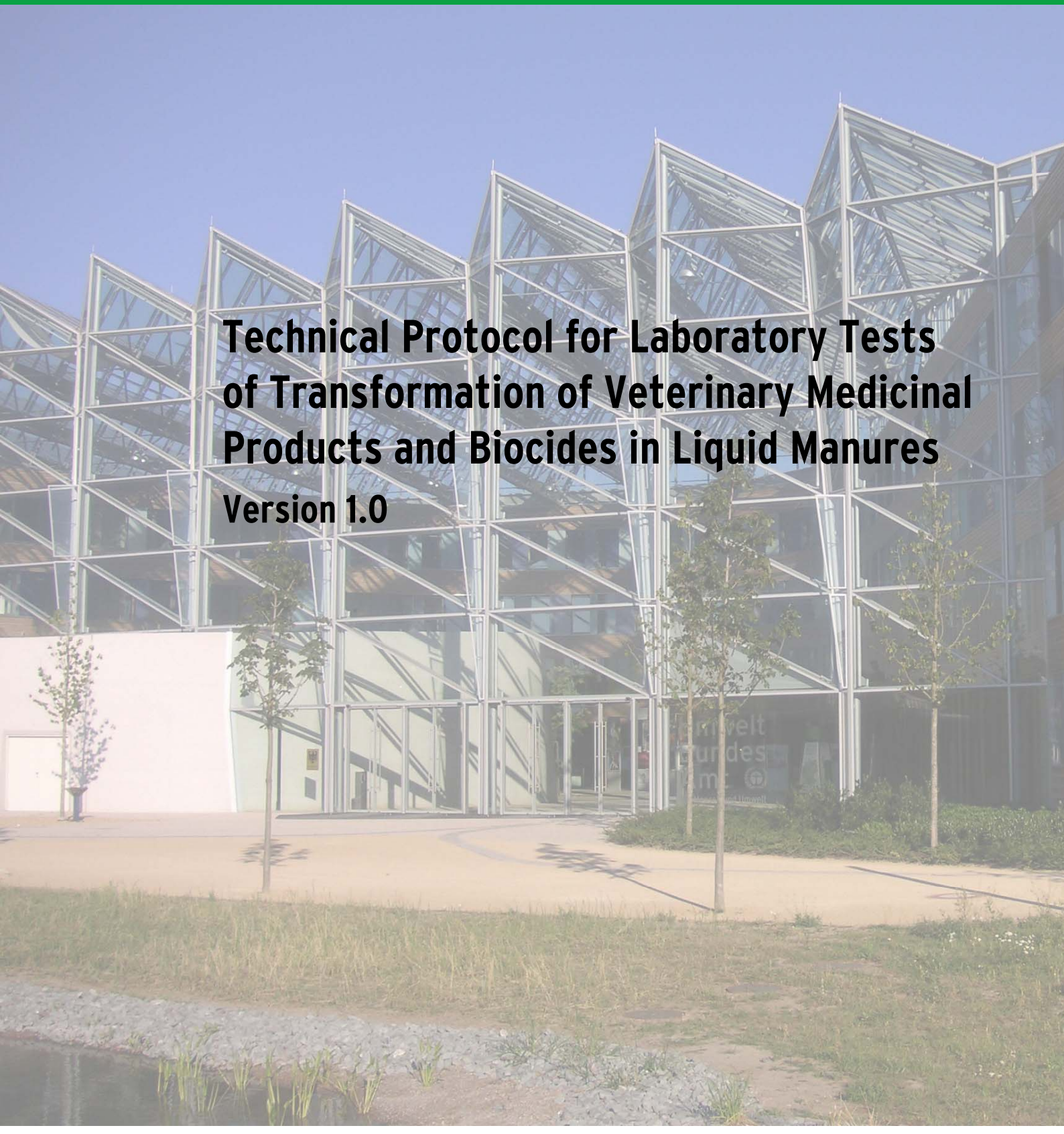

Technical Protocol for Laboratory Tests of Transformation of Veterinary Medicinal Products and Biocides in Liquid Manures

Version 1.0

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Technical Protocol for Laboratory Tests of Transformation of Veterinary Medicinal Products and Biocides in Liquid Manures

Version 1.0

by

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On behalf of the Federal Environment Agency (Germany)

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INTRODUCTION

1. This technical protocol describes a laboratory test method to evaluate the transformation of chemicals in liquid bovine and pig manures under anaerobic conditions and is primarily designed for veterinary medicinal products (VMP) and biocides. The environmentally relevant entry routes into liquid manures occur via urine and feces of cattle and pigs in stable housings after excretion of VMP (as parent compounds or metabolites) and after the application of biocides in animal housings (e.g., as disinfectants or insecticides). 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 farmland and grassland soils as organic fertilizers. Via this route, VMP and biocides may enter soil environments. Thus, the persistence of the chemicals 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 mainly aerobic storage conditions.

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 difficult. Therefore, this technical protocol focused on the sampling of excrements from cattle and pigs kept in stables and fed under standard nutrition conditions. This approach additionally ensures that excrement samples are operationally free of any contamination by VMP and biocides [1]. After the matrix characterization, reference-manure samples are prepared from the excrement samples by adding tap water to adjust 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. Testing of anaerobic transformation of chemicals in reference manures.

PRINCIPLE OF THE TESTS

4. The technical protocol consists of a tiered experimental design. In a first step, bovine and pig reference manures are prepared to guarantee reproducibility of the results. The excrements and the reference manure preparation are subjected to a matrix characterization to verify their suitability for laboratory testing. In a second step, the reference manure samples are fortified by the test substance and incubated in the dark under anaerobic conditions at constant temperature for up to 100 d. With respect to the high complexity of manure samples, it is highly recommended to apply the test substance as a ^{14}C-labeled radiotracer. After appropriate time intervals, mineralization, extractable residues, i.e., parent compound and metabolites, as well as non-extractable residues are to be determined in order to set detailed mass balances. Volatile products are also sampled for analysis using appropriate devices.

APPLICABILITY OF THE TESTS

5. These tests are principally applicable to every chemical (i.e., veterinary medicinal product or biocide) applied in animal houses for which an analytical method with sufficient accuracy and sensitivity is available.

INFORMATION ON THE TEST SUBSTANCE

6. The position of the ^{14}C-label (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}) has to be specified.

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

– Solubility in water according to OECD Guideline 105 [2].
– Solubility in organic solvents.
– Vapor pressure according to OECD Guideline 104 [3] and Henry´s law constant.

– n-Octanol/water partition coefficient according to OECD Guidelines 107 or 117 [4,5].
– Chemical stability in dark (hydrolysis) according to OECD Guideline 111 [6].
– pK_a if a molecule is liable to protonation or deprotonation according to OECD Guideline 112 [7].

8. Other useful information may include data on toxicity of the test substance to microorganisms.

9. Analytical methods (including extraction and clean-up methods) for identification and quantification of the test substance and its relevant 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

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

Recovery

12. Recoveries for the experiments are given by the respective mass balances that should range from 90 % to 110 % for radio-labeled test substances. When residue analytical methods are applied, recoveries are considered acceptable from 70 to 110 %.

Repeatability

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

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

long enough for formation of metabolites.

15. The limit of determination (LOD) of the analytical method for the test substance and for the transformation products should be at least 1 % of applied concentration. The limit of quantification (LOQ) should also be specified.

Accuracy of transformation data

16. The observed variability between replicates should be recorded and discussed.

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

CHARACTERIZATION OF THE EXCREMENT AND MANURE SAMPLES

Equipment and chemicals

17. Standard laboratory equipment is required:

- Plastic containers with lids, 20 L, 2 L and 1 L
- Mechanical mixing device (e.g., electric stirrer).
- Drying oven (105 $\pm$ 5 °C).
- Muffle furnace.
- Infrared heater.
- Incubator (5 to 20 $\pm$ 2 °C).
- Apparatus for determination of the total carbon content.
- Analytical balances (accuracy $\leq$ 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.

18. 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.

Sampling and feeding conditions

19. Excrements from conventionally fed single animals or groups of up to 8 individuals are taken over a period of 12 to 24 h. The race, feeding conditions (adequate to standard nutrition conditions) as well as the age of the individuals should be reported. The composition of the food should be given. The administration of VMP and the application of biocides have to be definitely excluded.

Conditioning, storage and matrix characterization of excrement samples

20. Directly after excretion, readily degradable organic compounds of the excrements undergo rapid decomposition enhancing the matrix heterogeneity [8,9]. 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 homogenized using an electric stirrer. Thereafter, the excrements samples are to be matrix characterized by the parameters as follows: dry substance content, total organic carbon, pH, redox potential, dissolved oxygen, ammonium and total nitrogen, biological oxygen demand. Constant conditions are indicated by the dissolved oxygen contents < 0.1 mg kg^{-1}, the redox potential Eh < 0 mV* and an ammonium content stable at $\pm$ 0.2 g kg^{-1}, are established [11-16]. 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. Then, matrix characterization has to be repeated.

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

Reference-manure preparation

21. For reference-manure preparation, the dry substance content (ds) of the excrement sample is a relevant parameter and has to be determined. The targeted mass of excrements is calculated using the following formula:

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

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

* Eh < 0 mV does not represent strictly anaerobic or methanogenic conditions but indicates facultative aerobically and anaerobically living microorganisms. To guarantee strictly anaerobic conditions of Eh < - 100 mV [10], the transformation tests are to be conducted under nitrogen atmosphere.

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]

22. 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. These dry substance contents correspond to values given for Europe in a range between 0.9 and 12 % [17-25]. In order to additionally study the effect of the dry substance content on the transformation of test substance, different dry substance contents (e.g., 2.5, 5 and 10 %) can be optionally tested.
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.

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 [26]. 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 % (*w*/*w*).

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 [27]. 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 appropriate methods. 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 stirred and homogenized excrement or manure sample (50 to 100 g) using a pH electrode [28].

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 [29].

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 [30].

Ammonium nitrogen (NH_4-N)

28. Under mildly alkaline conditions [31], 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 [32]. 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 has to be checked be-

fore transformation tests in reference manures are conducted. For this purpose, the biological oxygen demand† can be determined [33]. 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.

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:

† By means of the BOD_5 measurement, the activity of aerobic microorganisms is merely comprised. Thus, the validity of the biological oxygen demand in anaerobic manure samples may be limited. Certainly, there is not any alternative method without any interference. The determination of the dehydrogenase activity, feasible to determine the activities of aerobic and anaerobic microorganisms, may be limited by its final photometric measurement of triphenyl formazan at λ = 485 nm or λ = 546 nm because of the deeply colored excrement and manure extracts. The application of a readily degradable reference substance, e.g., sodium benzoate, in parallel batch experiments causes other inadequacies. In order to check the microbial activity of manure samples at the start of the transformation test series, this test is too time consuming due to its 4-week test period specified by the OECD guideline 311 [34]. Due to the different experimental designs, the degradability of this test substance is only measured by the gas production, this test is not appropriate to check the microbial activity at longer incubation intervals. So far the application of an external standard substance should be followed in the future, there is the need to identify an appropriate ^{14}C-labeled reference substance that shows a characteristic behavior in bovine and pig manure within incubation intervals up to 100 days.

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

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 $\geq$ 10 g kg^{-1} are reported without decimal places.

PART II: ANAEROBIC TRANSFORMATION TEST IN REFERENCE MANURES

Equipment, instruments and chemicals

31. For transformation tests of chemicals 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. A closed batch apparatus allowing for a discontinuous gas exchange can be used. This batch apparatus shown in Figure 1A (see Annex C), traces back to the biometer-type flask already mentioned in the OECD Guideline 304 [35] 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 [36] (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. The vulnerability of both laboratory-test systems is the direct determination of ^{14}C-methane released out of the ^{14}C-labeled test substance under methanogenic conditions. In both laboratory test-systems, the ^{14}C-carbon dioxide free headspaces of the incubations flasks have to be transferred into a combustion apparatus where ^{14}C-methane is oxidized to ^{14}C-carbon dioxide. The latter is to be trapped again in an absorbing scintillation cocktail and then scintillation counted.

34. For additional measurements, e.g., pH, redox potential, biological oxygen demand, parallel batch tests with unlabeled 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.

35. For the analytical procedures standard laboratory equipment is required, e.g., 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.

36. 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

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 31). 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 ± 2 °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.

Test substance application

40. For addition to manure and distribution in manure, the test substance can be dis-

solved in water or, when necessary, in minimum amounts of organic solvents 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 [36].
The use of solvents which inhibit the 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 [36].

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 account. The applied concentration should be based on the substance specific exposure assessment of the chemical under study [37]. 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 39. For each sampling time point separate sample flasks and additional control flasks are prepared in at least triplicate (duplicate samples for analytical purposes of the test compound, one sample for matrix characterization). The minimal set of flasks should allow sampling on days 0, 1, 3, 7, 30, 72 and 100. Optionally, the test can be continued up to day 180.

43. Parameters to be determined for excrements and reference manures are subsequently specified in the following. Excrements: dry substance. Reference manures: Full set of parameter (see above).
Duplicate incubation flasks are removed at appropriate time intervals and the manure samples extracted with appropriate solvents of different polarity and analyzed for the test substance and/or transformation products. A well-designed study includes sufficient flasks so that two flasks are sacrificed at each sampling event. Absorption solu-

tions or solid absorption materials are also removed at various time intervals (7-day intervals during the first month and after one month in 14-day intervals) during and at the end of incubation of each soil sample and analyzed for volatile products. Besides a reference-manure sample taken directly after application (0-day sample) at least 5 additional sampling points should be included. Time intervals should be chosen in such a way that pattern of decline of the test substance and patterns of formation and decline of transformation products can be established.

44. 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[‡] has to be investigated for every test substance.

45. When using ^{14}C-labeled test substance, non-extractable radioactivity is to 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 and cellulose, 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 is scintillation counted to quantify amounts of non-extractable residues.

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‡ 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

46. 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 amount and, where appropriate, as µg kg^{-1} manure (based on manure fresh weight) for each incubation interval. A mass balance should also be set up. If possible, metabolites should be identified and their concentrations should be plotted against time to show their rates of formation and decline. A relevant 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 differentiate between the formation of carbon dioxide or methane and other volatile compounds as well as the formation of extractable and non-extractable residues.

A graphical presentation of the test substance concentrations against time will allow an estimation of its transformation half-life or DT_{50}. The data are evaluated by using appropriate kinetic programs (see FOCUS degradation kinetics report) [38]. To evaluate the goodness of fit of the kinetic models, Chi-square test and t-test should be performed.

TEST REPORT

47. 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,
- 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.

Excrements: dry substance.

Reference manure: Full set of parameter (see above).

Results of transformation experiments:

- repeatability, recovery, LOD, LOQ of the analytical methods used,
- mass balances and recoveries should range between 90-110 % for radio-

labeled and 70-110 % for non-radiolabeled test substances, respectively (see paragraph 12),

- 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 $^{14}CO_2$, gaseous and volatile transformation products,
- plots of the concentrations for the test substance and, where appropriate, for relevant transformation products in manure versus time,
- half-life or DT_{50} (DT_{90}, if possible) for the test substance and, where appropriate, for relevant transformation products (> 10%) including confidence limits and parameters on goodness of fit of the kinetic models (i.e., error level chi^2, t-test, r^2),
- proposed pathways of transformation, where appropriate,
- 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 used for extraction of as parent compound or metabolite.

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, biological oxygen demand.

Mineralization (MIN) is the transformation of test substances to carbon dioxide and water under aerobic conditions. In the context of this technical protocol, mineralization means transformation during which a ^{14}C-labeled carbon atom is oxidized resulting in the release of ^{14}C-carbon dioxide. Under methanogenic conditions, ^{14}C-methane may be released, too.

Metabolites are substances resulting from the biotransformation of the test substance that occur in the extractable fraction.

Non-extractable residues (NER) represent compounds that are retained in the matrices of manures or manured soils as parent compound or corresponding transformation products after the extraction procedure. That method 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 water is added to adjust defined dry substance contents typical for bovine or pig manures.

Test substance is any substance that is applied in the laboratory test systems.

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 (carbon dioxide, methane or related volatiles).

Annex B

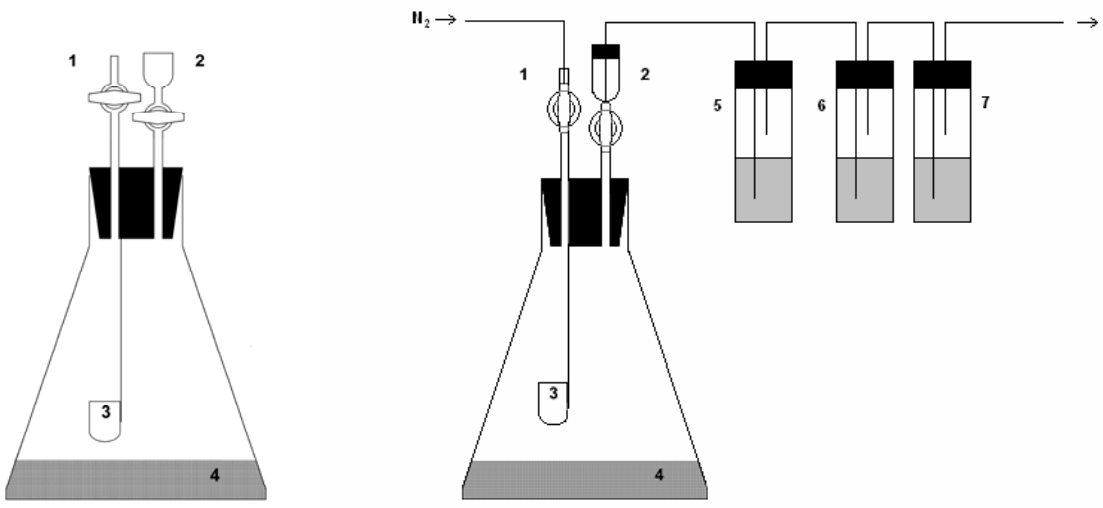
Methods of the matrix characterization of excrement and manure samples [26-33]

Parameter	Guideline
dry substance (ds)	ISO 11465 (1993)
total organic carbon (TOC)	ISO 10694 (1995)
pH value	DIN EN 12176 S5 (1998)
redox potential (Eh)	DIN 38404 C6 (1984)
dissolved oxygen (O_2)	ISO 5814 (1990)
ammonium nitrogen (NH_4-N)	ISO 5664 (1984)
total nitrogen (N_{total})	ISO 11261 (1995)
biological oxygen demand (BOD_5)	ISO 5815 (2003)

Annex C

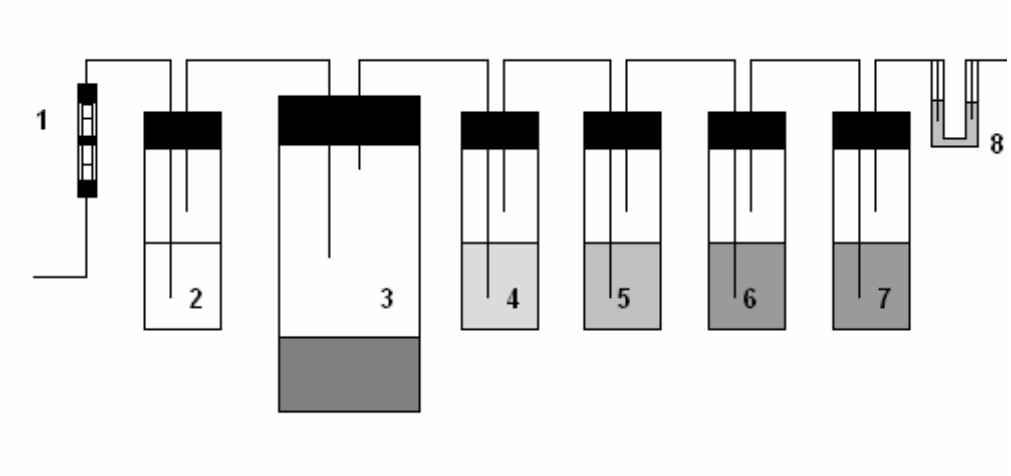
Laboratory-test systems

A B



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 test substance in liquid manures. A: without and B: with additional stripping device [39, modified in accordance to 35]



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 test substance in liquid manures [36]