Section 3

Environmental fate and behaviour

Test Guideline No. 320

Determining Anaerobic Transformation of Chemicals in Liquid Manure

30 June 2022

OECD Guidelines for the Testing of Chemicals

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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Anaerobic Transformation of chemicals in Liquid Manure

1. Introduction

1. This guideline describes methods to examine the transformation of chemicals in pig and cattle liquid manure under anaerobic conditions. The test guideline is based on method development and validation described in [1,2,3] and on existing guidelines [4,5]. The experiments are performed to determine the rate of transformation of the test chemical, the identity and rates of formation and decline of transformation products, the amount of test chemical that is mineralized to CO_2 or CH_4, or that is transformed to volatile transformation products, and the amount of non-extractable residues (NER). Such studies are relevant for chemicals that are administered to housed animals and are subsequently excreted (e.g. veterinary medicinal products or feed additives) or for chemicals that are applied in buildings for livestock and may also enter the manure collected from these animal housings (e.g. biocides). Pesticides may also be introduced into manure via contaminated animal feed.

2. Definitions

2. For definitions see Annex 1.

3. Initial considerations and limitations

3. The method is applicable to all chemicals for which an analytical method with sufficient recovery, accuracy and sensitivity is available. It is applicable to slightly volatile, non-volatile, water-soluble or poorly water-soluble chemicals. The method described in this test guideline is not suitable for volatile chemicals. When using a closed (semi-static or flow-through) test system it is possible to test slightly volatile substances (with Henry's law constants <100 Pa * m^3/mol or <1*10^{-3} atm * m^3/mol). When considering testing of mixtures, difficult test chemicals, or test chemicals not clearly within the applicability domain described in this guideline, upfront consideration should be given to whether such testing will yield results that are scientifically defensible. If the test guideline is used for the testing of a mixture, a UVCB (substances of unknown or variable composition, complex reaction products or biological materials) or a multi constituent substance, its composition should, as far as possible, be characterized, e.g. by the chemical identity of its constituents, their quantitative occurrence and their substance-specific

properties. Recommendations about the testing of difficult test chemicals (e.g. mixtures, UVCB or multi-constituent substances) are given in OECD Guidance Document No. 23 [6].

4. Principle of the test

4. Manure samples are treated with the test chemical and incubated in the dark under controlled laboratory conditions (at constant temperature under anaerobic conditions, see also paragraph 35). After appropriate time intervals, manure samples are removed, extracted and analysed for the parent substance and for transformation products. Volatile products are collected for analysis using appropriate trapping devices to quantify formation of CO_2 and CH_4; and if required further volatile products. Using ^{14}C-labeled material, mineralization rates of the test chemical can be measured and a mass balance, including the formation of NER, can be established. Based on the results DT_{50}, and, if appropriate, DT_{90} values can be calculated.

5. This guideline is meant to study the transformation of chemicals in liquid manure from pig or cattle, which is a mixture of urine, faeces and water and may also contain bedding material. Typical dry matter contents for pig and cattle manure have been found to be 5% and 10%, respectively [7].

5. Information on the test Chemical

6. Non-labelled or labelled test chemicals can be used to measure the rate of dissipation of the parent chemical. ^{14}C-radiolabeled material is required for studying the pathway of transformation, for quantifying CO_2- and CH_4-formation, formation of NER, screening for and quantification of transformation products and for establishing a mass balance. The label(s) should be positioned in the most stable part(s) of the molecule. For tracking the active moiety, label(s) should be positioned accordingly. For complex molecules (e.g. containing more than one aromatic ring system) or for extensively substituted molecules, labelling in different positions might be necessary. The test chemical should be labelled so that the transformation pathway can be traced as far as possible and transformation products can be tracked. The choice of the labelling position(s) should be justified and illustrated in a structural formula of the test chemical in the test report.

7. The use of stable isotopes such as ^{13}C, ^{15}N, or ^{2}D (non-exchangeable) may help in identification of transformation products or characterization of NER by various spectroscopic methods (e.g. mass spectrometry (MS) or nuclear magnetic resonance spectrometry (NMR)).

8. The purity of the test chemical should be at least 95%. Deviations should be justified.

9. Before carrying out the test on transformation in manure, it is recommended to consider the following information on the test chemical:

a) solubility in water (OECD TG 105; [8]),

b) solubility in organic solvents,

c) vapour pressure (OECD TG 104; [9]) and Henry's law constant,

d) n-octanol/water partition coefficient (OECD TG 107; [10]),

e) chemical stability in water (hydrolysis) (OECD TG 111; [11]),

f) pK_a if a molecule is liable to protonation or deprotonation (OECD TG 112; [12]),

g) sorption behaviour (OECD TG 106; [13]).

10. Other useful information may include data on the toxicity of the test chemical or transformation products to microorganisms, e.g. according to OECD TG 209 [14], OECD TG 216 [15], OECD TG 217 [16] or OECD TG 224 [17].

11. Analytical methods (including extraction and clean-up methods) for quantification and identification of the test chemical and its transformation products should be available. If available, analytical standards should be used for the characterization and/or identification of transformation products by spectroscopic and chromatographic methods. If analytical standards are unavailable, identification based on spectrometric techniques (high resolution mass spectrometry (HRMS), and nuclear magnetic resonance spectrometry (NMR)) may be attempted.

6. Reference substance and demonstration of proficiency

12. A reference substance may be included into the test to ensure an appropriate test setup and microbial activity in manure. A substance that is easily degraded under the test conditions, e.g. salicylic acid (as sodium salicylate CAS: 54-21-7 or as salicylic acid CAS: 69-72-7) may be useful for this purpose [3]. For the reference substance, the same results as for the test chemical should be reported. A description of an extraction scheme for salicylic acid can be found in Annex 2.

7. Validity of the test

13. For a test to be valid, the following criteria should be met:

- The test has to be conducted under redox conditions typically found in manure tanks[1]. As an indicator parameter the redox potential (reported as E_h) should be ≤ -100 mV throughout the entire test period[2]
- For radiolabelled chemicals, the mass balance in the beginning of the study should be within 90% to 110%. During the study, the mass balance for each individual replicate should fall within 85%-115%.[3]
- For labelled and unlabelled test chemicals, the analytical method should have been demonstrated to have a recovery of 70-110%[4].

[1] Typical values measured in manure tanks range from -230 mV to -400 mV [7]. During validation, mean E_h-values of -374 ± 54 mV for pig manure and -316 ± 110 mV for cattle manure were observed [2].

[2] (see also paragraph 35).

[3] If a replicate after time point zero does not meet the 85%-115% criterion, this replicate should be excluded from evaluation. The study can still be considered as valid, if a sound justification is provided and sufficient replicates at sufficient sampling time points remain for evaluation.

[4] Also refer to appropriate documents on validation of analytical methods e.g. OECD, 2014 [18].

8. Description of the method

8.1. Apparatus and chemical reagents

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

- Analytical instruments such as GC or HPLC, including the appropriate detection systems for analysing radiolabelled or non-labelled substances,
- Instruments for identification purposes (e.g. RAM, MS, HRMS, NMR, etc.),
- Liquid scintillation counter,
- Oxidizer for combustion of radioactive material,
- Centrifuge,
- Extraction apparatus (for example, centrifuge tubes for cold extraction, Soxhlet apparatus for continuous extraction under reflux, apparatus for accelerated solvent extraction, i.e. extraction under high pressure and temperature),
- Instrumentation for concentrating solutions and extracts (e.g. rotary evaporator),
- Water bath,
- Mechanical mixing device (e.g. kneading machine, rotating mixer, hand blender).

15. Chemical reagents used include, for example:

- NaOH, analytical grade (2 M), or other appropriate base (e.g. KOH, ethanolamine)
- $Ba(OH)_2$, analytical grade (0.25 M)
- H_2SO_4, analytical grade (0.05 M)
- HCl (10%), analytical grade,
- Organic solvents, analytical grade, such as acetone, methanol, etc.,
- Inorganic salts, analytical grade, such as KH_2PO_4 (for extraction solvents),
- Scintillation liquid.

8.2. Test set-up

16. The incubation is conducted in a suitable system. Examples of a semi-static and a flow-through incubation apparatus are shown in Annex 3 and Annex 4 respectively. Other incubation systems are described in references [4] and [5]. For substances with no or very low mineralization a continuous system or a semi-static system can be used. For substances showing higher mineralization (as known from literature or a pre-test), a semi-static system is preferred [3].

17. To ensure anaerobic conditions, humidified nitrogen is passed over the samples in the beginning and intermittently (for the semi-static system) or continuously (for the flow-through system) at a recommended flow rate of 50 to 200 mL/min. NaOH filled traps (or another appropriate trapping solution) are used to trap evolving CO_2. Potentially formed methane (CH_4) accumulates in the incubation flask (no excess pressure to be expected, semi-static system) or passes through the CO_2-traps (flow-through-system) and is subsequently combusted in an oven

(temperature details in the annexes) to form CO_2, and finally trapped in a CO_2-trap. A detailed description is given in Annex 3 (semi-static system) and Annex 4 (flow-through system). To verify that the radioactivity trapped in the CO_2-traps is $^{14}CO_2$ and not from potentially also formed volatile fatty acids (VFA), $Ba(OH)_2$ precipitation of the radioactivity can be conducted. Depending on the test chemical and its potential transformation products, further traps (e.g. ethylene glycol, sulphuric acid) might be required for collecting volatile organic compounds.

18. A pre-test might give valuable indications for the behaviour of the test chemical and transformation products (e.g. in some cases rapid dissipation of the test chemical may be observed and the sampling time points have to be adjusted accordingly, or dissipation may be slow, necessitating planning for a longer study duration).

8.3. Manure

8.3.1. Manure selection

19. Manure for testing purposes should be sampled from manure storage or pre-storage tanks or manure lagoons. Storage facilities may be above ground or below ground. The manure should not have been exposed to the test chemical or compounds from the same substance class within the six months prior to sampling. This should be demonstrated by obtaining information on the medication of the animals producing the manure (e.g. veterinary medicinal products or feed additives) and on biocide or other chemical applications in animal housings (e.g. disinfection or treatment against insects) within the six months' time period. Analytical verification of chemical residues is recommended, especially when residues in food sources from field applications are expected. The number, type and age of animals should be known as well as their feed. Studies on transformation in manure should be conducted in manure of the relevant species (e.g. pigs, cattle), extrapolation from one species to another is not possible. See also paragraph 24/25 on manure characterization. At least one manure[5] per species should be used. If several manures were sampled and turned out to be not suitable, e.g. due to matrix parameters not conforming to the specifications, then the results should nevertheless be reported.

8.3.2. Collection, handling and storage of manure

Sampling of pigs and cattle liquid manure

20. Prior to collection, the liquid manure should be homogenized by mixing in the manure tank. Devices installed in the tank or external devices may be used for this purpose. Manure should be mixed for at least one hour before sampling. This has proved sufficient for homogenization of manure independent of tank volume [19]. Concerning the time interval between mixing and sampling, it should be considered that pig manure separates into different phases more rapidly than cattle manure. Therefore, pig manure should be sampled immediately after mixing, whereas cattle manure can be mixed up to one day in advance before sampling.

21. Liquid manure can be collected from the tank using e.g. a ladle with a large beaker. Containers should be filled to a maximum of approximately 75% of their volume and closed tightly. Gaseous products will continue to be produced by microbial activity and it is therefore suggested that a tube with a fermentation air lock be connected to an outlet in the container.

22. The sampling site, the sampling procedure (time and duration of mixing), and the type (e.g. above/below ground, covered/open) and size of manure tank should be recorded in detail.

[5] The number of manures should meet the requirements of respective regulatory frameworks (e.g. VMP, biocides). See Annex 6 for more information.

Storage of liquid manure

23. The liquid manure for the test should preferably be freshly collected from the manure tank. If this is not possible, the manure may be stored at 4°C to 20°C (preferably at the test temperature) for up to two months. The same precautions as described in paragraph 21 for gas production should be applied. Storage should ensure anaerobic/methanogenic conditions (see paragraph 35).

8.3.3. Manure characterization

24. Key parameters that have to be measured and reported (with reference to the method used) and the stage of the test at which those parameters have to be determined are summarized in the table hereafter.

25. Measurement of parameters for characterization of liquid manure:

Parameter[6]	Stage of test procedure				
	Sampling	Sampling	Sampling	Sampling	Sampling
Ph[7]	X	X	X	X	X
Organic matter content [%][8]		X			
Nitrogen content [N_{total}; mg/kg][9]		X			
Nitrogen content [NH_4-N; mg/kg][10]		X			
Redox potential [mv][11]	X	X	X	X[12]	X
Dry matter content [%][13]	X	X	X		X
Manure wet weight[14]		X			X
Temperature [°C]	X	X	X	X	X

26. Data collected after sampling stage (i.e. start of acclimation, start of test, during test and at the end of the test) have to be reported based on the adjusted dry matter content and wet weight of the manure.

27. A background control or blank sample should be analysed, to exclude the presence of the test chemical in the manure.

28. Except for the dry matter content (see paragraph 29) no changes or adjustments should be made to the sampled manure.

8.3.4. Dry matter content and homogenization of manure

29. Prior to the start of the acclimation period (see paragraphs 32 and 33), the dry matter content of the manure should be determined and should be adjusted, if necessary. The recommended dry matter content in cattle and pig manure is 10 ± 1% (w:w) and 5 ± 1% (w:w), respectively [7]. If the dry matter content is below the recommended value, it can be concentrated by careful centrifugation (e.g. for 10 minutes at 740 x g). However, the initial dry matter content has to be ≥ 8% (cattle) or ≥ 3% (pig). If these minimum values are not met, fresh manure has to be collected and used for the test. If the dry matter content is too high, water (de-ionized water, bubbled with nitrogen for 30 minutes) should be added as needed.

30. Before determination of the dry matter content and before adjustment (if necessary), the manure should be homogenized. Pig manure should be homogenized under anaerobic conditions in order to obtain a fairly stable phase. This can be achieved, e.g. by filling the manure into a

[6] For all matrix parameters it has to be specified in the report whether they relate to dry or to wet mass of the sample.

[7] e.g. ISO 10390 „Soil, treated biowaste and sludge - Determination of pH“ [20]

[8] e.g. DIN 12879 „Charakterisierung von Schlämmen - Bestimmung des Glühverlustes der Trockenmasse” [21]

[9] e.g. ISO 11261 “Soil quality - Determination of total nitrogen - Modified Kjeldahl method” [22]. For conversion of mass based units in volume based units a density of 0.001 kg/m^3 is used

[10] e.g. ISO 5664 “Water quality - Determination of ammonium - Distillation and titration method” [23].

[11] e.g. ISO 11271 “Soil quality - Determination of redox potential - Field method” [24] and/or DIN 38404-6 “Determination of the oxidation reduction (redox) potential” [25]

[12] It has to be assured that the given specifications for the redox potential are met throughout the study. Therefore, at least three measurements equally spaced during the test period is recommended.

[13] e.g. DIN EN 12880 “Characterization of sludges - Determination of dry residue and water content” [26]

[14] for each incubation vessel

beaker, putting a mixer/homogenizer (e.g. hand blender) into the manure, and gently passing a nitrogen stream over the manure while mixing. Cattle manure may be homogenized by gently mixing (e.g. using a glass bar), in this case no additional measures to prevent introduction of oxygen are used. Alternatively, the same procedure described for pig manure can be applied for cattle manure to prevent introduction of oxygen.

31. After adjustment of the dry matter content, the manure is homogenized again (set up as above) and subsamples of 50 – 100 g (wet weight) each should be directly filled into the incubation vessels which are used for the acclimation and the transformation study.

8.3.5. Acclimation of manure

32. If a semi-static apparatus (see Annex 3) is used, the incubation system is flushed with nitrogen for 1 hour to maintain anaerobic conditions. Thereafter, the incubation system is closed by valves. If a flow-through apparatus (see Annex 4) is used, the incubation apparatus has to be closed and a constant, water saturated stream of nitrogen is passed over the manure at a rate in the range of approximately 50 - 200 mL/min.

33. The acclimation should be carried out for 21 ± 1 days at test temperature in the dark (preferred) or in diffuse light.

8.4. Test conditions

8.4.1. Test temperature and light conditions

34. Incubation should take place in the dark (preferred) or in diffuse light at a controlled (±2°C) temperature, which may be the field temperature or a standard temperature of 20°C. Field temperature may be either the actual temperature of the sample at the sampling time or an average field temperature at the sampling site [7]. Resulting DT_x values can be converted to environmentally relevant temperatures [27]. To determine the pathway of transformation, environmentally relevant temperatures might have to be used[15].

8.4.2. Anaerobic incubation conditions

35. Transformation studies in cattle and pig manure should be performed under redox conditions similar to the conditions observed in a manure tank. Typical redox potential values measured in pig and cattle manure have been found to range from -230 mV to -400 mV [7]. Redox potential should be measured[16] and reported regularly to ensure stable anaerobic conditions throughout the experiment (see paragraph 25). E_h should never surpass -100 mV [5] (see also validity criteria in paragraph 13).

8.4.3. Abiotic controls

36. For information on the abiotic transformation of the test chemical it is recommended to include sterile controls. For test chemicals undergoing rapid abiotic transformation sterile controls are obligatory. Sterile controls also may provide additional insight into the type of transformation occurring. Manure is sterilized, treated with sterile test chemical and flasks kept closed. Sampling of sterile controls should be done according to the sampling schedule but sampling can be restricted to fewer time points. Sterile controls should be sampled at least at the

[15] If transformation products cannot be identified at lower temperatures, higher temperatures might additionally be used for this specific purpose.

[16] A metal measuring electrode (e.g. platin electrode) against a reference electrode (e.g. silver-silver chloride electrode (Ag^+/AgCl) can be used to measure redox potential. The measured redox potential should be reported in relation to the standard hydrogen electrode as E_h together with pH and temperature of the matrix.

end of the test. Sterilization can be achieved by autoclaving at least twice following this protocol: Preheat the manure in the test vessels overnight (at least 12 h) to 100°C. Let the vessels cool to room temperature during the day. Start the first autoclaving cycle (15 min, 121°C) and let the test vessels again cool down to room temperature overnight to enable germination of bacterial spores. Then start the second autoclaving cycle (15 min, 121°C). This procedure helps to inactivate bacterial spores and prevents foaming. Other methods to stop the biological activity can be used, if appropriate (e.g. adding a toxicant or gamma irradiation)[17].

8.4.4. Treatment and application of test chemical

37. The test chemical should be dosed into the manure at a concentration that reflects the maximum expected manure concentration, which depends on substance specific exposure scenarios. Concentrations in the mg/kg range are commonly observed for e.g. veterinary pharmaceuticals in manure [1]. The rationale for using a certain test chemical concentration should be reported. If this concentration is not sufficient for detection and identification of transformation products, the test may be conducted at increased concentrations. However, excessively high concentrations potentially toxic to microorganisms should be avoided.

38. The test chemical should be applied as an aqueous solution or dissolved in an appropriate water miscible organic solvent and should be added into the acclimated manure in the respective incubation vessels followed by thoroughly mixing while maintaining anaerobic conditions. This can be achieved, e.g. by continuously passing a nitrogen stream over the samples during application. The required volume of stock solution should be pipetted into the manure under simultaneous stirring using the pipette tip to evenly distribute the solution in the manure. Afterwards, the pipette tip remains in the manure[18]. Other forms of application could be used (e.g. glass pipettes, microliter syringes) if homogenous distribution of the test chemical can be achieved with the method. The total volume of the solvent used for application should not exceed 1% by volume.

8.4.5. Test duration and sampling

39. Test duration will depend on the rate of transformation of the parent chemical and transformation products. The standard study duration is 90 days after the end of the acclimation period. This time was derived from a survey on typical manure storage times [28]. In certain cases, it might be reasonable to prolong the study. Ideally, the test chemical and transformation products should each be present in amounts below 10% of the applied amount at the end of the study.

40. At least duplicate incubation flasks are sacrificed per sampling. Sampling intervals should be selected in a way that the pattern of decline of the test chemical, and the pattern both of formation and decline of transformation products can be established (e.g. 0, 1, 3, 7 days; 2, 3 weeks; 1, 2, 3 months, etc.). Besides sampling directly after application, at least 7 additional sampling points should be included. More sampling time points may be necessary for kinetic modelling and to cover formation and decline of transformation products. A pre-test might give valuable indications for the behaviour of the test chemical and transformation products. In some cases, rapid dissipation of the test chemical may be observed and sampling time points have to be adjusted accordingly.

41. CO_2 and CH_4 are potential volatile transformation products which could be formed under anaerobic conditions. Beside these compounds, other volatiles (e.g. volatile fatty acids

[17] It should be considered that the sorption characteristics of the manure may be altered by sterilization.

[18] to avoid losses since manure may stick to the tip

(VFA)) might also be formed. The experimental set-up has to be such that the following requirements are fulfilled:

- quantitative capturing to avoid any losses of volatiles and enable establishment of a mass balance,
- differentiation between formed CO_2, CH_4 and other volatiles.

42. For this purpose, traps to measure mineralization are removed at least at the sampling time points and analysed for trapped $^{14}CO_2$ and other evolved gases, respectively. At first, absorption traps are removed, replaced by freshly filled traps, and analysed for radioactivity content. Thereafter, the manure incubation flasks to be removed at that particular sampling point are treated by addition of 10 mL 10% HCl in order to strip potentially dissolved CO_2 (or HCO_3^- / CO_3^{2-}). After addition of 10 mL 10% HCl, the incubation flasks are closed again and nitrogen is passed through for at least 24 hours. Thereafter, manure incubation flasks are removed and the samples are subjected to clean-up, extraction procedures and analyses. CO_2-traps installed directly before acidification are removed and radio-counted for additionally trapped $^{14}CO_2$. In case the test chemical or transformation products are not known to be stable to the acidification conditions, separate incubation flasks should be included for chemical analysis.

43. For detailed descriptions of the sampling procedure, see Annex 3 (semi-static system) and Annex 4 (flow-through system).

8.5. Test procedure

8.5.1. Mass balance, recovery, repeatability and sensitivity of analytical method

44. For non-labelled and labelled test chemicals the recovery and other quality criteria (e.g. repeatability) of the test chemical-specific analytical method should be determined e.g. according to [18] and should be at least 70% to 110%. These criteria also apply for analytical methods for transformation products. For radiolabelled test chemicals, the initial mass balance should range from 90%-110% and the mass balance for each individual replicate during the test should be in the range of 85% - 115% (see also validity criteria in paragraph 13).

45. The limit of detection (LOD) of the analytical method for the test chemical and for the transformation products should be at least 1% of the applied dose or at least 0.01 mg/kg wet weight, whichever is lower. The limit of quantification (LOQ) should also be specified.

8.5.2. Measurements and analysis

46. Manure samples are extracted directly after sampling, unless sample storage can be shown to not influence the extraction efficiency and analytical determination, e.g. by reporting this in the analytical method validation. The samples should be extracted with appropriate solvents. An extraction approach should be followed that ensures optimal recovery of parent substance and transformation products. Aqueous solvent mixtures and acid and base systems should be used as solvents to ensure extraction of more polar parent and transformation products. Exhaustive extraction methods should be applied. These methods comprise e.g. pressurized liquid extraction (e.g. ASE®, PLE), reflux, soxhlet etc. with appropriate solvents. When using ^{14}C-labeled test chemical, the residues remaining after exhaustive extraction (non-extractable residues, NER[19]) should be quantified by combustion and a mass balance should be calculated for each sampling interval. Analytes should not be altered by the respective extraction method.

[19] If the amount of NER cannot be quantitatively determined, e.g. in an unlabeled study, the outcome of the study can only be used in a qualitative way, e.g. as information on formed transformation products.

This can be demonstrated by appropriate controls for the known substances. Detailed descriptions of the used extraction methods should be included in the test report.

47. Concentration of the test chemical and the transformation products[20] at every sampling time should be determined and reported (see also paragraph 48). In general, transformation products detected at ≥ 10% of the applied radioactivity at any sampling time should be identified. Transformation products once detected at ≥ 5% of the applied radioactivity for which concentrations are increasing during the study should also be identified.

48. Typically, identification is accomplished either by co-chromatography of the transformation product with known standards using two dissimilar systems or by techniques capable of positive structural identification such as MS, NMR, etc. In the case of co-chromatography, chromatographic techniques utilizing the same stationary phase with two different solvent systems are not adequate for the verification of the transformation product identity, since the methods are not independent. Identification by co-chromatography should be obtained using two dissimilar, analytically independent systems, such as reverse and normal phase thin layer chromatography (TLC) or TLC and high-performance liquid chromatography (HPLC). Provided that the chromatographic separation is of suitable quality, then additional confirmation by spectroscopy is not required. Unambiguous identification can also be obtained using methods providing structural information such as gas chromatography/mass spectrometry (GC-MS), liquid chromatography/mass spectrometry (LC-MS), liquid chromatography/tandem mass spectrometry (LC-MS/MS), and NMR.

49. The stereochemistry of transformation products generally does not need to be determined unless a differing behaviour is observed.

50. Appropriate methods should be used to elucidate the transformation pathway as fully as possible.

51. Special attention should be paid to NER, as manure is a transitional compartment, i.e. the manure matrix itself is degraded after spreading onto soil. Accordingly, parent substance or transformation products entrapped in or associated with the manure matrix, which cannot be extracted by the exhaustive extraction methods mentioned above, will with high probability be released as a consequence of manure matrix degradation[21].

8.5.3. Kinetic evaluation of the test data

52. The quality of the fit of an appropriate kinetic model to the test data should be evaluated according to the appropriate regulatory recommendations (e.g. FOCUS kinetic guidance [31], EPA recommendations, etc.). More sampling time points might be required if transformation products are observed to be able to derive DT_{50} and DT_{90} values.

[20] Different regulatory frameworks might have different thresholds or criteria for transformation product identification. Make sure to comply with the relevant regulatory guidance during planning of the study.

[21] Guidance on further characterization and classification of NER is under development [29,30]. In the absence of further experimental information, NER could be considered as parent compound. Different regulatory frameworks might follow different concepts concerning NER, e.g. regarding analytical determinations, special extraction methods, classification of different types of NER and use of the results for risk assessment. Make sure to comply with the relevant regulatory requirements during planning of the study.

9. Data and reporting

9.1. Data treatment and evaluation of test results

53. The results of the manure matrix parameters should be reported as specified in the table in paragraph 25 (based on wet weight, if applicable).

54. The amounts of test chemical, transformation products, volatile substances, CO_2 and CH_4 and non-extractable residues should be given as % of applied initial amount and, where appropriate, as mg/kg manure (based on wet weight) for each sampling interval. A mass balance should be given in percentage of the applied initial amount for each sampling interval. Data should be reported separately for each replicate and as an arithmetic mean of all replicates (see Annex 5 for an example). A graphical presentation of the test chemical and transformation product concentration against time on a non-logarithmic scale should be included. Major transformation products 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 the applied dose at any time during the study. Additionally, transformation products detected at ≥ 5% of the applied radioactivity for which maximum formation has not been reached at the end of the study should also be identified (see paragraph 47).

55. DT_{50} and DT_{90} values for the test chemical and any transformation product should be determined where possible, by fitting the data with an appropriate kinetic model considering available guidance. The DT_x values should be reported together with the description of the model used, and a measure for the goodness of fit. Details for calculations can be found for example in reference [31].

9.2. Test report

56. The report should include:

Test chemical and transformation products (where applicable):

- common name, chemical name, CAS number, structural formula (indicating position of label(s) when radiolabelled material is used) and relevant physical-chemical properties,
- purity (impurities) of test chemical,
- radiochemical purity of labelled chemical and specific activity, certificate of analysis (where appropriate);

Standard substance(s) for identification of transformation products:

- chemical name and structure of analytical standards used for the characterization and/or identification of transformation products;

Analytical determinations:

- methods for determination of manure matrix parameters
- methods for quantification and identification of the test chemical, and transformation products
- results of analytical method validation, not limited to but including recovery, repeatability, LOD and LOQ (expressed as % of applied amount and in mg/kg) of the analytical methods used (see also [18]),

- mass balance
- detailed description of extraction procedure;

Test manure:

- details of sampling site (date, location, type and number of animals, feed, type and size of manure tank, information of use of chemicals, e.g. biocides or veterinary medicinal products, in the six months prior to sampling),
- date and procedure of manure sampling,
- matrix parameters of manure (pH, organic matter content, nitrogen content, redox potential, dry matter content, temperature, wet weight; see also table in paragraph 25),
- duration of manure storage and storage conditions (if stored in the lab);

Test conditions:

- dates of the performance of the studies,
- amount of test chemical applied,
- calculation of maximum expected manure concentration (incl. rationale)
- solvents used (if appropriate) and method of application for the test chemical,
- weight of manure treated (fresh weight),
- description of the incubation system used,
- nitrogen flow rates (for flow-through systems and semi-static systems only),
- temperature,
- matrix parameters of manure (pH, redox potential, dry matter content, manure wet weight, temperature; see also table in paragraph 25)
- number of replicates and number of controls including sterile controls;

Results:

- result of manure matrix characterization for each time point,
- tables of results expressed as % of applied initial dose and, where appropriate, as mg/kg manure (on a wet weight basis) given for each replicate and as mean of all replicates for the following parameters (see Annex 5 for an example):
  - characterization of non-extractable radioactivity,
  - quantification of released CO_2 and CH_4, and other volatile compounds,
  - mass balance for each sampling point,
  - substance specific quantification of test chemical and transformation products
- plots (non-logarithmic) of concentrations versus time for the test chemical and, where appropriate, for major transformation products,
- DT_{50}, and DT_{90} values for the test chemical and, where appropriate, for major transformation products including kinetic model used and procedure used for fitting,

- abiotic transformation under sterile conditions,
- substance storage stability, when samples are stored prior to chemical analysis,
- an assessment of transformation kinetics for the test chemical and, where appropriate, for transformation products,
- a proposed pathway for transformation including structural formula and consistent names throughout the report for transformation products
- discussion and interpretation of results,
- raw data (e.g. sample chromatograms, calculations of transformation rates and methods used to identify transformation products).

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Annex 1: Definitions and Abbreviations

Definitions

DT_{50} (Disappearance or Dissipation Time 50) is the time within which the initial concentration of the test chemical is reduced by 50%

DT_{90} (Disappearance or Dissipation Time 90) is the time within which the initial concentration of the test chemical is reduced by 90%

Flow-through test: Incubation system where a constant, water saturated stream of nitrogen is passed over the manure.

Limit of detection (LOD) and limit of quantification (LOQ): The limit of detection (LOD) is the concentration of a substance below which the identity of the substance cannot be distinguished from analytical artefacts. The limit of quantification (LOQ) is the concentration of a substance below which the concentration cannot be determined with an acceptable accuracy.

Manure: mixture of urine, faeces and water collected in a storage tank, high liquid content, may contain residual bedding material

Mass balance: The percentage of applied radioactivity initially added that is recovered from the test system at each sampling time point.

Mineralization (under anaerobic conditions) is the complete transformation of an organic compound to CH_4, CO_2 and H_2O.

Non-extractable residues (NER): represent compounds in manure, which persist in the matrix after extraction.

Recovery: The ratio (in %) in between initially added test chemical and test chemical recovered after extraction.

Semi-static test: Incubation system where humidified nitrogen is passed over the manure samples in the beginning and intermittently (e.g. once a week) throughout the test.

Transformation products: all substances resulting from biotic or abiotic transformation reactions of the test chemical including CO_2, CH_4 and non-extractable residues.

Abbreviations

ASE: accelerated solvent extraction

GC: gas chromatography

GC-MS: gas chromatography/mass spectrometry

HPLC: high performance liquid chromatography

HRMS: high resolution mass spectrometry

LC-MS: liquid chromatography/mass spectrometry

LC-MS/MS: liquid chromatography/tandem mass spectrometry

LOD: limit of detection

LOQ: limit of quantification

LSC: liquid scintillation counting

MS: mass spectrometry

NMR: nuclear magnetic resonance spectrometry

NER: non-extractable residues

PLE: pressurized liquid extraction

RAM: Radioactivity Monitoring

TFA: trifluoroacetic acid

TLC: thin layer chromatography

UVCB: Substances of Unknown or Variable composition, Complex reaction products or Biological materials.

VFA: volatile fatty acids

VMP: veterinary medicinal products

Annex 2: Sampling and analytical determination for salicylic acid

Besides the procedure described below, other appropriate analytical methods can be used (e.g. GC-MS or LC-MS).

50 g wet manure sample are extracted once by 80 mL methanol + 1% trifluoroacetic acid (TFA), and thereafter twice by 50 mL methanol + 1 % TFA. For extraction, the samples are shaken for 30 minutes on a horizontal shaker and centrifuged (e.g. for 10 minutes at 740 x g). After centrifugation, the supernatant extract is collected and the pellet is subjected to the next extraction step. The whole process is repeated twice. Extracts are combined, and further analysed by radio TLC. After the last extraction step, the pellet is air dried and aliquots are subjected to combustion and radioassaying to provide information on the amount of non-extractable residues (NER).

In addition to the described extraction further extraction steps using accelerated solvent extraction (ASE®, PLE) can be performed. The accelerated solvent extraction (ASE®), i.e. extraction under high pressure and temperature (100°C, 12000 kPa, heat up for 5 minutes, followed by a static time of 10 minutes) uses the same solvent mixture as for the first extraction step (80 mL methanol + 1 % TFA). Extraction is performed twice but extracts are not combined.

As the extracts without further clean-up might influence the performance of the HPLC resulting in broad peaks, radio-TLC is preferred over HPLC. The following TLC-system is suggested:

- stationary phase: silica gel KG60
- mobile phase: methanol / toluene / ethylacetate / acetic acid; 10/44/43 /3 (v/v/v/v)

The radioactive peaks obtained after the development of the TLC-plates are characterized by their R_f-values and allocation to the peaks of co-chromatographed salicylic acid and possible transformation products.

Typical R_f-values are:

- Salicylic acid: $R_f = 0.50 – 0.55$
- Salicyluric acid: $R_f = 0.31 – 0.36$
- Gentisinic acid: $R_f = 0.42 – 0.47$

If, in addition, peaks are observed which cannot be allocated to any of the used reference substances, they should be described by their R_f-values and named as transformation product T1, T2, etc.

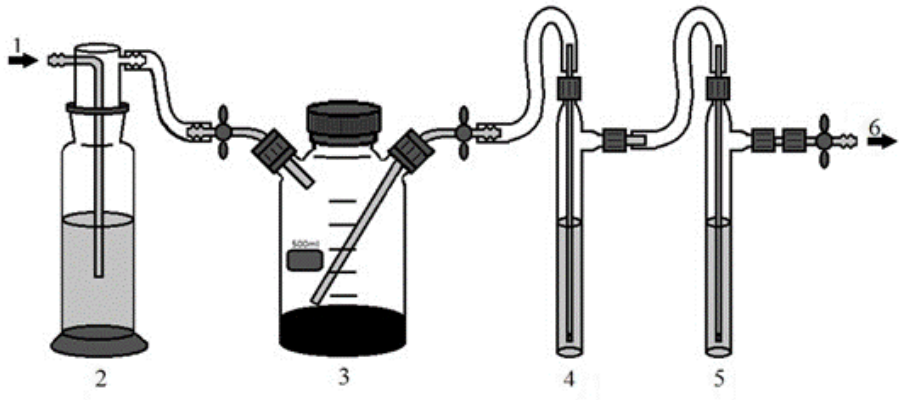
To determine mineralization to CO_2 and CH_4 please refer to Annex 3.

A mass balance can be established by adding the amounts of radioactivity given in [% of applied radioactivity; (% aR)] in the aqueous/organic extracts, carbon dioxide ($^{14}CO_2$), methane ($^{14}CH_4$) and non-extractable residues (NER):

Mass balance [% aR] = extractables [% aR] + $^{14}CO_2$ [% aR] + $^{14}CH_4$ [% aR] + NER [% aR].

Annex 3: Incubation in a semi-static system

Figure 1. Example of a semi-static apparatus Incubation



1) nitrogen inlet
2) gas washing bottle containing deionized water
3) incubation flask containing manure
4) CO_2-trap (e.g. containing 2 *M* NaOH)
5) CO_2-trap (e.g. containing 2 *M* NaOH)
6) outlet

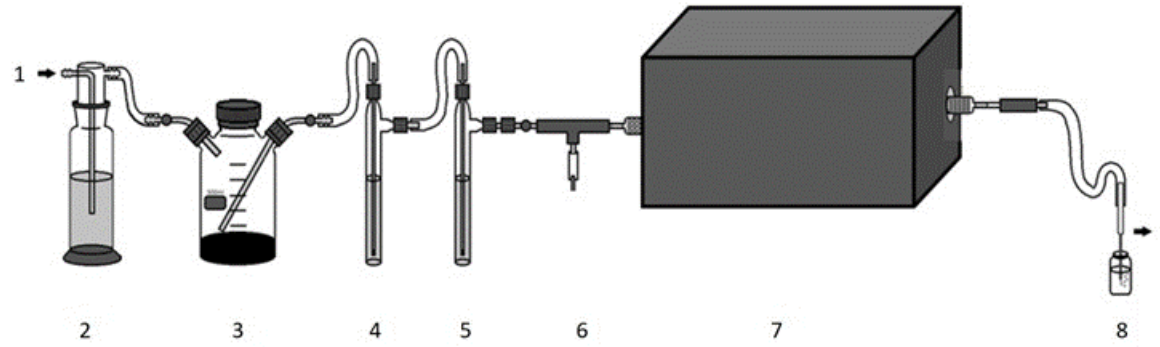
Manure samples are added to the incubation flask [3]. The flask is connected to a semi-static apparatus. For a period of 1 hour a gentle stream of moistened nitrogen is passed over the manure to exclude air from the system and to ensure anaerobic conditions. After flushing with moistened nitrogen, the system is closed by closing the two valves directly at the incubation flasks and the valve at the outlet of the second NaOH trap [5]. Depending on the test chemical and its potential transformation products, further traps (e.g. ethylene glycol, sulphuric acid) might be required for collecting volatile organic compounds.

Note: The valve at the outlet of the incubation flask might be left open to increase the headspace of the semi-static system and to enable absorption of evolved CO_2 in the first CO_2-trap [4] during incubation. In that case, a safety trap (empty washing bottle) should be inserted in between the incubation flask [3] and CO_2-trap [4] to prevent a backflow of absorbing solution into the incubation flask in case of pressure drop in the test system.

At the end of the incubation period, the respective incubation flasks are connected to the flow-through setup outlined below to detect formed CO_2, CH_4 and VFAs.

As a result of gas formation of the manure, the pressure in the closed test vessel increases during the incubation period. To avoid losses of volatiles resulting in an incomplete mass balance, the test vessels should be connected to the flow-through apparatus and purged with moistened nitrogen at regular intervals (e.g. once a week).

Figure 2. Detection of CO2, CH4 and VFAs



1) nitrogen inlet
2) gas washing bottle containing deionized water
3) incubation flask containing manure
4) CO2-trap (e.g. containing 2 M NaOH)
5) CO2-trap (e.g. containing 2 M NaOH)
6) tube as bypass for further air/oxygen inlet (O_2) containing silica gel or soda lime pellets
7) oven with quartz glass tube (filled with CuO as catalyst) at 800°C - 850°C
8) CO2-trap (e.g. containing 2 M NaOH)

Differentiation between CO_2, CH_4 and VFAs

Humidified nitrogen is bubbled through the manure samples at a rate in the range of approximately 50 – 200 mL/min for at least 1 hour. Evolved $^{14}CO_2$ is purged from the manure samples, transported and captured in traps ([4] and [5]) containing a CO_2-absorber (e.g. 2 *M* NaOH). If $^{14}CH_4$ is formed, it will pass through the CO_2-traps ([4] and [5]). After the addition of oxygen or ambient air [6] $^{14}CH_4$ is catalytically (= CuO) oxidized in an oven [7] at 800°C - 850°C to form $^{14}CO_2$. The formed $^{14}CO_2$ is trapped in the CO_2-trap [8] situated at the outlet of the oven.

Such a set-up enables the differentiation between evolved $^{14}CO_2$ (captured in traps [4] and [5]) and $^{14}CH_4$ (captured in trap [8]). Depending on the test chemical and its potential transformation products, further traps (e.g. ethylene glycol, sulphuric acid) might be required for collecting volatile organic compounds.

To verify that the radioactivity captured in the CO_2-traps [4] and [5] is $^{14}CO_2$ and not from volatile fatty acids (VFA) that may also be formed, $BaCl_2$ precipitation of the radioactivity can be conducted. The radioactivity in the trapping solutions [4] and [5] is counted. Thereafter, 20 mL 0.25 *M* $BaCl_2$ is added to 10 mL aliquots of trapping solution [4] and [5] each. Precipitation of $Ba^{14}CO_2$ occurs. The supernatant is then radio-counted again. The radioactive content in the supernatant after precipitation can be attributed to VFAs whereas the difference of radioactive content before precipitation minus radioactive content after precipitation can be attributed to evolved $^{14}CO_2$.

Quantification of volatiles

Quantification of trapped volatiles is by radio counting (liquid scintillation counting, LSC) of aliquots of the trapping solutions.

Particularly when using a semi-static setup, it is important to acidify the samples to release trapped CO_2 by addition of 10 mL 10% HCl, followed by further incubation and trapping of additionally released CO_2.

This can be achieved as follows: after humidified nitrogen has been bubbled through the manure samples for at least 1 hour, CO_2-traps ([4] and [5]) are removed and analysed for trapped $^{14}CO_2$ and other evolved gases, respectively, as described above. The removed traps are replaced by freshly filled ones.

Thereafter, the manure incubation flasks to be removed at that particular sampling point are treated by addition of 10 mL 10% HCl in order to strip potentially dissolved CO_2 (or HCO_3^- / CO_3^{2-}). After adding of 10 mL 10% HCl the incubation flasks are closed again and moistened nitrogen is bubbled through the manure for at least 24 hours[22]. Samples are not stirred in order to avoid foaming. If foaming is nevertheless observed, the acid should be added slowly (e.g. dropwise) over the incubation period. Thereafter, manure incubation flasks are removed and manure is cleaned-up and extracted. CO_2-traps are also removed and radio-counted for additionally trapped CO_2.

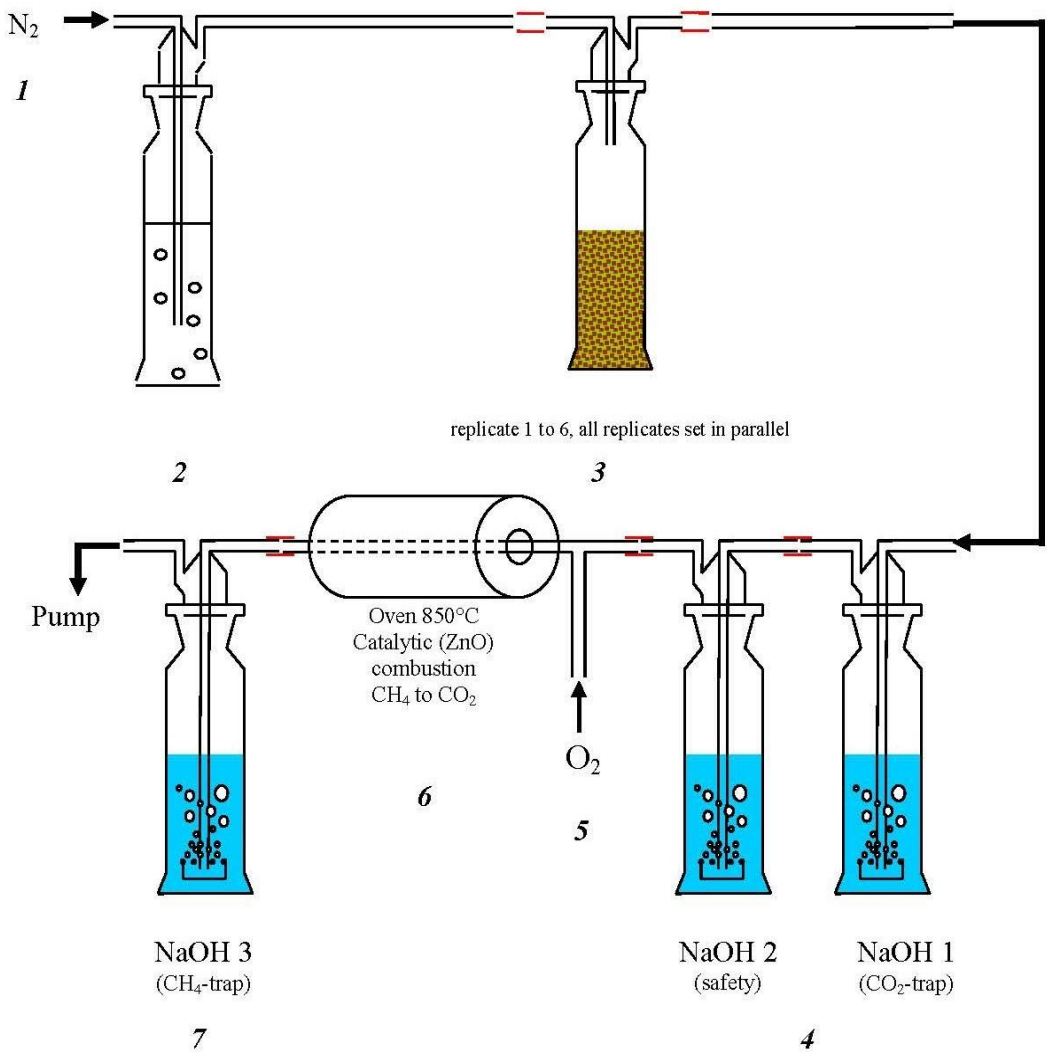
Note: Prior to the addition of 10% HCl to the manure, it has to be checked whether the test chemical and transformation products are stable under acidic conditions[23]. If this is not the case, further replicates have to be incubated for that purpose.

[22] The time needed for purging the $^{14}CO_2$ completely in the CO_2-traps, depends strongly on the amount of $^{14}CO_2$ formed and how fast this $^{14}CO_2$ formation is. For test chemicals with high mineralization purging for at least 24 h is recommended but time periods up to several days might be necessary to trap the $^{14}CO_2$ completely. If high mineralization is expected the optimal purging might be determined in preliminary investigations.

[23] In case of unknown transformation products, the stability check is not possible. A pre-test can be conducted to identify expected transformation products.

Annex 4: Incubation in a flow-through system

Figure 3. Example of a flow-through apparatus Incubation



32. nitrogen is gently passed over the manure samples
33. gas washing bottle containing water
34. manure transformation flasks filled with at least 50 – 100 g manure (fresh weight)
35. for anaerobic transformation two NaOH-traps in sequence are needed to trap evolving CO_2.
36. addition of oxygen or ambient air for subsequent catalytic combustion of CH_4
37. oven for combustion of CH_4 to form CO_2

38. NaOH-trap for CO_2 formed from CH_4

Differentiation between CO_2, CH_4 and VFAs

Humidified nitrogen is passed over the manure sub-samples at a rate in the range of approximately 50 – 200 mL/min. By such a constant N_2-stream evolved $^{14}CO_2$ is purged from the manure samples, transported and captured in traps 1 and 2 (safety trap) containing a CO_2-absorber (e.g. 2 *M* NaOH). Potentially formed $^{14}CH_4$ passes the CO_2-traps. After the addition of oxygen it is catalytically (= CuO) oxidized in an oven at 800°C - 850°C to form $^{14}CO_2$. The formed $^{14}CO_2$ is trapped in the CO_2-trap situated at the outlet of the oven.

Such a set-up enables the differentiation between evolved $^{14}CO_2$ (captured in traps 1 and 2) and $^{14}CH_4$ (captured in trap 3). Depending on the test chemical and its potential transformation products, further traps (e.g. ethylene glycol, sulphuric acid) might be required for collecting volatile organic compounds.

To verify that the radioactivity captured in the CO_2-traps 1 and 2 is $^{14}CO_2$ and not from potentially also formed volatile fatty acids (VFA) $BaCl_2$ precipitation of the radioactivity can be conducted. The radioactivity in the trapping solutions 1 and 2 is counted. Thereafter, 20 mL 0.25 M $BaCl_2$ is added to 10 mL aliquots of trapping solution from traps 1 and 2 each. Precipitation of $Ba^{14}CO_2$ occurs. The supernatant is then radio-counted again. The radioactive content in the supernatant after precipitation can be attributed to VFAs whereas the difference of radioactive content before precipitation minus radioactive content after precipitation can be attributed to evolved $^{14}CO_2$.

Quantification of volatiles

Quantification of trapped volatiles is by radio counting (liquid scintillation counting, LSC) of aliquots of the trapping solutions.

Furthermore, it should be proven whether evolved $^{14}CO_2$ is purged quantitatively when passing the humidified nitrogen over the manure samples. This can be verified by addition of HCl to the manure sub-samples in order to strip CO_2 (or HCO_3^- / CO_3^{2-}) being potentially dissolved in the manure matrix. Purging by addition of HCl should be applied in case the amount of $^{14}CO_2$ exceeds the level of 10% of the total radioactivity (TRR).

This can be achieved as follows: CO_2-traps 1 and 2 are removed at the particular sampling point and analysed for trapped $^{14}CO_2$ and other evolved gases, respectively, as described above. The removed traps are replaced by freshly filled ones.

Thereafter, the manure incubation flasks to be removed at that particular sampling point are treated by addition of 10 mL 10% HCl in order to strip potentially dissolved CO_2 (or HCO_3^- / CO_3^{2-}). After adding of 10 mL 10% HCl the incubation flasks are closed again and nitrogen is passed over for 24 hours. Samples are not stirred in order to avoid foaming. If foaming is nevertheless observed, the acid should be added slowly (e.g. dropwise) over the incubation period. Thereafter, manure incubation flasks are removed and manure is cleaned-up and extracted. CO_2-traps are also removed and radio-counted for additionally trapped CO_2. In order to avoid interferences and cross-contaminations by evolving gases sampling should start with samples next to the outlet.

Note: Prior to the addition of 10% HCl to the manure sub-samples it has to be checked whether the test chemical and transformation products is stable under acidic conditions[24]. If this is not the case, further replicates have to be incubated.

[24] In case of unknown transformation products the stability check is not possible. A pre-test can be conducted to identify expected transformation products.

Annex 5: Reporting scheme for results

Table A5. Example for a reporting scheme - distribution of radioactivity measured throughout an anaerobic transformation study in liquid manure [%aR]

Time [d]	Measured radioactivity [%aR]														
	Parent substance			Transformation product			Mineralization (CO_2 + CH_4)			NER			Mass balance		
	R1	R2	Mean	R1	R2	Mean	R1	R2	Mean	R1	R2	Mean	R1	R2	Mean
0	91.0	97.0	94.0	nd	nd	nd	nd	nd	nd	nd	nd	nd	91.0	97.0	94.0
2	80.0	82.0	81.0	nd	nd	nd	nd	nd	nd	nd	nd	nd	80.0	82.0	81.0
5	75.0	76.0	75.5	nd	nd	nd	nd	nd	nd	23.1	21.9	22.5	98.1	97.9	98.0
10	67.0	66.0	66.5	nd	nd	nd	nd	nd	nd	25.2	24.7	25.0	92.2	90.7	91.5
18	60.0	59.0	59.5	nq	nq	nd	nd	nd	nd	36.7	33.3	35.0	96.7	92.3	94.5
27	53.0	55.0	54.0	nq	nd	nq	nd	nd	nd	45.3	42.7	44.0	98.3	97.7	98.0
35	49.0	51.0	50.0	2.2	1.3	1.8	nd	nd	nd	36.7	33.9	35.3	87.9	86.2	87.1
50	38.0	40.0	39.0	5.7	6.0	5.9	nd	nd	nd	53.4	52.3	52.9	97.1	98.3	97.7
65	33.0	34.0	33.5	10.8	9.7	10.3	nd	nd	nd	47.5	45.9	46.7	91.3	89.6	90.5
78	29.0	33.0	31.0	12.5	15.7	14.1	nd	nd	nd	46.9	47.1	47.0	88.4	95.8	92.1
90	34.0	30.0	32.0	9.4	8.5	9.0	nd	nd	nd	44.9	43.7	44.3	88.3	82.2	85.3
LOD	0.3%			0.5%			0.3%			0.5%			-		
LOQ	0.5%			1.0%			0.5%			1.0%			-		

R1 = replicate 1, R2 = replicate 2, Mean = mean of R1 and R2, nd = not detected (< LOD, limit of detection), nq = not quantified (<LOQ, limit of quantification)

Annex 6: Variability in manure matrix parameters and DT_{50} values

Manure matrix parameters

Manure shows less variability in matrix parameters than other environmental matrices such as soil or sediment. Data from method development and validation show a narrow range for matrix parameters, although manures were chosen to be as diverse as possible [3].

Cattle manure: Dry matter: 9.9 ± 2.2% [3]

C_{org}: 4.1 ± 0.9% [3]

pH_{winter}: 7.3 ± 0.4 [19]

pH_{summer}: 6.9 ± 0.2 [19]

Pig manure: Dry matter: 5.2 ± 3.2% [3]

C_{org}: 2.1 ± 1.3% [3]

pH: 7.3 ± 0.1 [19]

DT_{50}-values

Variability for DT_{50} values (expressed as COV) between different soils has been estimated to be around 100% by FOCUS (2000). Based on information for manures derived from method development, validation studies and regulatory studies, a mean COV of 38% was observed.

	Variability different soils	Variability different manures
COV (FOCUS 2000)	100%	-
COV (mean; available studies)	89% (n = 43)*	37% (n = 9)**
COV (range available studies)	7%-600% (n = 43)*	20-57% (n = 9)**
COV (mean; direct comparison for eprinomectin; same laboratory, same substance) (EMA 2018)	109% (n = 4)	57% (n = 5)

* Data derived from evaluation of 43 regulatory studies

** Data derived from method development [19], validation studies [2] and marketing authorization application (EMA 2018)

The available data indicate lower variability of matrix parameters and DT_{50}-values for manure compared to soil.

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For all other references, see the literature section of the main guideline text.