough

for formation of metabolites. In general, the analytical method is considered repeatable, if the difference of duplicates does not exceed 10 %. Furthermore, the analytical quality is expressed by the mass balances of the conducted tests (see recovery chapter, paragraph 12).

15. The limit of determination (LOD) of the analytical method for the test substance and for the transformation products should be at least 0.1 mg kg^{-1} manure (fresh weight) or 1 % of applied concentration whichever is lower. The limit of quantification (LOQ) should also be specified.

Accuracy of transformation data

16. The data are evaluated by using appropriate statisics (e.g., modelmaker). Regression analysis of the concentrations of the test substance as a function of time gives the appropriate information on the reliability of the transformation curve and allows the calculation of the confidence limits for half-lives (in case of pseudo first order kinetics) or DT_{50} values and, if appropriate, DT_{90} values [10,11].

PART I. SAMPLING OF EXCREMENTS AND PREPARATION OF REFERENCE BOVINE AND PIG MANURES

INFORMATION ON THE EXCREMENT AND MANURE SAMPLES

Sampling and feeding conditions

17. To get the complexity and variability of liquid manures under control, excrements from conventionally fed single animals or groups of up to 8 individuals are taken in a period of 12 to 24 h. The race (e.g., diary cows: German Holstein – black and white, fattened pigs: German Federal Hybrid Breeding Program), feeding conditions (adequate to standard nutrition conditions) as well as the age of the individuals should be known. For the food, the percentage composition should be given, if possible, reflecting the herbivore nutrition type of cattle (e.g., grass silage, pellets and minerals) and the omnivore nutrition type of pigs (barley, soya pellets, soya oil, vitamins, minerals, trace elements and amino acids). The administration of VMP has to be definitely excluded.

Conditioning, storage and matrix characterization of excrement samples

18. Directly after excretion, readily degradable organic compounds of the excrements undergo rapid decomposition enhancing the matrix heterogeneity [12,13]. To minimize this effect, conditioning of excrement samples is necessary. For this purpose, the excrement samples are kept in plastic containers (approximately 20 L) at ambient temperature. Within a 21-d period, they are daily homogenised using an electric stirrer. To ensure a gas exchange, the lid loosely covers the container. Thereafter, the excrements samples are to be matrix characterized by the parameters as follows: dry substance, total organic carbon, pH, redox potential, dissolved oxygen, ammonium and total nitrogen, biological oxygen demand. If anaerobic conditions, indicated by the dissolved oxygen contents < 0.1 mg kg^{-1}, the redox potential Eh < +150 mV [13] and an ammonium content stable up to $\pm$ 0.2 g kg^{-1}, are established [14-19], the excrements can be directly used for the reference-manure preparation or long-term stored up to 360 d at -20 °C until analytical processing. After defrosting, the excrements are stored at 20 $\pm$ 2 °C for 3 d to remobilize the excrements inherent microorganisms. According to principles of the analytical quality assurance, matrix characterization has to be repeated.

Reference-manure preparation

19. For reference-manure preparation, the dry substance content (ds) of the excrement sample under study must be known. The targeted mass of excrements is calculated using the following formula:

$$m_{ex} = \frac{ds_{man} \cdot m_{man}}{ds_{ex}} \quad (1)$$

$$m_w = m_{man} - m_{ex} \quad (2)$$

m_{ex}: mass of required excrement sample [g]
ds_{ex}: dry substance content of the excrement sample [%]
m_{man}: mass of the resulted manure sample [g]
ds_{man}: targeted dry substance of the manure sample [%]
m_w: mass of required water [g]

20. Subsequently, the required quantity of the excrement is weighed into a sample bottle or directly into the flask of the laboratory-test system. After adding the tap water, the manure sample is to be homogenized and the matrix characterization is to be exemplarily carried out again. The prepared manure samples can be stored in closed sample bottles at 4 °C for 7 d, without any relevant change of the matrix parameters.

CHARACTERIZATION OF EXCREMENT AND MANURE MATRICES

Further detailed information about the manure matrix characterization procedure is given in the Annex B.

Equipment and chemicals

21. Standard laboratory equipment is required and especially the following:

- Plastic containers with lids, 20 L, 2 L and 1 L.
- Mechanical mixing device (e.g., electric stirrer).
- Drying oven (105 ± 5 °C).
- Muffle furnace (550 ± 25 °C).
- Infrared heater with analytical balances.
- Incubator (5 to 20 ± 1 °C).
- Apparatus for determination of the total carbon content.
- Analytical balances (accuracy ≤ 1 mg).
- pH-meter with pH electrode and appropriate test solution.
- Millivoltmeter with redox electrode and appropriate test solution.
- Oxygen meter with measuring probe.

- Distillation and digestion systems with distillation flasks or tubes.
- Incubation bottles (Karlsruher bottles).
- Spectral photometer, digestion stand with digestion flasks or tubes.

22. Chemicals used include, for example:

All used chemicals (e.g., NaOH, H_2SO_4, etc.) and organic solvents (e.g. acetone, methanol) should be of analytical grade.

Dry substance content (ds)

23. Excrements and manure samples (1 to 6 g) are dried in a drying oven to constant mass at 105 ± 5 °C [20]. Alternatively, an infrared heater can be used to drive out the water. The difference of an amount of excrement or manure before and after the drying procedure is used to calculate the dry substance content. The dry substance content is expressed in percentage, with an accuracy of ± 1 % (*m*/*m*).

Total organic carbon (TOC)

24. The carbon present in excrement or manure samples is oxidized to carbon dioxide by heating up at least 900 °C in a flow of oxygen-containing gas that is free from carbon dioxide [21]. Prior to combustion, carbonates are to be removed from the dried samples (50-100 mg) by an excess of hydrochloric acid (4 mol L^{-1}). Subsequently, the samples are to be dried at 105 °C, homogenized and mixed with aluminium oxide (1:20). The mixed samples are combusted and the released amount of carbon dioxide is measured by titrimetry, gravimetry, conductometry, gas chromatography or infrared detection method, depending on the apparatus used. The total organic carbon is expressed in % dry substance or in g kg^{-1} fresh weight, with an accuracy of 1 g kg^{-1}.

$$TOC_{ds} = \frac{m_c \cdot f}{m_a} \cdot 100 \qquad TOC_{fw} = \frac{m_c \cdot f}{m_a} \cdot 10 \cdot ds$$

ds: dry substance [%]
fw: fresh weight
m_c: amount of carbon [µg]
m_a: initial weight [µg]
f: dilution factor
100: conversion factor to percent
10: conversion factor to 1 kg excrement or manure

pH value

25. The pH value is measured directly in the homogenized excrement or manure sample (50 to 100 g) using a pH electrode. The pH value can be considered as stable when the pH measured over a period of 5 s varies by not more than 0.02 units. The results are expressed to one significant figure [22].

Redox potential (E_h)

26. The redox potential is measured directly in the homogenized excrement or manure sample (50 to 100 g) using a redox electrode system, related to the voltage of standard hydrogen electrode. The value of the redox potential is quoted rounded to nearest 10 mV [23].

Dissolved oxygen content (O_2)

27. The dissolved oxygen content is measured directly in the homogenized excrement or manure sample (50 to 100 g) using an electrochemical cell which is isolated from the sample by a gas permeable membrane [24]. The resulted oxygen content is given in mg O_2 kg^{-1} to the first decimal place for results > 0.1 mg O_2 kg^{-1}. Results less 0.1 mg oxygen kg^{-1} are reported as $\leq$ 0.1 mg kg^{-1}.

Ammonium nitrogen (NH_4-N)

28. Under mildly alkaline conditions [25], a distillation of the homogenized excrement or manure sample (1 to 4 g) is performed. The released ammonia is trapped in a receiving flask containing 50 mL boric acid solution (20 g L^{-1}) and an indicator solution (e.g., 200 µL mixed indicator No. 5). Titration of the ammonium in the distillate is conducted with standard volumetric hydrochloric acid solution (0.1 mol L^{-1}). The ammonium nitrogen concentration (NH_4-N), expressed in g NH_4-N kg^{-1} and rounded to one significant figure is calculated using the formula:

$$NH_4 - N = \frac{(V_1 - V_0) \cdot c \cdot M_N}{m}$$

V_1: volume of hydrochloric acid used in the titration of the sample [mL]

V_0: volume of hydrochloric acid used in the blank test [mL]

m: mass of the excrement or manure sample [g]

c: concentration of hydrochloric acid [0.1 mol L^{-1}]

M_N: molar mass of nitrogen [14.01 g mol^{-1}].

Total nitrogen (N_{total})

29. The total nitrogen content of homogenized excrement and manure samples (1 to 4 g)

is determined by Kjeldahl digestion that transfers the nitrogen containing compounds (proteins, amines, etc.) into ammonium compounds [26]. After the addition of bases, ammonia is released by distillation and titrated. The reaction is accelerated by Kjeldahl tablets (5 g) that contains sulfates and metallic salts. The sulfates increase the boiling point of the concentrated sulfuric acid (10 mL). The selenium, copper or titanium salts shorten the time of digestion. After a boiling period of at least 3 h, the distillation of the released ammonia follows. The distillate finally trapped in 50 mL boric acid (20 g L^{-1}) is titrated using a standard volumetric hydrochloric acid solution (0.1 mol L^{-1}) as well as an indicator solution (e.g., 200 µL mixed indicator No. 5). The total content of nitrogen expressed in g N kg^{-1} and rounded to one significant figure is calculated using the formula:

$$N_{total} = \frac{(V_1 - V_0) \cdot c \cdot M_N}{m}$$

V_1: volume of hydrochloric acid used in the titration of the sample [mL]

V_0: volume of hydrochloric acid used in the blank test [mL]

m: mass of the excrement or manure sample [g]

c: concentration of hydrochloric acid [0.1 mol L^{-1}]

M_N: molar mass of nitrogen [14.01 g mol^{-1}].

Biological oxygen demand in 5 d (BOD_5)

30. The microbial activity of excrement and manure samples have to be checked before transformation tests in reference manures are conducted. For this purpose, the biological oxygen demand[a] can be determined [27]. Excrement and manure samples are diluted with varying volumes (Table 1) of tap water nearly saturated with oxygen and containing allylthiourea (2 mg L^{-1}) to suppress nitrification.

[a] By means of the BOD_5 measurement, the activity of aerobic microorganisms is merely comprised. Thus, the dehydrogenase activity can be alternatively determined considering that nearly every microorganism is enabled to reduce triphenyltetrazolium chloride to triphenyl formazan. The photometric measurement of the latter at λ = 485 nm, however, may be interfered by coloured excrement or manure extracts. Then, the measurement should be alternatively carried out at λ = 546 nm.

According to the OECD Guideline 311 [28], the fermentation of test substances, e.g., sodium benzoate, can be studied. Those tests, however, take a 4-week period and appropriate test substances may not be available as ^{14}C-labeled radiotracers.

Table 1: Dilution factors for the BOD_5 determination in excrement and manure samples

Sample	dilution factor
bovine matrix, ≥ 15 % ds	1:4000
bovine matrix, < 15% ds	1:2000
pig matrix, ≥ 10 % ds	1:4000
pig matrix, < 10% ds	1:2000

The sample solutions are filled in airtight bottles (Karlsruher bottles) and incubated at 20 ± 1 °C in the dark for 5 d. The BOD_5 is calculated from the difference between the initial and final dissolved oxygen content, allowing for blank value:

$$BOD_5 = \left[(C_1 - C_2) - \frac{V_t - V_e}{V_t} \cdot (C_3 - C_4) \right] \cdot \frac{V_t}{V_e}$$

C_1: dissolved oxygen concentration in the sample solution at time zero [mg kg^{-1}]

C_2: dissolved oxygen concentration in the sample solution after five days [mg kg^{-1}]

C_3: dissolved oxygen concentration in the blank solution at time zero [mg kg^{-1}]

C_4: dissolved oxygen concentration in the blank solution after five days [mg kg^{-1}]

V_t: total volume [mL]

V_e: sample volume [mL]

Results less than 1 g kg^{-1} of oxygen are reported with two significant figures. Results between 1 g kg^{-1} and 10 g kg^{-1} are reported to one significant figure. Results ≥ 10 g kg^{-1} are reported without decimal places.

PART II: ANAEROBIC TRANSFORMATION TESTS OF VMP IN REFERENCE MANURES

Equipment, instruments and chemicals

31. For transformation tests of VMP in manure, the application of ^{14}C-labeled test substances is strongly recommended to set up mass balances differentiating between mineralization and the formation of extractable and non-extractable residues. For this purpose, suitable laboratory-test systems are to be used. Particularly considering that these transformation tests are to be performed under anaerobic conditions, typical for manure storage in tanks, a closed batch apparatus allowing for a discontinuous gas exchange can be used. Advantages are little required laboratory space, possible use of commercially available incubators instead of climatic chambers as well as effortless and cost-extensive handling. This batch apparatus shown in Figure 1A (see Annex C), traces back to the biometer-type flask already mentioned in the OECD Guideline 304 [29] that has been slightly modified by the installation of an internal ^{14}C-carbon dioxide trap and additionally equipped with an external stripping device (Figure 1B; see Annex C). The latter allows for the gas analysis of the incubation flask´s headspace to check for the release of volatile metabolites.

32. Alternatively, the flow-through system mentioned in the OECD Guideline 307 [30] (Figure 2; see Annex C) can be used. Here, nitrogen is to be used as stripping gas introduced in stop-flow mode only because there is not any necessity for a continuous gas exchange in the transformation tests in manures under anaerobic conditions.

33. For additional measurements, e.g., pH, redox potential, biological oxygen demand, parallel batch tests with non-labeled test substances are to be conducted. Here, the biological oxygen demand or alternative methods are important to check the microbial activity of manure under test conditions (see paragraph 30). This approach additionally facilitates to determine biological effects of the applied test substance and the used solvent on the manure inherent microorganisms.

34. For the analytical procedures standard laboratory equipment is required and especially the following:

– Sample preparation: Extractor, rotary evaporator, clean-up apparatus (e.g., solid phase extractor, gel permeation chromatograph).
– Radiotracer analysis: Liquid scintillation counter, radio-thin layer chromatograph or radio-high performance liquid chromatograph, oxidizer.
– Residue analysis: Gas chromatograph or high performance liquid chromatograph, mass spectrometer, nuclear magnetic resonance spectrometer.

35. For radiotracer analysis scintillation cocktails for organic and aqueous solutions as well as for trapping of $^{14}CO_2$ are necessary and every chemical (e.g. NaOH, H_2SO_4, etc.) and organic solvent (e.g. ethylene glycol, acetone, methanol etc.) should be of analytical grade. When residue analysis is applied, chemicals and solvents should be of residue analysis or HPLC grade quality.

Test conditions

36. The dry substance contents of bovine and pig manures of 10 % and 5 %, respectively, are adjusted by the addition of tap water to the corresponding excrement samples (see paragraph 20). These dry substance contents correspond to the average values given for Germany that ranged between 0.9 and 12 % [31-36] and should be used by default. In order to additionally study the effect of the dry substance content on the transformation of VMP and biocides in manure, different dry substance contents (e.g., 2.5, 5 and 10 %) can be optionally tested.

37. Anaerobic conditions are to be ensured permanently. In the closed laboratory-batch system, nitrogen is rinsed directly after the test-substance application and directly before incubation for at least 5 min. This procedure is to be repeated for every gas exchange (for trapping of stripping gases see paragraph 44). Using the flow-through system, nitrogen is discontinuously introduced in stop-flow mode to maintain anaerobic conditions.

38. The temperature is to be maintained constant at 20 ± 1 °C to study the transformation of VMP and biocides in manure under standard laboratory conditions. Since the temperature in manure tanks is dependent on ambient conditions, transformation tests in manure can be optionally carried out at lower temperature, too (e.g., 10 °C, 5 °C).

39. The duration of the transformation tests should be accounted for 100 d. Optionally, this incubation period may be extended up to 180 d in order to simulate the long-term manure storage [37].

Test substance application

40. For addition to manure and distribution in manure, the test substance can be dissolved in water (deionized or distilled) or, when necessary, in minimum amounts of organic solvents (e.g. acetone, acetonitrile, methanol) in which the test substance is sufficiently soluble and stable. However, the amount of the selected solvent should not have any relevant effect on manure inherent microorganisms. In order to ensure an even active substance distribution in the samples, the solvent volume should be 40 to 75 µL per sample [30].

The use of solvents which inhibit microbial activity, such as dimethyl sulfoxide, chloroform, dichloromethane and other halogenated solvents, should be avoided. If this is not possible, the test substance can also be added as a solid, e.g., mixed in quartz sand. If the test substance is added using a solvent, the solvent should be allowed to evaporate before the spiked carrier is added to the sample [30].

41. For the adjustment of the applied amounts of manure and radiotracer, the substance specific exposure assessment as well as the analytical feasibility mainly defined by the specific radioactivity of the radiotracer under study is to be taken into special account. The applied concentration should be based on the substance specific exposure assessment of the VMP or biocide under study [38]. If the corresponding detection limit is not achievable, the concentration may be enlarged up to a factor of 10.

Performance of the transformation tests in manure

42. About 50 to 100 g manure (fresh weight basis) are placed into each incubation flask of the laboratory-test systems illustrated in Figure 1 or 2 (see Annex C) and the test substance is applied as described in paragraph 40. When organic solvents are used for the application of the test substance, they should be removed from manure by evaporation. Then the sample is thoroughly mixed by shaking the flask.

43. The duplicate incubation flasks are incubated in the dark at 20 ± 1 °C for, e.g., 0, 3, 7, 30, 72, 100, and optional 180 d. In parallel, additional flasks with and without spiking of the non-labeled test substance are also incubated for matrix characterization purposes and additional tests.

44. In the laboratory-batch system, the headspaces of the incubation flasks are rinsed by a gentle stream of nitrogen every 7 d during the incubation period. The stripping gas is passed through 3 external traps filled with 10 mL ethylene glycol, 10 mL sulfuric acid (0.05 mol L^{-1}) and 10 mL scintillation cocktail to trap related volatiles and ^{14}C-carbon dioxide, respectively. Subsequently, the absorption solution of the internal ^{14}C-carbon dioxide trap is exchanged and every absorption solution is scintillation counted. In the flow-through system, the headspace of the incubation flask is exchanged every 7 d. Thereafter, the external traps are to be exchanged and analyzed as described before.
In order to measure ^{14}C-methane released out of the ^{14}C-labeled radiotracer intially applied or metabolites formed during the incubation period, $^{14}CO_2$ free headspace gases out the laboratory-test systems have to be transferred in a combustion apparatus, quantitatively oxidized in an oxygen stream at 900 °C. The released $^{14}CO_2$ is to be trapped in a $^{14}CO_2$ trapping scintil-

lation cocktail an scintillation counted.

45. Duplicate incubation flasks are removed at the appropriate incubation intervals (see paragraph 43). The manure samples are to be extracted exhaustively. In preliminary tests, therefore, the extraction efficiency of solvents of different polarity (sequential extraction technique) and of different extraction procedures[b] has to be investigated for every test substance.

46. Non-extractable radioactivity will be quantified by scintillation counting after combustion of the already extracted manure matrix. For homogenization, the extracted manure samples are mixed with a mixture of sea sand (20 g) and cellulose (5 g), dried in a desiccator and then thoroughly ground. Finally, aliquots of this mixture are combusted using an oxidizer. The released ^{14}C-carbon dioxide trapped in a scintillation cocktail and scintillation counted to quantify amounts of non-extractable residues.

[b] Besides a direct extraction procedure of the manure samples, the liquid phases of the manure samples can be removed by lyophilization and the dried materials can be treated by means of organic solvents (e.g., acetone, acetonitrile, ethyl acetate, methanol) in single or sequential extraction steps.

Alternatively, the extraction procedure may start by separating liquid and solid phases of the manure samples via centrifugation. Then, the liquid phases can be directly analyzed for radioactivity amounts by scintillation counting. The identification of corresponding metabolites in aqueous phases, however, may be interfered by time consuming enrichment procedures often accompanied by precipitation of co-extracted matrix components. The separated solid sample materials can be treated by means of organic solvents (e.g., acetone, acetonitrile, ethyl acetate, methanol) in single or sequential extraction steps.

DATA AND REPORTING

Treatment of results

47. The amounts of test substance, transformation products, gaseous and volatile substances and non-extractable residues in manure samples should be given as % of the initially applied radioactivity and, where appropriate, as µg kg^{-1} manure (based on manure fresh weight) for each incubation interval. A mass balance should be set up, too. A graphical presentation of the test substance concentrations against time will allow an estimation of its transformation half-life or DT_{50}. If possible, metabolites should be identified and their concentrations should also be plotted against time to show their rates of formation and decline. A major transformation product is any product representing ≥ 10% of applied dose at any time during the study.

For ^{14}C-labeled test substances, the mass balance should be differentiated between the formation of carbon dioxide or methane and if it is existent the formation of volatile compounds as well as the formation of extractable and non-extractable residues.

TEST REPORT

48. The report must include:

Test substance:

- common name, chemical name, CAS number, structural formula (indicating position of label when radiolabeled material is used) and relevant physical-chemical properties,
- purity (impurities) of test substance,
- radiochemical purity of labeled chemical and specific activity (where appropriate).

Excrements and manure:

- location of excrement sampling,
- age, number, race of animals under investigation,
- feeding conditions,
- date of sampling,
- length of the excrement preconditioning period,
- length of excrement or manure storage and storage conditions (if stored).

Test conditions:

- dates of the performance of the studies,
- amount of test substance applied,
- solvents used and method of application for the test substance,
- weight of manure samples treated initially and at each incubation interval for analysis,
- description of the incubation system used,
- air flow rates (for flow-through systems only),
- temperature of experimental set-up,
- method(s) of extraction,
- methods for identification and quantification of the test substance and metabolites in manure,
- number of replicates and number of controls.

Results of excrement and manure characterization:

- dry substance content, total organic carbon content, pH, redox potential, dissolved oxygen concentration, ammonium content, total nitrogen content, biochemical oxygen demand after 5 d, should be determined at least initially and at the end of the transformation tests. If possible, every parameter should be given for each incubation interval.

Results of transformation experiments:

- repeatability and sensitivity of the analytical methods used,
- mass balances and recoveries should range between 90-110 % and 70-110 %, respectively (see paragraph 21),
- tables of results expressed as % of applied initial dose and, if appropriate, as mg kg^{-1} manure (on a fresh weight basis),
- mass balances until the end of the studies,
- characterization of non-extractable radioactivity or residues in manures,
- quantification of released $^{14}CO_2$, $^{14}CH_4$ and related volatiles,
- plots of the concentrations for the test substance and, where appropriate, for major transformation products in manure versus time,
- half-life or DT_{50} (DT_{90}, if possible) for the test substance and, where appropriate, for major transformation products including confidence limits,
- discussion and interpretation of results.

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Annex A

Definitions

Excrements are complex and heterogeneous mixtures of urine and feces of cattle and pigs.

Extractable residues (ER) represent compounds occurring in the organic solvent as parent compound or metabolites.

Disappearance Time 50 (DT_{50}) is the time within which the concentration of the test substance is reduced by 50 %.

Disappearance Time 90 (DT_{90}) is the time within which the concentration of the test substance is reduced by 90 %.

Matrix characterization. Excrement and manure samples are characterized by numerous parameters, i.e. dry substance, total organic carbon, pH, redox potential, dissolved oxygen, ammonium nitrogen, total nitrogen and biological oxygen demand.

Mineralization (MIN) is the transformation of the veterinary medicinal products to CO_2 and H_2O under aerobic conditions. In the context of this technical protocol, mineralization means transformation during which a labeled carbon atom is oxidized resulting in the release of $^{14}CO_2$. Under strictly methanogenic conditions, $^{14}CH_4$ may be released, too.

Metabolites are substances resulting from the transformation of the test substance that are occurring in the extractable fraction.

Non-extractable residues (NER) represent compounds that are retained in the matrices of manure as parent compound or corresponding transformation products after the extraction procedure that must not substantially change the compounds themselves or the structure of the matrix.

Radiotracers denote ^{14}C-labeled test substances. Their application facilitates the set-up of mass balances considering the mineralization (MIN) and the formation of extractable (ER) and non-extractable residues (NER).

Reference manures are excrement samples to that tap water is added to adjust defined dry substance contents typical for bovine or pig manures.

Test substance is any veterinary medicinal product or biocide under study that is applied in the laboratory test system.

Transformation product is every substance resulting from biotic or abiotic transformation of the test substance occurring in the extractable or non-extractable fractions or in the gas phase (CO_2, CH_4 or related volatiles).

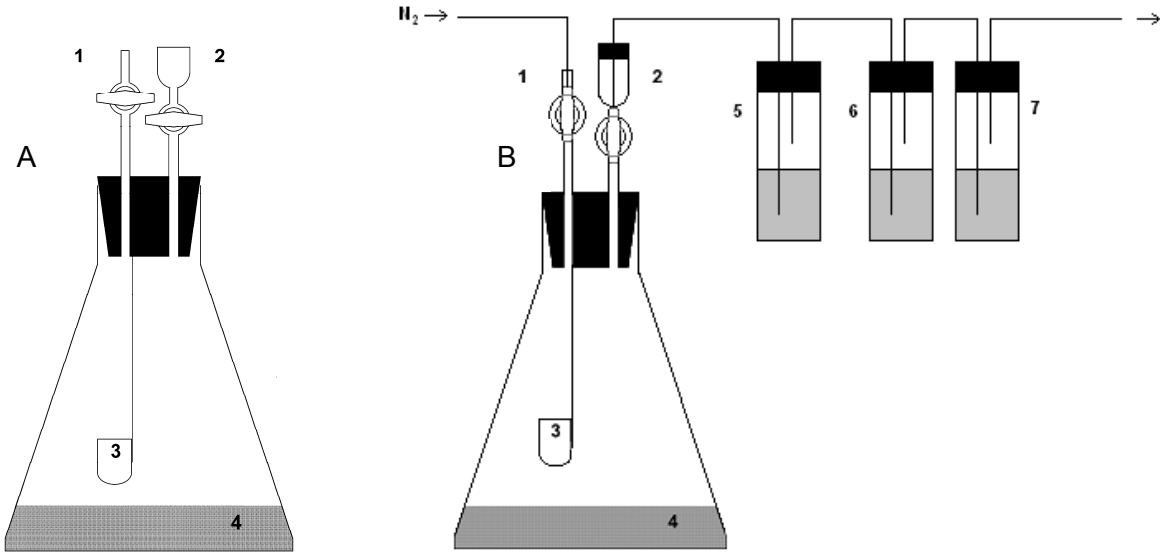
Annex B

Methods of the matrix characterization of excrement and manure samples

Parameter	guideline	equipment (examples)
dry substance (ds)	ISO 11465 (1993)	Ultra-X infra-red heater, (Gronert, Germany), or a drying oven
total organic carbon (TOC)	ISO 10694 (1995)	C-Analyser Dohrmann DC-90 (Dohrmann, Santa Clara, CA, USA)
pH value	DIN EN 12176 S5 (1998)	pH Multical 535 GLP with pH-glass electrode SenTix 61 (WTW Weilheim, Germany)
redox potential (Eh)	DIN 38404 C6 (1984)	pH Multical 535 GLP 8, (WTW Weilheim, Germany) with redox-electrode (Inolab Redox Einstabmesskette, Mettler Toledo, Giessen, Germany)
dissolved oxygen (O_2)	ISO 5814 (1990)	Oxi 340i with OxiCell 325 oxygen-electrode (Fa. WTW, Weilheim, Germany)
ammonium nitrogen (NH_4-N)	ISO 5664 (1984)	Distillation Unit 323 (both Büchi Labortechnik GmbH, Essen, Germany)
total nitrogen (N_{total})	ISO 11261 (1995)	Digestion Unit 430 and Distillation Unit 323 (Büchi Labortechnik GmbH, Essen, Germany)
biological oxygen demand (BOD_5)	ISO 5815 (2003)	Oxi 340i with OxiCell 325 oxygen-electrode (Fa. WTW, Weilheim, Germany), Karlsruher bottles, 250 mL (Schott, Mainz, Germany)

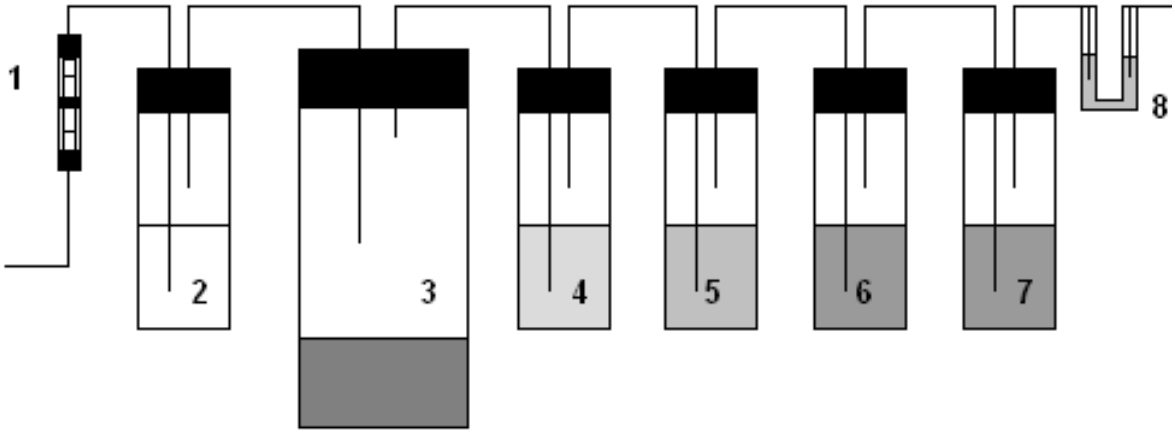
Annex C

Laboratory-test systems



1: inlet valve, 2: outlet valve with activated charcoal filter, 3: internal ^{14}C-carbon dioxide trap, 4: manure or manured soils sample, 5: external trap for ^{14}C-methane with ethylene glycol (10 mL), 6: external trap with sulfuric acid (10 mL, 0.05 M), 7: external ^{14}C-carbon dioxide trap with scintillation cocktail (10 mL)

Figure 1: Laboratory-batch system for transformation tests of ^{14}C-labeled VMP and biocides in liquid manures. A: without and B: with additional stripping device [39, modified in accordance to 29]



1: flow meter, 2: gas moistening flask, 3: incubation flask with the liquid manure sample, 4: ethylene glycol trap (30 mL), 5: sulfuric acid trap (30 mL, 0.05 M), 6, 7: potassium hydroxide solution traps (30 mL, 2 M), 8: bubble meter

Figure 2: Flow-through system for transformation tests of ^{14}C-labeled VMP and biocides in liquid manures [30]