Texte

Texte
45/07
ISSN 1862-4804

Veterinary Medicinal Products in Manures and Manured Soils: Development of a Technical Protocol for Laboratory Tests

- The Manure Project -

Umwelt Bundes Amt
Für Mensch und Umwelt

UMWELTFORSCHUNGSPLAN DES
BUNDESMINISTERIUMS FÜR UMWELT,
NATURSCHUTZ UND REAKTORSICHERHEIT

Forschungsbericht 204 67 455
UBA-FB 001086

Texte
45/07
ISSN
1862-4804

Veterinary Medicinal Products in Manures and Manured Soils: Development of a Technical Protocol for Laboratory Tests

– The Manure Project –

von

Robert Kreuzig
Sibylla Höltge
Julia Heise
Ilka Schmanteck
Florian Stein
Mufeed Batarseh

Technische Universität Braunschweig
Institut für Ökologische Chemie und Abfallanalytik

Im Auftrag des Umweltbundesamtes

Diese Publikation ist ausschließlich als Download unter
http://www.umweltbundesamt.de
verfügbar.

Herausgeber: Umweltbundesamt
Postfach 14 06
06813 Dessau-Roßlau
Tel.: 0340/2103-0
Telefax: 0340/2103 2285
Internet: http://www.umweltbundesamt.de

Redaktion: Fachgebiet IV 2.2
Dr. Jutta Klein-Goedicke

Dessau-Roßlau, Dezember 2007

Berichts-Kennblatt

1. Berichtsnummer UBA-FB	2.	3.

4. Titel des Berichts

Tierarzneimittel in Gülle und güllegedüngten Böden:
Die Erarbeitung eines Methodenkataloges für Labortests

– Das Gülle-Projekt –

5. Autor(en), Name(n), Vorname(n)

Kreuzig, R. (Projektleiter), Höltge, S., Heise, J., Schmanteck, I., Stein, F., Batarseh, M.

6. Durchführende Institution (Name, Anschrift)

Institut für Ökologische Chemie und Abfallanalytik
Technische Universität Braunschweig
Hagenring 30
D-38106 Braunschweig

7. Fördernde Institution (Name, Anschrift)

Umweltbundesamt
Wörlitzer Platz 1
D-06844 Dessau

8. Abschlussdatum
15.03.2007

10. UFOPLAN-Nr.
204 67 455

11. Seitenzahl
149

12. Literaturangaben
78

13. Tabellen
30

14. Abbildungen
46

15. Zusätzliche Angaben

16. Zusammenfassung

Im Rahmen des Gülle-Projektes wurde ein Methodenkatalog mit Richtliniencharakter erarbeitet, gemäß dem das Rückstandsverhalten von Veterinärpharmaka (VMP) in Gülle und güllegedüngten Böden untersucht werden kann. Da eine repräsentative und reproduzierbare Entnahme von Gülleproben in Güllesilos unmöglich ist, wurde hier ein neues Forschungskonzept entwickelt. Danach erfolgt die Probenahme von Exkrementen von Kühen und Schweinen aus der Einzeltierhaltung im Versuchsstall. Aus diesen Proben, die einer umfassenden Matrixcharakterisierung unterzogen werden, werden durch Zugabe von Wasser Referenzgülleproben mit definierten Trockensubstanzgehalten hergestellt. Diese werden schließlich in Abbautests für ^{14}C-markierte Testsubstanzen eingesetzt. Auf der Basis dieser Abbautests werden Testgülleproben mit 7 Tage gealterten VMP-Rückständen hergestellt und in Labortests zur Untersuchung von Abbau und Sorption von VMP in güllegedüngten Böden eingesetzt. Auf diesem Wege ist es möglich, Einflüsse während der Güllelagerung und darüber hinaus Matrixeinflüsse der Gülle auf das Rückstandsverhalten von VMP in Böden bereits unter Laborbedingungen zu erfassen. Diese Labortest orientieren sich weitestgehend an der landwirtschaftlichen Praxis, der analytischen Praktikabilität und der analytischen Qualitätssicherung. Letztere wurde bereits in ersten internen und externen Labortests geprüft. Schließlich wurden die erarbeiteten methodischen Aspekte im Entwurf eines Methodenkataloges (Technical Protocol) zusammengestellt.

17. Schlagwörter
Veterinärpharmaka, Exkrement, Referenzgülle, Testgülle, Boden, Abbau, Sorption

18. Preis	19.	20.

Report Cover Sheet

1. Report No. UBA-FB	2.	3.

4. Report Title

Veterinary Medicinal Products in Manures and Manured Soils: Development of a Technical Protocol for Laboratory Tests

– The Manure Project –

5. Author(s), Family Name(s), First Name(s)

Kreuzig, R. (Project leader), Höltge, S., Heise, J., Schmanteck, I., Stein, F., Batarseh, M.

6. Performing Organization (Name, Address)

Institute of Ecological Chemistry and Waste Analysis
Braunschweig University of Technology
Hagenring 30
D-38106 Braunschweig, Germany

204 67 455

7. Funding Agency (Name, Address)

Federal Environmental Agency
Wörlitzer Platz 1
D-06844 Dessau, Germany

8. Report Date
03/15/2007

11. No. of Pages
149

12. No. of Reference
78

13. No. of Tables
30

14. No. of Figures
46

16. Abstract

The Manure Project has been targeted at the development of a technical protocol for laboratory tests on fate and behavior of veterinary medicinal products (VMP) in liquid manures and manured soils. Since the representative and reproducible sampling of manure from high-volume tanks has been regarded impossible, an innovative research concept has been developed. First, excrement samples of cattle and pigs individually kept at an experimental stable were taken. These samples were thoroughly matrix characterized. Then, tap water was added to prepare reference manures of definite dry substance contents. Subsequently, the long-term degradation of VMP applied as ^{14}C-labeled radiotracers was investigated in these manure samples. On the basis of the degradation tests, test manures with 7-day aged VMP residues were prepared and applied in laboratory tests on degradation and sorption in manured soils. By means of this experimental approach, the impacts of aging processes during manure storage and of the manure matrix on the fate of VMP in soils can be assessed already under laboratory conditions. These laboratory tests have been directed as closely as possible to the principles of good agricultural practice, to the analytical practicability and to the analytical quality assurance checked in first intra- and interlaboratory tests. Finally, the methodological aspects have been compiled in the proposal of a Technical Protocol (draft version).

17. Keywords
Veterinary medicinal products (VMP), excrement, reference manure, test manure, soil, degradation, sorption

18. Price	19.	20.

Structure

Abbreviations

A-SMZ	Acetyl-sulfamethoxazole
BE	Bovine excrement
BHZP	German Federal Hybrid Breeding Program
BIO	Biology V, RWTH Aachen, Germany
BM	Bovine manure
BS	Laboratory-batch system
BOD_5	Biological oxygen demand within 5 days
COD	Chemical oxygen demand
DHA	Dehydrogenase activity
DT_{50}	Disappearance time in days for 50 % of the initially applied substance
ds	Dry substance
ECO	Institute of Ecological Chemistry and Waste Analysis, Braunschweig University of Technology, Germany
Eh	Redox potential
ER	Extractable residues
ERY	Erythromycin
FAL	Federal Agriculture Research Center, Braunschweig, Germany
FTS	Flow-through system
ICP-OES	Optical emission spectroscopy with inductively coupled plasma
IR heater	Infrared heater
ISO	International Organization for Standardization
K_d	Soil/water distribution coefficient
K_{OC}	Soil/water distribution coefficient standardized on the organic substance in soil
KET	Ketoprofen
M	mol L^{-1}
MET	Metabolite
MIN	Mineralization
NH_4-N	Ammonium-nitrogen content
N_{total}	Total nitrogen content
LSC	Liquid scintillation counting
LUFA	Landwirtschaftliche Untersuchungs- und Forschungsanstalt

NER	Non-extractable residues
OC	Organic carbon
OECD	Organization for Economic Cooperation and Development
PCM	Paracetamol
PE	Pig excrement
PM	Pig manure
RCC	RCC-Ltd., Itingen, Switzerland
R_f	Retention factor
R_{min}	Mineral content
RTLC	Radio thin layer chromatography
SIR	Substrate-induced respiration
SDZ	Sulfadiazine
SLT	Biometric-flask system with soda-lime trap
SMZ	Sulfamethoxazole
STD	Standard application
TM_B	Test manure prepared from bovine reference excrements
TM_P	Test manure prepared from pig reference excrements
TOC	Total oxygen content
TPF	Triphenyl formazane
TPT	Triphenyltetrazolium chloride
TUBS	Braunschweig University of Technology, Germany
UBA	Federal Environmental Agency, Dessau, Germany
UMS	Fraunhofer-Institut für Umwelt-, Sicherheits- und Energietechnik, Oberhausen, Germany
VICH	International Cooperation on Harmonization of Technical Requirements for Authorization of Veterinary Medicinal Products
WHC_{max}	Maximum water holding capacity
VMP	Veterinary medicinal products

1 Introduction

After the administration of veterinary medicinal products (VMP) in livestock husbandry, parent compounds and metabolites are excreted by production animals via urine and feces. In loose housing stables with slatted floors, the excrements are transferred to the manure aboveground silos or underground pits. After the storage of manures up to several months, they are applied to farmland or grassland soils as organic fertilizers. VMP that are not degraded during manure storage enter soil environments via this entry route (Halling-Sørensen et al., 1998). This fact is namely considered relevant by the authorization procedure of VMP (VICH, 2000, 2003). For the risk assessment in Phase I, the trigger of 100 µg VMP kg^{-1} soil is defined. During manure storage, however, degradation and sorption of VMP are not taken into account as concentration determining processes. If this trigger is exceeded for antibiotics, those substances have to undergo testing on acute ecotoxicological effects and on degradation and sorption in soils and surface waters (Koschorreck et al., 2002, Rönnefahrt et al., 2002). For antiparasitics, those laboratory tests are mandatory. In those Phase II tests, the test substances under study are applied in an appropriate solvent. This procedure is defined as the standard application. Thus, possible matrix effects of the manures are not considered.

Numerous studies on fate and behavior of VMP in bovine and pig manures clearly demonstrated possible effects of manure storage and manure application on the fate of VMP in soils (Höltge and Kreuzig, 2007, Kreuzig and Höltge, 2005, Kreuzig et al., 2007, Kühne et al., 2000, Loke et al., 2003). Nevertheless, there is a lack of a technical protocol for laboratory tests on the fate of VMP in manure and manured soils until today. That may be partly caused by the impossibility of taking representative and reproducible samples in manure silos up to a volume of 10000 m^3 and the preparation of test manures[1] applied in the laboratory tests to simulate the real entry route of VMP into soil environments. The objectives of the Manure Project, therefore, are targeted at the development of a technical protocol for laboratory tests on the degradation of VMP in bovine and pig manures as well as on degradation and sorption in manured soils. Therefore, an innovative research concept was developed focused on sampling of excrement samples of cattle and pigs individually kept at an experimental stable, matrix charac-

[1] "Test manures" define real manure samples that were fortified with the respective ^{14}C-labeled VMP and incubated to simulate aging processes of VMP residues during the storage of manure.

terization of the excrement samples, preparation of reference manures, carrying out of degradation tests for selected VPM in manures, preparation of test manures and fate monitoring in manured soils. These laboratory tests have been directed as closely as possible to the principles of good agricultural practice, to the analytical feasibility considering appropriate time and cost limits and to the analytical quality assurance checked in first intra- and interlaboratory tests. The methodological aspects have been compiled in the proposal of the Technical Protocol (draft version) and, together with the results of the Manure Project, they were presented in the UBA Expert Symposium held in Braunschweig, Germany, on 20 to 21 February 2007.

2 Excrements and reference manures

Liquid manures are heterogeneous matrices of high complexity and variability. This heterogeneity is dependent on the animal species, the animals´ age, feeding conditions and VMP administration. This fact has been clearly shown by the studies of LUFA Nord-West (Merkel, 2005) in that 2000 samples of bovine and pig manures each were analyzed from 1997 until 2004. Selected results are listed in **Tab. 2.1**.

Tab. 2.1: Differences in the composition of 2000 bovine and pig manure samples from 1997-2004 (Merkel, 2005)

Parameter	**ds** [%]	**P_2O_5** [g kg^{-1}]	**Cu** [mg kg^{-1}]	**NH_4-N** [g kg^{-1}]	**N_{total}** [g kg^{-1}]
Bovine manure					
minimum	0.4	0.05	0.08	0.01	0.43
median	8.7	1.7	3.9	1.7	4.0
maximum	12.3	2.7	12.1	2.9	5.7
Pig manure					
minimum	0.4	0.03	0.22	0.27	0.60
median	4.9	2.3	16.1	2.7	4.6
maximum	11.6	6.3	53.1	4.9	8.3

Additionally, residues of feeding stuff and straw as well as disinfection and cleaning agents may be released into the storage tanks by farming practices enhancing the heterogeneity of manures (Hoffmann and Hege, 1991; Montforts and Tarazona Lafarga, 2003; Schuchardt and Hahne, 1996). Finally, organic constituents and contaminants of manures undergo decomposition processes during the storage of manures up to several months (German Ordinance Concerning Fertilizers, 2006). Due to these aspects, the representative and reproducible sampling of manure from tanks up to a volume of 10000 m^3 is regarded impossible.

In order to develop the methodology of degradation tests of VMP in manure, therefore, the Manure Project is focused on a tiered research concept. At first, excrement samples were taken from individually kept production animals. On the basis of the matrix characterization, tap water was then added to those samples to prepare reference-

manure samples with defined dry substance contents. Finally, these samples were matrix characterized again.

2.1 Excrement sampling

The excrements were sampled from conventionally fed single cattle and pig groups of 6-8 individuals in the experimental stable of the Institute of Animal Nutrition, Federal Agricultural Research Centre (FAL), Braunschweig, Germany, and in a dairy cow farm as well as in a pig fattening farm in Ohrdorf and Erpensen, Lower Saxony, Germany. Six different excrements per animal species, cattle and pigs, were taken considering the different age of animals and different feeding conditions. The sampling periods averaged between 12 to 24 h.

Due to the 32-% contingent of diary cows within the cattle livestock in Germany (German Federal Statistical Office, 2006), excrements of diary cows were mainly selected for laboratory testing (BE-1, BE-3, BE-4, BE-5, BE-6) (**Tab. 2.2**).

Tab. 2.2: Origin of bovine excrements (BE)

Excrement	Origin	Age of animals	Feeding
BE-1	FAL, Braunschweig	5 years	maize silage, grass silage, wheat silage, pellet, mineral food
BE-2	FAL, Braunschweig	8 months	maize silage, grass silage, wheat silage
BE-3	Meyer, Farm Ohrdorf	5 years	maize silage, grass silage, hay, pellet, mineral food
BE-4	FAL, Braunschweig	5 years	maize silage, pellet, mineral food
BE-5	FAL, Braunschweig	4 years	grass silage, maize silage, pellet, mineral food
BE-6	FAL, Braunschweig	5 years	grass silage, maize silage, pellet, mineral food

Additionally, calf excrements (BE-2) were taken considering calves constitute the second important contingent (31 %) in cattle livestock and they are the major medically treated group. Except for the calf that did not get any pellet and mineral food, the feeding conditions of these animals were adequate to standard nutrition conditions reflecting the herbivore nutrition type of cattle. All animals belonged to the German Holstein Black and White Race. The feeding frequency was twice the day and the water uptake occurred autonomously.

Tab. 2.3: Origin of pig excrements (PE)

Excrement	Origin	Age of animals	Feeding
PE-1	FAL, Braunschweig	6 months	46 % barley, 35 % wheat, 15 % soya pellet,1.5 % soya oil, 2 % vitamins/ minerals/trace elements, 0.5 % amino acids
PE-2	FAL, Braunschweig	12 months	25 % barley, 50 % wheat, 20 % soya pellet, 2 % soya oil, 3 % vitamins/minerals/trace elements
PE-3	Beyer Farm, Erpensen	6-8 months	60 % potato refuse, 30 % wheat/barley, 7 % soya pellet/soya oil, 3 % vitamins/minerals/trace elements
PE-4	FAL, Braunschweig	4 months	46 % barley, 35 % wheat, 15 % soya pellet,1.5 % soya oil, 2 % vitamins/ minerals/trace elements, 0.5 % amino acids
PE-5	FAL, Braunschweig	4 months	37 % barley, 27.5 % wheat, 18 % soya pellet,12.5 % triticale, 2 % soya oil, 3 % vitamins/minerals/trace elements
PE-6	FAL, Braunschweig	7 months	46 % barley, 35 % wheat, 15 % soya pellet,1.5 % soya oil, 2.5 % vitamins/ minerals/trace elements/amino acids

Tab. 2.4: Matrix characterization of excrement samples

Parameter	Guideline	Equipment
Dry substance content (ds)	ISO 11465 (1993)	ultra-X infrared heater (Gronert, Germany) or a drying oven
Mineral content (R_{min})	DIN 19684-3 (2000)	muffle furnace (Haereus, Germany)
Copper (Cu), phosphate content (P)	ISO 11885 (1996)	Laboratory Microwave Equipment (Start system with Terminal 320, MLS, MWS-Vertrieb GmbH, Leutkirch, Germany), ICP-OES system (Vista-MPX CCD simultane ICP-OES, Varian, Palo Altra, CA, USA)
Total organic carbon (TOC)	ISO 10694 (1995)	C-Analyser Dohrmann DC-90 (Dohrmann, Santa Clara, CA, USA)
pH value	DIN EN 12176 S5 (1998)	pH Multical 535 GLP with pH-glass electrode SenTix 61 (WTW Weilheim, Germany)
Redox potential (Eh)	DIN 38404 C6 (1984)	pH Multical 535 GLP 8, (WTW Weilheim, Germany) with redox-electrode (Inolab Redox Einstabmesskette, Mettler Toledo, Giessen, Germany)
Dissolved oxygen (O_2)	ISO 5814 (1990)	Oxi 340i with OxiCell 325 oxygen-electrode (WTW, Weilheim, Germany)
Total nitrogen (N_{total})	ISO 11261 (1995)	Digestion Unit 430 and Distillation Unit 323 (Büchi Labortechnik GmbH, Essen, Germany)
Ammonium nitrogen (NH_4-N)	ISO 5664 (1984)	Distillation Unit 323 (Büchi Labortechnik GmbH, Essen, Germany)
Biological oxygen demand (BOD_5)	ISO 5815 (2003)	Oxi 340i with OxiCell 325 oxygen-electrode (WTW, Weilheim, Germany), Karlsruher bottles, 250 mL (Schott, Mainz, Germany)
Chemical oxygen demand (COD)	ISO 15705 (2002)	Cuvette test LCI 400, Spectral-Photometer Cadas 100 (both Hach Lange GmbH, Düsseldorf, Germany), Thermoblock TR 205 (Merck, Darmstadt, Germany)

Besides 13 millions of cattle, 27 millions of pigs are kept in Germany (German Federal Statistical Office, 2006). The main part of the latter accounted for fattened pigs (40 %). Due to the omnivore nutrition type of pigs, a major variability of feeding conditions had to be taken into account (**Tab. 2.3**). PE-1, PE-4, PE-5 and PE-6 excrements were excreted by pigs fed according to the standard feeding conditions, while PE-2 were from pigs fed with a definitely higher wheat amount (50 %). PE-3 were from pigs fed with 60 % potato refuse from a potato chips factory. The food uptake amounted approximately 3.5 kg per animal and day and the water uptake occurred autonomously. All excrements were taken from animals of the German Federal Hybrid Breeding Program (BHZP).

2.2 Characterization of excrement matrices

The characterization of the excrement samples under study was based on the determination of physico-chemical parameters listed in **Tab. 2.4**. These are frequently applied for the analysis of waste water, sewage sludge and soil samples or used to assess the biogas potential of different manures (Hahne, 2001; Hüther, 1999; Møller et al., 2004; Schuchhardt and Hahne, 1996). For quality assurance, the analyses were generally performed in duplicates.

For the determination of the **dry substance contents (ds)**, excrement samples (1 to 6 g) were dried in a drying oven to constant mass at 105 ± 5 °C (ISO 11465, 1993). The dry substance content, expressed as percentage by mass to an accuracy of 1 % (*m*/*m*) is calculated using the following formula:

$$ds = \frac{(m_c - m_a) \cdot 100\ \%}{(m_b - m_a)}$$

m_a: mass of the empty crucible [g]
m_b: mass of the crucible plus sample; initial weight [g]
m_c: mass of the crucible plus oven-dried sample; output weight [g].

Alternatively, an infrared heater was used to drive out the water. For this procedure, 3 to 5 g of the homogenized samples were equally distributed on the weighing scale and the infrared heater was then activated. At mass constancy, the dry substance content

was calculated according to:

$$ds = \frac{m_b \cdot 100\ \%}{m_a} \qquad \text{or} \qquad ds = 100\ \% - w_{H_2O}$$

m_a: initial weight [g]
m_b: output weight [g]
w_{H_2O}: water content [%].

The repeatability of both methods was tested for selected excrement and manure samples. As shown in **Tab. 2.5**, both methods supplied consistent results.

Tab. 2.5: Determination of dry substance contents by means of drying oven or infrared heater (n= 4)

Sample	**105 ± 5 °C** ds [%]	**IR heater** ds [%]
Bovine excrement	11 ± 0.6	12 ± 0.8
Bovine manure	9 ± 0.6	9 ± 0.5
Pig excrement	21 ± 0.4	23 ± 0.8
Pig manure	4 ± 0.9	5 ± 1.3

For the determination of the **mineral content (R_{min})**, the dried substance of excrements samples were tempered in a muffle furnace at 550 ± 25 °C according to DIN 19684-3 (2000). The difference of an amount of excrement before and after the tempering procedure is used to calculate the mineral content that is expressed with an accuracy of ± 1 % (*m*/*m*) according to

$$R_{min} = 100 - \frac{(m_c - m_a) \cdot 100}{(m_b - m_a)}$$

m_a: mass of empty crucible [g]
m_b: mass of crucible plus dry substance [g]
m_c: mass of crucible plus combusted dry substance [g]
100: conversion factor to percent.

For quality assurance, selected bovine excrements were analyzed in 4 replicates. With deviations ≤ 11 % the reproducibility of this method was given (**Tab. 2.6**).

Tab. 2.6: Determination of mineral content in selected excrements

Sample	R_{min}[1] [% ds]	R_{min}[2] [% ds]	R_{min}[3] [% ds]	R_{min}[4] [% ds]	∅ R_{min} [% ds]
BE-1	18	21	17	21	19 ± 2
BE-3	24	25	24	24	24 ± 0
BE-4	15	16	15	15	15 ± 1

BE: bovine excrement

The determination of **copper** and **phosphorus content** was performed according to ISO 11885 (1996). After a microwave digestion, a measurement of atomic emission by optical spectroscopy (ICP-OES) was performed. After the addition of 8 mL nitric acid, 2 mL hydrogen peroxide and 2 mL water, the digestion process with 100 to 200 mg excrement samples (ds) was conducted by means of microwaves at 200 °C for 1 h. Then, ICP-OES analyses followed. For external calibration, standard solutions from 0.01 to 100 mg L^{-1} were analyzed. The instrument performance was checked by analyzing a reference standard solution after every 10 samples. Furthermore, one blank of an acidified water sample was analyzed. The concentration of this sample was substracted from all samples. The element content was expressed in mg kg^{-1} fresh weight with an accuracy of 1 mg kg^{-1}. Results higher 1000 mg kg^{-1} were expressed in g kg^{-1} fresh weight with an accuracy of 1 g kg^{-1}.

The **total organic carbon (TOC)** in excrement samples was determined by means of combustion in an oxygen stream at 900 °C and subsequent infrared detection of carbon dioxide released. For this purpose, carbonate was removed from excrement samples by an excess of hydrochloric acid (4 M). Subsequently, they were dried, homogenized and mixed with aluminum oxide (1:20). The mixed samples were combusted and the combustion gas was analyzed by means of a non-dispersive infrared detector. The organic carbon amount was calculated on the basis of an external standardization in a range of 20 to 150 µg carbon, using an oxalic acid dihydrate ($C_2H_2O_4 \cdot 2\ H_2O$) standard, mixed with aluminum oxide (1:9). The repeatability was checked by analyzing potassium hydrogen phthalate ($C_8H_5KO_4$) standards. The average recovery was 99 % with a relative standard deviation of 6 %.

Besides the **pH values, redox potentials (Eh)** and **dissolved oxygen contents (O_2)** were measured directly in the excrements by means of electrodes. By means of the latter ones, anaerobic conditions, typical for storage conditions in manure tanks, were identified. For this purpose, threshold values of Eh < +150 mV and O_2 < 0.1 mg kg^{-1} have been defined according to Ndegwa et al. (2003) and Michels et al. (2000). They specified permanent aerobic conditions in pig manure and in waste water by minimum levels of Eh = +150 mV and Eh = +200 mV, respectively. On the other hand, the limit of microbial activity in liquid media under aerobic conditions was O_2 = 0.1 mg kg^{-1} (Strauch et al., 1977, Domsch, 1992).

For the **total nitrogen (N_{total})** determination by Kjeldahl digestion, 1 to 3 g of the excrement samples were weighted into digestion tubes. Subsequently, 1 Kjeldahl tablet (5 g, Hg- and Se-free; Merck, Darmstadt, Germany), 10 mL concentrated sulfuric acid and boiling chips were added. Digestion followed at maximum 410 °C for 3 h. After cooling down, the distillation was carried out using 30 mL demineralized water and 70 mL sodium hydroxide solution (32 %) for 10 minutes. Distillates were introduced into 50 mL boric acid solution (2 %) with 200 µL mixed Indicator V for ammonia titrations (Merck, Darmstadt, Germany) and titrated with 0.1 M hydrochloric acid from green to grey. A blank test in which the same procedure was performed without excrements was carried out, too. The total content of nitrogen in g N kg^{-1} sample rounded to one significant figure, was 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 sample [g]
c : Concentration of hydrochloric acid [0.1 M]
M_N: Molar mass of nitrogen [= 14.01 g M^{-1}].

For quality assurance, excrement and manure samples were fortified with phenylalanine. These fortification experiments revealed a limit of determination of 2 g kg^{-1} with an average recovery of 101 ± 6 %. Recoveries of 97 ± 8 % were obtained for phenylalanine standards analyzed without matrix to check the instrument performance.

According to the total nitrogen determination, the **ammonium nitrogen (NH_4-N)** was determined, however, without any digestion procedure. For distillation, 15 mL demineralized water and 30 mL sodium hydroxide solution (32 %) were used. Distillation time was 10 min, too. For quality assurance, excrement and manure samples were spiked with ammonium sulfate solution. In these fortification experiments, a limit of determination of 0.2 g kg^{-1} and recovery rates of 100 ± 12 % were obtained. The repeatability of this method was revealed by recovery rates of 99 ± 1 %, analyzed for ammonium sulfate standards without matrix.
In fresh matrices, readily degradable nitrogen containing substances, e.g., urea, are decomposed by ammonification. Thus, the ammonium nitrogen and the ammonium nitrogen/total nitrogen ratios increase. Hence, Hahne (2001) mentioned those ratios may indicate aging processes in excrement samples.

The **biological to chemical oxygen demand ratio (BOD_5/COD)** is frequently determined to characterize the degradability of waste water pollutants (Hütter, 1994). Rapid biodegradability is reflected by a ratio of 0.5 to 1, a ratio < 0.5 shows minor biodegradability and at < 0.1 biodegradability is excluded. Both parameters can be drawn on for the characterization of decomposition processes in excrements, too (Hahne, 2001). Furthermore, the BOD_5 is one indicator for the microbial activity in manure. Both parameters are analyzed as follows:
For the **BOD_5** determination, the excrement samples were diluted by adding tap water at 1:2000 for bovine excrements and at 1:4000 for pig excrements. To inhibit nitrification, 2 mL allylthiourea solution L^{-1} (0.1 %; Merck, Darmstadt, Germany) were added. The diluted excrement samples were then filled into 250-mL airtight bottles (Karlsruher bottles; Schott, Mainz, Germany). Those were incubated at 20 °C in the dark for 5 days. The BOD_5 was calculated from the difference between initial and final dissolved oxygen content, allowing for blank value:

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

C_1: dissolved oxygen concentration in the sample solution at time zero [mg kg^{-1}]
C_2: dissolved oxygen concentration in the sample solution after five days [mg kg^{-1}]
C_3: dissolved oxygen concentration in the blank solution at time zero [mg kg^{-1}]

C_4 : dissolved oxygen concentration in the blank solution after five days [mg kg^{-1}]
V_t : total volume [mL]
V_e : sample volume [mL].

Results below 1 g oxygen kg^{-1} excrement were reported with two significant figures. Results between 1 g kg^{-1} and 10 g kg^{-1} were reported to one significant figure. Results $\geq$ 10 g kg^{-1} were reported without decimal places.

The **COD** was determined by means of the cuvette test according to ISO 15705 (2002) (LCI 400; Hach Lange GmbH, Düsseldorf, Germany) applicable up to 1000 mg O_2 kg^{-1}. By means of the sulfuric acid/ potassium dichromate treatment, catalyzed by silver nitrate, organic substances of the excrement samples were completely oxidized during a digestion procedure at 148 °C for 2 h. Simultaneously, Cr^{6+} was reduced to Cr^{3+}. The extinction of the green colored Cr^{3+} solution was determined by photometric measurement at λ = 605 nm. For this purpose, bovine and pig excrement samples were diluted to ratios of 1:200 and 1:250, respectively. The performance of this method was checked using potassium hydrogen phthalate solution (COD: 200 mg L^{-1}) and spiked samples. Recovery rates were 105 $\pm$ 10 % and 77 $\pm$ 9 %, respectively, demonstrating the efficiency of the cuvette test.

Tab. 2.7: Determination of chemical oxygen demand with different methods

Sample	**COD ISO 15705** [g kg^{-1}]	**COD ISO 6060** [g kg^{-1}]
Standard	101	96
Bovine excrement	56	42
Bovine manure	37	38
Pig excrement	111	82
Pig manure, PM-1	29	26
Pig manure, PM-2	37	35

Alternatively the COD was determined according to ISO 6060 (1989). A sample aliquot was digested under reflux in the presence of mercury(II) sulfate with a known amount

of potassium dichromate and silver catalyst in strong sulfuric acid. After the digestion process, the titration of the remainder of the dichromate with ammonium iron(II) sulfate was conducted. The COD of selected samples was determined using both methods. revealing their consistency (**Tab. 2.7**).

Tab. 2.8: Characterization of bovine and pig excrements within the preconditioning period

Parameter	BE-4	BE-5	PE-4	PE-5
ds [%]	13 ± 1	12 ± 0	17 ± 1	16 ± 0
TOC [g kg^{-1}]	51 ± 3	40 ± 3	64 ± 4	72 ± 9
pH	6.2 ± 0.2	6.5 ± 0.2	6.2 ± 0.2	6.1 ± 0.2
Eh [mV]	< 150	< 150	< 150	< 150
O_2 [mg kg^{-1}]	d 0-5: > 0.1 d 5-20: < 0.1	d 0 to 7: > 0.1 d 5 to 21: < 0.1	d 0- 6: > 0.1 d 6-21: < 0.1	d 0- 7: > 0.1 d 7-28: < 0.1
NH_4-N [g kg^{-1}]	d 0-8: 1.0-1.6 d 8-20: 1.6	d 0-14: 0.8-1.2 d 14-21: 1.2	d 0-13: 3.6-5.6 d 13-21: 5.6	d 0-22: 2.8-5.8 d 22-28: 5.8
N_{total} [g kg^{-1}]	3.6 ± 0.2	3.1 ± 0.2	9.0 ± 0.2	8.9 ± 0.4
NH_4-N / N_{total}	0.3 to 0.5	0.3 to 0.4	0.4 to 0.6	0.3 to 0.6
BOD_5 [g kg^{-1}]	23 ± 4	8 ± 2	24 ± 2	22 ± 1
COD [g kg^{-1}]	83 ± 8	62 ± 8	120 ± 8	147 ± 11
BOD_5 / COD	0.3	0.1	0.2	0.2

Changes in the excrement matrices were mainly reflected by the dissolved oxygen the and ammonium content. As shown by the dissolved oxygen contents, bovine and pig

excrements were not strictly anaerobic until a preconditioning period of 7 days, respectively. The redox potentials remained at Eh < +150 mV. Ammonium contents increased from 0.9 to 1.4 g kg^{-1} and from 3.2 to 5.6 g kg^{-1} in bovine and pig manure, respectively. That indicated the decomposition from readily degradable nitrogen containing substances, e.g., urea, resulting in an increasing NH_4-N/N_{total} ratio. The other parameters remained nearly constant displayed by the standard deviations listed in **Tab. 2.8**.
Taking these results into special consideration, preconditioning periods of 14 days for bovine excrements and 22 days for pig excrements were maintained to assure constant anaerobic conditions and ammonium nitrogen contents. Thereafter, excrement samples could be stored at 4 to 7 °C in the dark without further changes and then applied for laboratory testing until analyrical processing.

2.3 Excrement storage tests

Directly after excretion, readily degradable organic compounds of the excrements undergo rapid decomposition enhancing the matrix heterogeneity (Strauch et al., 1977). To reduce this effect, excrement samples were preconditioned. For this purpose, they were stored in 20-L containers at ambient temperature up to 28 days after sampling. Within this period, they were regularly homogenized. Aliquots were taken every 2 to 7 days for matrix characterization.
The application of manure samples for testing the VMP degradation requires the repeatability in sampling of excrements and preparation of the reference manure samples. Furthermore, providing of reserve samples is a relevant aspect of analytical quality assurance.
For this purpose, the long-term storage up to 378 days was tested. Therefore, BE-1 and PE-1 already matrix characterized were stored at -20 °C in 1- to 2-L plastic containers. After defrosting, the excrements were stored at ambient temperature for 3 days to reactivate the excrement inherent microorganisms. Then, these samples were matrix characterized again to check for changes. The comparison of excrement parameters before and after freezing did not show relevant differences (**Tab. 2.9**). Thus, reserve samples can be used after long-term storage at -20 °C for further analytical processing without any impacts on the analytical quality.

Tab. 2.9: Effects of long-term storage at -20 °C on the quality of reserve samples of preconditioned bovine and pig excrement samples

	Bovine excrement (BE-1) preconditioned for 8 days storage at -20 °C				**Pig excrement (PE-1)** preconditioned for 13 days storage at -20 °C			
Storage period [d] **/ Parameter**	**0**	**19**	**185**	**378**	**0**	**7**	**110**	**322**
ds [%]	13	13	14	13	22	23	21	22
TOC [g kg^{-1}]	53	47	39	46	70	60	73	96
pH	6.8	6.5	6.5	7.4	7.3	7.3	7.2	7.3
Eh [mV]	60	100	130	-140	-140	-10	-270	-30
O_2 [mg kg^{-1}]	< 0.1				< 0.1			
NH_4-N [g kg^{-1}]	1.7	1.5	1.7	1.7	8.3	9.0	9.3	9.0
N_{total} [g kg^{-1}]	4.3	4.0	4.0	4.0	14.0	13.8	13.7	13.8
NH_4-N/ N_{total}	0.4	0.4	0.4	0.4	0.6	0.7	0.7	0.7
BOD_5 [g kg^{-1}]	7.6	7.6	11	9.1	29	28	26	23
COD [g kg^{-1}]	80	73	88	63	182	135	169	179
BOD_5 / COD	0.1	0.1	0.1	0.1	0.2	0.2	0.2	0.1

Tab. 2.10: Composition of bovine excrements (BE) and reference manures (BM)

	Excrements						Reference manures					
Matrix / Parameter	BE-1	BE-2	BE-3	BE-4	BE-5	BE-6	BM-1	BM-2	BM-3	BM-4	BM-5	BM-6
ds [%]	13	13	10	13	12	13	adjusted to 10 %					
R_{min} [% TS]	19	15	24	15	12	13	---	---	---	---	---	13
Cu [mg kg^{-1}]	13	7	6	12	6	7	10	5	6	9	5	5
P [g kg^{-1}]	0.9	0.7	1.1	1.0	0.7	0.9	0.7	0.6	1.1	0.8	0.6	0.8
TOC [g kg^{-1}]	47	54	40	50	42	57	39	42	39	39	37	44
pH	6.9	8.4	8.0	6.2	6.5	6.3	7.0	8.1	8.0	6.6	6.6	6.7
Eh [mV]	40	10	-20	-40	-40	-100	-40	-80	-20	10	-100	-160
O_2 [mg kg^{-1}]	< 0.1						< 0.1					
NH_4-N [g kg^{-1}]	1.6	4.5	4.0	1.6	1.2	2.3	1.3	3.2	4.0	1.3	0.9	1.6
N_{total} [g kg^{-1}]	4.1	6.4	6.5	3.5	3.1	4.4	3.2	5.0	6.5	2.6	2.5	3.8
BOD_5 [g kg^{-1}]	9.4	11	6.0	23	8.5	18	8.3	7.3	6.0	14	9.3	9.9
COD [g kg^{-1}]	76	70	65	83	62	120	71	60	65	50	59	112

Tab. 2.11: Composition of pig excrements (PE) and manures (PM)

	Excrements						Reference manures					
Matrix / Parameter	PE-1	PE-2	PE-3	PE-4	PE-5	PE-6	PM-1	PM-2	PM-3	PM-4	PM-5	PM-6
ds [%]	23	18	21	17	16	13	adjusted to 5 %					
R_{min} [% TS]	22	19	15	21	17	16	---	---	---	---	---	16
Cu [mg kg^{-1}]	25	29	29	16	16	8	6	8	7	5	5	3
P [g kg^{-1}]	4.4	3.8	2.2	2.3	3.0	2.4	1.0	1.1	0.5	0.7	0.9	1.0
TOC [g kg^{-1}]	74	93	103	66	70	56	19	20	21	22	20	18
pH	7.4	7.3	5.7	6.3	6.1	6.8	7.7	7.0	5.8	7.5	6.7	7.3
Eh [mV]	- 130	-90	40	-50	-100	-180	-180	-90	60	-170	-110	-180
O_2 [mg kg^{-1}]	< 0.1						< 0.1					
NH_4-N [g kg^{-1}]	9.2	6.2	3.4	5.8	5.7	4.5	1.9	2.0	0.9	2.0	1.7	1.8
N_{total} [g kg^{-1}]	13.8	9.9	9.4	9.0	8.9	6.8	3.0	3.0	2.3	2.6	2.7	2.8
BOD_5 [g kg^{-1}]	27	23	28	25	21	21	10	10	10	12	9.5	9.1
COD [g kg^{-1}]	173	98	153	124	147	103	40	41	41	32	49	48

2.4 Preparation of reference manures

On the basis of the characterized excrement samples, reference-manure samples were prepared for laboratory tests on degradation of VMP. For this purpose, the dry substance contents of bovine and pig manures of 10 % and 5 %, respectively, were adjusted by adding tap water to the related excrement samples. These dry substance contents corresponded to the average values given for Germany (Buning, 1997; Merkel, 2005; Møller et al., 2004; Schuchardt and Hahne, 1996).

Consequently, the prepared manure samples were matrix characterized to identify differences to the primary excrement samples. For bovine excrements and manures, the physico-chemical parameters under study are listed in **Tab. 2.10**. Considering single parameters, minor differences occurred between the excrement and manure matrices under study (Merkel, 2005). Starting from excrements of dry substance contents ranging from 10 to 13 %, it became obvious that the primary different total organic carbon contents were compensated by the addition of tap water to prepare the manure samples. pH values, redox potentials, dissolved oxygen contents and the ammonium nitrogen/total nitrogen ratios as well as the biological/chemical oxygen demand ratios remained nearly unchanged.

The comparison between pig excrements and manures also showed differences for both matrices. The defined preparation of corresponding references manures, however, reduced the heterogeneity of pig manures from manure tanks (**Tab. 2.11**). By means of this innovative approach of reference manure preparation, bovine and pig manures become matrices that are available for reproducible laboratory testing. There is a further relevant advantage that is not expressed by both tables: It is assured that these reference manures are free of any VMP and biocide residues which may interfere the laboratory tests due to their bioactive properties.

2.5 Soil sampling

For testing on degradation and sorption of VMP in manured soils, topsoil samples were taken from two farmlands (**Tab. 2.12**). Soil samples were sieved < 2 mm and stored at -20 °C for 90 days at maximum. Prior to the start of the mobility tests, soil samples were air dried. For degradation tests, however, soil samples were reconditioned at am-

bient temperature for 3 days. Microbial activity was determined by substrate induced respiration (SIR) (ISO, 2003). Thus, the oxygen demand ranged from 1.9 to 2.3 mg O_2 100 g^{-1} dry soil h^{-1} for the microbially active clay soil and from 0.3 to 0.5 mg O_2 100 g^{-1} dry soil h^{-1} for the sand soil, independent if sampled field-fresh or reconditioned.

Tab. 2.12: Soil properties of soils under investigation

Investigation site	**Adenstedt**	**Nienwohlde**
Landscape	Ambergau	Lüneburger Heath
Vegetation	wheat	potato
Soil type	Luvisol	Cambisol
Texture:	silty clay	silty sand
sand [%]	5.0	76.8
silt [%]	55.5	19.1
clay [%]	38.5	4.2
pH $_{(CaCl_2)}$	6.9	5.4
WHC_{max} [%]	42.5	26.6
OC [%]	1.6	0.8
Microbial biomass [%]	3.7	1.4
SIR [mg O_2 100 g^{-1} h^{-1}]	2.1	0.4

WHC_{max} : maximum water holding capacity
OC : organic carbon
SIR : substrate-induced respiration

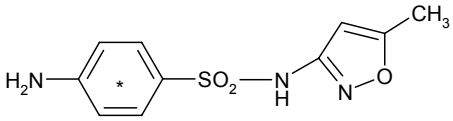
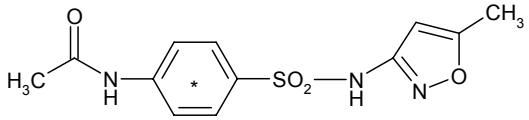
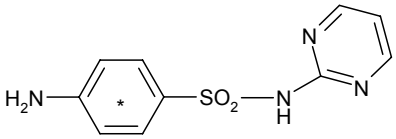
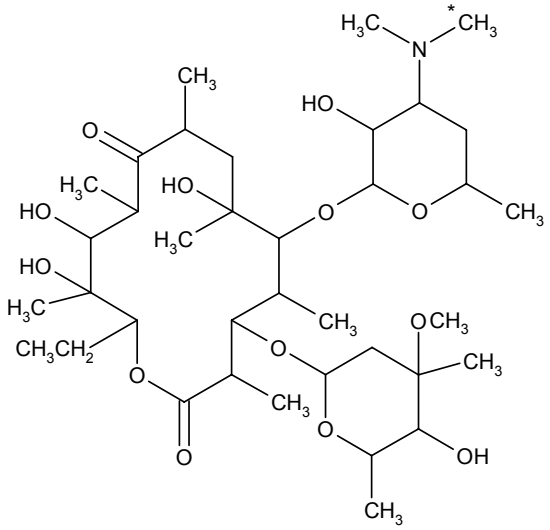
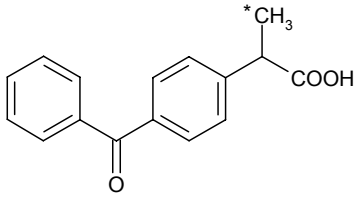
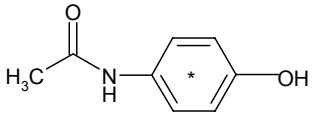
3 Test substances

Besides the matrix characterization of bovine and pig excrements and reference manures, the development of the technical protocol for degradation tests of VMP in manure as well as for degradation and sorption tests in manured soils focused on the application of test substances. Due to the undeniable advantage of setting up detailed mass balances with special consideration of mineralization and formation of extractable and non-extractable residues, the test substances were applied as ^{14}C-labeled radiotracers. Although the extractable fractions were screened for parent compounds and metabolites by means of radio thin layer chromatography (RTLC), these laboratory tests were merely targeted at the reproducible application of the reference manure samples. It was not the goal of the Manure Project to elucidate the metabolic pathways of the test substances by the concrete identification of their metabolites.

VMP under study belonged to different pharmaceutical groups. Their chemical structures are shown in **Tab. 3.1**. From the group of sulfonamide antibiotics, sulfamethoxazole (SMZ) and sulfadiazine (SDZ), which are frequently used in veterinary medicine to prevent and treat bacterial diseases (Boxall et al. 2004; Löscher et al. 2003), were tested. In animal as well as in human metabolism, sulfonamides are partly decomposed to the corresponding acetyl-metabolites. After sulfamethoxazole treatment of cattle, approximately 70 % were excreted via urine as the unchanged parent compound accompanied by 28 % of acetyl-sulfamethoxazole and 2 % further conjugates (Institut für Veterinärpharmakologie und -toxikologie, 2006). In water, manure and soil, this metabolite undergoes deacetylation and sulfamethoxazole is regenerated (Göbel et al., 2005). Therefore, the degradation of ^{14}C-acetyl-sulfamethoxazole (A-SMZ) in manures was tested, too.

The macrolide antibiotic erythromycin (ERY) is widely used in veterinary medicine against numerous bacterial infections (Schluesener et al., 2006). Thus, this macrolide was included in the fate monitoring studies in manures and manured soils. Additionally, the degradation of ketoprofen (KET) was examined in bovine manure. Calves and dairy cattles are medicated with this non-steroidal anti-inflammatory drug to control pain after dehorning (Stafford and Mellor, 2005; Milligan et al. 2004; Faulkner and Weary, 2000). Furthermore ketoprofen is used for the treatment of mastitis (Kluge and Ungemach, 1998).

Tab. 3.1: Chemical structures of test substances under study

Substance	Chemical structure
Sulfamethoxazole (SMZ) N^1-(5-methyl-3-isoazolyl)-sulfanilamide	 [phenyl ring-U-^{14}C]-sulfamethoxazole
Acetyl-sulfamethoxazole (A-SMZ) N^4-(3-acetyl-)-N^1-(5-methyl-3-isoxazolyl)-sulfanilamide	 [phenyl ring-U-^{14}C]-acetyl-sulfamethoxazole
Sulfadiazine (SDZ) N^1-(2-pyrimidinyl)-sulfanilamide	 [phenyl ring-U-^{14}C]-sulfadiazine
Erythromycin (ERY) 6-(4-dimethylamino-3-hydroxy-6-methyl-oxan-2-yl)oxy-14-ethyl-7,12,13-trihydroxy- 4-(5-hydroxy-4-methoxy-4,6-dimethyl-oxan-2-yl)oxy-3,5,7,9,11,13-hexamethyl-1-oxacyclotetradecane-2,10-dione	 [N-methyl-^{14}C]-erythromycin
Ketoprofen (KET) 2-(3-benzoylphenyl) propionic acid	 [propionic acid-3-^{14}C]-ketoprofen
Paracetamol (PCM) 4-(acetamidophenol)	 [phenyl ring-U-^{14}C]-paracetamol

Paracetamol (PCM) completed the patterns of test substances. Besides the wide application in human medicine, this antiphlogistic is used in pig herds to inhibit the anorexia of infection and improves the clinical efficacy of the antiinfectious therapy against bacterial respiratory diseases (del Castillo et al., 2006; Emmerich and Ungemach, 2006).

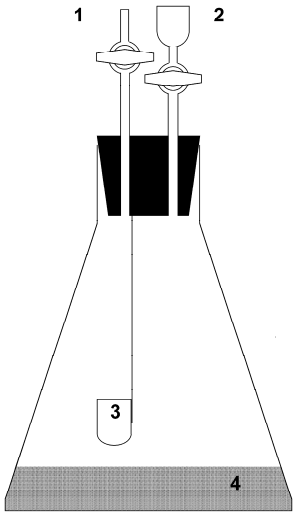
4 Experimental design

The application of ^{14}C-labeled radiotracers provides the undeniable advantage of balancing fate and behavior of applied test substances in complex environmental samples. In order to differentiate between mineralization (MIN), extractable (ER) and non-extractable residues (NER), the use of closed laboratory-batch systems allowing for a discontinuous gas exchange was required. Alternatively, flow-through systems were applied. Both systems have been described in OECD guidelines (1981a, 2002) for testing on biodegradability of chemicals in soils under predominantly aerobic conditions typical for terrestrial topsoils. However, storage of bovine and pig manure in tanks is dominated by anaerobic conditions. Hence, there is not any necessity for a continuous gas exchange in degradation tests of VMP in manures. The requirement that laboratory testing has to be conducted at definite time and cost limits thus favored the application of the laboratory-batch systems. Compared to the flow-through systems, the first ones have the advantages of the less required laboratory space, the possible use of incubators instead of climatic chambers as well as effortless and cost-extensive handling. Nevertheless, the performance of both systems was compared by parallel laboratory-batch tests and matrix characterization.

4.1 Degradation tests of VMP in manures

Laboratory-batch tests

The degradation tests were conducted in the laboratory-batch systems (**Fig. 4.1**). For this purpose, 50 to 100-g aliquots of bovine or pig manure with 10 and 5 % dry substance content, respectively, and matrix characterized as mentioned before, were filled into the 300-mL Erlenmeyer flasks. Then, the manure samples were fortified with 50 µL of one ^{14}C-labeled test substance dissolved in an appropriate solvent. Initial concentrations of 34 to 1380 µg VMP kg^{-1} manure were applied (**Tab. 4.1**).



1: inlet valve, **2:** outlet valve with activated charcoal filter, **3:** internal ^{14}C-carbon dioxide trap (8 mL of 0.1 M potassium hydroxide solution), **4:** manure sample

Fig. 4.1: Laboratory-batch system for degradation tests of ^{14}C-labeled VMP in manures

The adjustment of the applied amounts of the radiotracers focused on the substance specific exposure assessment as well as on the analytical feasibility mainly defined by the specific radioactivity of the radiotracer under study. According to the OECD guideline 307 (2002), the radiochemical purity was ≥ 95 %. Due to the instability of ^{14}C-erythromycin (Biotrend, 2005), however, its purity was 75 %. The standard solution contained a second substance of 25 %. Probably, the decomposition product ^{14}C-erythromycin-H_2O was formed by an intramolecular dehydration (Hirsch et al., 1999). A second substance (14 %) was also detected in the standard solution of ^{14}C-ketoprofen. Nevertheless, the use of these test substances was appropriate to investigate the reproducible applicability of reference or test manures. In parallel tests, the effects of the VMP and solvent (all solvents: Merck, Darmstadt, Germany) on the manure inherent microbial activity were checked. Furthermore, those tests were used for the matrix characterization of the manure samples of every incubation interval.

Tab. 4.1: ^{14}C-labeled radiotracer standard solutions under study

Radio-tracer	Solvent	Specific radioactivity [MBq mmol^{-1}]	Radio-chemical purity [%]	Concen-tration [Bq µL^{-1}]	Concen-tration [µg µL^{-1}]	Concen-tration [µg kg^{-1}]
SMZ[#]	CH_3CN	148	98	1470	2.5	1250
SMZ[#]	CH_3CN	148	98	650	1.1	560
A-SMZ	CH_3CN	148	100	1380	2.8	1380
SDZ	CH_3CN	814	94	1420	0.4	220
ERY	C_2H_5OH	2035	75	1400	0.5	500
PCM	CH_3OH	200	100	1040	0.8	390
KET	$(CH_3)_2CHOH$	2110	86	560	0.1	34

[#]: The degradation of SMZ in manure was tested in 2 different concentrations

Subsequently, the laboratory-batch systems were stoppered with glass stoppers equipped with inlet and outlet valves to allow for a discontinuous gas exchange. Additionally, the stoppers were equipped with an internal trap filled with 8 mL 0.1 M potassium hydroxide solution (Kreuzig and Höltge, 2005) to absorb ^{14}C-carbon dioxide, potentially released by mineralization. After rinsing the batch systems with nitrogen to realize anaerobic conditions, they were incubated in the dark at 20 ± 1 °C for 0, 3, 7, 30, 72, 100 and 177 days. All degradation tests were conducted in duplicates. The potassium hydroxide solutions were exchanged every 7 days and the 8-mL aliquots were mixed with Quicksafe A (10 mL; Zinsser, Frankfurt, Germany) in order to determine the mineralization via liquid scintillation counting (LSC) (Tri-Carb 2500 TR, Packard, Meriden, CT, USA). After the potassium hydroxide exchange, the batch systems were rinsed with nitrogen for 5 min to save anaerobic conditions and incubated again.

At the end of every incubation interval, the manure samples were directly extracted on a horizontal shaker (Type 3020; Gesellschaft für Labortechnik, Burgwedel, Germany) at 200 rpm overnight or alternatively stored at -20 °C until the start of the analytical procedure. The solvent used was ethyl acetate (150 mL). Its exhaustive extraction effi-

ciency was tested before (Kreuzig and Höltge, 2005, Kreuzig et al., 2005a, b). For the extraction of erythromycin, 50-g manure samples were mixed with 16.7 g urea. Then, 20 mL of phosphate buffer solution (33.5 g dipotassiumhydrogen phosphate L^{-1} and 1.1 g potassium dihydrogen phosphate L^{-1}, adjusted to pH 8.0) and 150 mL ethyl acetate were added (Schluesener et al., 2006).

The suspensions were decanted through filters and the manure matrix was threefold rinsed with 50 mL ethyl acetate each. Extracts were pooled and rotary evaporated. Subsequently, 10 to 100-µL aliquots were mixed with Quicksafe N (10 mL; Zinsser, Frankfurt, Germany) and scintillation counted to determine the amounts of extractable residues. Selected extracts were screened for the parent compound and metabolites by means of radio thin layer chromatography (RTLC) (Tracemaster 20 Automatic TLC-linear analyzer B 284; Berthold, München, Germany). For this purpose, the extracts were applied onto silica gel plates (20 x 20 cm^2, Merck, Darmstadt, Germany) and developed by using two different solvents or solvent mixtures, respectively (**Tab. 4.2**).

Tab. 4.2: Solvents for radio-thin layer chromatography

Radio-tracer	Identification		Confirmation	
		R_f		R_f
SMZ	ethyl acetate	0.82	chloroform/n-butanol (8:1)	0.70
A-SMZ	ethyl acetate	0.50	chloroform/n-butanol (8:1)	0.42
SDZ	ethyl acetate	0.53	chloroform/n-butanol (8:1)	0.41
ERY	dichloromethane/methanol/ 25 % ammonium hydroxide	0.40	methanol/acetone (1:1)	0.25
PCM	ethyl acetate/acetone/ acetic acid	0.62	toluene/isopropanol/25% ammonia (30:60:10)	0.72
KET	hexane/diethyl ether/ formic acid (50:50:1)	0.42	toluene/acetone/ formic acid (60:39:1)	0.60

The manure matrix, already extracted and mixed with 20 g of sea sand and 5 g cellulose, was air dried and thoroughly ground. Finally, 150-mg aliquots were combusted using an oxidizer (OX-500; Harvey Instruments, Hillsdale, NJ, USA). The released ^{14}C-carbon dioxide was trapped in Oxysolve-C400 (15 mL; Zinsser, Frankfurt, Germany) and scintillation counted to determine amounts of non-extractable residues.

Modification of the test parameters

The heterogeneity and complexity of bovine and pig manures is still reflected by their dry substance contents ranging between 0.4 and 12 % (Bouwman and Reus, 1994; Lallai et al., 2002; Merkel 2005; Møller et al., 2004; Schuchhardt and Hahne, 1996; Shah et al., 2004). Therefore, bovine and pig reference-manure samples under study were adjusted to 2.5, 5 and 10 % dry substance content to investigate the impact of the dry substance on the degradation of ^{14}C-sulfamethoxazole and ^{14}C-erythromycin during the incubation period up to 30 days.

The storage conditions in aboveground manure silos are particularly affected by ambient temperatures that were measured in Germany at 8 to 10 °C on average (Deutscher Wetterdienst, 2005). For the Netherlands, annual average temperatures of 12 and 15 °C are given for bovine and pig manures, respectively (Monforts and Tarazona Lafarga, 2003). To study the temperature impact, therefore, incubation temperatures were 5, 10, and 20 °C.

Tests on the release of ^{14}C-carbon dioxide

In order to study whether test substances undergo mineralization under anaerobic conditions in manure, indicated by the release of ^{14}C-carbon dioxide, ^{14}C-ibuprofen was included into the degradation tests in pig manure. This test substance, frequently administered as an analgesic drug in human medicine, is a structural analogue of ketoprofen. From testing its degradation in sewage sludge and soils (Kreuzig et al., 2005a), it was well known for its relevant mineralization rates. The results of the respective degradation test of ^{14}C-ibuprofen in sewage sludge are illustrated in **Fig. 4.2** showing dynamic processes with decreasing amounts of extractable residues and decreasing amounts of non-extractable ones within 102 days. Additionally, the amounts of released ^{14}C-carbon dioxide were 15 % within the first 28 days of the incubation period reaching 29 % after 102 days.

Within the 30-day degradation tests of ^{14}C-ibuprofen in pig manure, the release of ^{14}C-carbon dioxide only amounted to 0.4 % of the initially applied radioactivity (0.03 MBq in 50 µL; 33 µg ibuprofen kg^{-1} manure) confirming that mineralization principally occurred (**Fig. 4.3**). In contrast to the degradation test in sewage sludge, however, extractable and non-extractable residues remained nearly constant. Dynamic decomposition processes seemed to be largely suppressed emphasizing the lower degradation of ^{14}C-ibuprofen in manure than found in sewage sludge or soil.

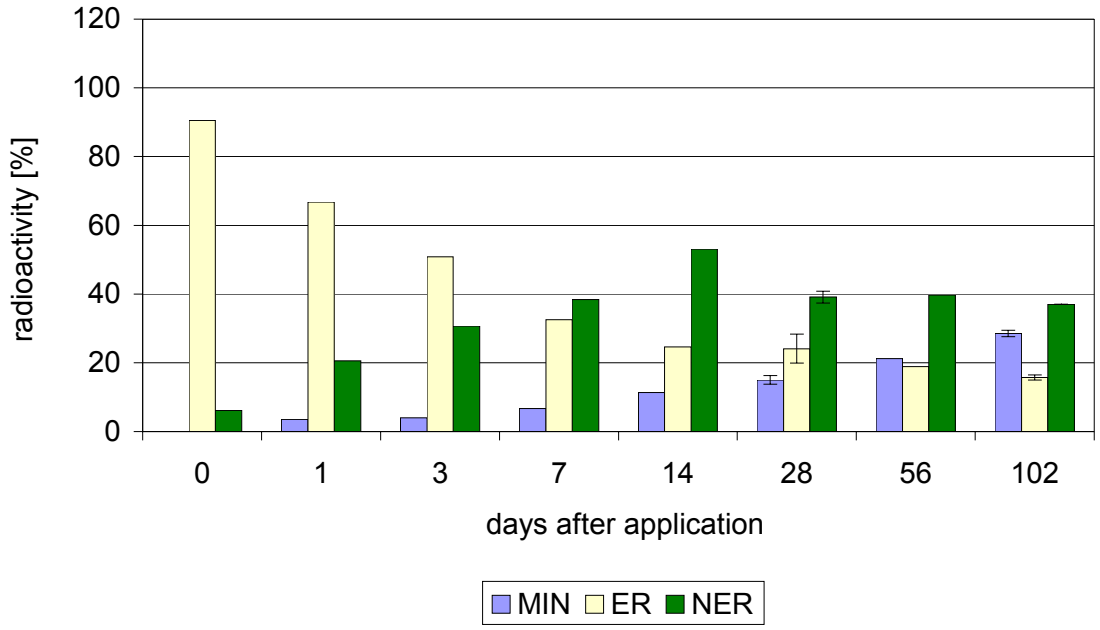


Fig. 4.2: Degradation of ^{14}C-ibuprofen in sewage sludge (balances: 85 ± 7 %) (Kreuzig et al., 2005a)

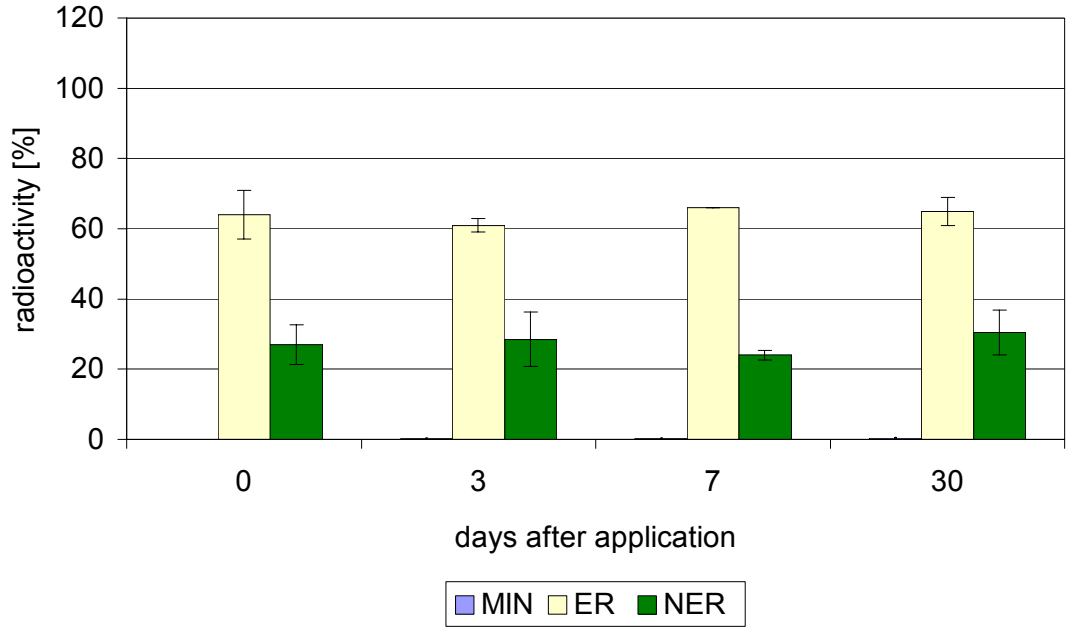
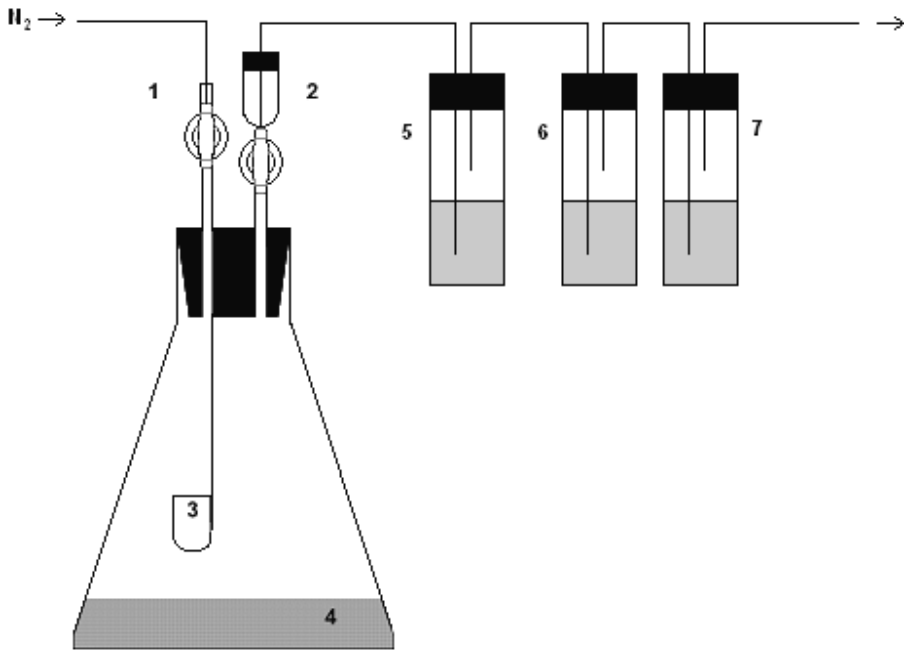


Fig. 4.3: Degradation of ^{14}C-ibuprofen in pig manure (balances: 92 ± 3 %)

Tests on the release of ^{14}C-methane and related volatiles

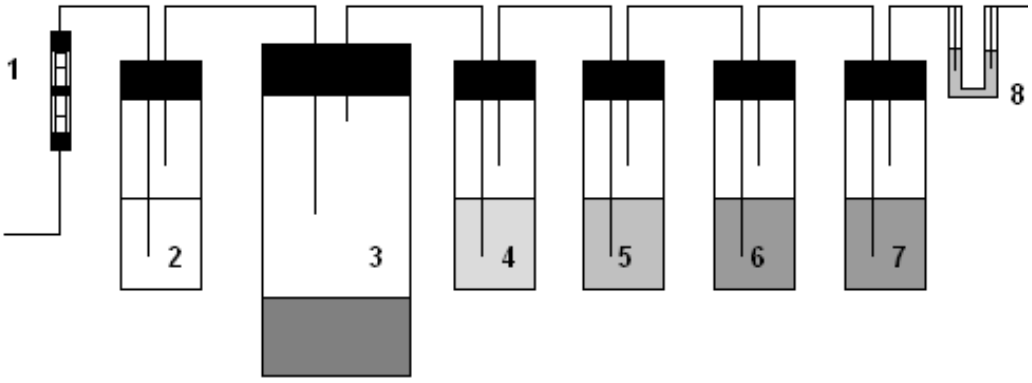
During manure storage, the organic biomass is decomposed by facultatively and obligatorily anaerobic microorganisms (Lallai, 2002; Loke et al. 2003; Møller et al. 2004). Under strictly methanogenic conditions, revealed by redox potentials of Eh < -200 mV, biogas is formed containing approximately 70 % methane and 30 % carbon dioxide. According to this principle, the decomposition of the applied ^{14}C-labeled radiotracer may be accompanied by the release of ^{14}C-methane. To check for this process, selected degradation tests of VMP, i.e., 177-day samples preferred, were used for additional gas analyses of the incubation flasks´ headspace. Hence, the laboratory-batch systems were equipped additionally with special stripping devices (**Fig. 4.4**).



1, 2: inlet-/outlet valves, **3:** internal ^{14}C-carbon dioxide trap (8 mL of 0.1 M potassium hydroxide solution), **4:** manure sample, **5:** ethylene glycol trap, **6:** sulfuric acid trap, **7:** scintillation cocktail Oxysolve-C400 trap

Fig. 4.4: Laboratory-batch system with stripping device

Directly before the exchange of the potassium solution every 7 days, the headspaces were degassed by a gentle stream of nitrogen. The stripping gas was passed through 3 external traps filled with 10 mL ethylene glycol, 10 mL sulfuric acid (0.05 M) and 10 mL Oxysolve C-400 (Zinsser Analytic GmbH, Frankfurt), to trap ^{14}C-methane or volatile metabolites and ^{14}C-carbon dioxide, respectively. Subsequently, the absorption solutions were scintillation counted. However, radioactivity was not detected in any test. These results were exemplarily confirmed by tests in the flow-through systems.



1: flow meter, **2:** gas moistening flask, **3:** incubation flask with the 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

Fig. 4.5: Flow-through system for degradation tests of ^{14}C-labeled VMP in manures

Flow-through systems

As described in the OECD guideline 307 (2002), a constant air flow passes the incubation flask and the appended absorption traps of the flow-through systems when the biodegradability of chemicals in aerobic topsoils is studied (**Fig. 4.5**). However, to realize anaerobic conditions typically for manure tanks, the system was streamed by means of nitrogen. Indeed, it has to be considered that manure tanks are not passed by a permanent gas flow. There, the gas exchange only occurred at the manure´s surface controlled by diffusion. In the 500-mL incubation flasks, 100-g bovine or pig ma-

nure samples were filled in and fortified with ^{14}C-labeled VMP as described before. A minor nitrogen stream (1750 cm^3 min^{-1}), moistened by demineralized water, passed through the incubation flasks and the appended traps for ^{14}C-methane or volatile metabolites and ^{14}C-carbon dioxide. The incubation experiments were conducted in duplicates in the dark at 20 ± 2 °C for 7 days. At the end of the incubation period, 10-mL aliquots of the absorption solutions of the traps were mixed with Quicksafe A (10 mL; Zinsser, Frankfurt, Germany) and scintillation counted. However, radioactivity was not determined in the traps. These results matched those of the batch-system tests with discontinuous gas exchange. Subsequently, the manure samples were extracted and analyzed as described before.

4.2 Preparation of test manures

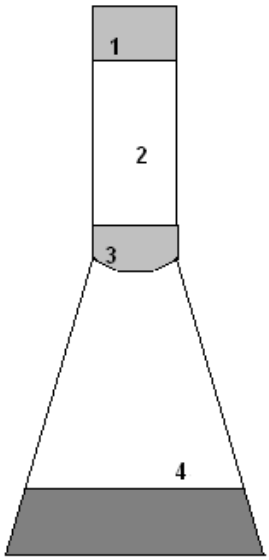
In order to mimic the VMP entry route into soils already under laboratory conditions, test manures were prepared. For degradation tests, 10 to 20 g manure samples (fresh weight basis; matrix characterized) were filled into each incubation flask and spiked with one test substance as described for the degradation tests in manure. To study the sorption behavior, 5-g manure samples were used. In accordance to the degradation tests in manure, the incubation flasks were incubated under anaerobic conditions for 7 days. Parallel tests were conducted to set up mass balances and to quantify the amount of the test substance by means of RTLC screening.

4.3 Laboratory tests on degradation in manured soils

To investigate the impact of manure matrix on the metabolic fate, degradation tests were performed after test-manure application. For this purpose, 50 to 100 g microbially active soil samples, matrix characterized and adjusted to approximately 40 % of the maximum water-holding capacity, were added directly into the incubation flasks of the test-manure preparation. This was done to avoid losses that would inevitably occur when test-manure samples were transferred into flasks containing soil samples. The used manure/soil ratio did not exceed a ratio of 5:1, corresponding to the fourfold of the maximum manure amount accepted by the German Ordinance Concerning Fertilizers

(2006). The calculation of the manure amount was based on the following parameters: 170 kg N ha^{-1}, 5 kg N kg^{-1} manure, 1500 kg soil m^{-3} and 0.05 m soil depth. Each batch was incubated in the dark at 20 ± 1 °C for at least 28 days and up to 102 days. For quality assurance, batch experiments were performed in duplicates and mass balances were set up. In the laboratory batch-systems (**Fig. 4.1**), potassium hydroxide solutions and air were simultaneously exchanged every 3 to 4 days to conserve aerobic conditions. In the flow-through systems these conditions were received by a constant air flow (**Fig. 4.5**).

In both systems, the absorption solutions, i.e., potassium hydroxide, ethylene glycol or sulfuric acid, were exchanged every 7 days. Additionally, the biometric-flask system, also described in the OECD guideline 307 (2002), was exemplarily tested. There, the continuous gas exchange through the soda-lime trap is controlled by diffusion (**Fig. 4.6**).



1: glass wool, **2:** soda-lime trap (15 g), **3:** oil treated glass wool, **4:** soil sample

Fig. 4.6: Biometric-flask system with soda-lime trap for degradation tests in manured soils

As described for the degradation tests, acetyl-sulfamethoxazole, ketoprofen and paracetamol samples were extracted with ethyl acetate (150 mL), while a mixture of urea,

phosphate buffers and ethyl acetate was used for the extraction of ^{14}C-erythromycin. The soil matrices already extracted were air dried and thoroughly ground. Finally, 150-mg aliquots were mixed with 10 mg cellulose (Merck, Darmstadt, Germany) and combusted using an oxidizer to determine amounts of non-extractable residues.

In order to understand the relevance of manure matrix induced degradation processes, tests after standard application were exemplarily conducted, too. In accordance to guidelines of OECD (1981a, 2002) and BBA (1986, 1998) on the biodegradability of chemicals in soils, aliquots of the ^{14}C-test substance standard solution (50 µL) were directly fortified to 50-100 g microbially active soil samples.

4.4 Laboratory tests on sorption in manured soils

For testing on sorption of VMP in manured soils, the 5-g test manure samples were transferred into 100-mL centrifuge tubes using the 35-mL calcium chloride (0.01 M) solution. Then, 25-g soil samples were added according to a soil/water ratio of 1:1.4 (Boesten, 1990, von Oepen et al., 1991, Rütters et al., 1999). The closed centrifuge tubes were shaken on a horizontal shaker at 220 rpm and 22 ± 2 °C for 24 hours. Subsequently, the suspensions were centrifuged at 4000 rpm (Megafuge, 1.0, Heraeus, Hanau, Germany) for 30 min. The aqueous phases were removed and 10-mL aliquots were added to 10 mL of Quicksafe A (Zinsser, Frankfurt, Germany) and liquid scintillation counted. To determine the radioactivity in the solid phase, 150-mg aliquots of the moist soil samples were mixed with 10 mg cellulose and then combusted.

According to the OECD guideline106 (2000), soil samples should be equilibrated first using 90 % of the total volume of calcium chloride for 12 hours. To adjust a soil/water ratio of 1:1, 45 mL calcium chloride solution (0.01 M) were added to 50-g soil samples and equilibrated on a horizontal shaker at 220 rpm and 22 ± 2 °C for 12 hours. Thereafter, 5-g test manure samples containing ^{14}C-erythromycin residues were transferred into the centrifuge tube by using 5 mL calcium chloride solution. Then, the closed centrifuge tubes were shaken on a horizontal shaker at 220 rpm and 22 ± 2 °C for 24 h and analyzed as described before.

Additional tests were carried out to understand the manure matrix effects without any aging processes during the manure storage. For this purpose, 5-g manure samples were spiked with 50 µL standard solutions of one test substance each and immediately

mixed with 25-g soil samples and 35 mL calcium chloride solution. The analytical processing was carried out as described before.

In further experiments, the K_d values were determined in batch-equilibrium tests after standard application. According to OECD guidelines 106 (1981b, 2000), standard solutions (50 µL) were introduced into 100-mL centrifuge tubes, filled with 5 mL 0.01 M calcium chloride solution. The mixtures were homogenized by sonication for 5 min. After the addition of the 25 to 50-g soil samples and further 30 to 45 mL calcium chloride solution, according to a soil/water ratio of 1:1.4 and 1:1, respectively, the closed centrifuge tubes were shaken. Samples were subsequently analyzed as described for the tests after test-manure application. For quality assurance, all soil/water distribution coefficients were determined in 4 replicates.

4.5 Preparation of intra- and interlaboratory tests

The development of the analytical methods from the matrix characterization of excrement samples to the preparation of reference-manure samples and the degradation tests of VMP in manures was accompanied by the compilation of standard operating procedures. Their applicability was checked in intra- and interlaboratory tests. For the first methodological check on the matrix characterization, scientific and technical staff members of the Institute of Ecological Chemistry and Waste Analysis (ECO) with different experiences in the field of analytical chemistry participated in the first intralaboratory test series.

On the basic of those results, a workshop was held in Braunschweig on 5 July 2006 to introduce the methods of the matrix characterization and of the degradation tests in manures to the external participants: Fraunhofer-Institut für Umwelt-, Sicherheits- und Energietechnik, Oberhausen, Germany (UMS), RCC-Ltd., Itingen, Switzerland (RCC) and Biology V, RWTH Aachen, Germany (BIO). Thus, bovine and pig excrements were matrix characterized by UMS, ECO and RCC, while the degradation tests of ^{14}C-sulfadiazine were performed by RCC, ECO and BIO. For those purposes, excrement samples were taken at the FAL Institute of Animal Nutrition, Braunschweig, preconditioned and intermediately stored at -20 °C at ECO. Then, the excrement samples, the ^{14}C-labeled test substance, the standard operation procedures and the data sheets were dispatched to the participants by ECO. The concrete schedule of the interlaboratory

tests is listed in **Tab. 4.3**. On the participants site, due to the appointed assignment of tasks, the excrement samples were matrix characterized for ds, R_{min}, TOC, Cu, P, pH, Eh, O_2, NH_4-N, N_{total}, BOD_5 and COD. Reference manures were prepared at 10 % ds for bovine manure and 5 % for pig manure. The degradation tests were conducted at 20 °C in the dark for 0 and 7 days of incubation under discontinuous nitrogen exchange to guarantee anaerobic conditions.

Tab. 4.3: Schedule of the first interlaboratory tests on the matrix characterization of excrement samples and degradation tests of ^{14}C-sulfadiazine in reference manures

Date	Measure
06/20/2006	sampling of excrements at FAL Institute of Animal Nutrition
07/12/2006	end of preconditioning and start of storage at -20 °C at ECO
08/22/2006	dispatch of test materials - bovine and pig excrement samples (2.5 kg in dry ice) - ^{14}C-labeled sulfadiazine dissolved in acetonitrile - standard operation procedures - data sheets
08/28/2006	possible start of the interlaboratory tests
09/30/2006	data transfer to ECO

Finally, intralaboratory tests on the degradation of ^{14}C-erythromycin in manured soils were performed at ECO with three test persons of different experiences. For this purpose, bovine and pig reference manures were used to prepare test manures containing 7-day aged ^{14}C-erythromycin residues. After test-manure application, the manured soil samples were incubated for 7 days. Then, mass balances were set up for mineralization, extractable and non-extractable residues.

5 Degradation tests of VMP in bovine and pig reference manures

The degradation tests in bovine and/or pig manures were focused on ^{14}C-sulfamethoxazole, ^{14}C-acetyl-sulfamethoxazole, ^{14}C-sulfadiazine, ^{14}C-erythromycin, ^{14}C-ketoprofen and ^{14}C-paracetamol. First, long-term degradation tests with incubation periods up to 177 days were conducted to simulate the long-term manure storage up to 6 months as required by the German Ordinance Concerning Fertilizers (2006). Those tests targeted at the evaluation of the reproducible applicability of the reference manure samples. Indeed, extractable fractions were RTLC screened for parent compounds and metabolites. However, the detected metabolites could not be identified in the frame of the Manure Project.

Additionally, parallel matrix characterization tests under respective conditions were conducted. The non-labeled test substances were applied in appropriate standard solutions to determine toxic effects to the manure inherent microorganisms by the test substances themselves or the solvents. These tests were furthermore used for the simultaneous characterization of the manure matrices under study. Thus, the dissolved oxygen contents and the redox potentials as well as the ammonium contents and the biological and chemical oxygen demands were determined in order to pursue decomposition processes of organic constituents of the manure matrices under anaerobic conditions. The microbial activity was observed in the manure samples under the respective test conditions by means of the biological oxygen demand, fully aware that this parameter is mainly focused on aerobic or facultative anaerobic microorganisms.

Supplementary short-term degradation tests with 30-day incubation periods were carried out to monitor the effects of different dry substance contents (2.5, 5, 10 %) of manures and different incubation temperatures (5, 10, 20 °C). Finally, different bovine and pig manure matrices were included into the test series to consider different animal species, animal ages and feeding conditions.

5.1 Sulfamethoxazole

Long-term degradation tests in bovine manure

In the first tests series in 2005, ^{14}C-sulfamethoxazole was applied at an initial concentration of 560 µg kg^{-1} manure. The balances of those tests are illustrated in **Fig. 5.1**.

Within the 177-day incubation period, the extractable ^{14}C-sulfamethoxazole residues continuously dropped from 96 to 3 % of the initially applied radioactivity while the non-extractable ones simultaneously increased up to 95 %. The relevance of non-extractable sulfonamide residues in bovine manure has been already found in the Runoff Project (Kreuzig et al., 2005b, Kreuzig et al., 2007, Höltge and Kreuzig, 2007). Mineralization was < 0.1 %.

In the second test series on sulfamethoxazole in 2006, bovine excrement samples, stored before at -20 °C for longer than 180 days, were used to prepare the respective reference-manure samples. The latter were then spiked with 1250 µg sulfamethoxazole kg^{-1} manure to improve the boundary conditions for the RTLC metabolite screening. However, the degradation of this test substance in bovine was not relevantly affect manure by the modified test conditions. Despite the enhanced concentration of sulfamethoxazole, the ethyl acetate extracts could only be RTLC screened up to the samples of day 0, 3, and 7 because of the rapid formation of non-extractable residues. Within this period, sulfamethoxazole disappeared rapidly.

In the extractable fraction of the 0-day sample, 67 % of the initially applied radioactivity accounted for sulfamethoxazole as the unchanged parent compound. This amount dropped within 7 days to 6 %. In the same period, the amounts of unidentified metabolites increased from 19 % to 25 %. These results obtained after the ethyl acetate development of the silica gel plates were confirmed by the use of a chloroform/n-butanol (8:1) mixture as the second solvent. In the 7-day sample, 4 % of the radioactivity initially applied was identified as the parent compound, accompanied by 26 % unidentified metabolites.

Besides the application of the laboratory-batch systems, flow-through systems were used in parallel short-term tests. The results listed in **Tab. 5.1** clearly depicted the equivalence of both laboratory-tests systems. Furthermore, the release of ^{14}C-methane and the formation of related volatile metabolites by the decomposition of the applied radiotracer was monitored. However, radioactivity could not be detected, neither in the stripped headspace of the laboratory-batch systems nor in the traps of the flow-through systems.

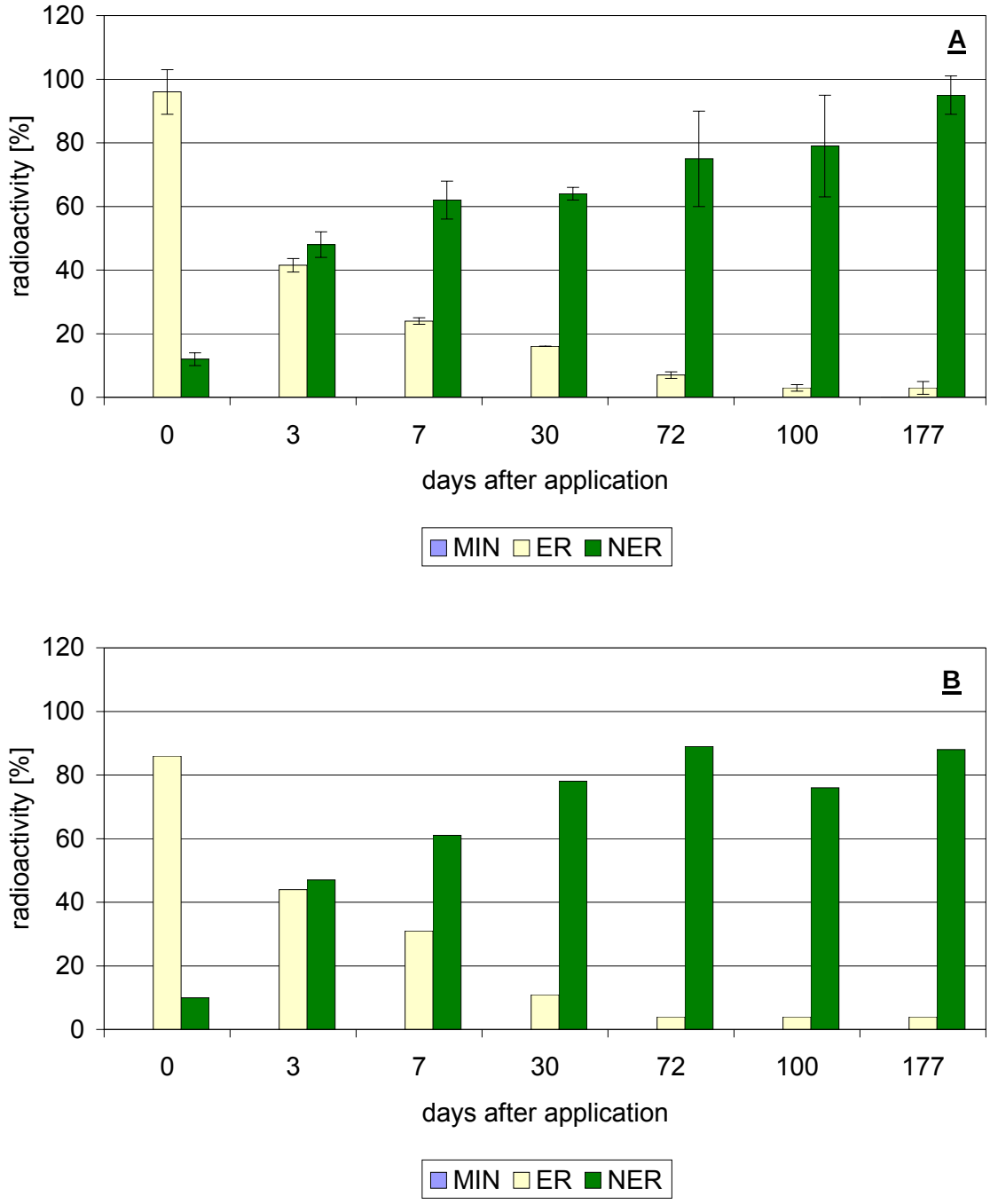


Fig. 5.1: Degradation of ^{14}C-sulfamethoxazole at A: 560 µg kg^{-1} (balances: 89 ± 10 %) and B: 1250 µg kg^{-1} bovine manure BM-1 (balances: 90 ± 5 %)

Tab. 5.1: Degradation tests of ^{14}C-sulfamethoxazole in manures within 7 days comparing laboratory-batch and flow-through systems

Matrix	**Bovine manure**		**Pig manure**	
Test system	batch system	flow-through system	batch system	flow-through system
MIN	< 0.1	< 0.1	< 0.1	< 0.1
ER	27	23	42	47
NER	65	73	58	54
Balances	92	96	100	101

In parallel matrix characterization tests with non-labeled sulfamethoxazole, the boundary conditions of the degradation tests were determined. As revealed by the dissolved oxygen contents of O_2 < 0.1 mg kg^{-1} and the redox potentials of Eh < +150 mV, test conditions remained permanently anaerobic within 177 days (**Fig. 5.2**).

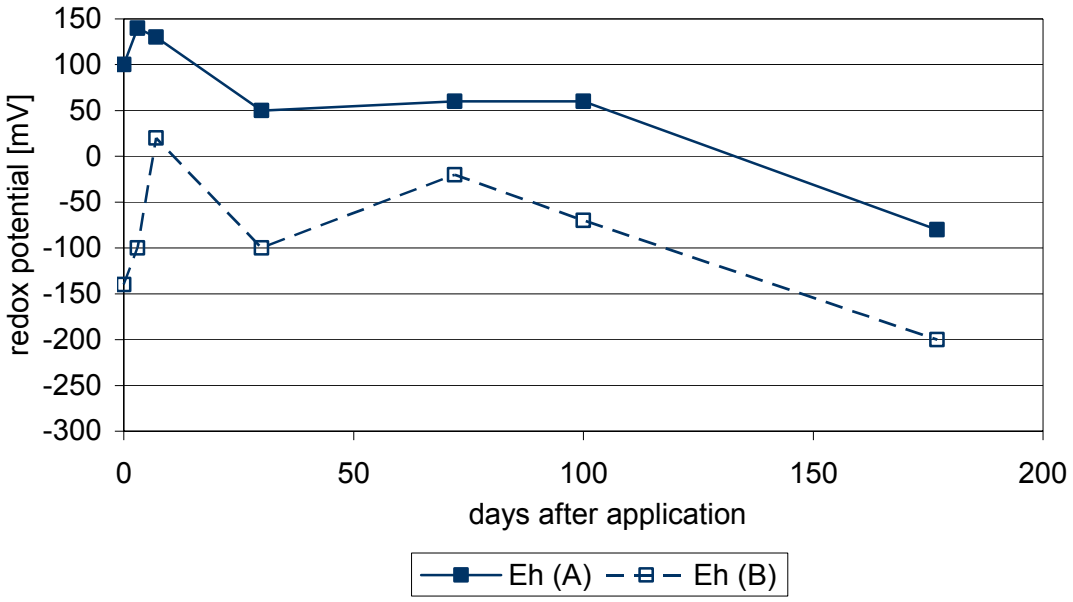


Fig. 5.2: Redox potentials during the degradation tests of sulfamethoxazole at A: 560 µg kg^{-1} and B: 1250 µg kg^{-1} bovine manure BM-1

Further information on processes in the manure itself was given by the ammonium contents that ranged from 1.2 to 1.7 g kg^{-1} (**Fig. 5.3**). With the content of total nitrogen between 3.2 and 3.8 g kg^{-1}, NH_4-N/N_{total} ratios from 0.4 or 0.5 were calculated. The biological oxygen demand acted as an indicator for the microbial activity in the manure samples. As indicated by values of 3.0 and 1.7 g BOD_5 kg^{-1} manure, the 177-day samples remained microbially active during the long-term incubation. This result was consistent with the dynamic transformation of extractable into non-extractable ^{14}C-sulfamethoxazole residues found in the corresponding degradation test.

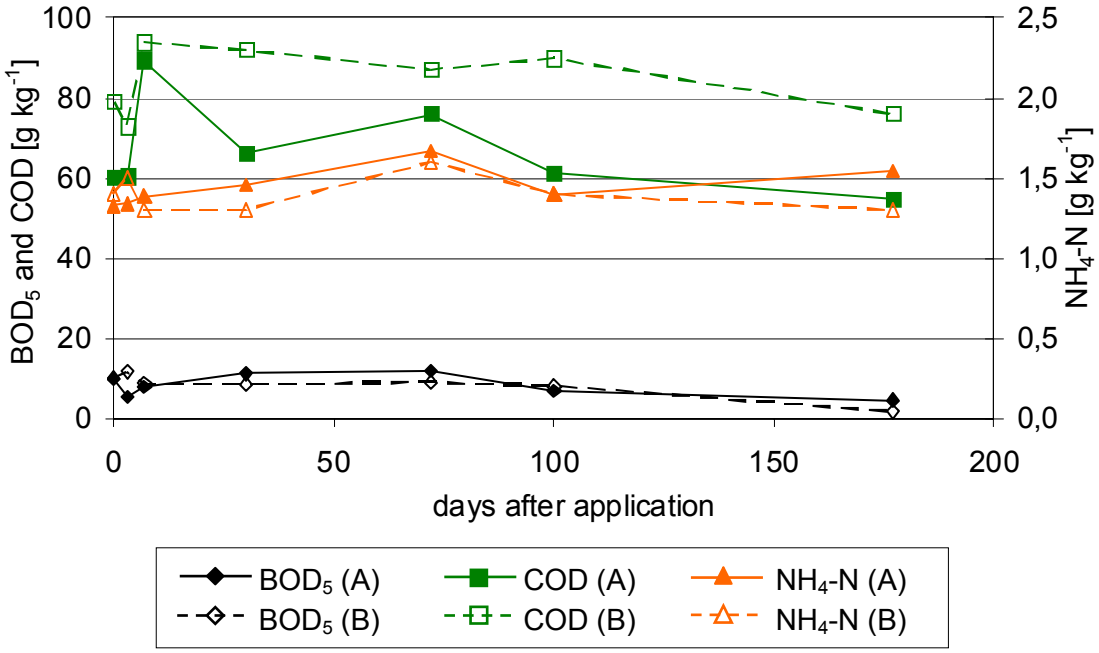


Fig. 5.3: BOD_5, COD and NH_4-N during the degradation tests of sulfamethoxazole at A: 560 µg kg^{-1} and B: 1250 µg kg^{-1} in bovine manure BM-1

In addition to the long-term degradation tests, matrix characterization tests were carried out to monitor effects by the test substance itself and by the used spiking solvent. Besides tests in which non-labeled sulfamethoxazole (560 and 1250 µg kg^{-1} bovine manure) was spiked in acetonitrile (50 µL), the effects of pure acetonitrile versus a non-spiked manure sample were monitored. In these tests, nearly the same results of the parameters under study were found (**Tab. 5.2**). Thus, effects of acetonitrile and the test substance, independent on its concentration, could be excluded.

Tab. 5.2: Effects of the spiking of sulfamethoxazole in acetonitrile on matrix parameters of bovine manure in the 177-day degradation tests

Parallel test / Parameter	**Matrix test**	**Solvent test** [50 µL]	**Substance test** [560 µg kg^{-1}]	**Substance test** [1250 µg kg^{-1}]
ds [%]	9	10	9	9 ± 0.7
TOC [g kg^{-1}]	38	44	37	40 ± 0.7
pH	7.4	7.5	7.7	8.0 ± 0.1
Eh [mV]	-40	-90	-80	-200 ± 10
O_2 [mg kg^{-1}]	< 0.1	< 0.1	< 0.1	< 0.1
NH_4-N [g kg^{-1}]	1.6	1.7	1.7	1.3 ± 0
N_{total} [g kg^{-1}]	3.3	3.1	3.2	3.4 ± 0.1
BOD_5 [g kg^{-1}]	4.7	6.0	3.0	1.7 ± 0
COD [g kg^{-1}]	55	49	49	69 ± 10

Impacts of dry substance content and incubation temperature in bovine manure

In short-term degradation tests with incubation intervals of 3, 7 and 30 days, the impact of dry substance contents on the transformation of extractable into non-extractable ^{14}C-sulfamethoxazole residues was studied. As depicted in **Fig. 5.4**, this process slowed down within the first 7 days when dry substance contents were adjusted at 2.5 and 5 %. The application of lower dry substance contents stood for a lower amount of solid manure material and a lower biomass possibly resulting in a decline of sorption and degradation, respectively. In the 30-days samples, however, the level of the batch tests with manure of 10-% dry substance content was nearly reached. Similar tendencies were found when the manure samples BM-1 were incubated at different temperatures (**Fig. 5.5**). At 5 and 10 °C, the transformation also slowed down due to the temperature depending activity of manure inherent microorganisms. After 30 days, however, these differences were leveled, too. Both test series under modified boundary conditions clearly showed that the formation of non-extractable residues is influenced by the microbial activity of the manure samples, particularly a sufficient sorption capacity of the manure matrices also at low dry substance contents has to be assumed.

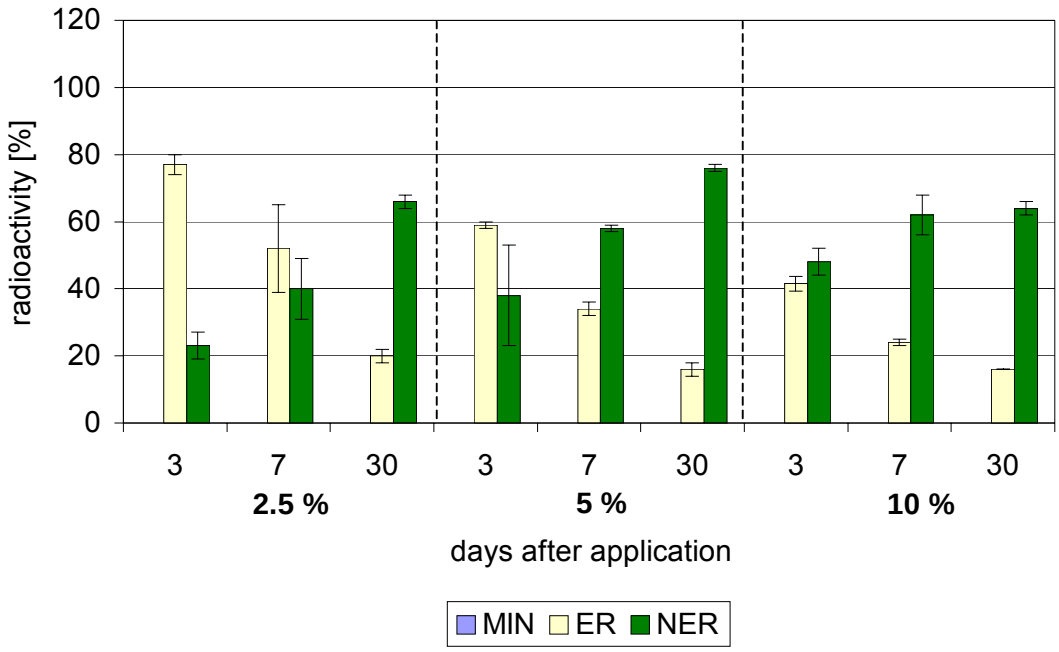


Fig. 5.4: Degradation of ^{14}C-sulfamethoxazole in bovine manure BM-1 at different dry substance contents (ds) and at 20 °C (balances: 91 ± 6 %)

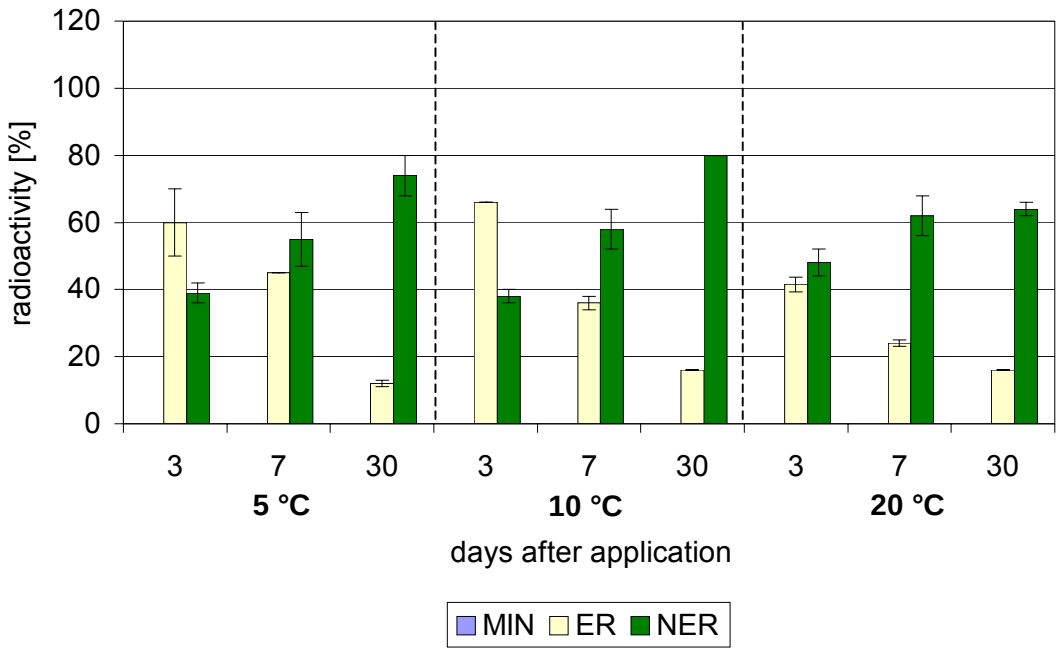


Fig. 5.5: Degradation of ^{14}C-sulfamethoxazole in bovine manure BM-1 at 10 % dry substance content and different incubation temperatures (balances: 93 ± 8 %)

Impacts of different bovine manure matrices

Within the different degradation tests, 3 different manure matrices were included taking different ages of cattle and different feeding conditions into account. Due to the herbivore nutrition type of cattle, only minor differences in pH values, ammonium and total nitrogen contents as well as in biological and chemical oxygen demands were found (cf. Tab. 2.10, page 16).

The results of the matrix characterization were also reflected by the corresponding degradation tests. Taking the analytical deviations between replicates of one manure matrix into account, relevant differences between different bovine manure matrices were not found (**Fig. 5.6**). The extractable ^{14}C-sulfamethoxazole residues of approximately 40 % after 3 days dropped continuously to 13 % on average while the non-extractable residues increased up to approximately 80 % after 30 days.

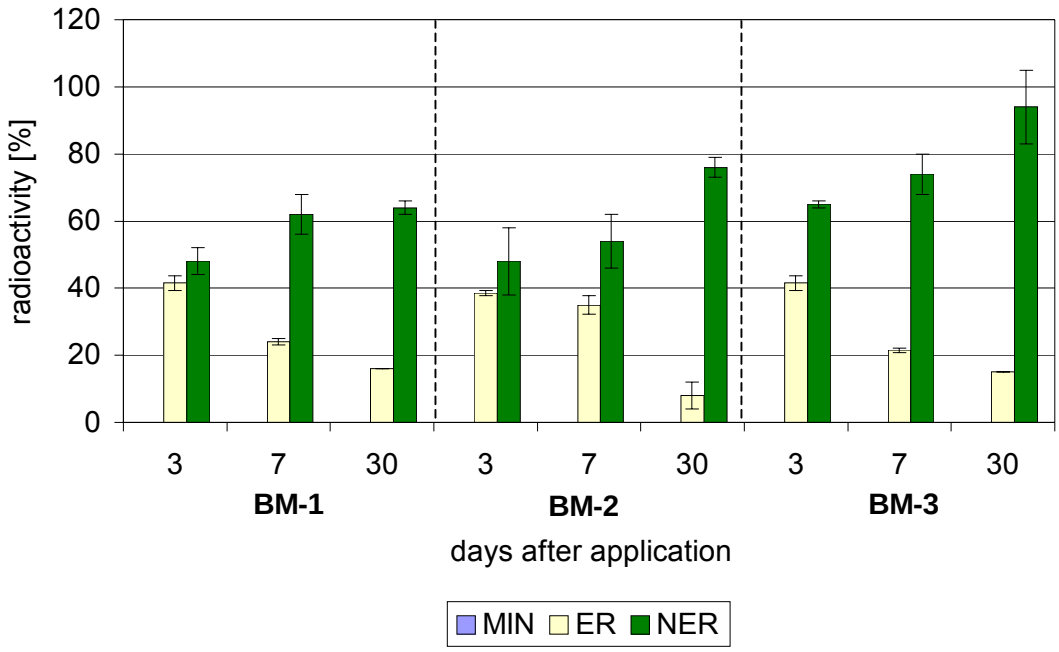


Fig. 5.6: Degradation of ^{14}C-sulfamethoxazole in different bovine manures (balances: 92 ± 10 %)

Long-term degradation tests in pig manure

The comparison between the degradation tests in bovine (cf. Fig. 5.1, page 38) and pig manures (**Fig. 5.7**) clearly showed the higher affinity of ^{14}C-sulfamethoxazole residues

to the bovine than to the pig manure matrices. Thus, a slower decrease of extractable ^{14}C-sulfamethoxazole residues was found in pig manure.

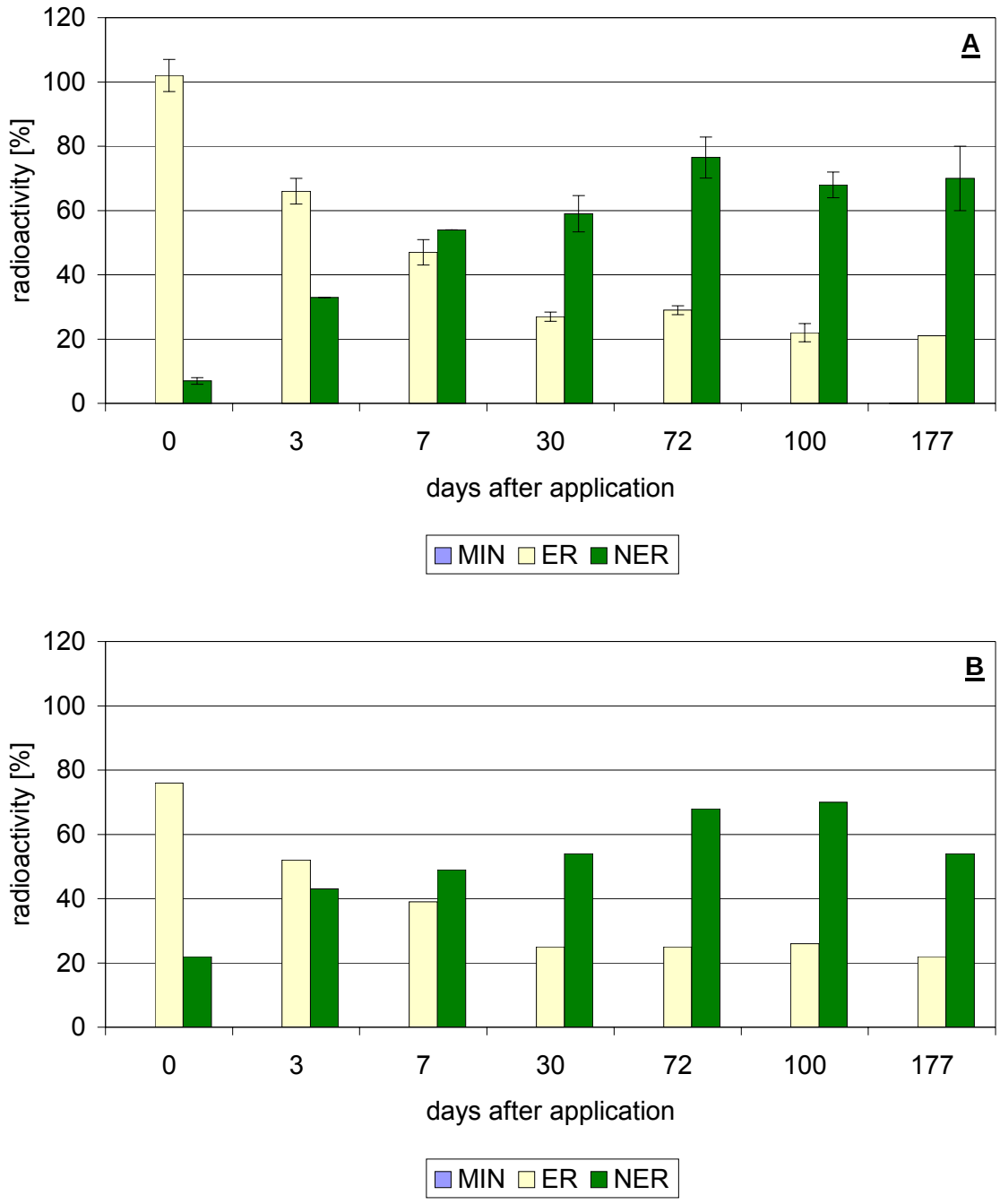


Fig. 5.7: Degradation of ^{14}C-sulfamethoxazole at A: 560 µg kg^{-1} (balances: 97 ± 9 %) and B: 1250 µg kg^{-1} pig manure PM-1 (balances: 89 ± 9 %)

During the 177-day incubation period in 2005, the extractable fraction never fell below 20 % of the initially applied radioactivity. Simultaneously, non-extractable residues increased until 72 days after application exceeding the 70-% level only once. Thereafter, the dynamics of this transformation process declined revealed by the nearly constant ratios of extractable and non-extractable fractions.

In the reference-manure samples prepared in 2006 from pig excrements that were stored at -20 °C longer than 180 days, similar tendencies of the degradation of ^{14}C-sulfamethoxazole were observed. As already described for corresponding tests with bovine manure samples, the concentration of the test substance and long-term storage of manure samples did not considerably affect the fate of ^{14}C-sulfamethoxazole in the degradation tests.

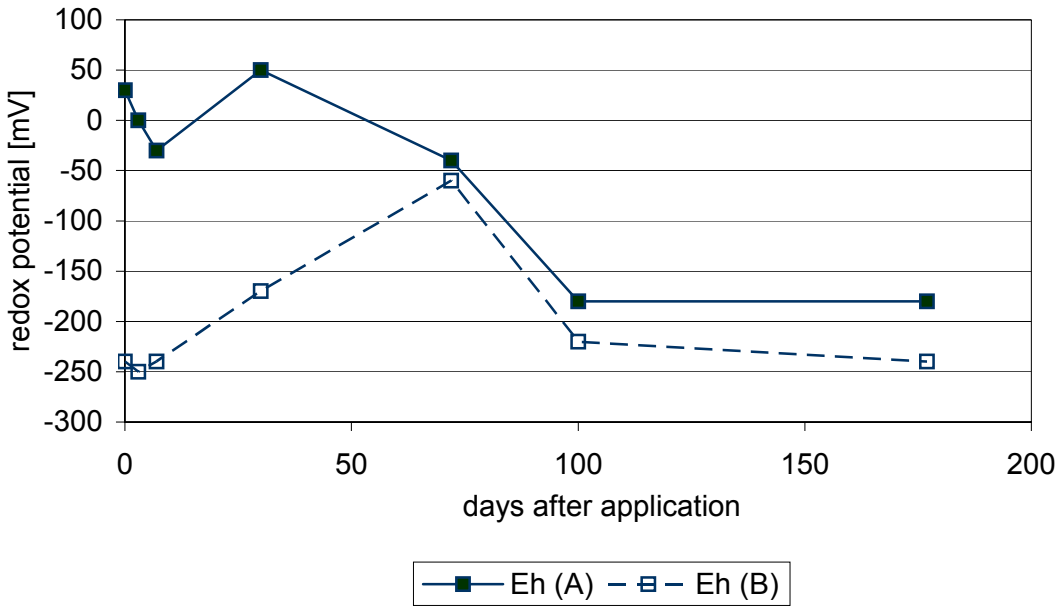


Fig. 5.8: Redox potentials during the degradation tests of sulfamethoxazole at A: 560 µg kg^{-1} and B: 1250 µg kg^{-1} pig manure PM-1

The RTLC metabolite screening performed for the extracts of the degradation test at 1250 µg sulfamethoxazole kg^{-1} pig manure revealed a rapid disappearance of the parent compound. In the 0-day sample, 71 % of the radioactivity initially applied were identified as the unchanged test substance. Within 7 days, this amount dropped to 18 %. In the extracts of the 30-day samples, only unidentified metabolites were detected.

The above mentioned decline of the dynamic transformation of extractable into non-extractable sulfamethoxazole residues was also reflected by the matrix characterization continuously conducted in parallel tests. There, the redox potentials and the biological and chemical oxygen demands dropped during the long-term incubation period while the NH_4-N contents remained nearly constant (**Fig. 5.8, 5.9**).

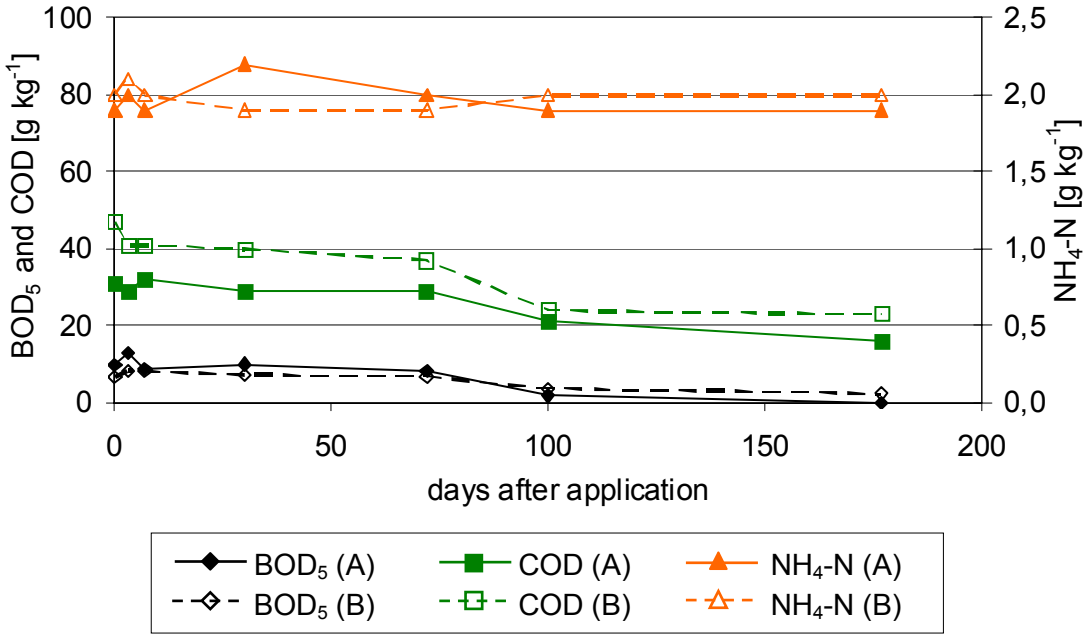


Fig. 5.9: BOD_5, COD and NH_4-N during the degradation test of sulfamethoxazole at A: 560 µg kg^{-1} and B: 1250 µg kg^{-1} pig manure PM-1

In contrast to the parameter characteristics in bovine manure, however, the decreases flattened between the 100-days and 177-days intervals. Particularly, the breakdown of the biological oxygen demand to 0.02 g O_2 kg^{-1} in the 177-day batch tests at 560 µg kg^{-1} pig manure indicated a considerable inhibition of the manure inherent microorganisms. In the degradation test at 1250 µg kg^{-1}, however, the biological oxygen demand of 2.4 g kg^{-1} indicated that microbial activity was remained until the end of the long-term degradation tests in pig manure.

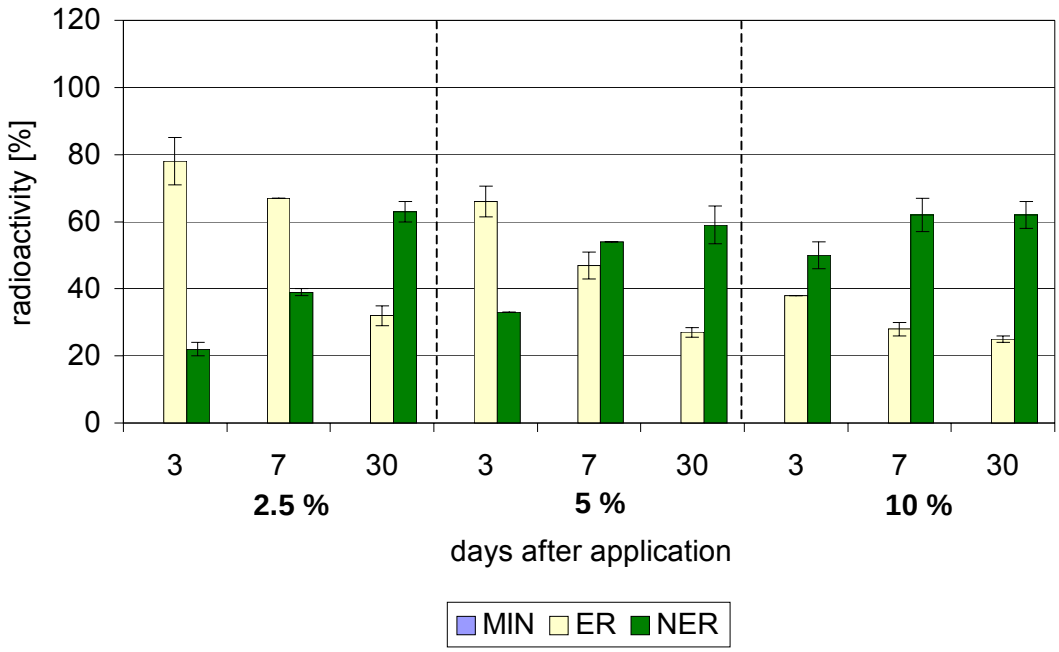


Fig. 5.10: Degradation of ^{14}C-sulfamethoxazole in pig manure PM-1 at different dry substance contents (ds) and at 20 °C (balances: 95 ± 7 %)

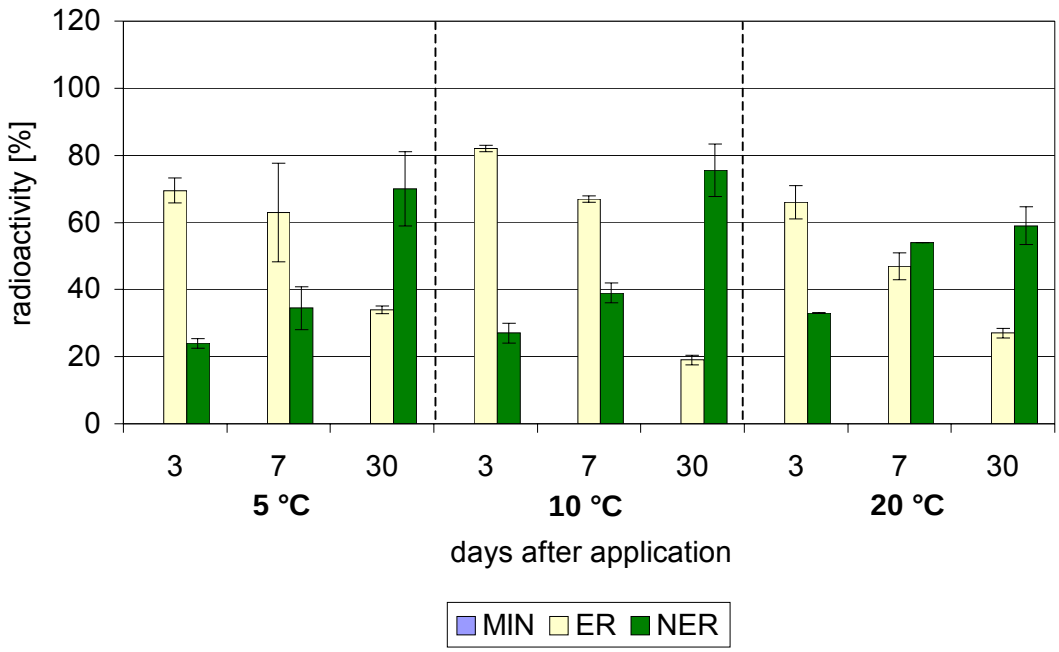


Fig. 5.11: Degradation of ^{14}C-sulfamethoxazole in pig manure PM-1 at 5 % dry substance content and at different incubation temperatures (balances: 99 ± 7 %)

Despite the interferences of the BOD measurements in both series of the degradation tests, there were merely minor effects on the metabolic dynamics of ^{14}C-sulfamethoxazole in pig manure. On the one hand, this fact confirmed that the experimental design of the Manure Project is appropriate to assess the long-term degradation of VMP in manures. On the other hand, there is a need of further method development for the determination of the microbial activity under permanently anaerobic conditions.

Impacts of dry substance content and incubation temperature in pig manure

The tendencies already described for these additional degradation tests in bovine manure were also reflected in those conducted in pig manure. The higher relevance of extractable ^{14}C-sulfamethoxazole residues is illustrated in **Fig. 5.10** and **5.11**. At 2.5-% dry substance content and at 5 and 10 °C, the extractable fractions definitely dominated the non-extractable ones including the 7-day incubation intervals. After 30 days, however, the differences were leveled and non-extractable residues achieved highest intensities.

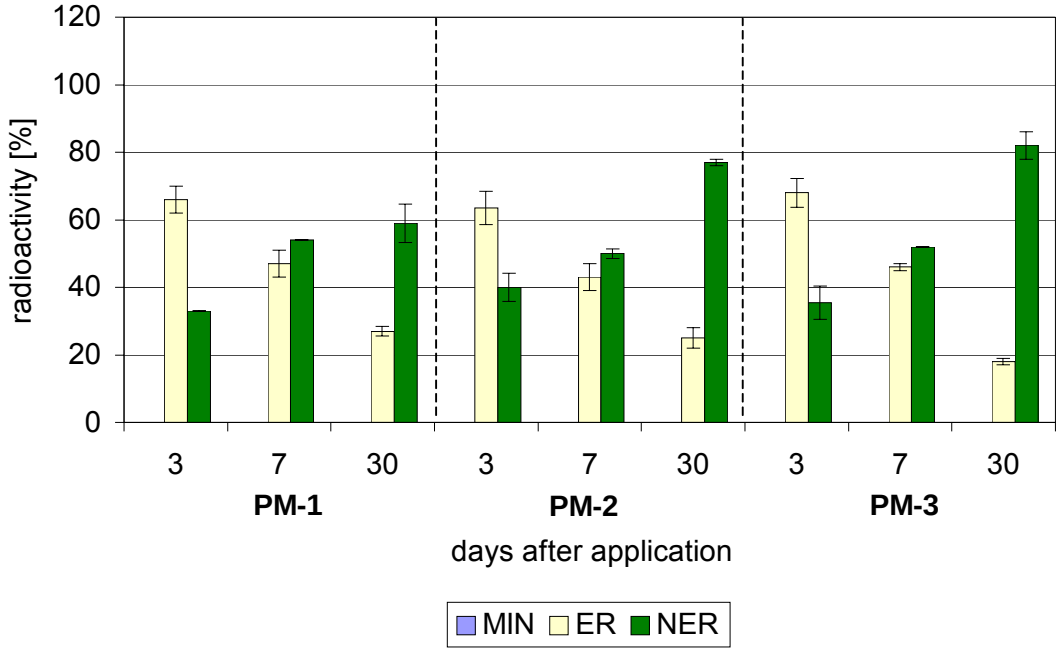


Fig. 5.12: Degradation of ^{14}C-sulfamethoxazole in different pig manures (balances: 99 ± 6 %)

Impacts of different pig manure matrices

Due to the omnivore nutrition type of pigs, higher differences in the parameter characteristics of the pig excrements were determined (cf.Tab. 2.11). However, those were minimized by adding tap water to prepare reference-manure samples with the 5-% dry substance content. Their application in the degradation tests of ^{14}C-sulfamethoxazole thus led only to slightly different intensity ratios of extractable and non-extractable residues (**Fig. 5.12**). After 3 days of incubation, the extractable residues dominated the non-extractable ones while after 30 days, the initially applied radioactivity was mainly transferred into the non-extractable fraction.

5.2 Acetyl-sulfamethoxazole

Due to the availability of ^{14}C-labeled test substances, numerous fate monitoring studies on VMP are exclusively focused on the parent compounds. The VMP administered to production animals, however, undergo metabolic decomposition resulting in the formation of corresponding metabolites. Those ones were then excreted together with unchanged parent compounds. Therefore, ^{14}C-acetyl-sulfamethoxazole was included in the Manure Project as the corresponding metabolite of the sulfonamide sulfamethoxazole. The metabolic fate of this metabolite in manure is of particular relevance because acetyl-sulfamethoxazole undergoes deacetylation and sulfamethoxazole is regenerated (Höltge and Kreuzig, 2007).

Long-term degradation tests in bovine manure

During the 177-day incubation period, the metabolic fate of ^{14}C-acetyl-sulfamethoxazole was predominated by the formation of non-extractable residues revealing again the high affinity of sulfonamide residues to the bovine manure matrix.

In comparison to sulfamethoxazole, however, the metabolic dynamics slowed down. Albeit, the extractable residues continuously dropped from 82 to 5 % (**Fig. 5.13**), the radioactivity amounts up to the 30-day samples were appropriate for the RTLC metabolite screening. Up to the 7-day incubation interval, the amount of acetyl-sulfamethoxazole as the unchanged parent compound decreased from 82 to 12 % of the radioactivity initially applied. Thereafter, the test substance was detected in amounts $\leq$ 3 %.

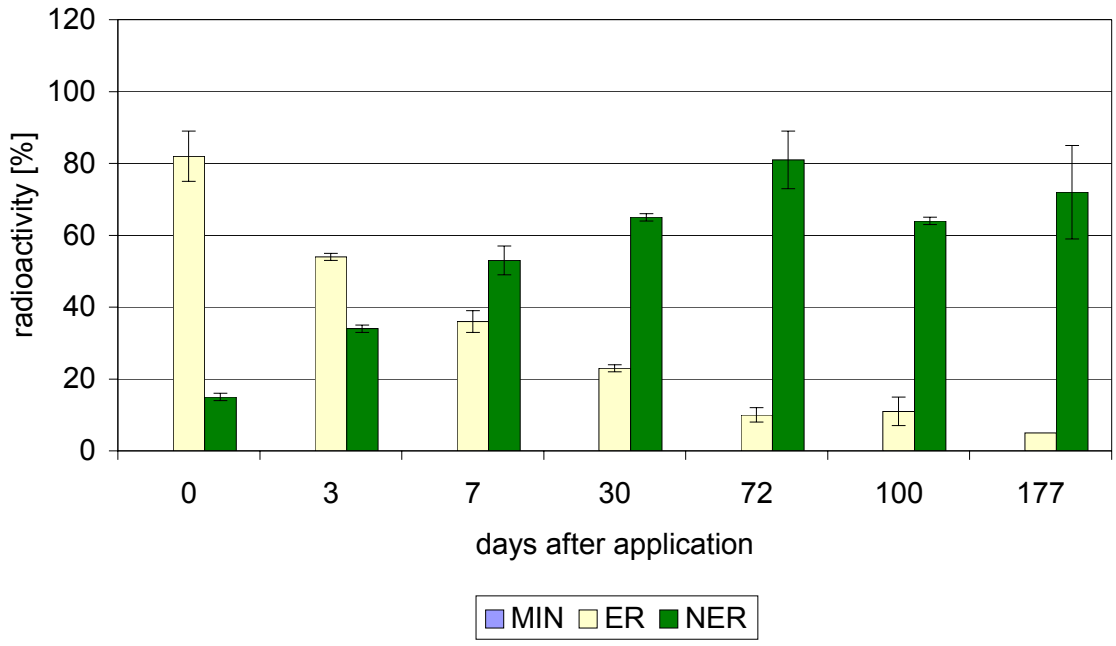


Fig. 5.13: Degradation of ^{14}C-acetyl-sulfamethoxazole in bovine manure BM-1 (balances: 86 ± 8 %)

Up to 7 days, ^{14}C-sulfamethoxazole occurred in the ethyl acetate extracts at amounts of 3 % accompanied by further unidentified metabolites. The differentiation of both compounds by RTLC analysis using 2 different solvents for the development of the silica gel plates is illustrated in **Fig 5.14**.

The mineralization of < 0.1 % within 100 days and the formation of non-extractable residues > 70 % within 72 days indicated the persistent nature of acetyl-sulfamethoxazole, if the persistence criteria of pesticides in soil are applied to the risk assessment for veterinary medicinal products and corresponding metabolites.

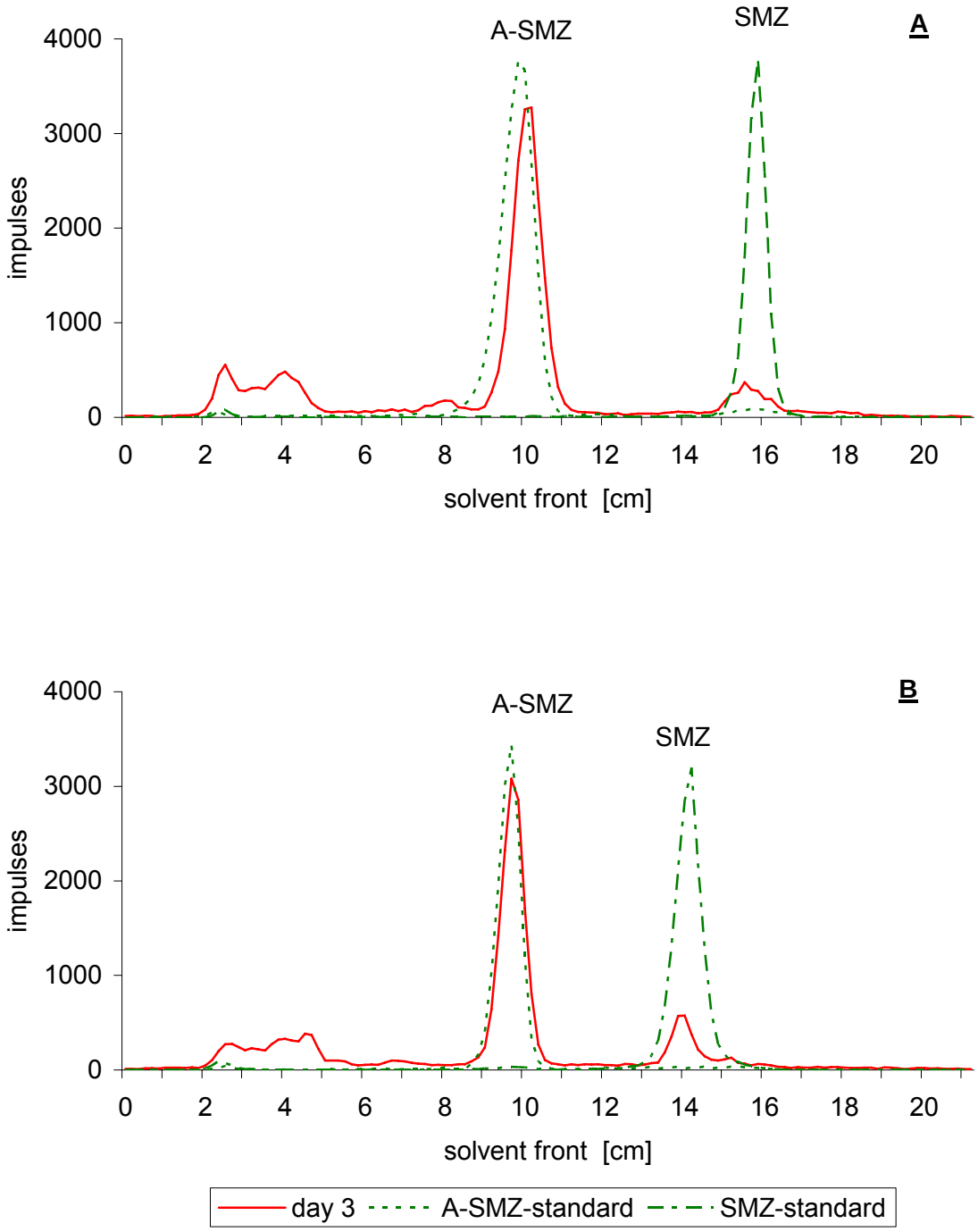


Fig. 5.14: RTLC chromatograms of the ethyl acetate extract from the 3-day sample of the degradation test on ^{14}C-acetyl-sulfamethoxazole in bovine manure. A: ethyl acetate and B: chloroform/n-butanol (8:1) used as solvent for development of the silica gel plates.

Parallel matrix characterization tests with non-labeled acetyl-sulfamethoxazole were carried out, too. As revealed by dissolved oxygen contents of O_2 < 0.1 mg kg^{-1} and redox potentials of Eh ≤ -80 mV, test conditions remained permanently anaerobic within 177 days. Most parameters remained constant. Merely, BOD_5 increased primary from 7.9 up to 9.8 g kg^{-1}. From the 72-day incubation interval, BOD_5 dropped to 2.3 g kg^{-1} on average (**Tab. 5.3**). This value was definitely higher than the limit of determination of 0.02 g kg^{-1} revealing that the bovine manure matrix was microbially active after the 177-day incubation. For the 177-day matrix characterization tests, duplicates were analyzed. Results for all parameters coincided revealing the reproducible applicability of reference manure in the long-term degradation tests.

Tab. 5.3: Matrix characterization during the degradation tests of acetyl-sulfamethoxazole in bovine manure

Day / Parameter	**0**	**3**	**7**	**30**	**72**	**100**	**177.A**	**177.B**
ds [%]	11	11	9	9	9	9	8	8
TOC [g kg^{-1}]	37	37	40	63	45	41	40	37
pH	7.4	6.8	6.8	6.6	7.1	7.4	7.6	7.6
Eh [mV]	-180	-170	-160	-80	-80	-100	-200	-220
O_2 [mg kg^{-1}]	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
NH_4-N [g kg^{-1}]	1.3	1.5	1.4	1.2	1.4	1.5	1.2	1.0
N_{total} [g kg^{-1}]	3.3	3.3	3.3	3.2	3.2	3.3	3.6	3.5
BOD_5 [g kg^{-1}]	7.9	9.8	9.2	9.4	9.8	2.3	2.8	2.4
COD [g kg^{-1}]	57	79	81	89	82	73	67	62

Long-term degradation tests in pig manure

For the degradation of ^{14}C-acetyl-sulfamethoxazole in pig manure, the same tendencies were observed than it was described for ^{14}C-sulfamethoxazole. The decrease of extractable residues thus slowed down confirming the lower affinity of sulfonamide residues to the pig manure matrix. Within 177 days, 18 % of the initially applied radioactivity remained extractable. This process was accompanied by the increasing formation of non-extractable residues that amounted to 81 % at maximum after the ethyl acetate extraction procedure. Mineralization was < 0.1 % (**Fig. 5.15**).

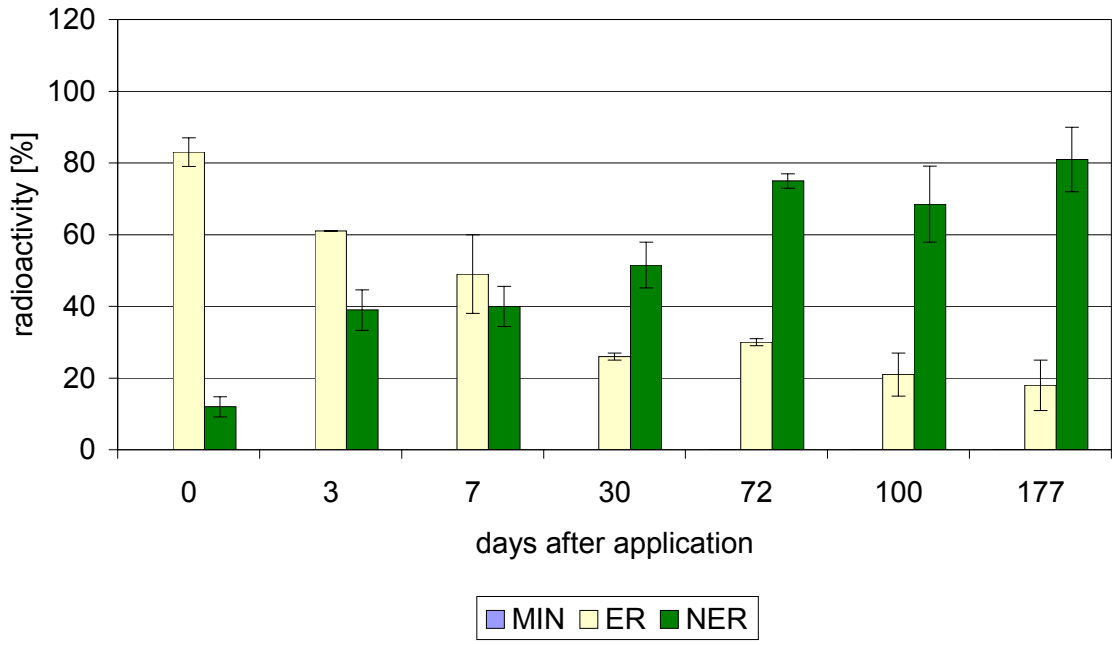


Fig. 5.15: Degradation of ^{14}C-acetyl-sulfamethoxazole in pig manure PM-1 (balances: 94 ± 9 %)

As depicted by the RTLC metabolite screening, the extracts of the 0-day samples exclusively contained ^{14}C-acetyl-sulfamethoxazole as the unchanged parent compound. Up to the 7-day sample, ^{14}C-sulfamethoxazole was found at amounts of ≤ 3 % of the radioactivity initially applied. By this fact, the deacetylation of acetyl-sulfamethoxazole was also confirmed for pig manure.

In pig manure, strictly anaerobic conditions were also reflected by oxygen contents < 0.1 mg kg^{-1} and redox potentials ≤ -110 mV. Total organic carbon, pH-value, ammonium nitrogen and total nitrogen content remained nearly constant during the 177-day tests, while the biological oxygen demand decreased. Thus, the decrease of the biological oxygen demand indicated a slowdown of aerobic or facultative anaerobic microorganisms while the activity of the anaerobic ones could not assessed by this method. Hence, there is a need of further method development.

5.3 Sulfadiazine

Besides ^{14}C-sulfamethoxazole and its corresponding metabolite ^{14}C-acetyl-sulfamethoxazole, ^{14}C-sulfadiazine was additionally monitored in order to assess the impact of different substituents of structural analogues on their degradation in bovine and pig manures.

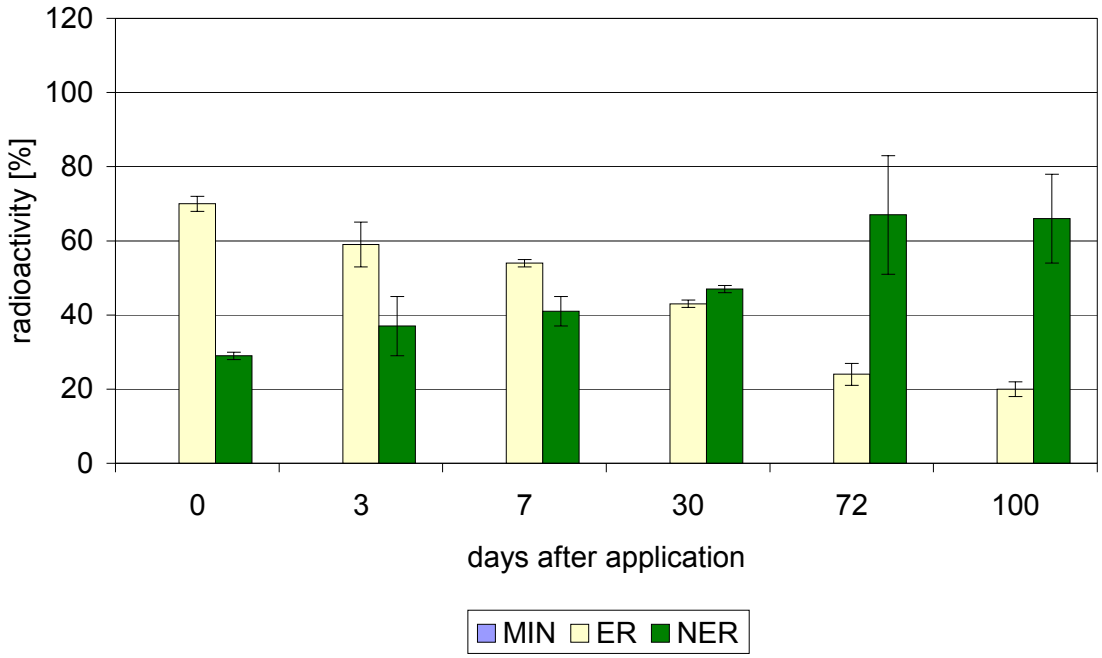


Fig. 5.16: Degradation of ^{14}C-sulfadiazine in bovine manure BM-1 (balances: 93 ± 5 %)

Long-term degradation tests in bovine manure

In comparison to ^{14}C-sulfamethoxazole, slower dynamic processes in bovine manure were observed for ^{14}C-sulfadiazine (**Fig. 5.16**). After the application of the test substance, extractable residues thus decreased from 70 % to 20 % within 100 days. The slowdown of the metabolic dynamics of ^{14}C-sulfadiazine in bovine manure was also reflected by the RTLC metabolite screening. Thus, the unchanged ^{14}C-sulfadiazine was the dominant constituent in the extracts of the degradation tests up to the 100-day intervals. The parent compound was accompanied by an unidentified metabolite that simultaneously decreased while the non-extractable residues increased to 66 % at

maximum. Mineralization was < 0.1%.

In contrast to ^{14}C-sulfadiazine, ^{14}C-sulfamethoxazole and ^{14}C-acetyl-sulfamethoxazole, were detectable only up to the 7-day interval. Thereafter, unidentified metabolites occurred in the extracts. The different degradation of the sulfonamides under study, however, could not be attributed to different manure matrix effects as shown by means of the parallel matrix characterization tests (**Tab. 5.4**). This fact clearly emphasized the relevance of the matrix characterization procedures for the excrement and the reference manure samples and during the degradation tests. In the frame of the method development, important information was thus gained about the applicability of the sample matrices and the test conditions under study.

Tab. 5.4: Matrix characterization during the degradation tests of sulfadiazine in bovine manure

Day / Parameter	**0**	**3**	**7**	**30**	**72**	**100**
ds [%]	9	10	10	9	10	8
TOC [g kg^{-1}]	47	49	43	35	47	40
pH	6.9	6.6	6.3	6.8	7.6	7.7
Eh [mV]	- 60	- 80	- 140	- 160	- 210	- 160
O_2 [mg kg^{-1}]	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
NH_4-N [g kg^{-1}]	1.4	1.4	1.6	1.3	1.4	1.4
N_{total} [g kg^{-1}]	3.2	3.2	3.1	3.3	3.5	3.3
BOD_5 [g kg^{-1}]	9.2	9.3	11.0	8.3	4.5	6.4
COD [g kg^{-1}]	98	94	82	67	50	80

Long-term degradation tests in pig manure

A slower disappearance of ^{14}C-sulfadiazine was found in pig manure. Here, the mass balances of the degradation tests were predominated by the extractable fractions up to the 100-day incubation interval. The extractable residue slightly dropped from 72 % to only 59 % of the radioactivity initially applied (**Fig. 5.17**).

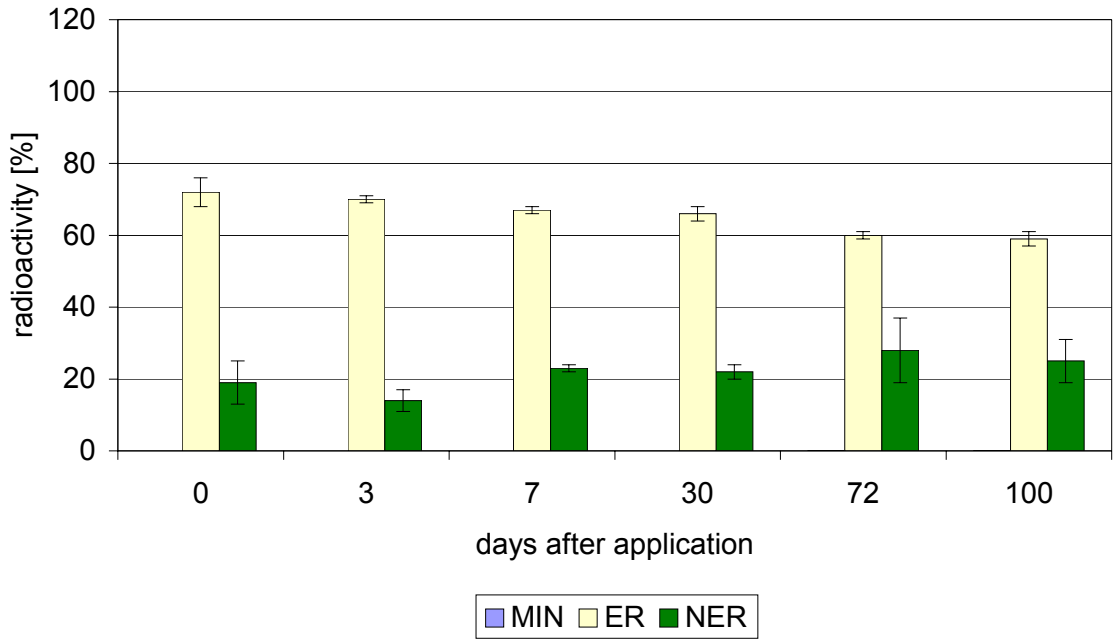


Fig. 5.17: Degradation of ^{14}C-sulfadiazine in pig manure PM-1 (balances: 88 ± 3 %)

As shown by RTLC metabolite screening, the amounts of ^{14}C-sulfadiazine as the unchanged parent compound decreased from 69 % to 46 % while an unidentified metabolite increased from 3 % to 13 % at maximum. The formation of non-extractable residues remained nearly constant during the 100-day incubation period. Finally, they amounted to 25 %. Mineralization was < 0.1 % again. The results were in accordance with those of Grote (2005). There, the concentrations of sulfadiazine and acetyl-sulfadiazine were determined in pig manure samples from tanks within a period of 8 months. While the acetyl-sulfadiazine concentration decreased rapidly, a rise of the sulfadiazine concentration was observed in the first month. Afterwards, a slow decrease up to nearly the initial concentration was noticed. The different degradation of sulfadiazine versus those of the other sulfonamides was not reflected by the matrix characterization. Here, the same tendencies, described for sulfamethoxazole and acetyl-sulfamethoxazole were obtained for the pig manure matrix. Despite the biological oxygen demand that decreased from 9.9 to 3.9 g kg^{-1}, the other parameters remained nearly constant.

Short-term degradation tests in bovine and pig manures

In order to check the performance of the applied laboratory-batch system, additional 7-day degradation tests of ^{14}C-sulfadiazine were conducted by means of the flow-through systems. The results listed in **Tab. 5.5** are based on the mass balances. Additionally, the extractable fractions were differentiated for ^{14}C-sulfadiazine and an unidentified metabolite to take this metabolic decomposition into account, too. In accordance to the OECD guideline 307 (2002), the radioactivity balances of degradability tests may vary between 90 to 100 %. On this basis, both laboratory-test systems supply consistent results.

Tab. 5.5: Degradation tests of ^{14}C-sulfadiazine in bovine manures within 7 days comparing laboratory-batch and flow-through systems

Matrix	**Bovine manure**				**Pig manure**			
Test system	batch system		flow-through system		batch system		flow-through system	
MIN [%]	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
SDZ [%]	36	37	33	35	62	66	71	69
MET [%]	17	14	19	21	8	7	4	4
NER [%]	33	53	58	53	15	19	29	36
Balances [%]	86	104	110	109	85	92	104	109

Retention factors determined by RTLC metabolite screening:
R_F: 0.60 for sulfadiazine (SDZ) and R_F: 0.05 for the unidentified metabolite (MET)

5.4 Ketoprofen and paracetamol

The development of the technical protocol for degradation tests of VMP in manure required the application of different bovine and pig manure matrices. In order to identify differences caused by feeding conditions and age of the production animals, the excrement and reference-manure samples were thoroughly characterized by numerous matrix parameters. Furthermore, the degradation of numerous test substances were tested in the reference manures. Those VMP belong to different chemical classes and cover a wide range of different physico-chemical properties. Therefore, the degradation

of ketoprofen in bovine manure and of paracetamol in pig manure were studied although their application patterns in veterinary medicine are of lower relevance. Nevertheless, these degradation tests contributed evaluating the reproducible applicability of the reference manures.

Long-term degradation tests of ^{14}C-ketoprofen in bovine manure

Within the frame of the Soil Project[2], the biodegradability of ^{14}C-ketoprofen has been already studied in soil, sewage sludge and sludge amended soil (Kreuzig et al., 2005). In those sample matrices, ketoprofen was readily degradable. After 102-day incubation periods, mineralization rates exceeded 27 % in sewage sludge and even 40 % in soils. In bovine manure, however, the determined mass balances were predominated by the unchanged parent compound that slightly decreased from 68 % to 59 % within 100 days. Non-extractable residues slightly increased from 13 % to 25 % while mineralization was < 0.1 % (**Fig. 5.18**).

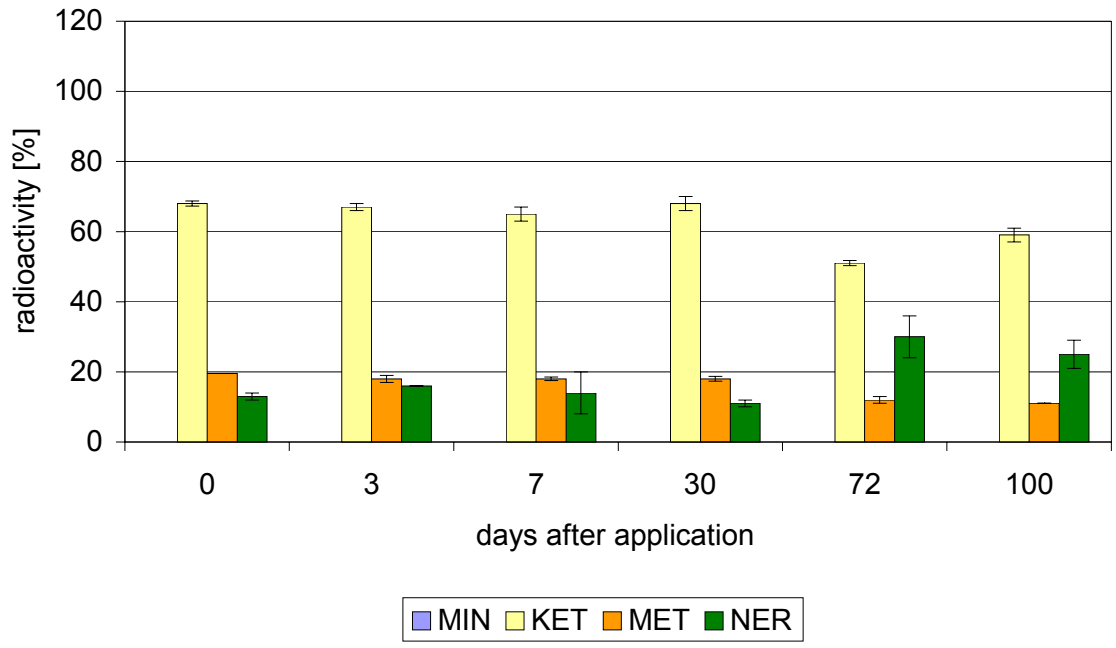


Fig. 5.18: Degradation of ^{14}C-ketoprofen in bovine manure (BM-1) (balances: 100 ± 3 %)

[2] "Environmental Behavior of Selected Pharmaceutical Medicines and Relevant Metabolites in Soils" funded by the German Federal Environmental Agency (Soil Project, FKZ 201 67 40/02)

The differences of the metabolic dynamics in bovine manure and sewage sludge are of particular interest for biocides that are applied in animal husbandry and undergo anaerobic conditions during manure storage. For this purpose, a degradability test in digested sludge is required (EC, 1998, TNsG, 2000, ISO, 1995). By means of this approach, however, the differences in the composition of sludge and manure matrices with their possible effects on the metabolic fate of chemicals are disregarded.

In accordance with the other degradation tests in bovine manure, most matrix parameters remained nearly constant over a period of the 100-day incubation period. Even the biological oxygen demand remained nearly constant at 12 g kg^{-1} within 72 days. Thereafter, it dropped to 4.3 g kg^{-1} (**Fig. 5.19**).

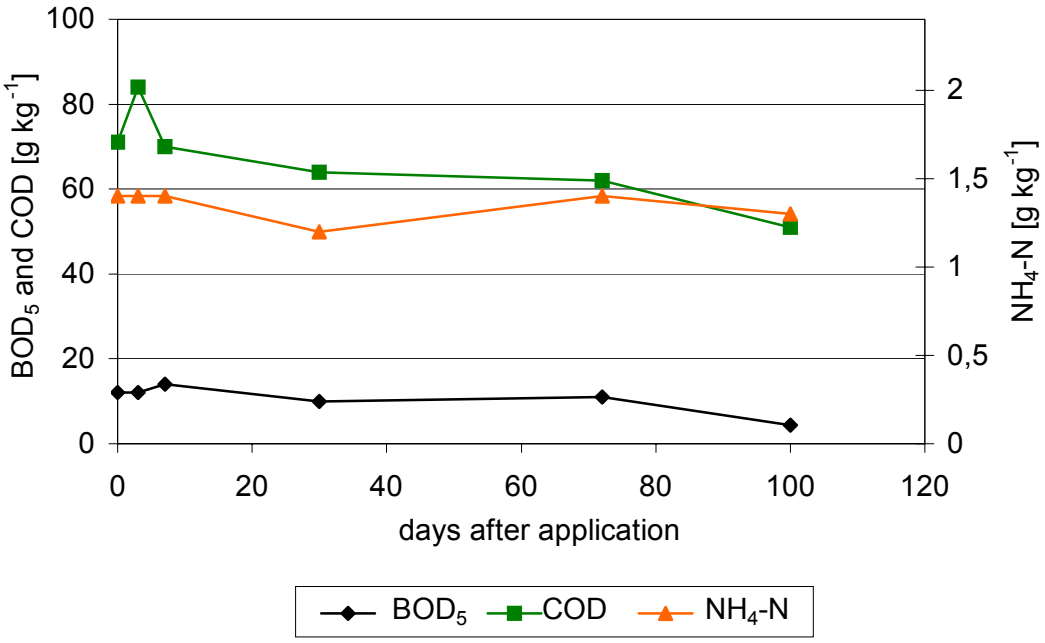


Fig. 5.19: BOD_5, COD and NH_4-N during the degradation test of ketoprofen in bovine manure BM-1

Long-term degradation tests of ^{14}C-paracetamol in pig manure

Fate and behavior of ^{14}C-paracetamol was monitored in the Soil Project, too. In contrast to ketoprofen, paracetamol showed a strong affinity to sewage sludge and soil matrices. Thus, > 60 % of the initially applied radioactivity was found in the non-extractable fraction already 1 day after the application. The degradation test in pig ma-

nure was also predominated by the non-extractable fractions. However, they increased more slowly from 40 % to 96 %. This process was accompanied by the slowdown of extractable residues from 79 % to 17 %. Mineralization was again < 0.1 % (**Fig. 5.20**).

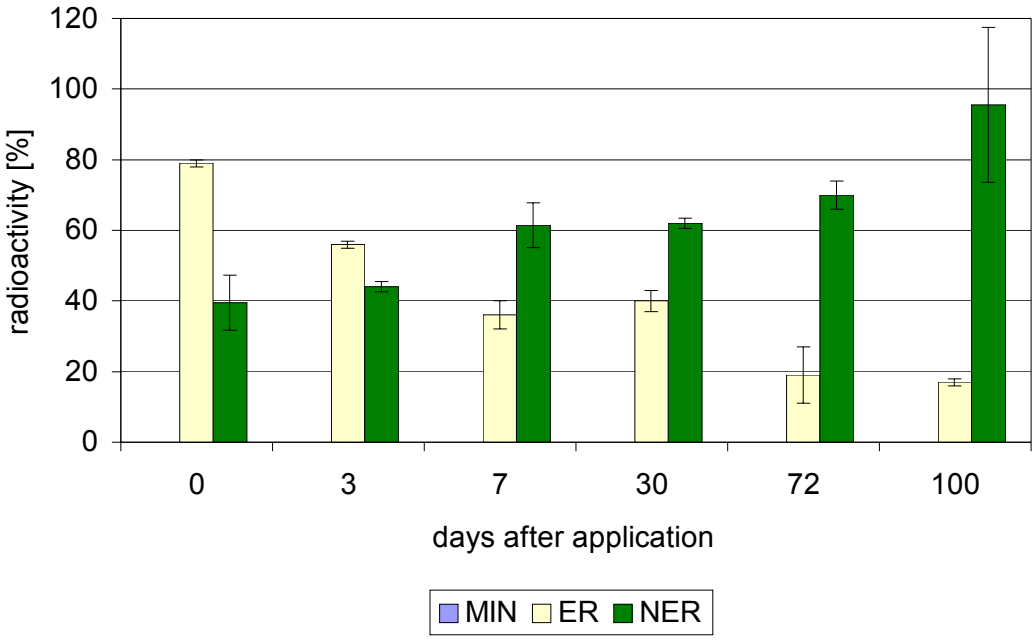


Fig. 5.20: Degradation of ^{14}C-paracetamol in pig manure (PM-1) (balances: 103 ± 11 %)

During the degradation tests in pig manure, relevant changes of the matrix parameters did not occur. Merely, the biological oxygen demand dropped from 11 g kg^{-1} in the 72-day sample to 5.7 g kg^{-1} in the 100-day sample. These matrix characterization tests clearly showed again that the degradation of VMP in bovine and pig manure can be reproducibly monitored under strictly anaerobic conditions typical for the long-term manure storage in tanks.

5.5 Erythromycin

For the development of the technical protocol, the testing of VMP from different chemical classes was inevitably required. Besides the sulfonamides, therefore, the second

focus was put on the macrolide antibiotic erythromycin. Long-term degradation tests in bovine and pig reference manures were conducted in the dark at 20 ± 1 °C within incubation periods up to 177 days. Additional short-term tests with different dry substance contents of the manure samples and different incubation temperatures were performed. Parallel batch tests were simultaneously carried out for the detailed matrix characterization during the incubation periods.

Long-term degradation tests in bovine manure

The degradation of ^{14}C-erythromycin was tested in bovine manures BM-1 and BM-5. The feeding conditions of the single kept cattle were nearly the same reflected by similar manure compositions (cf. Tab. 2.10, page 16). Nevertheless, the dynamic processes in these tests differed up to 100 days of incubation. In the bovine manure BM-1, extractable residues thus dropped from 86 to 7 % in 100 days. Non-extractable residues increased from 22 to 93 % (**Fig. 5.21**). In the bovine manure BM-5, however, these dynamic processes slowed down. The initial amount of extractable residues was only 54 % while non-extractable residues amounted to 39 %. These amounts remained nearly unchanged up to the 30-day samples. The extractable residues then dropped to 29 % while non-extractable ones reached 59 %. Beyond the 100-day incubation intervals of both test series, the extractable fractions were quantitatively transferred into non-extractable residues. Mineralization rates were < 0.1 %, respectively.

The extracts were screened for parent compound and metabolites up to the 100-day samples. Thereafter, the extractable amounts were far too low for the RTLC screening. In this period, the concentration of ^{14}C-erythromycin dropped continuously, whereas the concentration of the second substance, probably ^{14}C-erythromycin-H_2O (cf. page 24), increased until day 30. In the 0-day samples of the bovine manure BM-1, 65 % of the initially applied radioactivity was identified as the unchanged parent compound. The corresponding value in the bovine manure BM-5 was 37 %. After 100 days, however, 3 and 4 % of the initially applied radioactivity accounted for the unchanged ^{14}C-erythromycin. On the basis of the RTLC-screenings, DT_{50} values were 23 and 33 days, respectively.

Despite the differences of the metabolic dynamics in both test series, the results were congruent after 177 days. For the BM-5 batches, the manure samples were obviously infected by molds. This side-effect could not be observed in any other test series neither in other degradation tests nor in the parallel matrix characterization tests. In the

latter, strictly anaerobic conditions were revealed by dissolved oxygen contents of O_2 < 0.1 mg kg^{-1} and redox potentials of Eh < -70 mV (**Tab. 5.6**).

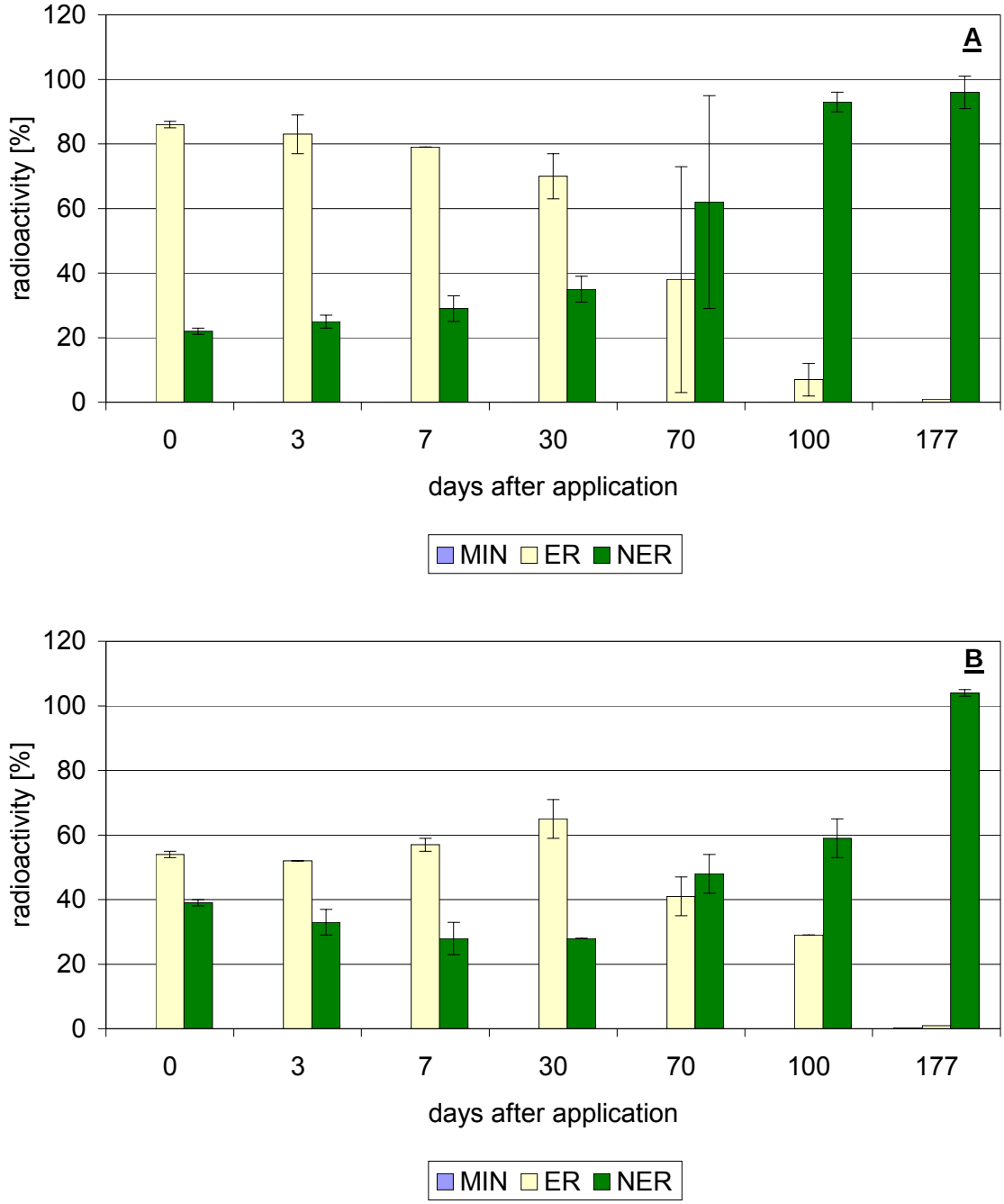


Fig. 5.21: Degradation of ^{14}C-erythromycin in bovine manure A: BM-1 (balances: 104 ± 4 %) and B: BM-5 (balances: 91 ± 7 %)

Furthermore, the matrix parameters were not effected neither by the spiking solvent nor by the test substance. Within the 177-day incubation periods, the parameters under study remained approximately unchanged, except the biological oxygen demand that decreased from 7.3 to 0.8 g kg^{-1}. Hence, the different intensities of extractable and non-extractable ^{14}C-erythromycin residues of the degradation tests in BM-1 and BM-5 could not be elucidated by the matrix characterization tests.

Tab. 5.6: Composition of different bovine manure samples before and at the end of the degradation tests of erythromycin

Test / Parameter	**Matrix test** BM-5		**Solvent test** BM-5		**Substance test** BM-5		**Substance test** BM-1	
Day	**0**	**177**	**0**	**177**	**0**	**177**	**0**	**177**
ds [%]	10	8	9	9	10	10	10	10
TOC [g kg^{-1}]	42	42	41	43	42	42	47	37
pH	6.9	7.8	7.0	7.6	6.7	7.8	7.3	8.4
Eh [mV]	-110	-160	-160	-170	-70	-160	-120	-170
O_2 [mg kg^{-1}]	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
NH_4-N [g kg^{-1}]	0.8	0.9	0.8	1.0	1.0	0.9	1.4	1.2
N_{total} [g kg^{-1}]	2.5	2.1	2.5	3.0	2.5	2.1	3.4	3.1
BOD_5 [g kg^{-1}]	7.6	0.4	5.2	0.7	9.1	0.4	7.1	1.7
COD [g kg^{-1}]	84	60	62	39	62	60	78	84

Impacts of dry substance content and incubation temperature in bovine manure

The impact of dry substance contents on the formation of non-extractable ^{14}C-erythromycin residues was demonstrated in short-term degradation tests. In bovine manure with 2.5 and 5 % of dry substance contents, 17 and 25 % of the initially applied radioactivity was analyzed as non-extractable residues after 30 days (**Fig. 5.22**). The corresponding value was 35 % at 10-% dry substance content. Furthermore, slightly higher mineralization rates of 4 % and 3 % were determined at 2.5-% or 5-% dry substance contents, respectively, after 30 days.

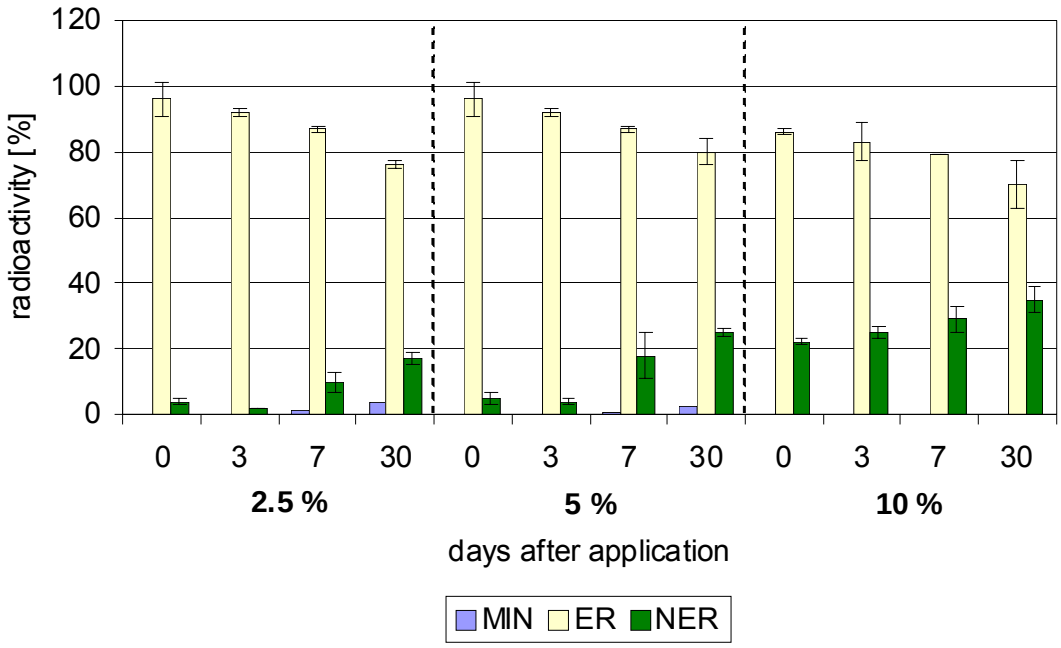


Fig. 5.22: Degradation tests of ^{14}C-erythromycin in bovine manure (BM-1) at different dry substance contents and at 20 °C (balances: 102 ± 5 %)

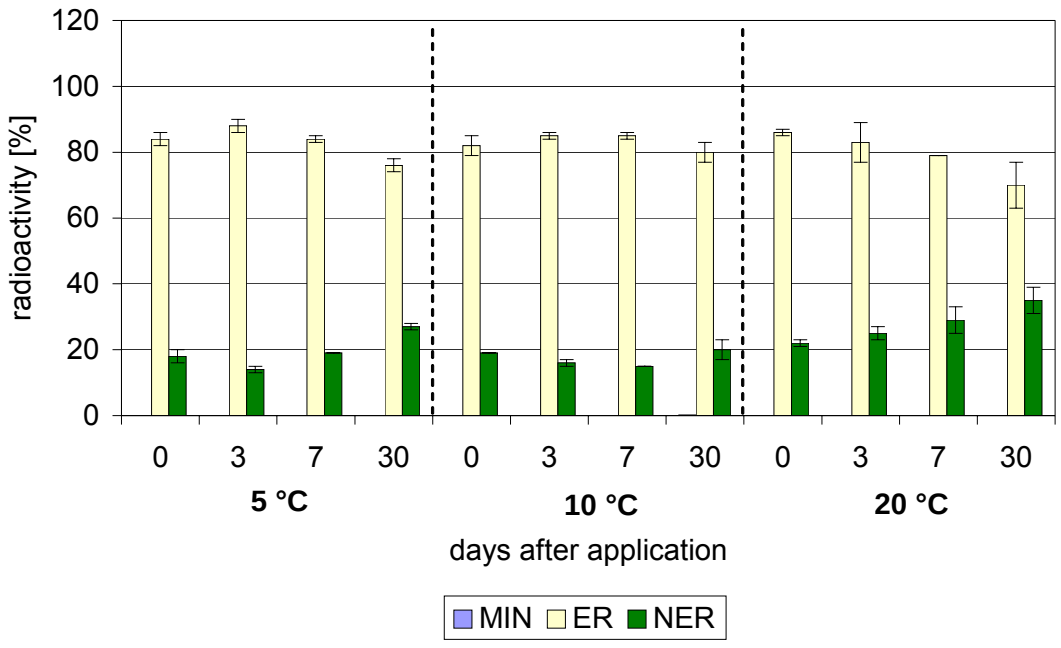


Fig. 5.23: Degradation tests of ^{14}C-erythromycin in bovine manure (BM-1) at 10 % dry substance content and at different incubation temperatures (balances: 100 ± 5 %)

In contrast to the degradation of ^{14}C-sulfamethoxazole in bovine manure BM-1, only minor impacts of the temperature on the metabolic dynamics of ^{14}C-erythromycin could be found. Within the 30-day incubation periods, the extractable residues remained nearly constant at 84 ± 4 % at 5 and 10 °C (**Fig. 5.23**). At 20 °C, the extractable residues dropped slowly from 86 to 70 % of the initially applied radioactivity. Simultaneously, the non-extractable residues increased to 27 %, 20 % or 35 %, respectively. In these short-term tests, mineralization was < 0.1 %.

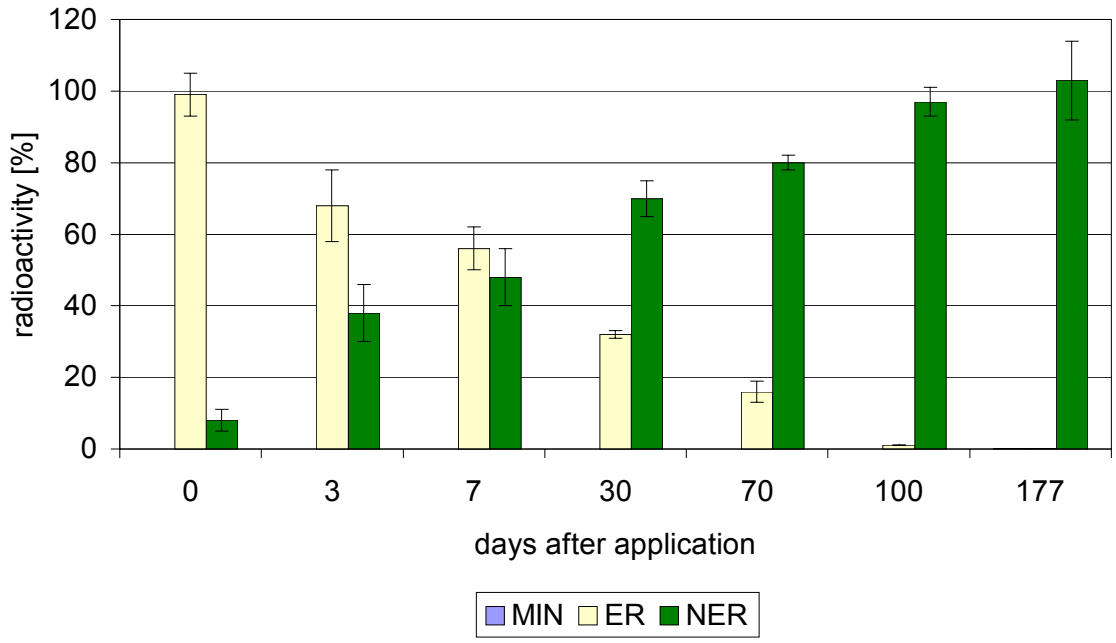


Fig. 5.24: Degradation of ^{14}C-erythromycin in pig manure PM-1 (balances: 102 ± 4 %)

Long-term degradation tests in pig manure

Extractable ^{14}C-erythromycin residues rapidly dropped from 99 % to 1 % of the initially applied radioactivity within 100 days. Thereafter, a decrease to 0.2 % occurred. This process was accompanied by a continuous formation of non-extractable residues. Already after 30 days, the crucial trigger of 70 % was exceeded first. This fraction finally reached 96 %. Mineralization rates of 0.1 % were of minor relevance (**Fig. 5.24**). In contrast to the sulfonamides under study, a higher affinity of ^{14}C-erythromycin to the pig manure matrix was observed although pig manure was adjusted at 5 % while bovine

manure contained 10 % dry substance. The formation of non-extractable residues thus is not necessarily dependent on the dry substance content, but on the matrix and test substance properties.

Until the 70-day intervals, the radioactivity amounts in the extracts were appropriate for the RTLC metabolite screening. Within this period, the unchanged ^{14}C-erythromycin dropped from 77 to 3 %. DT_{50} was 3 days. The unidentified metabolite simultaneously decreased from 24 to 14 %.

The matrix characterization tests clearly showed permanently anaerobic conditions up to the 177-day incubation interval. The redox potentials mainly fell below ≤ -200 mV indicating strictly methanogenic conditions (**Fig. 5.25**). Nevertheless, ^{14}C-methane or related volatiles could not be detected in the corresponding degradation tests by head-space analysis of the incubation flasks.

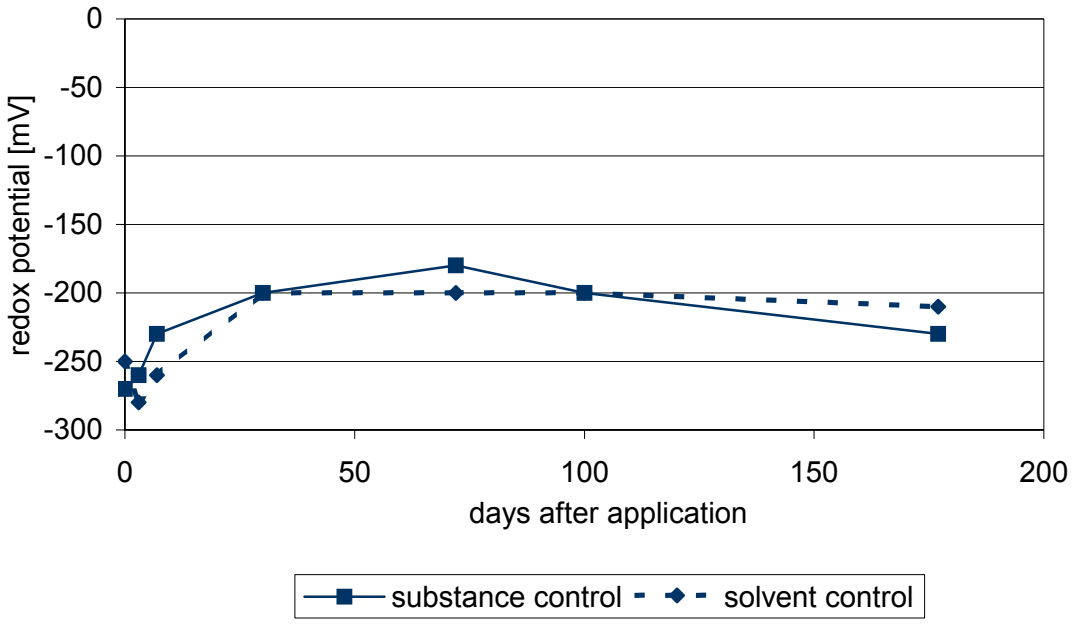


Fig. 5.25: Redox potentials in the matrix characterization tests using pig manure PM-1

In contrast to the degradation tests of sulfonamides in pig manure, here the biological oxygen demand was ≤ 4 g kg^{-1} already after 30 days. Furthermore, the chemical oxygen demand slightly dropped while the ammonium nitrogen content slightly increased

(**Fig. 5.26**). The courses of these parameters might indicate minor decomposition processes of the organic substances in pig manure.

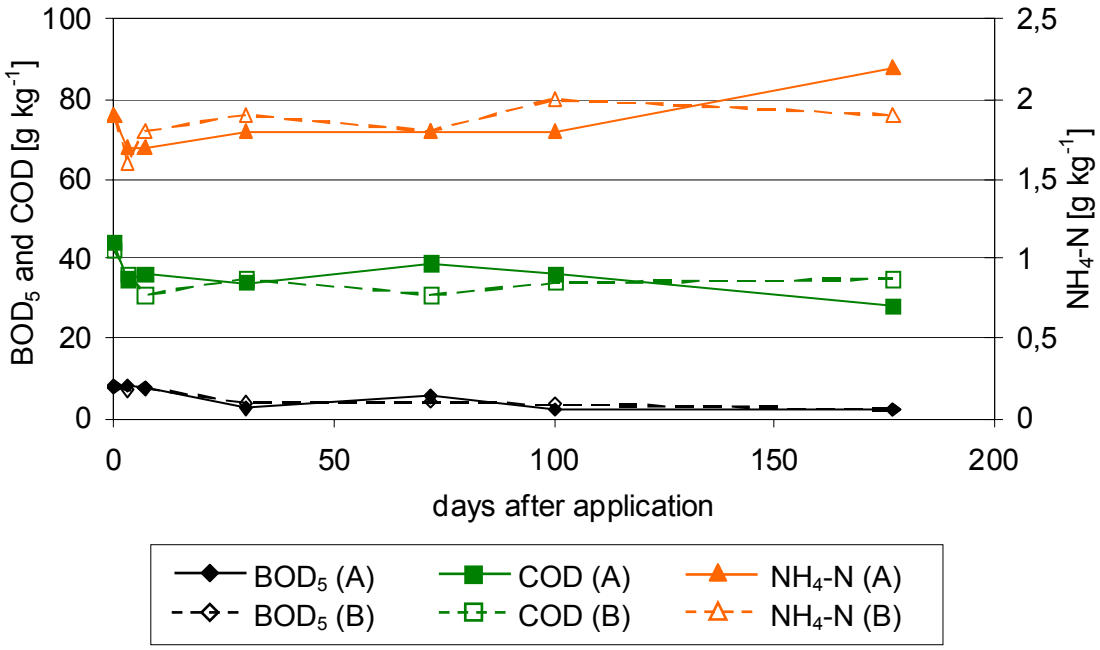


Fig. 5.26: BOD_5, COD and NH_4-N during the degradation test in pig manure PM-1
A: erythromycin substance control B: solvent control

Impacts of dry substance content and incubation temperature in pig manure

Different dry substance contents of manures determined the specific surfaces of the solid manure matrix and the microbial biomass of manures. The effects of both parameters were reflected during the first incubation intervals of the degradation tests of ^{14}C-erythromycin in pig manure. In the 0-day samples at 2.5 % and 5 % dry substance content, thus 7 % and 8 % remained non-extractable, while, at 10 % dry substance content, non-extractables amounted to 42 % at the same time (**Fig. 5.27**). Up to the 30-day incubation intervals, the initial differences leveled by the increase of the non-extractable ^{14}C-erythromycin fractions in pig as well as in bovine manure.

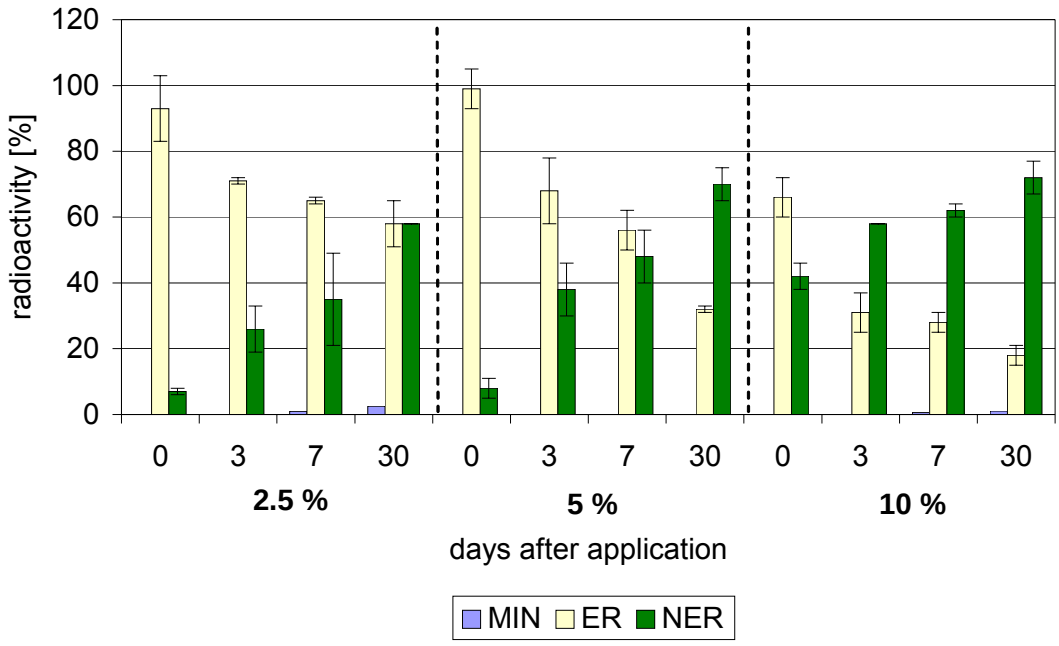


Fig. 5.27: Degradation of ^{14}C-erythromycin in pig manure (PM-1) at different dry substance contents (balances: 101 ± 8 %)

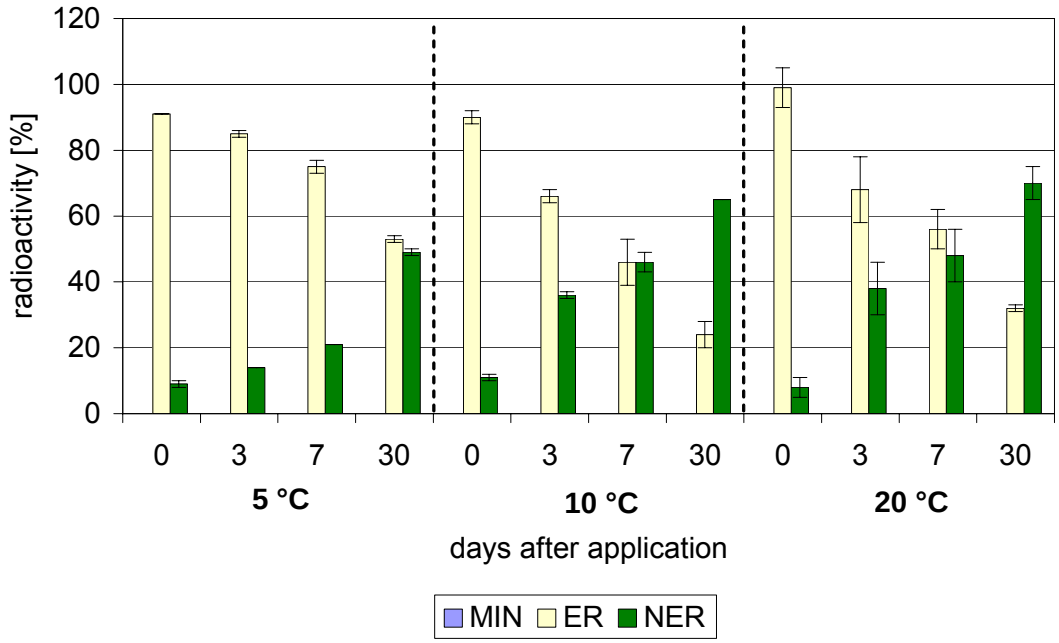


Fig. 5.28: Degradation of ^{14}C-erythromycin in pig manure (PM-1) at different incubation temperatures (balances: 100 ± 5 %)

Besides the effects of the microbial biomass, the degradation of VMP in manure depends on the microbial activity that is affected by the incubation temperature. Hence, the tests were conducted at 5, 10 and 20 °C. Particularly at 5 °C, extractable residues slowly dropped within 7 days of incubation. Even in the 30-day samples, those (53 %) predominated the non-extractable ones (49 %). At 10 and 20 °C, this degradation was accelerated (**Fig. 5.28**). Already in the 3-day samples, 36 and 38 % have accounted for non-extractable residues that finally increased to 65 and 70 %, respectively.

6 Fate monitoring in manured soils

The laboratory and field studies already carried out in the Runoff Project[3] clearly showed that fate and behavior of VMP in manured soils were affected by the manure application. Thus, sulfonamides rapidly disappeared in the superficial layer (0-15 cm) of a silty-clay soil under test-plot conditions without any indication of substance losses by leaching (Kreuzig and Höltge, 2005, Kreuzig et al., 2007a). In laboratory-batch experiments on the degradation of ^{14}C-sulfadiazine, the formation of non-extractable residues was identified as the major concentration determining process (Kreuzig and Höltge, 2005, Heise et al., 2006).

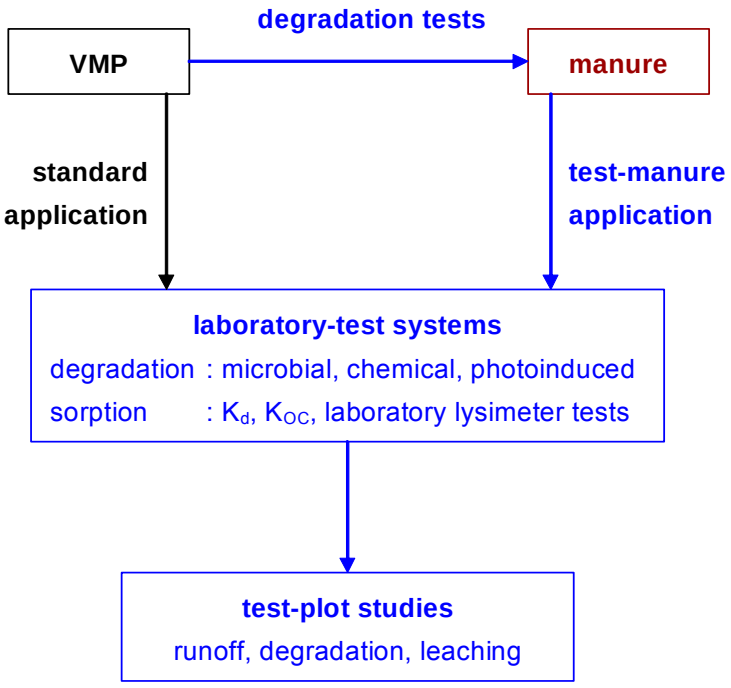


Fig. 6.1: Experimental design of the fate monitoring on VMP in manured soils

Within 50 days after application of the benzimidazole antiparasitics flubendazole and fenbendazole, both VMP steadily disappeared in the same soil. Thereafter, fenbenda-

[3] "Investigations on Runoff of Veterinary Medicinal Products from Farmland and Grassland after Manure Application" funded by the German Federal Environmental Agency (Runoff Project, FKZ 202 67 435, 2002-2004).

zole could not be determined above the limit of determination (20 µg kg^{-1} dry soil) caused by the formation of the corresponding sulfoxide and sulfone metabolites. The latter was exclusively formed in the clay soil after manure application (Kreuzig et al., 2007 a, b). As a result of these experiments, therefore, an experimental design was developed for fate monitoring on VMP in manured soils already under laboratory conditions (**Fig. 6.1**). On the basis of the degradation tests of VMP in manures, test manures with short-term aged VMP residues were prepared and fortified to soil samples to investigate degradation and sorption of VMP under laboratory and field conditions (Kreuzig and Höltge, 2005). In this way, the methodology of test-manure application was introduced, first.

6.1 Degradation of VMP in manured soils

In accordance to the development of the technical protocol for testing the degradation of VMP in manures, this experimental design for fate monitoring in manured soils was checked for analytical practicability and analytical quality assurance. Starting with matrix characterized reference-manure samples, test manures were prepared and applied in the degradation tests. For this purpose, the performance of three different laboratory-test systems was compared. These tests focused on ^{14}C-acetyl-sulfamethoxazole standard or test-manure applied to microbially active clay soil. Besides the set-up of mass balances considering mineralization, extractable and non-extractable residues, the rapid decomposition of this test substance into sulfamethoxazole could thus be pursued. Additionally, the metabolic fate in soil of ^{14}C-ketoprofen and ^{14}C-paracetamol was monitored in short-term tests. Subsequently, the macrolide antibiotic ^{14}C-erythromycin was long-term tested in two soils of different physico-chemical properties.

6.1.1 Performance of laboratory-test systems

The laboratory-batch system traced back to the OECD guideline 304 A (1981a) has already been applied for the degradation tests in manures. The flow-through system and the biometric-flask system with soda-lime trap were used, too. Both are specified in the OECD guideline 307 (2002). The goal of those different methodological approaches

is testing on the degradation of chemicals in topsoil samples under permanently aerobic conditions. Those are realized either by a discontinuous air exchange every 3-4 days, by a permanent oxygen flow rate or by a diffusion-controlled air exchange. Besides radiotracer analysis, parallel matrix characterization tests were conducted applying the non-labeled test substance in order to characterize the test conditions. Parameters under study were pH, redox potential, substrate-induced respiration and dehydrogenase activity.

Taken into account that the OECD guideline 307 (2002) accepts balances varying from 90 to 110 %, the mass balances did not considerably differ in those short-term batch experiments after standard application (**Tab. 6.1**). This was the same for the deacylation of ^{14}C-acetyl-sulfamethoxazole resulting in the regeneration of ^{14}C-sulfamethoxazole. Both performance criteria clearly showed the equivalent applicability of the three laboratory-test systems for monitoring the degradation of VMP in soils.

Tab. 6.1: Comparison of laboratory-test systems for the degradation of ^{14}C-acetyl-sulfamethoxazole in silty-clay soil after standard application

Day	**0**	**3**		**7**		
Test system		SLT	BS	SLT	BS	FTS
MIN [%]	nd	0.2	0.2	0.2	0.2	< 0.1
A-SMZ [%]	98	37	37	14	15	12
SMZ [%]	nd	33	33	33	32	30
NER [%]	7	24	30	46	51	61
Balances [%]	105	94	100	93	98	103

SLT : Biometric-flask with soda-lime trap and diffusion-controlled air exchange
BS : Laboratory-batch system with discontinuous air exchange every 3-4 days
FTS : Flow-through system with permanent oxygen stream
nd : not detected

This matter of fact was confirmed by the parallel matrix characterization tests (**Tab. 6.2**). As reflected by the redox potentials, aerobic conditions typical for topsoil samples prevailed in every test system after 3 and 7 days of incubation. Furthermore, the microbial activity in the soil samples under study was retained although differences were depicted by the substrate-induced respiration and the dehydrogenase activity. The first

indicating the activity of aerobic microorganisms maintained nearly constant while the latter dropped from 102 to 35 µg TPF g^{-1} dry substance 16 h^{-1}. However, this decrease reflecting the activity of the entirety of the soil inherent microorganisms was the same for every test system. After standard application, the applicability of the laboratory-batch system versus the others under study was proven again.

Besides the spiking of the aged residues of the test substance, readily degradable organic substances were introduced into the laboratory test-systems by the test-manure application to the soil samples under study. Therefore, it was relevant that aerobic conditions were retained in the laboratory-batch system devoid of an oxygen stream permanently passing through the incubation flask.

Tab. 6.2: Comparison of laboratory-test systems by matrix characterization

Day	**0**	**3**		**7**		
Test system		SLT	BS	SLT	BS	FTS
pH	6.2	6.2	6.2	6.3	6.4	6.4
Eh	490	430	450	520	530	510
SIR	1.2	1.0	1.3	---	1.0	1.2
DHA	102	99	102	34	35	35

SLT : Biometric-flask with soda-lime trap and diffusion-controlled air exchange
BS : Laboratory-batch system with discontinuous air exchange every 3-4 days
FTS : Flow-through system with permanent oxygen stream
Eh : Redox potential [mV]
SIR : Substrate-induced respiration [mg 100 g^{-1} h^{-1}]
DHA : Dehydrogenase activity [µg TPF g^{-1} 16 h^{-1}]

In **Tab. 6.3**, balances for ^{14}C-acetyl-sulfamethoxazole in manured clay soil and selected matrix parameters are compared. Considering the acceptable variation range of mass balances from 90 to 110 %, the results of the long-term tests were consistent for both test systems. This was confirmed by the matrix characterization tests, too. The redox potentials substantiated aerobic conditions up to the 100-day incubation interval. In contrast to the batch experiments after standard application, a definite higher microbial activity was revealed by the dehydrogenase activity after test-manure application.

Here, the values increased from the 7-day to the 28-day incubation interval. Finally, the microbial activity decreased up to 100 days of incubation, however, for both systems.

Tab. 6.3: Comparison of laboratory-test systems for the degradation of ^{14}C-acetyl-sulfamethoxazole in silty-clay soil after test-manure application

Day	**7**		**28**		**100**	
Test system	BS	FTS	BS	FTS	BS	FTS
MIN [%]	< 0.1	< 0.1	0.2	0.1	2	1
ER [%]	17	18	2	2	2	2
NER [%]	82	87	97	91	97	99
Balances [%]	99	105	99	104	101	102
pH	7.2	6.5	6.7	6.4	6.7	6.5
Eh	440	470	460	460	430	470
DHA	238	224	305	206	150	123

BS : Laboratory-batch system with discontinuous air exchange every 3-4 days
FTS : Flow-through system with permanent oxygen stream
Eh : Redox potential [mV]
DHA : Dehydrogenase activity [µg TPF g^{-1} 16 h^{-1}]

For a second series of performance tests, ^{14}C-erythromycin was applied to clay-soil samples via the application of test-manure that was prepared on the basis of reference-bovine manure. As already found for ^{14}C-acetyl-sulfamethoxazole, the metabolic fate of the macrolide antibiotic was consistently reflected by both laboratory-test systems (**Fig. 6.2**). Thus, these tests showed the equivalent applicability of both laboratory-test systems with advantages for the low-cost laboratory-batch system due to less required laboratory space, possible use of incubators instead of climatic chambers and effortless handling.

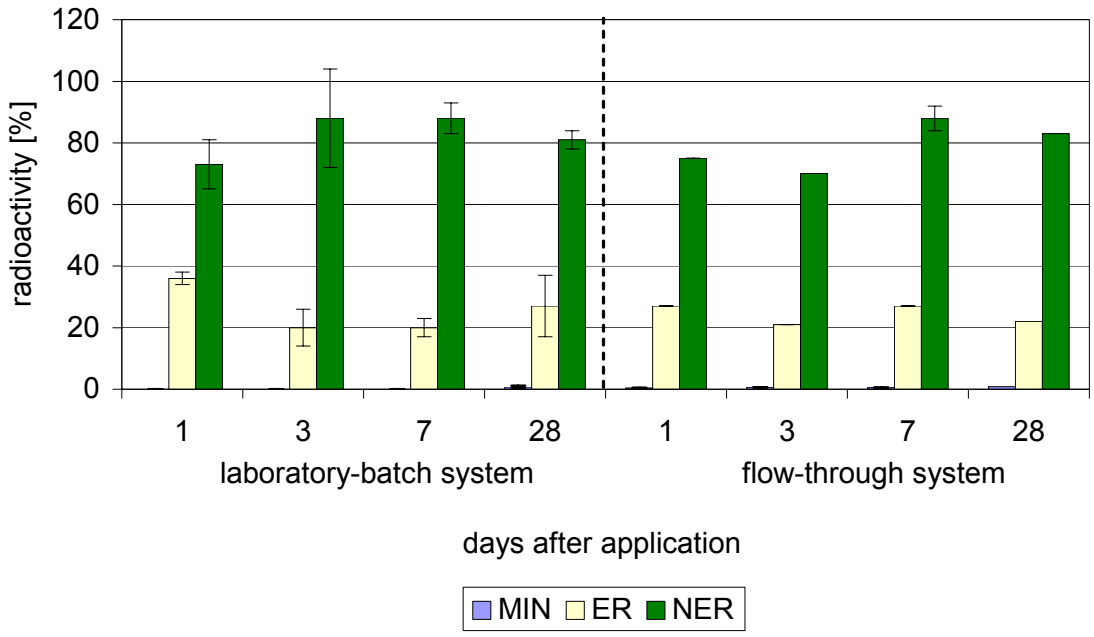


Fig. 6.2: Comparison of laboratory-test systems for the degradation of ^{14}C-erythromycin in silty-clay soil after test-manure application (BM-1) (balances: 106 ± 8 %)

6.1.2 Short-term tests on ketoprofen and paracetamol

Fate and behavior of ^{14}C-ketoprofen and ^{14}C-paracetamol in soils were investigated first in the Soil Project after standard and test-sludge application (Kreuzig et al., 2005a). There, both test substances were considered relevant as human pharmaceuticals that entered soil environments after irrigation of contaminated surface water for artificial ground water enrichment or after application of sewage sludge as organic fertilizer. Since both pharmaceuticals are applied in veterinary medicine, their entry via manure application in soil was also taken into account. Thus, short-term tests after test-manure application were conducted and compared to batch tests after standard application.

Fate of ^{14}C-ketoprofen in manured soils

For this purpose, ^{14}C-ketoprofen was fortified to matrix characterized bovine manure samples and incubated for 7 days. In those test-manure samples, 82 % accounted for ^{14}C-ketoprofen as the unchanged parent compound, while 19 % accounted for an uni-

dentified metabolite. Mineralization was < 0.1 % and non-extractable residues amounted to 2 %. By the addition of the 100-g microbially active soil samples, initial concentrations were thus adjusted at 24 µg ketoprofen kg^{-1} soil. During the 28-day incubation period, the extractable residues decreased from 83 % to 15 % of the initially applied radioactivity. The extracts of the latter still contained 5 % of ketoprofen. Simultaneously, non-extractable residues increased from 22 % to 87 % while mineralization finally reached 4.2 % (**Fig. 6.3**).

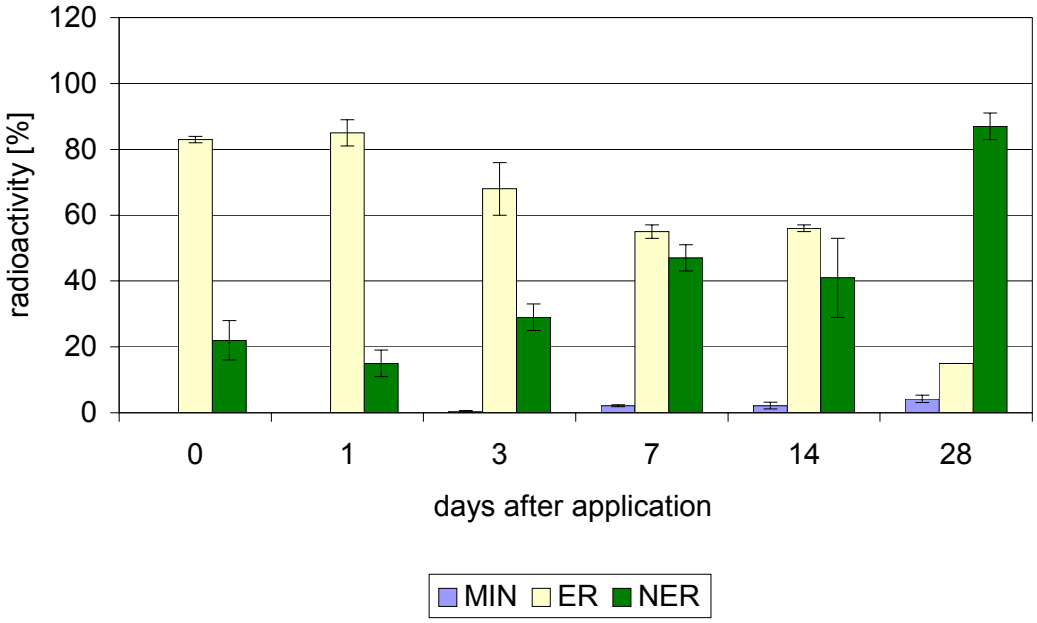


Fig. 6.3: Degradation of ^{14}C-ketoprofen in silty-clay soil after test-manure application (balances: 101 ± 3 %)

After standard application of ^{14}C-ketoprofen, however, the mass balances reflected considerable differences in the metabolic dynamics (**Fig. 6.4**). There, the extractable residues dropped from 92 % to 32 % already after 7 days of incubation. At the same time, 15 % of the initially applied radioactivity accounted for ^{14}C-carbon dioxide released by mineralization. At the end, this increased up to 26 % accompanied by the increase of non-extractable residues from 13 % to 51 %. This comparison showed again that the metabolic fate of VMP in soil is dependent on their entry route into soil environments. Thus, fate under field conditions can only be assessed when the manure

application is considered relevant already under laboratory conditions.

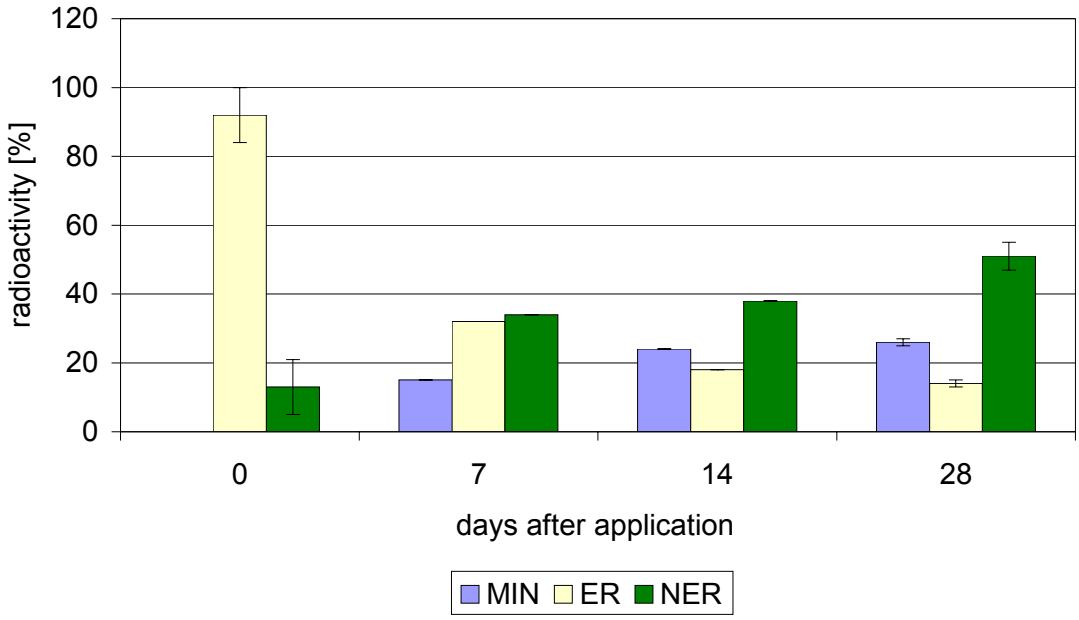


Fig. 6.4: Degradation of ^{14}C-ketoprofen in silty-clay soil after standard application (average: 89 ± 12 %)

Fate of ^{14}C-paracetamol in manured soils

Already in the Soil Project, the metabolic fate of ^{14}C-paracetamol was particularly noticeable due to its very rapid formation of non-extractable residues. However, this process that was intensified by the test-sludge application could only be observed in microbially active soil samples. In microbially inactive soil samples, however, the unchanged paracetamol remained extractable.

According to its application pattern in veterinary medicine, test-manure samples of ^{14}C-paracetamol were prepared on the basis of reference-pig manure. After 7 days, extractable residues amounted to 31 % containing 23 % of the unchanged parent compound. Besides two unidentified metabolites (approximately 4 % each), the non-extractable fraction accounted for 64 %. Mineralization was < 0.1 %. After the addition of 100-g microbially active soil samples, the initial concentration was 110 µg paracetamol kg^{-1} soil.

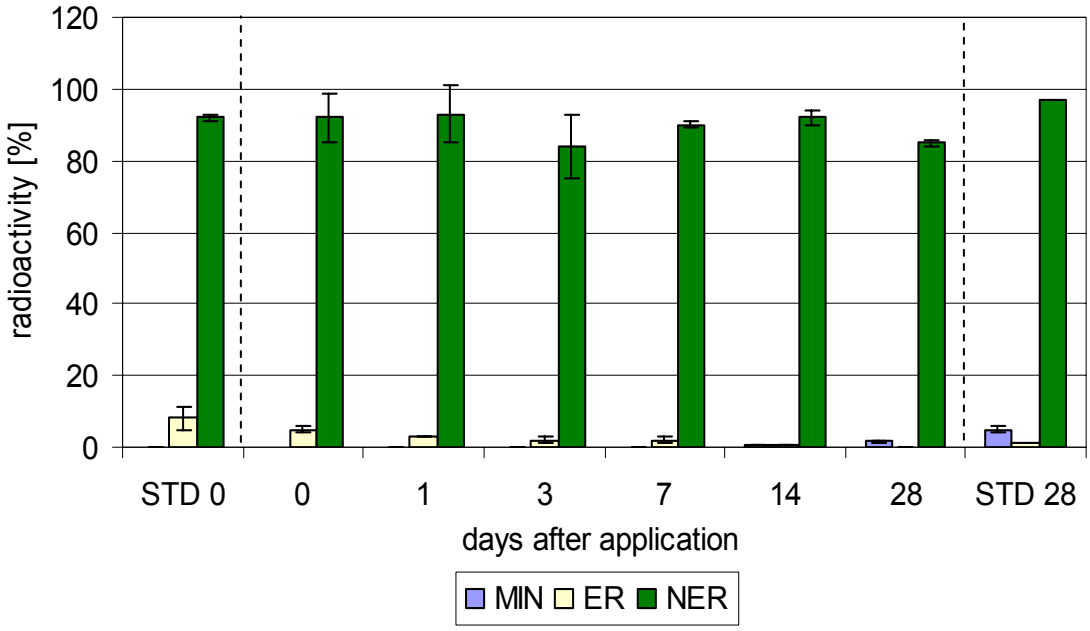


Fig. 6.5: Degradation of ^{14}C-paracetamol in silty-clay soil after test-manure application (balances: 91 ± 5 %) in comparison to standard application (STD)

The metabolic fate of ^{14}C-paracetamol in manured soil was predominated by the formation of non-extractable residues (**Fig. 6.5**). Already in the 0-day sample, those amounted to 92 % of the initially applied radioactivity. Subsequently, the non-extractable residues maintained nearly constant while the extractable ones dropped from 5 % to 0.2 %. After 28 days of incubation, the mineralization reached 2 % at maximum indicating that ^{14}C-carbon dioxide was released out of the non-extractable fraction. A similar tendency was found after test-sludge application (Kreuzig et al., 2005a).

After standard application, slightly differences in the intensity patterns of the mass balances occurred. In the 0-day samples, extractable residues still accounted for 8 % and finally dropped to 1 % while mineralization simultaneously increased up to 5 %. Non-extractable residues maintained nearly constant emphasizing again the high affinity of ^{14}C-paracetamol to the soil matrix.

6.1.3 Long-term tests on erythromycin

Since the Runoff Project, long-term tests on the degradation of VMP in manured soils have been performed for ^{14}C-sulfadiazine, ^{14}C-sulfamethoxazole, ^{14}C-acetyl-sulfamethoxazole, ^{14}C-flubendazole, ^{14}C-fenbendazole and ivermectine. The latter belonging to the class of macrolide endectocides is not available as a ^{14}C-labeled radiotracer and was thus applied as a non-labeled test substance. This experimental approach only allowed to determine the disappearance of the initially applied parent compound. Therefore, the macrolide antibiotic ^{14}C-erythromycin was involved into the degradation tests of the Manure Project.

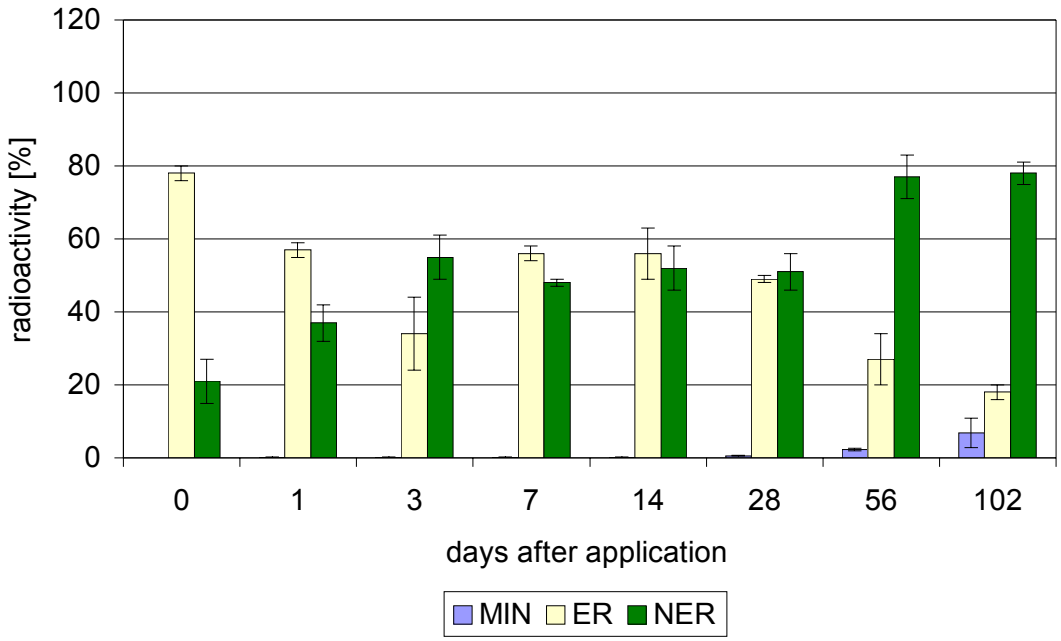


Fig. 6.6: Degradation of ^{14}C-erythomycin in silty-clay soil after standard application (balances: 101 ± 6 %)

Standard application

Preliminary, the metabolic fate in soil of ^{14}C-erythromycin in clay soil was investigated after standard application. Within the 102-day incubation period, the extractable residues decreased successively from 78 % to 18 % of the initially applied radioactivity. Along with this decrease, a release of ^{14}C-carbon dioxide by mineralization was found

after 14 days. Starting at 0.2 %, finally 7 % was reached. Simultaneously, the non-extractable residues increased up to 78 % after 102 days (**Fig. 6.6**).

Test-manure application

For the preparation of the test-manure samples, reference-pig manure was used that was fortified using ^{14}C-erythromycin. After the 7-day incubation period, silty-sand or silty-clay soil samples were added. The initial concentration thus was 155 µg erythromycin kg^{-1} dry soil. The degradation of ^{14}C-labeled erythromycin in silty-sand soil after test-manure application is illustrated in **Fig. 6.7**. In contrast to the standard application, here non-extractable residues were rapidly formed after mixing of test-manure and soil samples. Within the 102-day incubation period, they slightly decreased to 89 % accompanied by a decrease of extractable residues from 8 % to 4 %. Nevertheless, the release of ^{14}C-carbon dioxide could be observed first after 14 d. Mineralization was 0.1 % and subsequently increased up to 3 %.

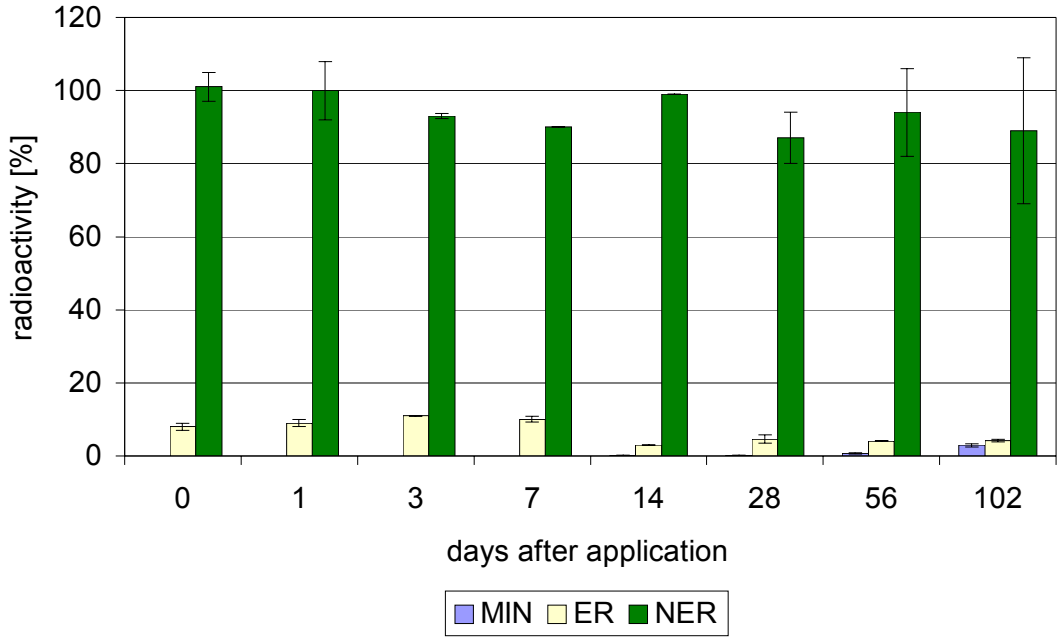


Fig. 6.7: Degradation of ^{14}C-erythomycin in silty-sand soil after test-manure application (PM-1) (balances: 101 ± 6 %)

These dynamic processes, rather unusual for the less microbially active silty sand (SIR: 0.4 mg O_2 100 g^{-1} h^{-1}), were reflected more clearly in the batch experiments using silty-clay soil samples (SIR: 2.1 mg O_2 100 g^{-1} h^{-1}). Within 56 days after test-manure application, the extractable fraction amounted to 10 % containing 4 % of the unchanged parent compound as shown by the RTLC screening. The non-extractable residues reached 92 % at the same time (**Fig. 6.8**).

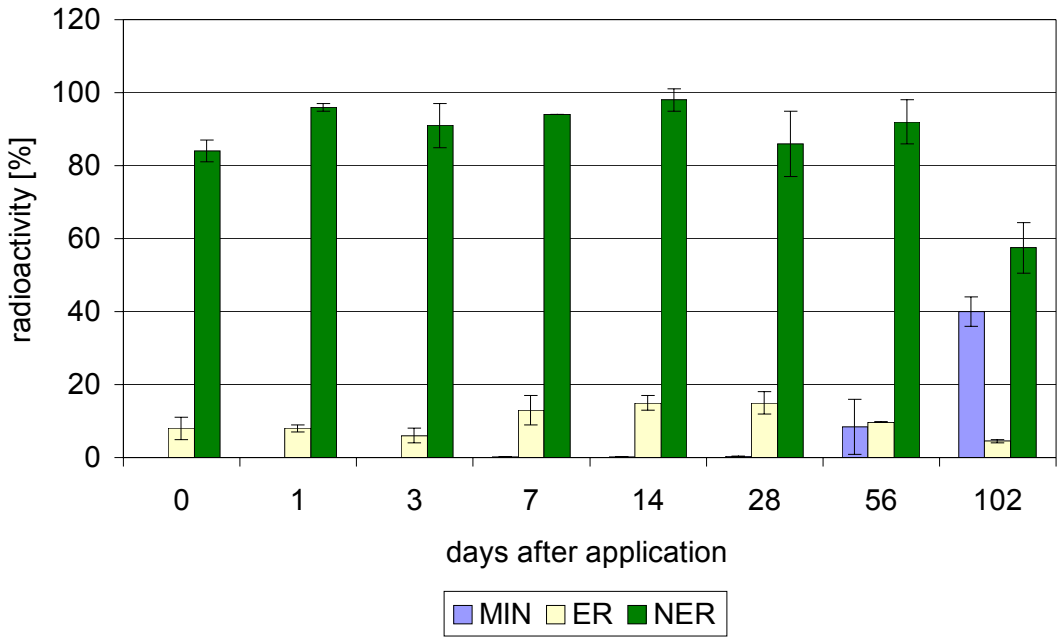


Fig. 6.8: Degradation of ^{14}C-erythomycin in silty-clay soil after test-manure application (PM-1) (balances: 103 ± 7 %)

Mineralization increased from 0.1 % after 7 days up to 8 %. Thereafter, mineralization escalated up to 40 % accompanied by the decrease of non-extractable residues up to 58 %. This process indicated that ^{14}C-carbon dioxide was released out of the non-extractable fraction. The extractable fraction simultaneously underwent only a less considerable decrease to 5 % containing 2 % of the unchanged parent compound. From that, however, the enhanced mineralization rates could not be derived.

6.2 Sorption in manured soils

In order to assess the mobility tendencies of VMP in soil under field conditions, batch equilibrium tests were carried out under laboratory conditions in order to determine soil/water distribution coefficients (K_d or K_{OC} values). The methodology of these tests has been primarily developed for pesticides. According to the OECD guideline 106 (1981b), the test substance under study was fortified to one portion of the calcium chloride solution that simulates the soil solution. Then, air-dried soil and the second portion of calcium chloride solution were added. This suspension was shaken for 24 h and, thereafter, separated by centrifugation. Subsequently, the concentration of the test substance in the aqueous phase was analytically determined.

Later, there was a methodological change reflected in the new OECD guideline 106 (2000). Here, the soil samples under study were equilibrated first in one portion of the calcium chloride solution overnight. Subsequently, the test substance under study and the second portion of the calcium chloride solution were added. The suspension was shaken for 24 h and the aqueous phase was analyzed.

Both methodological approaches have been applied for the assessment of the mobility of VMP in soils since 1997 (EMEA, 1998, VICH, 2003). However, both disregarded that VMP enter soil environments after manure application and that the manure matrix may affect sorption and mobility tendencies of VMP in soils. The latter effect was already shown within the Runoff Project. Although an enhanced mobility in soils was expected, e.g., for sulfadiazine due to its conventionally determined K_d value of 2 L kg^{-1} in silty-clay soil (Kreuzig and Höltge, 2005), relevant leaching could not be found after test-manure application, neither in laboratory-lysimeter experiments nor in test-plot studies under field conditions. On the basis of these results, the test-manure application was consequently adapted for the determination of soil/water distribution coefficients.

Impact of the standard and test-manure application on K_d values

According to the OECD guideline 106 (1981b), the impacts of standard and test-manure application on the K_d values of selected test substances were studied. The pure manure effect was studied, too. For this purpose, manure was added to soil after the test substance application via standard solution. The assessment of mobility tendencies was based on the trigger value defined by Fichter and Holden (1992). Thus, values < 5 L kg^{-1} indicate an enhanced mobility and leaching tendencies in soil.

The K_d values of the test substances under study, i.e., ^{14}C-sulfadiazine, ^{14}C-erythromycin, ^{14}C-ketoprofen and ^{14}C-paracetamol, were differently effected by the VMP application techniques (**Tab. 6.4**).

Tab. 6.4: K_d values of selected VMP after standard and test-manure application to silt-clay and silty-sand soil (n = 4)

Soil	Application technique	SDZ [L kg^{-1}]	ERY [L kg^{-1}]	KET [L kg^{-1}]	PCM [L kg^{-1}]
Silty clay	standard	2 ± 0	24 ± 2	3 ± 0	47 ± 2
	standard + BM	3 ± 0	33 ± 4	3 ± 0	---
	test manure (TM_B)	14 ± 1	3 ± 1	1 ± 0	---
	standard + PM	---	17 ± 2	---	42 ± 2
	test manure (TM_P)	---	1 ± 0	---	13 ± 2
Silty sand	standard	2 ± 0	14 ± 3	2 ± 0	35 ± 12
	standard + BM	15 ± 3	7 ± 1	3 ± 1	---
	test manure (TM_B)	84 ± 12	1 ± 0	1 ± 0	---
	standard + PM	---	7 ± 1	---	14 ± 2
	test manure (TM_P)	---	0.1 ± 0	---	7 ± 1

SDZ: sulfadiazine, ERY: erythromycin, KET: ketoprofen, PCM: paracetamol
BM: bovine manure, TM_B : test manure prepared from reference-bovine manure
PM : pig manure, TM_P : test manure prepared from reference-pig manure

After standard application, ^{14}C-sulfadiazine was still classified as a potential leacher while its mobility was definitely reduced after test-manure application. This effect especially concerned the silty-sand soil where a K_d of 84 L kg^{-1} was reached indicating a definitely lower leaching potential. As shown by the pure manure application, this impact could not be understood as an exclusive matrix effect. After test-manure application, the K_d values did not represent sorption of the initially applied test substance, but also of the metabolites and non-extractable residues formed during the 7-day incubation period of the test-manure preparation. Nevertheless, this innovative experimental approach contributed to the understanding of mobility of sulfadiazine in manured clay soil under field conditions (Kreuzig and Höltge, 2005, Kreuzig et al., 2005).

Complementary effects were found for ^{14}C-erythromycin. After standard application, K_d values were 24 L kg^{-1} for the clay soil and 14 L kg^{-1} for the sand soil, resulting in K_{OC} values of 1500 and 875 L kg^{-1}, respectively. According to the classification of Hollis (1991), both revealed only a slight mobility in soil. After test-manure application, however, K_d values dropped to 3 and 1 L kg^{-1}, respectively. Thus, the leaching potential of erythromycin would be underestimated by the conventional K_d value determination. A similar, but not so distinctive tendency was found for ^{14}C-paracetamol while ketoprofen was identified as a potential leacher, independent on neither standard nor test-manure application.

Effects of different experimental techniques on the K_d value determination

In additional tests, the effects of different experimental techniques were investigated. These differences were caused by different initial weights of the soil samples and different volumes of the calcium chloride solution. Despite the different boundary conditions in these test series, consistent mobility tendencies of ^{14}C-erythromycin were found (**Tab. 6.5**).

Tab. 6.5: Effects of different experimental techniques on the determination of K_d values of ^{14}C-erythromycin

Method		**A**			**B**		
Soil/water ratio		**1:1.4**			**1:1**		
Soil	**Application technique**	**OC** [%]	**K_d** [L kg^{-1}]	**K_{OC}** [L kg^{-1}]	**OC** [%]	**K_d** [L kg^{-1}]	**K_{OC}** [L kg^{-1}]
Silty clay	standard	1.6	24 ± 2	1500	1.6	8 ± 3	500
	test manure (TM_B)	4.5	3 ± 1	67	2.8	1 ± 0	36
	test manure (TM_P)	3.4	1 ± 0	29	2.9	1 ± 0	34
Silty sand	standard	1.1	14 ± 3	1273	1.6	4 ± 0	364
	test manure (TM_B)	3.4	1 ± 0	29	2.7	1 ± 0	37
	test manure (TM_P)	2.6	0.1 ± 0	4	2.3	1 ± 0	38

Method A: 25 g soil (ds) and 35 mL $CaCl_2$ (0.01 M) (OECD, 1981)
Method B: 50 g soil (ds) and 50 mL $CaCl_2$ (0.01 M) (OECD, 2000)

Highest K_d values were found after standard application. Thus, erythromycin could be

classified as slightly mobile in the silty-clay soil and as moderately mobile in the silty-sand soil (**Tab. 6.6**). The soil/water distribution coefficients dropped after test-manure application revealing ^{14}C-erythromycin residues as mobile in manured soils. The K_d value determination after test-manure application seemed to be more robust against the differing boundary conditions. As shown for this test substance, both methods can be applied to investigate the sorption behavior of VMP in manured soils.

Tab. 6.6: Mobility classes of pesticides in soils (Hollis, 1991)

Mobility class	**K_{OC}** [L kg^{-1}]
Immobile	> 4000
Slightly mobile	500-4000
Moderately mobile	75-499
Mobile	15-74
Very mobile	< 15

7 Intra- and interlaboratory tests

The development of the technical protocol for fate monitoring of VMP in manure and manured soil requires a tiered experimental design. That includes representative sampling of bovine and pig excrements, matrix characterization, preconditioning of excrement samples, preparation of reference-manure samples, performance of degradation tests, preparation of test-manure samples and performance of degradation and sorption tests of VMP in manured soils. Every step demands specific measures of analytical quality assurance. Thus, the first quality criterion focused on the reality to agricultural practice simulating the real entry route of VMP into soils via manuring as closely as possible. Thus, the natural composition of manures and their storage conditions have to be implemented already under laboratory conditions. The second criterion focused on the analytical practicability. Thus, developed tests have to be feasible in a defined frame of time and costs. The third criterion focused on the analytical quality assurance. Thus, the tests were conducted in duplicates and the test substances were applied as ^{14}C-labeled radiotracers allowing for the set-up of detailed mass balances. Those considered mineralization and formation of extractable and non-extractable residues. Finally, repeatability and reproducibility were checked in first intra- and interlaboratory tests, respectively.

7.1 Intralaboratory test on matrix characterization

A first intralaboratory test on the matrix characterization of bovine and pig excrements and reference manures was conducted at the Institute of EcoChemistry (ECO). The goal was to check the applicability of the standard operating procedures for the manifold sequence of different matrix parameters. The results are listed in **Tab. 7.1**. The determination of the dry substance contents was consistent though two different methods were applied. Results of mineral contents and total organic carbon matched, too. In contrast to the pH values, the use of electrodes for the determination of redox potentials resulted in a higher variability of the results. Nevertheless, anaerobic conditions were also revealed by the dissolved oxygen contents. Despite the complexity of the analytical methods for the determination of total nitrogen and ammonium nitrogen contents as well as the biological and chemical oxygen demands, those results were also

consistent.

Taking the complexity of those excrement and reference-manure samples and the multitude of different analytical methods into special account, the sequence of different matrix parameters was determined with high accuracy and precision although the experiences of the four test persons were quite different.

Tab. 7.1: Results of the intralaboratory test on matrix characterization of bovine (BE) and pig excrements (PE) and reference manures (BM, PM)

Matrix / Parameter	**BE-5**	**BM-5**	**PE-1**	**PM-1**
ds_{oven} [%]	10 ± 0.2	9 ± 0.3	21 ± 0.4	5 ± 0.5
ds_{IR} [%]	12 ± 0.9	10 ± 0.5	23 ± 0.8	6 ± 1.3
R_{min} [%]	13 ± 1	na	22 ± 2	na
TOC [g kg^{-1}]	40 ± 4	37 ± 3	80 ± 17	19 ± 4
pH	6.4 ± 0.1	6.5 ± 0.1	7.4 ± 0.1	7.7 ± 0.2
Eh [mV]	- 80 ± 50	- 100 ± 30	- 190 ± 70	- 220 ± 30
O_2 [mg kg^{-1}]	< 0.1	< 0.1	< 0.1	< 0.1
NH_4-N [g kg^{-1}]	1.2 ± 0.0	0.9 ± 0.1	9.3 ± 0.2	2.0 ± 0.1
N_{total} [g kg^{-1}]	2.9 ± 0.0	2.5 ± 0.1	13.7 ± 0.1	3.0 ± 0.0
BOD_5 [g kg^{-1}]	12 ± 2.0	na	20 ± 3.5	na
COD [g kg^{-1}]	61 ± 5	59 ± 6	179 ± 10	41 ± 3

na: not analyzed, n = 4

7.2 Interlaboratory tests on matrix characterization

Besides the repeatability of the matrix characterization of the excrement and reference-manure samples in one laboratory, the reproducibility in three different laboratories was also investigated (**Tab. 7.2**). Corresponding to the intralaboratory test, the analyses of dry substance and mineral contents, copper and phosphorus amounts, total organic carbon contents and pH values led to consistent results.

Tab. 7.2: Results of the interlaboratory test on matrix characterization of bovine and pig excrement samples (n = 3)

Matrix / Parameter	**Bovine excrement**			**Pig excrement**		
Laboratory	RCC	ECO	UMS	RCC	ECO	UMS
ds [%]	12 ± 0.1	12 ± 0.4	12 ± 0.0	12 ± 0.0	12 ± 0.1	12 ± 0.7
R_{min} [%]	13 ± 0.5	14 ± 0.5	16 ± 0.1	17 ± 0.4	18 ± 0.8	21 ± 1.2
Cu [mg kg^{-1}]	9 ± 0.3	7 ± 0.8	11 ± 0.5	9 ± 0.2	8 ± 0.2	13 ± 0.6
P [mg kg^{-1}]	1.0 ± 0.0	0.9 ± 0.0	1.3 ± 0.1	2.3 ± 0.1	2.6 ± 0.1	2.8 ± 0.1
TOC [g kg^{-1}]	52 ± 1	56 ± 5	46 ± 0	50 ± 0	52 ± 4	40 ± 0
pH	6.5	6.6	6.4	7.1	7.0	6.9
Eh [mV]	-60 ± 20	-160 ± 10	150 ± 40	-180 ± 0	-210 ± 10	-140 ± 0
O_2 [mg kg^{-1}]	< 0.1	< 0.1	1.2 ± 0.1	< 0.1	< 0.1	0.5 ± 0.1
NH_4-N [g kg^{-1}]	2.6 ± 0	2.5 ± 0	2.1 ± 0.1	6.2 ± 0	4.7 ± 0	4.3 ± 0
N_{total} [g kg^{-1}]	3.9 ± 0	4.5 ± 0	5.2 ± 0.2	5.6 ± 0.1	6.7 ± 0.1	6.7 ± 0.5
BOD_5 [g kg^{-1}]	32	15 ± 0	26 ± 0	45	19 ± 0	38 ± 2.1
COD [g kg^{-1}]	168	136 ± 1	143 ± 15	167	117 ± 13	107 ± 3

However, the measured redox potentials and dissolved oxygen contents clearly showed the difficulties of the use of electrodes particularly in the complex bovine excrement samples. Thus, one laboratory measured values of 150 ± 40 mV for the redox potential and 1.2 ± 0.1 mg kg^{-1} for the dissolved oxygen content in the bovine excrement samples. Those indicated rather aerobic conditions while the corresponding values of both other laboratories definitely reflected anaerobic conditions. Concerning the dissolved oxygen content, similar tendencies were found in the pig excrement samples although the redox potentials matched the values of both other laboratories. Despite those difficulties, anaerobic conditions during the incubation intervals of the degradation tests of VMP in reference manures up to 177 days can be guaranteed by the experimental procedure. After the manure samples are introduced and spiked by the ^{14}C-labeled test substance, therefore, the laboratory-batch systems are directly rinsed by means of a gentle nitrogen stream. Later on, this nitrogen rinsing is repeated after every exchange of the potassium hydroxide solution used for the absorption of ^{14}C-carbon dioxide. Hence, the anaerobic conditions could be operationally defined.

Furthermore, ammonium and total nitrogen contents were reliably analyzed at every laboratory. The variability of the biological oxygen demand may be attributed to different analytical techniques applied by two laboratories. One laboratory measured the oxygen contents in Karlsruher bottles using the oxygen electrode (ECO) while the other laboratory applied the Sapromat method (UMS). Finally, the results for the chemical oxygen demand were nearly consistent.

7.3 Interlaboratory test on degradation of ^{14}C-sulfadiazine in reference manures

Besides the matrix characterization of bovine and pig excrements, the methodology of the degradation tests of VMP in reference manures was involved in a first interlaboratory test. For this purpose, bovine and pig excrements and standard solutions of ^{14}C-sulfadiazine were dispatched to the test laboratories. There, the excrement samples were conditioned at ambient temperature. After matrix characterization, reference manures were prepared and used for degradation tests with incubation periods up to 7 days.

As principally shown for the degradation tests in bovine manure, the mass balances recorded in the 0-day samples were consistently predominated by the extractable fraction (**Fig. 7.1)**. Non-extractable residues were already found after application. Mineralization rates were < 0.1 %. Replicates matched for each laboratory. On closer examination, however, differences were obvious for the results of the three laboratories. Although the balances of RCC and ECO matched the acceptable variability range of 90 to 110 % (OECD, 2002), there was an intensity shift between extractable and non-extractable fractions. Thus, RCC found on average 88 % extractable and 12 % non-extractable residues while ECO found on average 72 % and 22 %, respectively. For these differences, different extraction procedures may be the crucial factor. On the one hand, deviating from the standard operating procedure given for this interlaboratory test, the bovine manure suspension was separated by centrifugation. Thereafter, the aqueous phase was directly scintillation counted and the solid phase was extracted by means of acetonitrile. On the other hand, the manure suspension was directly treated by means of ethyl acetate.

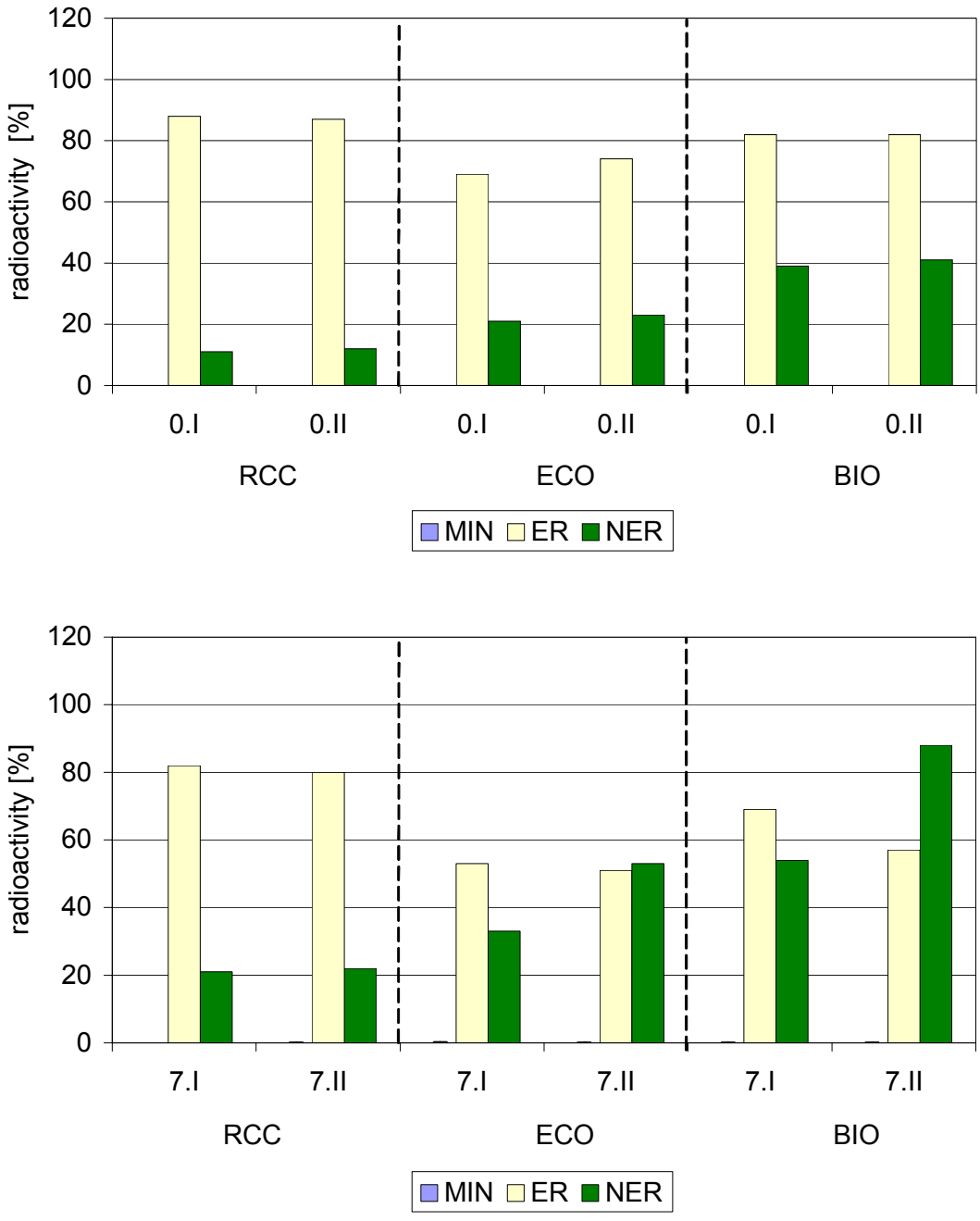


Fig. 7.1: Degradation of ^{14}C-sulfadiazine in bovine manure after 0- and 7-day incubation periods monitored at three laboratories

The RCC procedure thus led to the higher amounts of extractable residues. However, that was combined with the difficulties of a preliminary metabolite screening in the aqueous phase. At low concentrations of the formed metabolites, a direct HPLC/MS analysis is excluded because of too low sensitivity. The development of a method for

the analytical determination of unknown compounds, including enrichment and clean-up procedures, may turn out to be time and cost consuming.

The BIO data could be hardly included into the discussion of those results because the acceptable range of the recorded balances was exceeded. There, the non-extractable fractions with amounts of 40 % on average seemed to be overestimated. Particularly for extracted bovine manure samples, the solid phase has to be mixed with sea sand and cellulose before drying, grinding and combusting. Otherwise, an unequal distribution of non-extracted radioactivity may occur due to brittleness and hardness of this dried solid material. These tendencies were also found after the 7-day incubation periods. However, an increase of non-extractable residues was found at every laboratory reflecting the high affinity of ^{14}C-sulfadiazine to manure matrices.

In comparison to the interlaboratory tests for bovine manure, four aspects attracted attention in the tests with pig manure (**Fig. 7.2**). First, highest amounts of extractable residues and lowest amounts of non-extractable ones were found again by RCC. Second, ^{14}C-sulfadiazine has a lower affinity to pig manure than to bovine manure. Third, the formation of non-extractable residues increased with the increasing incubation period. Fourth, the mass balances recorded by the three laboratories matched the acceptable variability range. Hence, pig manure samples were easier to handle than the bovine manure samples.

The first interlaboratory test clearly showed that the innovative experimental design of the Manure Project principally facilitated the appropriate applicability of reference-manure samples for monitoring the degradation of VMP. However, the occurred experimental variability requires the improvement of single analytical procedures. Due to the matrix characterization, inaccuracies were found for the application of electrodes to measure redox potentials and dissolved oxygen contents. Nevertheless, those vulnerabilities can be compensated by the analytical procedure of these degradation tests in manures that operationally define anaerobic conditions. Further efforts should be focused on the determination of the microbial activity in manure samples. Thus, a change-over from the measurement of the biological oxygen demand to the dehydrogenase activity is preferred although the method development for the latter has to be optimized for the boundary conditions in manure samples.

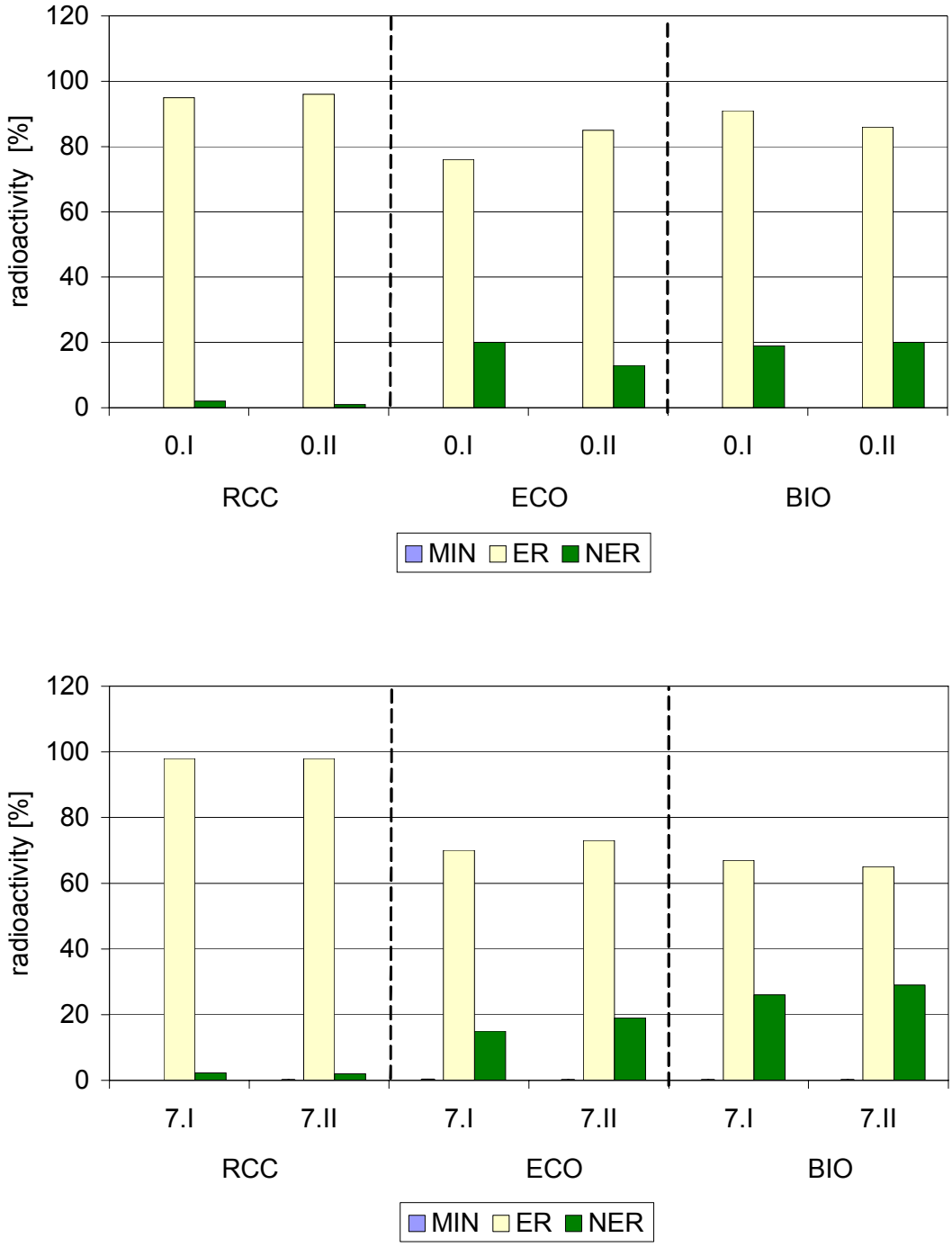


Fig. 7.2: Degradation of ^{14}C-sulfadiazine in pig manure after 0- and 7-day incubation periods monitored at three laboratories

As shown by these degradation tests of ^{14}C-sulfadiazine in bovine and pig manures, the extraction procedure for each test substance and its corresponding metabolites have to be optimized. The separation of the manure suspension may be an alternative approach to enhance the extraction efficiency. Due to the unsolved problem of the direct metabolite screening in aqueous solutions at low concentrations, the method development has to be also advanced taken the required time and cost frame for routine analysis into account. A further alternative to the direct solvent extraction may be the pretreatment of the manure samples by lyophilization.

7.4 Intralaboratory test on degradation of VMP in manured soils

For the completion of the Manure Project, a further intralaboratory test was conducted by three differently experienced test persons of ECO. For this purpose, test-manure samples containing ^{14}C-erythromycin were prepared first by conducting 7-day incubation tests. These results are illustrated in **Fig. 7.3**.

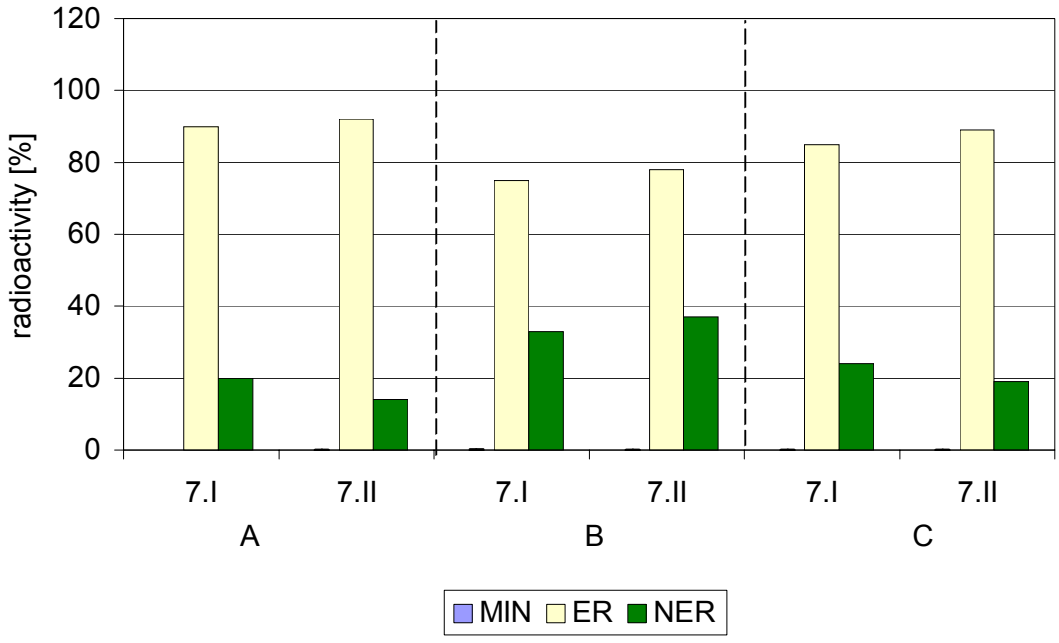


Fig. 7.3: Degradation of ^{14}C-erythromycin in bovine manure (BM-1) after 7 days monitored by three test persons (A-C)

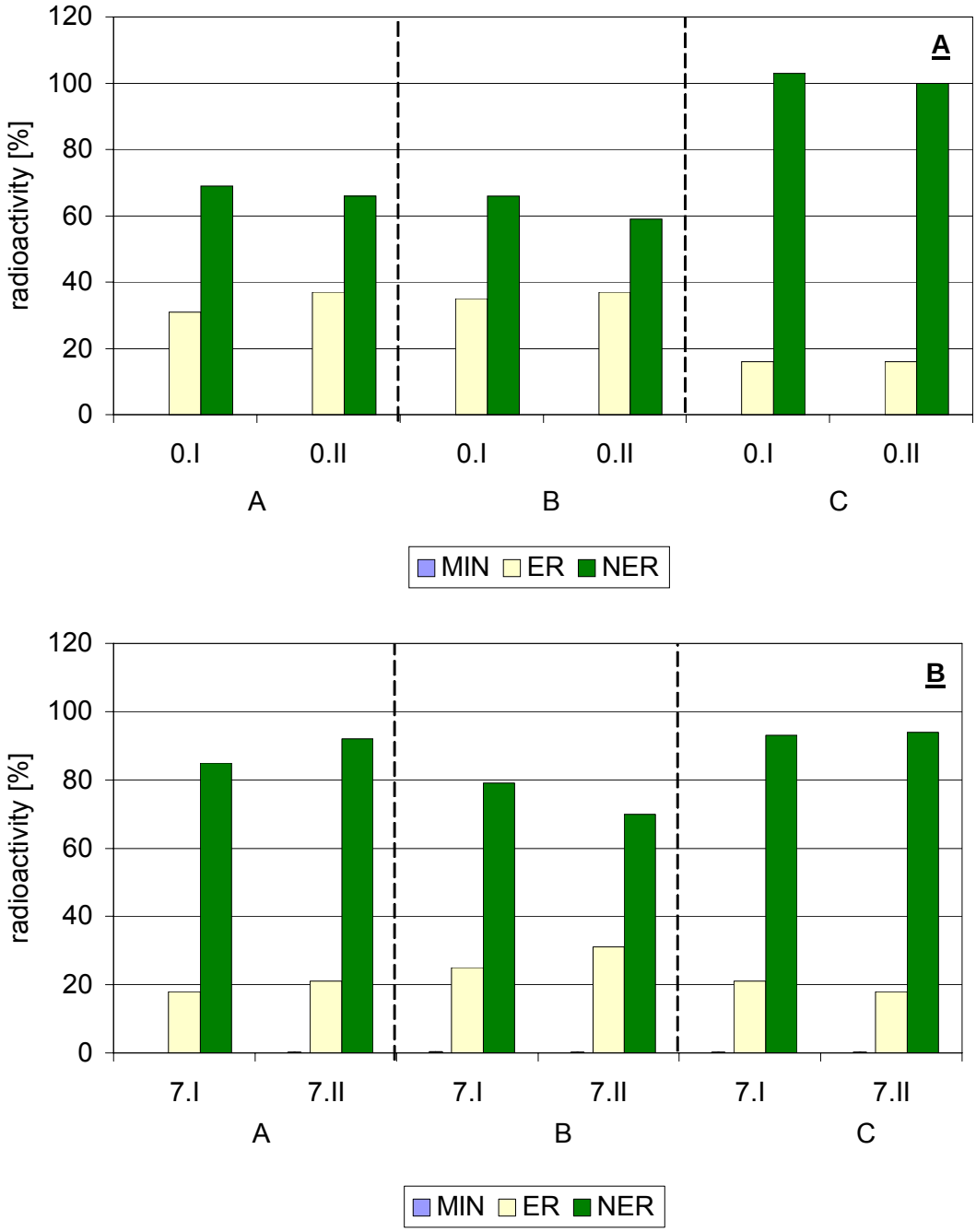


Fig. 7.4: Degradation of ^{14}C-erythromycin in silty-clay soil after test-manure application monitored by three test persons (A-C). Mass balances in the A: 0- and B: 7-day samples

The recorded mass balances were on average 109 ± 3 % and the single values of the duplicates of each test person were consistent. Nevertheless, there was a slight intensity shift of extractable and non-extractable fractions found for test person B. The test persons A and C found definitely lower amounts of non-extractable residues.

Slight differences between the results of the three test persons were also found in the degradation tests of ^{14}C-erythromycin in manured clay soil. Here, test person C determined the highest amounts of non-extractable residues in the 0-day samples (**Fig. 7.4**). In contrast, those differences were not recorded after 7 days of incubation.

These results clearly discover two aspects. First, the analytical quality is depending on the experiences of the analytical technicians or chemists. Second, the matrix characterization of excrement and manure samples as well as the degradation of VMP in manures and the degradation in manured soils can be reproducibly performed, if the technical protocol is strictly complied.

8 Summary

The initial situation

The entry route of VMP into soil environments is well understood. After the administration to production animals, VMP are excreted as parent compounds or metabolites. Together with the excrements, they are transferred into manure tanks. During the long-term manure storage, i.e., up to 6 months in Germany, VMP may undergo degradation or sorption under anaerobic conditions. Those are typical for the storage of bovine and pig manures. Non-degradable substances will reach soil environments when manures are applied as organic fertilizers. There, fate and behavior of VMP may be furthermore affected by the manure matrix. These aspects are partly considered by the authorization procedure. There, the entry of VMP is only calculated by exposure assessment. Due to the precautionary principles, degradation and sorption of VMP during manure storage are not considered as concentration minimizing processes. Thus, antibiotics above the trigger of 100 µg VMP kg^{-1} soil and antiparasitics have to be tested on ecotoxicological effects as well as on degradation and sorption in soils. For the latter laboratory tests, the test substances are applied in appropriate solvents to soil samples of different physico-chemical properties. Possible manure effects are thus disregarded.

The innovative concept

In order to close this lack, the Manure Project has been targeted at the development of a technical protocol for laboratory tests on the fate of VMP in manures and manured soils. Taking into account that bovine and pig manures are heterogeneous sample matrices of high complexity and variability, reproducible sampling activities in high-volume manure tanks is considered impossible. Therefore, an innovative research concept was developed that started in the experimental stable. There, excrements of individually kept cattle and pigs were sampled first. To understand the factors responsible for the variability of the excrement samples, different ages of production animals and different feeding conditions were taken into account. By means of this innovative approach, influences of disinfection or cleaning agents, commonly defined as biocides, and administered VMP could be principally excluded. Second, the excrement samples were thoroughly characterized by matrix parameters often used to analyze waste water, sewage sludge and soil samples or to assess the biogas potential of different manures. Parameters under study were dry substance, mineral content, copper and phosphate con-

tent, total organic carbon, pH, redox potential, dissolved oxygen content, total nitrogen and ammonium nitrogen content, biological and chemical oxygen demand. Third, tap water was added to prepare reference-manure samples of defined dry substance contents. These matrices underwent matrix characterization, too. Forth, selected VMP were applied as ^{14}C-labeled test substances, i.e., sulfamethoxazole, acetyl-sulfamethoxazole, sulfadiazine, ketoprofen, paracetamol and erythromycin, in order to monitor their degradation during the manure storage up to 177 days. Additionally, the matrix characterization was continued to identify the boundary conditions of the degradation tests. Furthermore, impacts of different dry substance contents and different incubation temperature were also considered. Fifth, test manures containing 7-day aged VMP residues were prepared and applied to soil samples to study degradation and sorption in manured soils. Via test-manure application, the real entry route of VMP into soil environments was simulated. Hence, this concept will improve the assessment of fate and behavior of VMP in soil under field conditions.

The evaluation of the concept

Three principles have been considered relevant for the development of the technical protocol. First, the degradation of VMP in manures as well as their entry route via manuring and manure effects on the fate of VMP in soils should be simulated under laboratory conditions. Second, the development of the experimental design has to be geared to the analytical practicability. Those laboratory tests have to be performed within a defined frame of time and costs. Third, the laboratory tests have to comply with the standards of the analytical quality assurance checked in preliminary intra- and inter-laboratory tests.

In contrast to heterogeneous manure samples from high-volume tanks, the innovative concept definitely enhanced the applicability of reference manures for laboratory testing. This fact is clearly revealed by the thorough matrix characterization of excrement and manure samples and the performed degradation tests of the test substances under study. These tests showed that considerable differences occurred for the degradation of VMP in bovine or pig manure. Therefore, that manure matrix should be tested that corresponds to the application patterns of the VMP in veterinary medicine. The impact of different ages of animals and different feeding conditions is of lower relevance. The latter can be largely reduced, when diets for the test species of cattle and pigs will be finally specified in the technical protocol. Different dry substance contents of the refer-

ence manures or different incubation temperatures affected the metabolic fate of VMP during the first incubation intervals. Thereafter, these effects were leveled out. For the long-term degradation tests, therefore, one dry substance content, i.e., 10 % for bovine manure and 5 % for pig manure, and one incubation temperature, i.e., 20 °C, are defined in the technical protocol. Boundary conditions may be optionally modified to study specific effects. Furthermore, the matrix characterization tests, carried out in parallel to the degradation tests of VMP in manures, showed the equivalence of the laboratory-test system and the flow-through system for monitoring the degradation of VMP in manures under anaerobic conditions.

On the basis of those degradation tests, test manures containing 7-day aged residues of the test substances were prepared. Via test-manure application, parent compounds initially applied and metabolites as well as non-extractable residues formed during the short-term incubation period were thus applied to soil samples in order to study degradation and sorption of VMP in manured soils. In contrast to the standard application, where only a single test substance is applied, the test-manure application enhances the fuzziness of the substance patterns as it may occur in manure tanks. Nevertheless, this approach ensured the simulation of the real entry route via manuring and the consideration of manure matrix effects on the fate of VMP in soil.

Need of further research activities

A first intralaboratory test on the matrix characterization of bovine and pig excrement and reference manures clearly showed that manures became applicable sample matrices for laboratory testing. This fact was confirmed by the first interlaboratory test. However, both also showed that the analytical determination of several matrix parameters may be difficult. Particularly, the use of electrodes to check for anaerobic conditions by the measurement of the redox potential and the dissolved oxygen content may be affected by some interferences. Nevertheless, anaerobic conditions are operationally guaranteed by the experimental design of the degradation tests in manures. Further research activities should focus on the determination of the microbial activity in excrements and manures. The determination of the biological oxygen demand merely comprised the activity of the aerobic microorganisms. Thus, the dehydrogenase activity could be alternatively determined. The final photometric analysis of this procedure may be interfered by the colored excrement and manure extracts at $\lambda = 485$ nm. Here, the method development should be continued to facilitate the substitution of the BOD

measurement. Furthermore, the copper content and the chemical oxygen demand can be canceled from the parameter patterns. Then, both only provide limited information.

The interlaboratory test also included the degradation tests of VMP in bovine and pig manure. In these short-term tests, the different laboratories found similar tendencies in the metabolic fate of ^{14}C-sulfadiazine, revealing the reproducible applicability of the test design. However, further research is necessary to develop a sophisticated extraction procedure. This should consider the exhaustive extraction of the parent compound and its corresponding metabolites. Thus, different extraction techniques should be compared for selected test substances, i.e., direct solvent extraction of the manure suspension, separation of the manure suspension by centrifugation followed by the solvent extraction of the solid phase or lyophilization of the manure sample followed by the solvent extraction. Finally, the extraction technique has to be adapted for each single VMP under study.

The second intralaboratory test dealt with fate monitoring of ^{14}C-erythromycin in manured soil. Although the test persons were differently experienced in analytical chemistry, this test clearly showed that the experimental sequence from the preparation of reference manures and test-manures to the degradability tests can be reproducibly performed. Nevertheless, further interlaboratory tests are needed to establish this technical protocol. For this purpose, further test substances should be included. Here, biocides that are applied in husbandry stables and enter manures are of particular interest. In contrast to VMP, those may differ in their physico-chemical properties because they belong to different chemical classes and their application patterns, e.g., direct application into manure or spray application in the stable. Finally, a consistent technical protocol should be established for laboratory testing on VMP and biocides in manures and manured soils.

9 Zusammenfassung

Die Ausgangssituation

Der Eintragspfad für Veterinärpharmaka in Böden ist eindeutig vorgezeichnet. Nach Verabreichung an Nutztiere werden die Veterinärpharmaka als unveränderte Ausgangssubstanzen oder gebildete Metaboliten ausgeschieden und gelangen in die Gülle. Während der langfristigen Güllelagerung, die z.B. in Deutschland bis zu 6 Monate betragen kann, unterliegen diese Abbau- und Sorptionsprozessen unter anaeroben Bedingungen, welche typisch für die Lagerung von Rinder- und Schweinegülle sind. Mit der Ausbringung der Gülle gelangen dann die während der Lagerung nicht abgebauten Substanzen in Böden, wo ihr Rückstandsverhalten wiederum von Matrixeffekten der Gülle beeinflusst werden kann. Diese Aspekte werden vom Zulassungsverfahren teilweise berücksichtigt. So wird die Eintragssituation mittels Expositionsabschätzung ermittelt. Gemäß den Vorsorgeprinzipien werden allerdings Abbau und Sorption während der Güllelagerung nicht als konzentrationsminimierende Prozesse betrachtet. Damit sind Antibiotika oberhalb des Schwellenwertes von 100 µg kg^{-1} Boden und Antiparasitika auf ökotoxikologische Effekte und auf Abbau sowie Sorption in Böden zu testen. In diesen Labortests werden die Testsubstanzen gelöst in einem geeigneten Lösungsmittel dotiert, so dass hiermit mögliche Einflüsse der Güllematrices nicht berücksichtigt werden können.

Das innovative Konzept

Genau hier setzte das Gülle-Projekt an, um diese Lücke mit der Erarbeitung eines Methodenkataloges mit Richtliniencharakter für Labortests zum Rückstandsverhalten von Veterinärpharmaka in Gülle und güllegedüngten Böden zu schließen. Aufgrund der Heterogenität von Gülle ist eine reproduzierbare Entnahme von Proben aus Güllesilos nicht gegeben. Daher begann dieses innovative Konzept im Versuchsstall, in dem Exkrementproben von Kühen und Schweinen aus der Einzeltierhaltung entnommen wurden. Um hier unterschiedliche Einflussgrößen auf die Zusammensetzung der Exkrementproben zu erkennen, wurden Tiere unterschiedlichen Alters sowie unterschiedliche Fütterungsbedingungen einbezogen. Diese Vorgehensweise bot vor allem den Vorteil, dass Einflüsse über eingetragene Desinfektions- und Reinigungsmittel sowie andere Biozide und verabreichte Veterinärpharmaka grundsätzlich ausgeschlossen werden konnten. Danach wurden die Exkrementproben einer umfassenden Matrixcha-

rakterisierung unterzogen. Berücksichtigte Parameter waren Trockensubstanz, Mineralgehalt, Kupfer- und Phosphatgehalt, Gesamtgehalt an organischem Kohlenstoff, pH, Redoxpotential, gelöster Sauerstoffgehalt, Ammonium- und Gesamtstickstoffgehalt sowie biologischer und chemischer Sauerstoffbedarf, die ebenfalls in der Analyse von Abwässern, Klärschlämmen oder Böden sowie zur Abschätzung des Biogaspotentials von Gülle herangezogen werden. Im nächsten Schritt wurden dann durch Wasserzugabe die Referenzgülleproben mit definierten Trockensubstanzgehalten hergestellt, die wiederum der Matrixcharakterisierung unterzogen wurden. Diese Referenzgülleproben wurden schließlich in den Abbautests der ^{14}C-markierten Testsubstanzen Sulfamethoxazole, Acetyl-sulfamethoxazole, Sulfadiazine, Ketoprofen, Paracetamol und Erythromycin eingesetzt. Die Inkubationsperiode von 177 Tagen simulierte dabei eine langfristige Güllelagerung. Auch hier erfolgte die Matrixcharakterisierung anhand der oben aufgeführten Parameter, um so Veränderungen der Güllematrices während dieser Tests erkennen zu können. Darüber hinaus wurden unterschiedliche Trockensubstanzgehalte und verschiedene Inkubationstemperaturen bezüglich ihres Einflusses auf den Abbau der Testsubstanzen in Gülle betrachtet. Auf der Basis dieser Abbautests wurde dann Testgülle mit 7 Tage gealterten Veterinärpharmaka-Rückständen hergestellt, um so Abbau und Sorption in Böden zu untersuchen. Mit dieser Testgülle-Applikation gelingt es, den realen Eintragspfad bereits unter Laborbedingungen nachzuvollziehen und somit die Abschätzung des Rückstandsverhaltens von Veterinärpharmaka in güllegedüngten Böden unter Freilandbedingungen zu verbessern.

Die Bewertung des Konzeptes

Der Entwicklung des Methodenkataloges beruhte auf drei grundsätzlichen Bedingungen. Dabei galt es, das Rückstandsverhalten von Veterinärpharmaka in Gülle und güllegedüngten Böden unter der besonderen Berücksichtigung des realen Eintragspfades bereits unter Laborbedingungen zu erfassen. Diese Labortests mussten sich ferner an der analytischen Machbarkeit in einem definierten Zeit- und Kostenrahmen und an den Standards der analytischen Qualitätssicherung orientieren. Letztere wurde in ersten internen und externen Labortests überprüft.

Im Gegensatz zu heterogenen Gülleproben aus Güllesilos ermöglicht dieses innovative Konzept die reproduzierbare Anwendbarkeit von Referenzgülleproben in Labortests. Dieses wird durch die umfassende Matrixcharakterisierung sowie durch die Abbautests eindeutig belegt. In diesen Tests ergaben sich deutliche Unterschiede für die einge-

setzten Rinder- und Schweinegülleproben. Daraus ergibt sich die Forderung, Veterinärpharmaka entsprechend ihres Anwendungsmusters in der entsprechenden Güllematrix zu testen. Einflüsse des Tieralters oder der Fütterungsbedingungen waren dagegen von untergeordneter Bedeutung. Letzterer kann ferner durch die Festschreibung definierter Fütterungsbedingungen weitgehend vermindert werden. Unterschiedliche Trockensubstanzgehalte und unterschiedliche Inkubationstemperaturen beeinflußen in den ersten Inkubationsintervallen der Abbau der untersuchten Veterinärpharmaka. Zu späteren Intervallen gleichen sich diese Unterschiede weitestgehend an. Damit ist es ausreichend, für die Abbautests im Methodenkatalog einen Trockensubstanzgehalt, z.B. 10 % für Rindergülle und 5 % für Schweinegülle, und eine Inkubationstemperatur, z.B. 20 °C, vorzuschreiben. Zur Ermittlung spezieller Effekte können diese Randbedingungen dann optional modifiziert werden. In den Matrixcharakterisierungstests, parallel zu den Abbautests durchgeführt, zeigte sich ferner die Gleichwertigkeit verschiedener Labortestsysteme, so dass für die Abbautests in Gülle unter anaeroben Milieubedingungen sowohl das Laborbatchsystem als auch das Durchflusssystem eingesetzt werden können.

Auf der Basis dieser Abbautests wurde Testgülle mit 7 Tage gealterten Veterinärpharmaka-Rückständen hergestellt und zur Untersuchung des Abbau- und Sorptionsverhaltens von Veterinärpharmaka in güllegedüngten Böden eingesetzt. Mit der Testgülle-Applikation werden so neben der anfangs eingesetzten Ausgangsverbindung auch gebildete Metaboliten und nicht-extrahierbare Rückstände zu den Bodenproben dotiert. Im Gegensatz zur Standard-Applikation, bei der gezielt nur eine Testsubstanz appliziert wird, resultiert aus dem dotierten Stoffspektrum eine gewisse Unschärfe, wie sie auch in Güllesilos auftreten kann. Nur mit diesem Ansatz können somit der reale Eintragspfad und möglich Güllematrixeffekte nachvollzogen werden.

Notwendigkeit weiterer Forschungsaktivitäten

In einem ersten internen Labortest wurde gezeigt, dass mit den Referenzgülleproben repräsentative Probenmatrizes vorliegen, die reproduzierbar in Labortests eingesetzt werden können. Das wurde auch in einem ersten externen Labortest belegt. Allerdings zeigten sich dort auch Schwierigkeiten bei der Messung einzelner Parameter. Insbesondere der Einsatz von Elektroden zur Messung von Redoxpotentialen und Gehalten des gelösten Sauerstoffes zur Überprüfung der anaeroben Milieubedingungen in Exkrement- sowie in Referenzgülleproben führte teilweise zu abweichenden Resultaten.

Allerdings sind anaerobe Milieubedingungen durch die Versuchsdurchführung operationell sichergestellt. Weitere Forschungsaktivitäten verlangt dagegen die Ermittlung der mikrobiellen Aktivität unter anaeroben Milieubedingungen der Gülle. Der biologische Sauerstoffbedarf erfasst hier bevorzugt aerobe Mikroorganismen. Die Gesamtheit der Mikroorganismen lässt sich zwar mittels der Dehydrogenaseaktivität bestimmen. Doch führen die teilweise stark gefärbten Extrakte der Exkrement- und Gülleproben bei der photometrischen Analyse bei $\lambda = 485$ nm zu Interferenzen. Hier sind damit weitere Schritte der Methodenentwicklung angezeigt. Aufgrund des eher geringen Informationsgehaltes können dagegen die Bestimmung der Kupfer-Gehaltes und des chemischen Sauerstoffbedarfes aus dem Methodenkatalog gestrichen werden.

Dieser erste externe Labortest beinhaltete auch die Abbautests von ^{14}C-Sulfadiazin in Rinder- und Schweinegülle. In diesen Kurzzeittests ermittelten die verschiedenen Laboratorien weitgehend übereinstimmende Tendenzen zum Abbau der eingesetzten Testsubstanz. Allerdings zeigte sich hier auch die Notwendigkeit, die Extraktionstechnik für die Gülleproben weiterzuentwickeln, um applizierte Testsubstanzen und korrespondierende Metaboliten erschöpfend extrahieren zu können. Somit sollten verschieden Extraktionstechniken näher untersucht werden, wie z.B. die direkte Extraktion der Güllesuspension mit einem organischen Lösungsmittel, die Trennung der Güllesuspension mittels Zentrifugation und anschließender Lösungsmittelextraktion der Feststoffmatrix sowie die Gefriertrocknung der Güllesuspension und anschließender Lösungsmittelextraktion der Feststoffmatrix. Nichtsdestotrotz muss die Extraktionstechnik an die Testsubstanz der jeweiligen Studie angepasst werden.

In einem zweiten internen Labortest wurde das Abbauverhalten von ^{14}C-Erythromycin in güllegedüngtem Boden untersucht. Auch wenn an diesem Labortest Testpersonen mit unterschiedlichen Erfahrungen in analytischer Chemie teilnahmen, konnte deutlich gezeigt werden, dass die experimentelle Versuchsabfolge von der Herstellung von Referenz- und Testgülleproben bis zu den Abbautests in güllegedüngten Böden weitgehend reproduzierbar durchgeführt werden kann. Allerdings sind für die Etablierung dieses Methodenkataloges noch weitere externe Labortest notwendig. Hierfür ist auch die Einbeziehung weiterer Testsubstanzen erforderlich. Von besonderer Bedeutung sind hier Biozide, die ebenfalls im Stall Anwendung finden und schließlich in die Gülle eingetragen werden. Im Gegensatz zu den Veterinärpharmaka zeichnen sich diese Substanzen oftmals durch andere physiko-chemische Eigenschaften aus, da sie aus anderen Stoffklassen stammen und andere Anwendungsmuster, z.B. direkte Anwendung in

der Gülle oder Sprayapplikation im Stall, haben. Schließlich sollte dieser Methodenkatalog mit Richtliniencharakter für Veterinärpharmaka und Biozide in Gülle und güllegedüngten Böden Anwendung finden können.

10 Acknowledgment

The authors gratefully acknowledge the financial support of the Federal Environmental Agency, Dessau, Germany, and the scientific supervision of Dr. J. Klein-Goedicke. Special thanks to Prof. Dr. G. Flachowsky, Dr. A. Berk, Dr. U. Meyer, Institute of Animal Nutrition, Federal Agricultural Research Center (FAL), for their cooperation in every aspect of animal husbandry and animal nutrition and their support during the excrement sampling at the experimental stable. Thanks also to Dr. J. Hahne, Institute of Technology and Biosystems Engineering, FAL, for many helpful discussions about the matrix characterization of excrement and manure samples. Special thanks also to Dr. U. Morgenroth and Dr. W. Völkel, RCC-Ltd., Itingen, Switzerland, Prof. Dr. A. Schäffer, J. Ebert and Dr. B. Schmidt, Institute of Environmental Biology and Chemodynamics, Biology V, RWTH Aachen, Germany, and Dr. U. Merrettig-Bruns, Fraunhofer-Institut für Umwelt-, Sicherheits- und Energietechnik, Oberhausen, Germany, for their participation in the interlaboratory tests and the corresponding workshops. Finally, thanks to all colleagues for their participation in the UBA Expert Symposium held in Braunschweig, Germany, on 20 to 21 February 2007.

11. References

BBA (1986): Verbleib von Pflanzenschutzmitteln im Boden - Abbau, Umwandlung und Metabolismus. Richtlinien für die amtliche Prüfung von Pflanzenschutzmitteln, Teil IV, 4-1. Biologische Bundesanstalt für Land- und Forstwirtschaft, ACO Druck, Braunschweig, Germany.

BBA (1998): Data requirement and criteria for decision making in the European Union and the Federal Republic of Germany for the authorization procedure of plant protection products. Mitteilungen aus der Biologischen Bundesanstalt, 358, 63–68.

Biotrend (2005): Technical communication.

Boesten, J.J., T.I. (1990): Influence of solid/liquid ratio on the experimental error of sorption coefficients in pesticide/soil systems. Pest. Sci. 12, 484-494.

Bouwman, G., Reus, J. (1994): Persistence of medicines in manure. Report CLM-163-1994.

Boxall, A.B.A., Fogg, L.A., Kay, P., Blackwell, P.A., Pemberton, E.J., Croxford, A. (2004): Veterinary medicines in the environment. Rev. Environ. Contam. Toxicol. 180, 1-91.

Buning, S. (1997): Ein Beitrag zur Optimierung der Längsverteilung von Flüssigmist. PhD Thesis, Braunschweig University of Technology, Germany.

Castillo del, J.R., Laroute, V. Pommier, P., Zemirline, C., Keita, A., Concordet, D., Toutain, P. L. (2006): Interindividual variability in plasma concentrations after systemic exposure of swine to dietary doxycycline supplied with and without paracetamol: a population pharmacokinetic approach. J. Anim. Sci., 84, 3155 – 3166.

Deutscher Wetterdienst (2005): http://www.dwd.de (Oktober 2005)

DIN 19684-3 (2000): Methods of soil investigations for agricultural water engineering - Chemical laboratory tests - Part 3: Determination of the loss on ignition and the residue of soil after ignition.

DIN 38404 C6 (1984): German standard methods for the examination of water, waste water and sludge; physical and physico-chemical parameters (group C); determination of the oxidation reduction (redox) potential (C 6).

DIN EN 12176 S5 (1998): Characterization of sludge - Determination of pH-value.

Domsch, K. (1992): Pestizide im Boden, mikrobieller Abbau und Nebenwirkungen auf Mikroorganismen. VCH Wiley, Weinheim, Germany, ISBN 3-527-28431-1.

EC (1998): Directive 98/8/EC of the European Parliament and of the Council of 16 February 1998 concerning the placing of biocidal products on the market. Off. J. EC, L 123/1.

Emmerich, I. U., Ungemach, F. R. (2006): Neue Arzneimittel für Pferde und landwirtschaftliche Nutztiere 2004/2005. Tierärztl. Prax., 34, 209-218.

Faulkner, P. M., Weary, D. M. (2000): Reducing pain after dehorning in dairy calves. J. Dairy Sci. 83, 2037-3041.

Fichter, P.M., Holden, P.W. (1992): A field study to meet United States Environmental Protection Agency regulatory for measurement of movement of pesticides to ground water. Brighton Crop Protect. Conf. Pests and Diseases, 7C (15), 853-858.

German Federal Statistical Office (2006): Statistische Jahrbuch 2006 für die Bundesrepublik Deutschland, 352.

German Ordinance Concerning Fertilizers (2006): Verordnung über die Grundsätze der guten fachlichen Praxis beim Düngen. BGBI. 181.

Göbel A., Thomsen A., Cardell Ch. S. M. , Joss A., Giger W. (2005): Occurence and sorption behavior of sulfonamides, macrolides, and trimethoprim in activated sludge treatment, Environ. Sci. Technol., 39, 3981-3989.

Grote, M. (2005): Antiinfektivaeinträge aus der Tierproduktion in terrestrische und aquatische Kompartimente. Abschlußbericht, Forschungsauftrag: IIA5-2038.06. 06.01-B/T 2/01. Herausgeber: Ministerium für Umwelt und Naturschutz, Landwirtschaft und Verbraucherschutz des Landes Nordrhein-Westfalen (MUNLV).

Hahne, J. (2001): Untersuchungen zu den stofflichen Umsetzungen bei der aerob-thermophilen Belüftung und Einsatz des Verfahrens zur Nährstoffabtrennung aus Schweinegülle. PhD Thesis, Technical University of Braunschweig, Germany.

Halling-Sørensen, B., Nors Nielsen, S., Lanzky, P., Ingerslev, F., Holten Lutzhøft, H., Jøregensen, S. (1998): Occurrence, fate and effect of pharmaceutical substances in the environment - a review. Chemosphere, 36, 357-393.

Heise, J., Höltge, S., Schrader S., Kreuzig, R. (2006): Chemical and biological characterization of non-extractable residues of sulfonamides in soil. Chemosphere, 65, 2352-2357.

Hirsch, R., Ternes, T., Haberer, K., Kratz, K.-L. (1999): Occurrence of antibiotics in the aquatic environment. Sci. Total Environ., 225, 109-118.

Höltge, S., Kreuzig, R. (2007): Laboratory testing of sulfamethoxazole and its metabolite acetyl-sulfamethoxazole in soil. Clean, 35, 104-110.

Hoffmann, H., Hege, U. (1991): Gülle – ein wertvoller Wirtschaftsdünger, ADI 1149, J.P: Bachem GmbH & Co. KG. Köln.

Hollis, J.M., (1991): Mapping the vulnerability of aquifers and surface waters to pesticide contamination at the national/regional scale. In: Walker, A. (Ed.), Monograph No. 47. Pesticides in soils and water: Current perspectives. Proceedings of a symposium organised by the British Crop Production Council. University of Warwick, Coventry, UK.

Hüther, L. (1999): Entwicklung Bodenanalytischer Methoden und Untersuchungen von Einflussfaktoren auf Ammoniak-, Methan- und Distickstoffmonoxidemissionen aus Flüssig- und Festmist. PhD Thesis, Technische Universität Braunschweig, Germany.

Hütter, L. (1994): Wasser und Wasseruntersuchung. 6. Auflage, Salle und Sauerländer, Frankfurt/Main.

Institut für Veterinärpharmakologie und –toxikologie (2006): Universität Zürich, Schweiz, http://www.vetpharm.unizh.ch/wir/00000072/3466__F.htm.

ISO 5664 (1984): Water quality. Determination of ammonium: Distillation and titration method.

ISO 5814 (1990): Water quality – Determination of dissolved oxygen – Electrochemical probe method.

ISO 5815 (2003): Water quality – Determination of biological oxygen demand after *n* days (BOD_n) – Part 1: Dilution and seeding method with allylthiourea addition.

ISO 6060 (1989): Water quality – Determination of chemical oxygen demand.

ISO 10694 (1995): Soil quality – Determination of organic and total carbon after dry combustion (elementary analysis).

ISO 11261 (1995): Soil quality – Determination of total nitrogen – Modified Kjeldahl method.

ISO 11465 (1993): Soil quality – Determination of dry matter and water content on a mass basis – Gravimetric method.

ISO 11734 (1995): Water quality of the ultimate anaerobic biodegradability of organic compounds in digested sludge – Method by measurement of the biogas production.

ISO 11885 (1996): Water quality – Determination of 33 elements by inductively coupled plasma atomic emission spectroscopy.

ISO 15705 (2002): Water quality – Determination of the chemical oxygen demand (ST-

COD) – small-scale sealed-tube method.

ISO 17155 (2003): Soil quality – Determination of abundance and activity of soil microflora using respiration curves.

Kluge, K., Ungemach, F. R. (1998): Neue Arzneimittel für Pferde und landwirtschaftliche Nutztiere und Veränderungen auf dem Arzneimittelmarkt seit 1996. Tierärztl. Prax., 26, 301-306.

Koschorreck, J., Koch, C., Rönnefahrt, I. (2002): Environmental risk assessment of veterinary medicinal products in the EU: A regulatory perspective. Toxicol. Lett., 131, 117-124.

Kreuzig, R., Blümlein, K., Höltge, S. (2007b): Fate of the Benzimidazole Antiparasitics Flubendazole and Fenbendazole in Manure and Manured Soils. Clean (in print).

Kreuzig, R., Höltge, S. (2005): Investigations on the fate of sulfadiazine in manured soil: Laboratory experiments and test plot studies. Environ. Toxicol. Chem. 24, 260-265.

Kreuzig, R., Höltge, S., Heise, J., Kolb, M., Berenzen, N., Hahn, T., Jergentz, S., Wogram, J., Schulz, R. (2007a): Untersuchungen zum Abflussverhalten von Veterinärpharmaka bei Ausbringung von Gülle auf Ackerland und Weide. UBA-Texte, ISSN 0722-186X. Umweltbundesamt, Berlin, 1-162.
http://www.umweltdaten.de/publikationen/fpdf-l/3237.pdf

Kreuzig, R., Höltge, S., Brunotte, J., Berenzen, N., Wogram, J., Schulz, R. (2005b): Test plot studies on runoff of sulfonamides from manured soils after sprinkler irrigation. Environ. Toxicol. Chem., 24, 777-781.

Kreuzig, R., Kullmer, Ch., Matthies, B., Plaga, B., Dieckmann, H., Höltge, S. (2005a): Verhalten von in der Umwelt vorkommenden Pharmaka und ihren Metaboliten in Modelltestsystemen – Teil 2: "Modellsystem Boden". UBA-Texte 11/05, ISSN 0722-186X. Umweltbundesamt, Berlin, 1-122.
http://www.umweltdaten.de/publikationen/fpdf-l/2897.pdf

Kühne, M., Ihnen, D., Möller, G., Agthe, O., 2000. Degradation of tetracyclines in water and liquid manure. J. Vet. Med. A47, 379-384.

Kullmer (2005): Humanpharmaka-Rückstände in Böden – Labortests zu Abbau und Verlagerung. PhD Thesis, TU-Braunschweig, ISBN 3-86537-615-0.

Lallai, A., Mura, G., Onnis, N. (2002): The effects of certain antibiotics on biogas production in the anaerobic digestion of pig waste slurry. Bio. Techn., 82, 205-208.

Loke, M., Jespersen, S., Vreeken, R., Halling-Sørensen, B., Tjørnelund J. (2003): De-

termination of oxytetracycline and its degradation products by high-performance liquid chromatography-tandem mass spectrometry in manure-containing anaerobic test systems. J. Chromatogr. B, 783, 11-23.

Löscher, W., Ungemach, F., Kroker, R. (2003): Pharmakotherapie bei Haus- und Nutztieren. 6. Auflage, Parey Verlag. ISBN: 3830441606.

Merkel, D. (2005): Persönliche Mitteilung.

Michels, J., Track, T., Gehrke, U. Sell D. (2000): Biologische Verfahren zur Bodensanierung. Grün-Weiße-Reihe des Umweltbundesamts.

Milligan, B. N., Duffied, T., Lissemore, K. (2004): The utility of ketoprofen for alleviating pain following dehorning in young dairy calves. Can. Vet. J., 45, 140-143.

Møller, H., Sommer, S., Ahring, B. (2004): Biological degradation and greenhouse gas emission during pre-storage of liquid animal manure In: J. Environ. Qual., 33, 27-36.

Montforts, M., Tarazona Lafarga, J. (2003): Environmental risk assessment for veterinary medicinal products. Part 4. Exposure assessment scenarios. RIVM report 601450017.

Ndegwa, P., Zhu, J., Luo, A. (2003): Effects of bioreactor temperature and time on odour-related parameters in aerated pig manure slurries In: Environ. Technol., 24, pp. 1007 - 1016.

OECD (1981a): Guideline for testing of chemicals. Inherent biodegradability in soil. 304 A, 1–11.

OECD (1981b): Guideline for testing of chemicals. Adsorption/Desorption. 106, 1-18.

OECD (2000): Guideline for testing of chemicals. Adsorption-desorption using a batch equilibrium method. 106, 1-45.

OECD (2002): Guideline for testing of chemicals. Aerobic and anaerobic degradation in soil. 307, 1–17.

Oepen, von, B., Kördel, W., Klein, W. (1991): Sorption of nonpolar and polar compounds to soils: processes, measurements and experience with the applicability of the modified OECD Guideline 106. Chemosphere, 22 (3-4), 285-304.

Rönnefahrt, I., Koschorreck, J., Kolossa-Gehring, M. (2002): Arzneimittel in der Umwelt – Teil 2: Rechtliche Aspekte und Bewertungskonzepte. Mitt. Umweltchem. Ökotox., 6-9.

Rütters, H., Höllrigl-Rosta, A., Kreuzig, R., Bahadir, M (1999): Sorption behaviour of prochloraz in different soils. J. Agric. Food Chem., 47 (3), 1242-1246.

Schlüsener, M. P., Arb, M. A., Bester, K. (2006): Elimination of macrolides, tiamulin and salinomycin during manure storage. Arch. Environ. Contam. Toxicol., 51, 21-28.

Schuchhardt, F., Hahne, J. (1996): Aerobe Behandlung landwirtschaftlicher Reststoffe, Nr. 5657. - In: Hösel, G., Kumpf, W.: Müll-Handbuch: Sammlung und Transport, Behandlung und Ablagerung sowie Vermeidung und Verwertung von Abfällen, ergänzbares Handbuch für die kommunale und industrielle Abfallwirtschaft. Schmidt Verlag, Berlin. ISBN 0176-4969.

Shah, S., Miller, J., Basden, T. (2004): Mechanical aeration and liquid dairy manure application impacts on grassland runoff water quality and yield. ASAE, 47 (3), 777-788.

Spaepen, K., Van Leemput, L., Wislocki, P., Verschueren, C. (1997): A uniform procedure to estimate the predicted environmental concentration of the residues of veterinary medicines in soil. Environ. Toxicol. Chem. 16, 1977 - 1982.

Stafford, K. J., Mellor, D. J. (2005): Dehorning and disbudding distress and its alleviation in calves. Vet. J., 169, 337-349.

Strauch, D, Baader, W., Tietjen C. (1977): Abfälle in der Tierhaltung. Verlag Eugen Ulmer, Stuttgart. ISBN 3-8001-4328-3.

Timme, G., Frehse, H., Laska, V. (1986): Zur statistischen Interpretation und graphischen Darstellung des Abbauverhaltens von Pflanzenschutzmittel-Rückständen. II. Pflanzenschutz-Nachr. Bayer, 39, 188-204.

TNsG (2000): Technical notes for guidance on data requirements for active substances and biocidal products. Final Draft Version 4.3.2.

van Vlaardingen, P., A. de Kneet, Janssen P. (2001): Degradation of veterinary drugs in manure. In: Luttik, R., von Raaij, M. (edt): Factsheets for the (eco)toxicological risk assessment strategy of the National Institute of Public Health and the Environment (RIVM). RIVM report 601516007.

VICH (2000): Environmental impact assessment (EIAs) for veterinary medicinal products (VMPs) – Phase I. VICH GL6, Ecotoxicity Phase I, Bruxelles, Belgium.

VICH (2003): Environmental impact assessment (EIAs) for veterinary medicinal products (VMPs) – Phase II Draft Guidance. VICH GL38, Ecotoxicity Phase II, Bruxelles, Belgium.

Proposal for a Technical Protocol (Draft Version)

Degradation of Veterinary Medicinal Products in Bovine and Pig Manure and Degradation and Sorption in Manured Soils

INTRODUCTION

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

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

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

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

II. Anaerobic degradation tests of VMP in reference manures.

III. Preparation of test manures.

IV. Aerobic degradation in manured soils.

V. Sorption tests in manured soils.

PRINCIPLE OF THE TESTS

5. The principle of the degradation test in manure is based on the tiered experimental design starting with the reproducible sampling of excrements. After a sophisticated matrix characterization, water is added to the excrement samples to prepare the reference-manure samples for these degradation tests under controlled experimental conditions. Test substances should be fortified as ^{14}C-labeled radiotracers with special respect to the high complexity of the manure samples. Mass balances are set up considering the mineralization and the formation of extractable and non-extractable residues. Extracts were additionally screened for the parent compound initially applied and metabolites formed during appropriate incubation intervals up to 180 d.

On the basis of these degradation tests in reference manure, test manures with 7-d aged VMP are prepared and applied in batch tests on degradation and sorption of VMP in manured soils in accordance to the OECD Guidelines 307 and 106 [1,2].

APPLICABILITY OF THE TESTS

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

INFORMATION ON THE TEST SUBSTANCE

7. The ^{14}C-labeled test substance is to be specified by the position of the labeling (most stable moiety in the molecule is to be preferred), the radiochemical purity (≥ 95 %) and the specific radioactivity.

8. Before carrying out degradation tests in manure and degradation and sorption tests in manured soils, the following information on the test substance should be available:

– Solubility in water according to OECD Guideline 105 [3].
– Solubility in organic solvents.
– Vapour pressure according to OECD Guideline 104 [4] and Henry´s law constant.
– n-Octanol/water partition coefficient according to OECD Guidelines 107 and 117 [5,6].
– Chemical degradation in dark (hydrolysis) according to OECD Guideline 111 [7].
– pK_a if a molecule is liable to protonation or deprotonation according to OECD Guideline 112 [8].

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

10. Analytical methods (including extraction and clean-up methods) for identification and quantification of the test substance and its metabolites should be available or have to be elaborated. Furthermore, reference substances should be used for the characterization and /or identification of degradation products by spectroscopic and chromatographic methods.

DEFINITIONS

11. See Annex A.

QUALITY CRITERIA

Recovery

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

13. The analyses of at least duplicate manure samples as well as manured soil samples directly after the addition of the test substance give a first indication of the repeatability of the analytical methods and of the uniformity of the application procedure for the test substance. Recoveries for the experiments are given by the respective mass balances that should range from 90 % to 110 %, if possible. Taking the high complexity of manure matrices and manured soil samples into special account, recoveries are considered acceptable at 70-110 %, especially when residue analytical methods are applied.

Repeatability

14. Differences of independently analyzed duplicates should not exceed 10 %, if possible.

15. Repeatability of the analytical method to quantify test substance and metabolites can be checked by duplicate analysis of the same extract of the manure and manured soils incubated long enough for formation of metabolites. In general, the analytical methods are considered repeatable, if the difference of duplicates does not exceed 10 %. Furthermore, the analytical quality is expressed by the mass balances of the conducted tests (see recovery chapter, paragraph 12).

16. The limit of determination (LOD) of the analytical method for the test substance and for the degradation products should be at least 0.1 mg kg^{-1} manure (fresh weight) and 0.01 mg kg^{-1} manured soils (dry weight) or 3 % of applied concentration whichever is lower. The limit of quantification (LOQ) should also be specified.

Accuracy of degradation data

17. The data are evaluated by using appropriate statistics (e.g., modelmaker). Regression analysis of the concentrations of the test substance as a function of time gives

the appropriate information on the reliability of the degradation curve and allows the calculation of the confidence limits for half-lives (in case of pseudo first order kinetics) or DT_{50} values and, if appropriate, DT_{90} values [11,12].

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

INFORMATION ON THE EXCREMENT AND MANURE SAMPLES

Sampling and feeding conditions

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

Preconditioning, storage and matrix characterization of excrement samples

19. Directly after excretion, readily degradable organic compounds of the excrements undergo rapid decomposition enhancing the matrix heterogeneity [13,14]. To minimize this effect, the preconditioning of excrement samples is necessary. For this purpose, the excrement samples are kept in plastic containers (approximately 20 L) at ambient temperature. Within a 21-d period, they are daily homogenised using an electric stirrer. To ensure a gas exchange, the lid loosely covers the container. Thereafter, the excrements samples are to be matrix characterized by the parameters as follows: dry substance, mineral content, total phosphorus, total organic carbon, pH, redox potential, dissolved oxygen, ammonium and total nitrogen, biological oxygen demand. If anaerobic conditions, indicated by the dissolved oxygen contents < 0.1 mg kg^{-1}, the redox potential Eh < +150 mV [14] and an ammonium content stable up to $\pm$ 0.2 g kg^{-1}, are established [15-20], 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 °C for 3 d to remobilize the excrements inherent microorganisms.

Reference manure preparation

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

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

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

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

21. 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 change in parameters.

CHARACTERIZATION OF EXCREMENT AND MANURE MATRICES

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

Equipment and chemicals

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

- Plastic containers with lid, 20 L, 2 L and 1 L,
- Mechanical mixing device (e.g., electric stirrer),
- Drying oven (105 ± 5 °C) or infrared heater with analytical balances,
- Muffle furnace (550 ± 25 °C),

- Laboratory microwave system,
- Inductively coupled plasma optical emission spectrometry (ICP-OES),
- Incubator (5 to 20 ± 1 °C),
- Apparatus for determination of the total carbon content,
- Analytical balances (accuracy ≤ 1 mg),
- pH-meter with pH electrode and appropriate test solution,
- Millivoltmeter with redox electrode and appropriate test solution,
- Oxygen meter with measuring probe,
- Distillation and digestion systems with distillation flasks or tubes,
- Incubation bottles (Karlsruher bottles),
- Heating system (148 ± 1 °C) for COD cuvettes,
- Spectral photometer, digestion stand with digestion flasks or tubes.

23. Chemicals used include, for example:
All used chemicals (e.g. NaOH, H_2SO_4, etc.) and organic solvents (e.g. acetone, methanol) should be of analytical grade.

Dry substance content (ds)

24. Excrements and manure samples (1 to 6 g) are dried in a drying oven to constant mass at 105 ± 5 °C [21]. Alternatively, an infrared heater can be used to drive out the water. The difference of an amount of excrement or manure before and after the drying procedure is used to calculate the dry substance content. The dry substance content is expressed in percentage, with an accuracy of ± 1 % (*m*/*m*).

Mineral content (R_{min})

25. Excrements and manure samples are glowed in a muffle furnace at 550 ± 25 °C to constant mass [22]. The difference of an amount of excrement or manure before and after the glowing procedure is used to calculate the mineral content that is expressed with an accuracy of ± 1 % (*m*/*m*).

Phosphate content (P)

26. After a microwave digestion, a measurement of atomic emission by an optical spectroscopic technique (ICP-OES) is performed. After the addition of 8 mL of nitric

acid, 2 mL of hydrogen peroxide and 2 mL of water, the digestion process with 100 to 200 mg dried substance of the excrement or manure samples is conducted in the microwave at 200 °C for 1 h. According to [23] the ICP-OES analysis is accomplished. For external calibration standard solutions in concentrations ranged from 0.01 to 100 mg L^{-1} are used. The element content is expressed in mg kg^{-1} fresh weight, with an accuracy of 1 mg kg^{-1}. Results higher than 1000 mg kg^{-1} are expressed in g kg^{-1} fresh weight, with an accuracy of 1 g kg^{-1}.

Total organic carbon (TOC)

27. 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 [24]. Prior to combustion, carbonates are to be removed from the dried samples (50-100 mg) by an excess of hydrochloride acid (4 mol L^{-1}). Subsequently, the samples are to be dried at 105 °C, homogenized and mixed with aluminum oxide (1:20). The mixed samples are combusted and the released amount of carbon dioxide is measured by titrimetry, gravimetry, conductometry, gas chromatography or infrared detection method, depending on the apparatus used. The total organic carbon is expressed in % dry substance or in g kg^{-1} fresh weight, with an accuracy of 1 g kg^{-1}.

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

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

pH value

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

Redox potential (Eh)

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

Dissolved oxygen content (O_2)

30. 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 [27]. The resulted oxygen content is given in mg O_2 kg^{-1} sample to the first decimal place for results > 0.1 mg O_2 kg^{-1}. Results less 0.1 mg oxygen kg^{-1} are reported as $\leq$ 0.1 mg kg^{-1}.

Ammonium nitrogen content (NH_4-N)

31. Under mildly alkaline conditions [28], 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, expressed in g NH_4-N kg^{-1} and rounded to one significant figure is calculated using the formula:

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

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 content (N_{total})

32. 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 [29]. After the addition of bases, ammonia is released by distillation and titrated. The reaction is accelerated by Kjeldahl

tablets (5 g) that contain sulfates and metallic salts. The sulfates increase the boiling point of the concentrated sulfuric acid (10 mL). The selenium, copper, titanium or mercury 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 days (BOD_5)

33. The microbial activity of excrement and manure samples have to be checked before degradation tests in reference manures are conducted or test-manure samples are prepared. For this purpose, the biological oxygen demand[4] can be determined [30]. 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.

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

According to the OECD Guideline 311 [31], the fermentation of test substances, e.g., sodium benzoate, can be studied. Those tests, however, take a 4-week period.

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

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

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

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

C_1: dissolved oxygen concentration in the sample solution at time zero [mg kg^{-1}]
C_2: dissolved oxygen concentration in the sample solution after five days [mg kg^{-1}]
C_3: dissolved oxygen concentration in the blank solution at time zero [mg kg^{-1}]
C_4: dissolved oxygen concentration in the blank solution after five days [mg kg^{-1}]
V_t: total volume [mL]
V_e: sample volume [mL]

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

PART II: ANAEROBIC DEGRADATION TESTS OF VMP IN REFERENCE MANURES

Equipment, instruments and chemicals

34. For degradation tests of VMP in manure, the application of ^{14}C-labeled test substances is strongly recommended to set up mass balances differentiating between mineralization and the formation of extractable and non-extractable residues. For this purpose, suitable laboratory-test systems are to be used. Particularly considering that these degradation tests are to be performed under anaerobic conditions, typical for manure storage in tanks, a closed batch apparatus allowing for a discontinuous gas exchange can be used. Advantages are little required laboratory space, possible use of commercially available incubators instead of climatic chambers as well as effortless and cost-extensive handling. This batch apparatus shown in Figure 1A (see Annex C), traces back to the biometer-type flask already mentioned in the OECD Guideline 304 [32] 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 ^{14}C-methane and volatile metabolites.

35. Alternatively, the flow-through system mentioned in the OECD Guideline 307 [1] (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 degradation tests of VMP in manures under anaerobic conditions.

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

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

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

38. All used chemicals (e.g. NaOH, H_2SO_4, etc.) and organic solvents (e.g. ethylene glycol, acetone, methanol etc.) should be of analytical grade. For radiotracer analysis scintillation cocktails are necessary.

Test conditions

39. The dry substance contents of bovine and pig manures of 10 % and 5 %, respectively, are adjusted by the addition of tap water to the corresponding excrement samples (see paragraph 12). These dry substance contents correspond to the average values given for Germany that ranged between 0.9 and 12 % [34-38] and should be used by default. In order to additionally study the effect of the dry substance content on the degradation of VMP in manure, different dry substance contents (e.g., 2.5, 5 and 10 %) can be facultatively tested.

40. 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 47). Using the flow-through system, nitrogen is discontinuously introduced in stop-flow mode to maintain anaerobic conditions.

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

42. The duration of the degradation tests should be accounted for approximately 180 d to simulate the long-term manure storage of 6 months [39].

Test substance application

43. For addition to manure and distribution in manure, the test substance can be dissolved in water (deionized or distilled) or, when necessary, in minimum amounts of organic solvents (e.g. acetone, acetonitrile, methanol, etc.) in which the test substance is sufficiently soluble and stable. However, the amount of the selected solvent should not have a 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 [1].

The use of solvents which inhibit microbial activity, such as dimethyl sulfoxide, chloroform, dichloromethane and other halogenated solvents, should be avoided. If this is not possible, the test substance can also be added as a solid, e.g. mixed in quartz sand. If the test substance is added using a solvent, the solvent should be allowed to evaporate before the spiked carrier is added to the sample [1].

44. For the adjustment of the applied amounts of manure and radiotracer, the substance specific exposure assessment as well as the analytical feasibility mainly defined by the specific radioactivity of the radiotracer under study is to be taken into special account. The applied concentration should be based on the substance specific exposure assessment of the VMP under study [40]. If the corresponding detection limit is not achievable, the concentration may be enlarged up to a factor of 10.

Performance of the degradation tests in manure

45. 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 43. When organic solvents are used for the application of the test substance, they should be removed from manure by evaporation. Then the sample is thoroughly mixed by shaking the flask.

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

further tests.

47. In the laboratory-batch system, the headspaces of the incubation flasks are rinsed by a gentle stream of nitrogen every 7 d during the incubation period. The stripping gas is passed through 3 external traps filled with 10 mL ethylene glycol, 10 mL sulfuric acid (0.05 M) and 10 mL scintillation cocktail to trap ^{14}C-methane or related volatiles and ^{14}C-carbon dioxide, respectively. Subsequently, the absorption solution of the internal ^{14}C-carbon dioxide trap is exchanged and every absorption solution is scintillation counted. In the flow-through system, the headspace of the incubation flask is exchanged every 7 d. Thereafter, the external traps are to be exchanged and analyzed as described before.

48. Duplicate incubation flasks are removed at the appropriate incubation intervals (see paragraph 46). 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[5] may be investigated for every test substance.

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

---

[5] Besides a direct extraction procedure of the manure samples, the liquid phase of the manure samples can be removed by lyophilization. Alternatively, the extraction procedure may start by separating liquid and solid phases of the manure samples via centrifugation. The liquid phases can be directly analyzed for radioactivity amounts by scintillation counting. For the identification of relevant metabolites, residue analytical methods have to be applied. Then, the solid manure samples may be treated by means of a sequential extraction technique.

PART III. PREPARATION OF TEST MANURES

50. In order to mimic the VMP entry route in soils already under laboratory conditions, test manures are prepared [41-44]. For this purpose, 10 to 20 g manure samples (fresh weight basis; matrix characterized) are placed into each incubation flask (see Figure 1 or 2; Annex C) and spiked with the test substance (see paragraphs 43). In accordance to the degradation tests in manure (see paragraphs 34 ff), the incubation flasks are incubated under anaerobic conditions for 7 d.

51. Parallel batch tests are to be prepared and treated as described in paragraph 50. By setting up mass balances and screening the extracts of the manure samples, the amounts of the parent compound initially applied and metabolites as well as non-extractable residues formed are to be determined. Thus, the concentrations of residues are to be quantified for the following laboratory tests on degradation and sorption in manured soils.

PART IV. AEROBIC DEGRADATION TESTS OF VMP IN MANURED SOILS

52. The aerobic degradation tests of VMP in manured soils are carried out in accordance to the principles of the OECD Guideline 307 [1]. Differences concern only the application of the test substance via test-manure application. Thereafter, the degradation tests follow the OECD Guideline 307 [1].

INFORMATION ON THE SOILS

Soil selection

53. For determining the degradation and sorption of VMP in manured soils, 3 different soils should be used representing a relevant range of agriculturally used soils. These soils should vary in their organic carbon content, pH, clay content and microbial biomass. The soils should be characterized, at least, for texture (% sand, % silt, % clay), according to FAO or USDA classification, pH, cation exchange capacity, organic carbon, bulk density, water holding capacity and microbial biomass. Additional information on soil properties may be useful in interpreting the results. For determination of the soil characteristics, the methods recommended in the OECD Guideline 307 [1] can be used. Microbial biomass should be determined by using the substrate-induced respiration (SIR) method [18,19] or alternative methods [20].

Sampling, handling and storage of soils

54. Detailed information on the history of the field site from where the test soil is sampled should be available. Details include location, vegetation cover, treatments with chemicals, treatments with organic and inorganic fertilizers, additions of biological materials or other contamination. Soils treated with the test substance or its structural analogues within the previous 4 years should not be used for degradation studies.

55. The soil should be freshly sampled from the field (from the A horizon or top 20-cm layer) with a soil water content which facilitates sieving. The soil should be processed as soon as possible after sampling. Vegetation, larger soil fauna and stones should be removed prior to passing the soil through a 2 mm sieve. Extensive drying and crushing of the soil before sieving should be avoided.

56. Studies with soils freshly sampled from the field are strongly preferred but, if the sampled and processed soil has to be stored prior to the start of the study, storage conditions must be adequate and for a limited time only (4 ± 2 °C for a maximum of 3 months or -20 °C for longer periods) to maintain microbial activity. Before the stored soil is used for these tests, it should be pre-incubated to allow germination and removal of seeds, and to re-establish equilibrium of microbial metabolism following the change from sampling or storage conditions to incubation conditions. A pre-incubation period between 2 and 28 d, approximating the temperature and moisture conditions of the actual test, is generally adequate. Before analytical processing, soil samples are to be principally controlled for microbial activity by tests mentioned before (see soil selection chapter, paragraph 53).

TEST MANURE APPLICATION TO SOIL

57. Matrix characterized soil samples (25 to 100 g; OECD 307 [1]) are to be added directly into the incubation flasks in that test-manure samples has been prepared (see paragraph 50). This is done to avoid losses of the radioactivity initially applied, i.e., parent compound and formed metabolites and non-extractable residues. Those losses would inevitably occur when test-manure samples are transferred into flasks containing soil samples. The manure amounts were calculated on the following parameters: 170 kg N ha^{-1}, 5 g N kg^{-1} manure, 1500 kg soil m^{-3} and 0.05 m soil depth. The manure/soil ratio should not exceed 1:5 that corresponds with fourfold of the maximum manure amount accepted by the German Ordinance Concerning Fertilizers [39]. The concentration of the test substance should reflect its exposure assessment as closely as possible [40]. If this concentration is not high enough to identify major degradation products, the application of test manures containing higher concentrations may be helpful. However, excessive amounts influencing manure or soil microbial functions should be avoided. Soil and manure samples are to be mixed thoroughly. Thereafter, aerobic degradation tests follow the OECD guideline 307 [1].

PART V. SORPTION TESTS OF VMP IN MANURED SOILS

58. The adsorption/desorption tests of VMP in manured soils are carried out in accordance to the principles of the OECD Guideline 106 [2]. Detailed information and references about soil selection, sampling, handling and storage are given there. Differences concern only the application of the test substance via test-manure application. Thereafter, sorption tests follow the OECD Guideline 106 [2].

DATA AND REPORTING

Treatment of results

59. The amounts of test substance, degradation products, gaseous and volatile substances and non-extractable residues in manure and manured soil samples should be given as % of the initially applied radioactivity and, where appropriate, as µg kg^{-1} manure (based on manure fresh weight) and µg kg^{-1} soil (based on soil dry weight), respectively, for each incubation interval. A mass balance should be set up, too. A graphical presentation of the test substance concentrations against time will allow an estimation of its degradation half-life or DT_{50}. If possible, metabolites should be identified and their concentrations should also be plotted against time to show their rates of formation and decline. A major degradation product is any product representing $\geq$ 10 % of applied dose at any time during the study.
For ^{14}C-labeled test substances, the mass balance should be differentiated between the formation of carbon dioxide or methane and if it is existent the formation of volatile compounds as well as the formation of extractable and non-extractable residues.

TEST REPORT

60. 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 manures:
- 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 soils:
- details of sampling site,
- date and procedure of soil sampling,
- properties of soils, such as pH, organic carbon content, texture (% sand, % silt, % clay), cation exchange capacity, bulk density, water retention characteristic and microbial biomass,
- length of soil storage and storage conditions (if stored),
- manured soil moisture content during incubation,
- pH, oxygen content and redox potential initially, during and at the end of the aerobic studies in manured soil.

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 and manured soil, respectively, treated initially and at each incubation interval for analysis,
- description of the incubation system used,
- air flow rates (for flow-through systems only),
- temperature of experimental set-up,
- method(s) of extraction,
- methods for identification and quantification of the test substance and metabo-

lites in manure and manured soils and absorption materials,
- number of replicates and number of controls.

Results of excrement and manure characterization:
- dry substance content, mineral content, total phosphorus, 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 degradation tests. If possible, every parameter should be given for each incubation interval.

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

Interpretation and evaluation of results

Although the studies are carried out in an artificial laboratory system, the results will allow estimation of the degradation of VMP in manure and degradation and sorption of VMP in manured soils under field conditions [1].

LITERATURE

[1] OECD (2002): Guideline for the testing of chemicals. Aerobic and anaerobic degradation in soil. Guideline 307. Paris, France, 1–17.

[2] OECD (2000): Guideline for the testing of chemicals. Adsorption-desorption using a batch equilibrium method, Guideline 106, 1-45.

[3] OECD (1995): Guideline for the testing of chemicals. Water solubility, Guideline 105, 1-7.

[4] OECD (2006): Guideline for the testing of chemicals. Vapor pressure. Guideline 104, 1-18.

[5] OECD (1995): Guideline for the testing of chemicals. Partition coefficient (n-octanol/water): Shake Flask Method. Guideline 107, 1-4.

[6] OECD (2004): Guideline for the testing of chemicals. Partition coefficient (n-octanol/water): HPLC Method. Guideline 117, 1-11.

[7] OECD (2004): Guideline for the testing of chemicals. Hydrolysis as a function of pH. Guideline 111, 1-16.

[8] OECD (1981): Guideline for the testing of chemicals. Dissociation constants in water. Guideline 112, 1-7.

[9] OECD (2000): Guideline for the testing of chemicals. Soil microorganisms: Nitogen degradation test. Guideline, 216, 1-10.

[10] OECD (2004): Guideline for the testing of chemicals. Soil microorganisms: Carbon degradation test. Guideline, 217, 1-10.

[11] Timme, G., Frehse, H., Laska, V. (1980): Statistical interpretation and graphic representation of the degradational behaviour of pesticide residues. I. Pflanzenschutz-Nachrichten Bayer, 33, 47-60.

[12] Timme, G., Frehse, H., Laska, V. (1986): Statistical interpretation and graphic representation of the degradational behaviour of pesticide residues. II. Pflanzenschutz-Nachrichten Bayer, 39, 188-204.

[13] Strauch, D., Baader, W., Tietjen, C. (1977): Abfälle in der Tierhaltung. Verlag Eugen Ulmer, Stuttgart, Germany. ISBN: 3-8001-4328-3.

[14] Ndegwa, P., Zhu, J., Luo, A. (2003): Effects of bioreactor temperature and time on odour-related parameters in aerated pig manure slurries. Environ. Technol., 24, 1007-1016.

[15] Michels, J., Track, T., Gehrke, U., Sell, D. (2000): Biologische Verfahren zur Bo-

densanierung. Grün-Weiße-Reihe des Umweltbundesamtes, Berlin, Germany.

[16] Domsch, K. (1992): Pestizide im Boden, mikrobieller Abbau und Nebenwirkungen auf Mikroorganismen. VCH Wiley, Weinheim, Germany, ISBN 3-527-28431-1.

[17] Methods of Soil Analysis (1986): Part 1, Physical and Mineralogical Methods. A. Klute, Ed., Agronomy Series No 9, 2nd Edition.

[18] Anderson, J.P.E., Domsch, K.H. (1978): A physiological method for the quantitative measurement of microbial biomass in soils. Soil Biol. Biochem., 10, 215-221.

[19] ISO 14240-1 and 2 (1997): Soil Quality - Determination of soil microbial biomass – Part 1: Substrate-induced respiration method. Part 2: Fumigation-extraction method.

[20] Methods of Soil Analysis (1982): Part 2, Chemical and Microbiological Properties. A.L. Page, R.H. Miller and D.R. Kelney, Eds. Agronomy Series No 9, 2nd Edition.

[21] ISO 11465 (1993): Soil quality – Determination of dry matter and water content on a mass basis – Gravimetric method.

[22] DIN 19684-3 (2000): Methods of soil investigations for agricultural water engineering - Chemical laboratory tests - Part 3: Determination of the loss on ignition and the residue of soil after ignition.

[23] ISO 11885 (1996): Water quality – Determination of 33 elements by inductively coupled plasma atomic emission spectroscopy.

[24] ISO 10694 (1995): Soil quality – Determination of organic and total carbon after dry combustion (elementary analysis).

[25] DIN EN 12176 S5 (1998): Characterization of sludge - Determination of pH-value

[26] DIN 38404 C6 (1984): German standard methods for the examination of water, waste water and sludge; physical and physico-chemical parameters (group C); determination of the oxidation reduction (redox) potential (C 6).

[27] ISO 5814 (1990): Water quality – Determination of dissolved oxygen – Electrochemical probe method.

[28] ISO 5664 (1984): Water quality. Determination of ammonium: Distillation and titration method.

[29] ISO 11261 (1995): Soil quality – Determination of total nitrogen – Modified Kjeldahl method.

[30] ISO 5815 (2003): Water quality – Determination of biological oxygen demand after *n* days (BOD_n) – Part 1: Dilution and seeding method with allylthiourea addition.

[31] OECD (2006): Guideline for the testing of chemicals. Anaerobic biodegradability of organic compounds in digested sludge: By measurement of gas production. Guideline 311, 1-20.

[32] OECD (1981): Guideline for the testing of chemicals. Inherent biodegradability in soil. Guideline 304 A. Paris, France, 1–11.

[33] Buning, S. (1997): Ein Beitrag zur Optimierung der Längsverteilung von Flüssigmist. PhD Thesis, Technical University of Braunschweig, Germany.

[34] Møller, H., Sommer, S., Ahring, B. (2004): Biological degradation and greenhouse gas emission during pre-storage of liquid animal manure. J. Environ. Qual., 33, 27-36.

[35] Schuchhardt, F., Hahne, J. (1996): Aerobe Behandlung landwirtschaftlicher Reststoffe, Nr. 5657. - In: Hösel, G., Kumpf, W.: Müll-Handbuch: Sammlung und Transport, Behandlung und Ablagerung sowie Vermeidung und Verwertung von Abfällen, ergänzbares Handbuch für die kommunale und industrielle Abfallwirtschaft. Schmidt Verlag, Berlin, Germany. ISBN 0176-4969.

[36] Bouwman, G., Reus, J. (1994): Persistence of medicines in manure. Report CLM-163-1994.

[37] Lallai, A., Mura, G., Onnis, N. (2002): The effects of certain antibiotics on biogas production in the anaerobic digestion of pig waste slurry. Bio. Techn., 82, 205-208.

[38] Shah, S., Miller, J., Basden, T. (2004): Mechanical aeration and liquid dairy manure application impacts on grassland runoff water quality and yield. ASAE, 47, 777-788.

[39] German Ordinance Concerning Fertilizers (2006): Verordnung über die Anwendung von Düngemitteln, Bodenhilfsstoffen, Kultursubstraten und Pflanzenhilfsmitteln nach den Grundsätzen der guten fachlichen Praxis beim Düngen. BGBl., I, Nr. 2.

[40] Spaepen, K.R.I., Van Leemput, L.J.J., Wislocki, P.G., Verschueren, Ch. (1997): A uniform procedure to estimate the predicted environmental concentration of the residues of veterinary medicines in soil. Environ. Toxicol. Chem., 16, 1977-1982.

[41] Kreuzig, R., Höltge, S., Heise, J., Kolb, M., Berenzen, N., Hahn, T., Jergentz, S., Wogram, J., Schulz, R. (2006): Untersuchungen zum Abflussverhalten von Veterinärpharmaka bei Ausbringung von Gülle auf Ackerland und Weide. ISSN 0722-186X. Umweltbundesamt, Berlin, Germany.

[42] Kreuzig, R., Heise, J., Höltge, S. (2006): Das Gülle-Projekt: Ausarbeitung eines Methodenkataloges zur Untersuchung des Rückstandsverhaltens von Tierarzneimitteln in Gülle und Boden. Mitt. Umweltchem. Ökotox., 2, 39-43.

[43] Kreuzig, R., Kullmer, C., Matthies, B., Höltge, S., Dieckmann, H. (2003): Fate and behaviour of pharmaceutical residues in soils. Fresenius Environ. Bull., 12, 550-558.

[44] Kreuzig R., Höltge S. (2005): Investigations on the fate of sulfadiazine in manured soil: Laboratory experiments and test-plot studies. Environ. Toxicol. Chem., 24, 771-776.

Annex A

Definitions

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

Extractable residues (ER) represent compounds occurring in the organic solvent as parent compound or 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, copper, total phosphorus, mineral content, total organic carbon, pH, redox potential, dissolved oxygen, ammonium nitrogen, total nitrogen, biological and chemical oxygen demand.

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

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

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

Soil is a mixture of mineral and organic constituents, the latter containing compounds of high carbon and nitrogen content. Microbially active soil is animated by small (mostly micro-) organisms. In the context of this guideline, soil means samples taken from arable or grassland, sieved $\leq$ 2 mm.

Standard application. The manure or soil sample under study is directly fortified with the test substance dissolved in an appropriate solvent (i.e. water or an organic solvent non-effective on the manure or soil inherent microorganisms).

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 system.

Test-manure Manure samples are fortified with the test substance dissolved in an appropriate solvent (i.e. water or an organic solvent non-effective on the manure inherent microorganisms) and incubated to simulate aging processes of the test substance during the storage of manure.

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

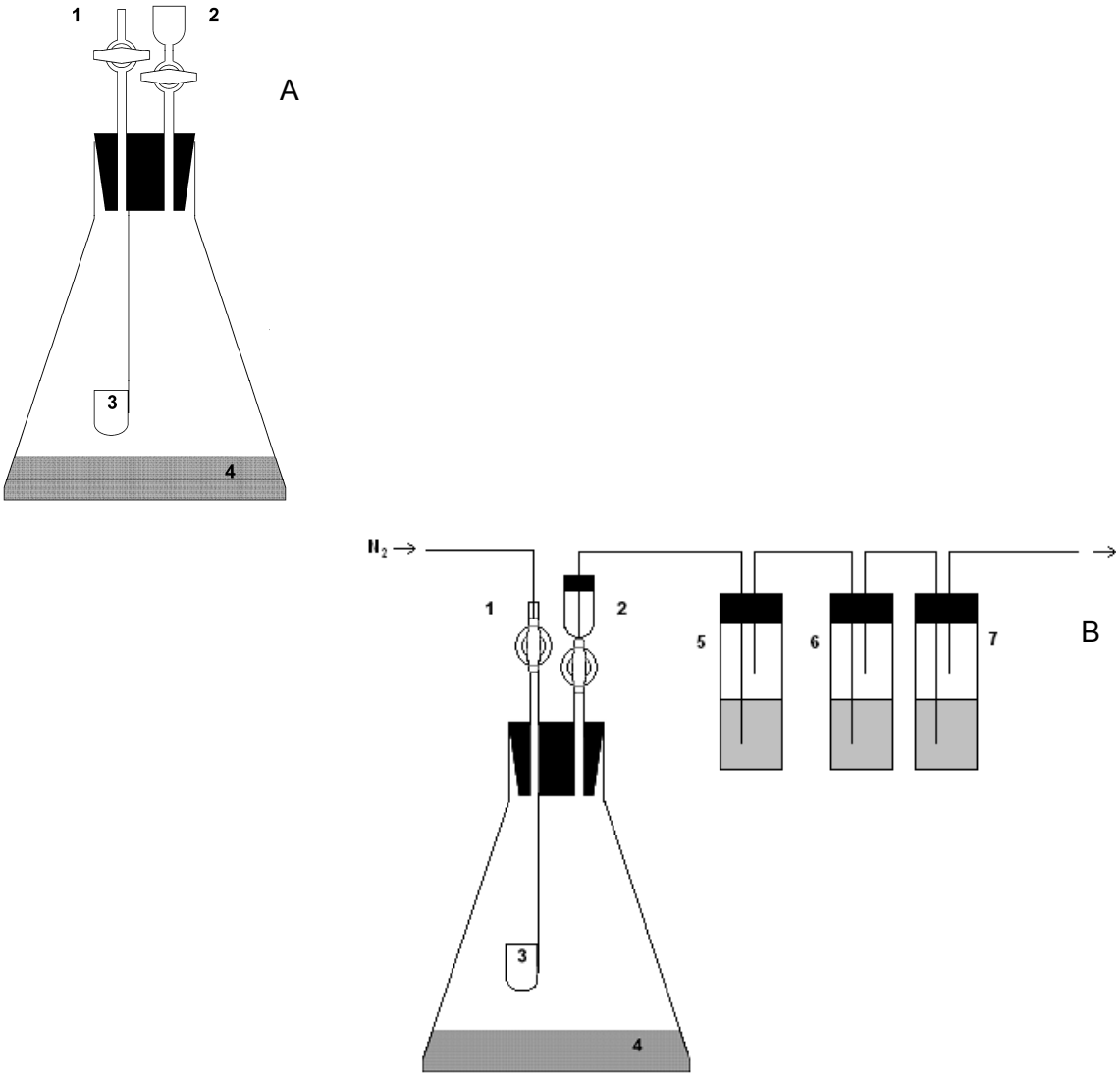
Annex B

Methods of the matrix characterization of excrement and manure samples

Parameter	Reference	Equipment
Dry substance (ds)	[21]	Ultra-X infra-red heater, (Gronert, Germany), or a drying oven
Mineral content (R_{min})	[22]	Muffel furnace (Haereus, Germany)
Phosphate content (P)	[23]	Laboratory Microwave Equipment (Start system with Terminal 320, MLS, MWS-Vertrieb GmbH, Leutkirch, Germany) ICP-OES system (Vista-MPX CCD simultane ICP-OES, Varian, Palo Altra, CA, USA)
Total organic carbon (TOC)	[24]	C-Analyser Dohrmann DC-90 (Dohrmann, Santa Clara, CA, USA)
pH value	[25]	pH Multical 535 GLP with pH-glass electrode SenTix 61 (WTW Weilheim, Germany)
Redox potential (Eh)	[26]	pH Multical 535 GLP 8, (WTW Weilheim, Germany) with redox-electrode (Inolab Redox Einstabmesskette, Mettler Toledo, Giessen, Germany)
Dissolved oxygen (O_2)	[27]	Oxi 340i with OxiCell 325 oxygen-electrode (Fa. WTW, Weilheim, Germany)
Ammonium nitrogen (NH_4-N)	[28]	Distillation Unit 323 (Büchi Labortechnik GmbH, Essen, Germany)
Total nitrogen (N_{total})	[29]	Digestion Unit 430 and Distillation Unit 323 (Büchi Labortechnik GmbH, Essen, Germany)
Biological oxygen demand (BOD_5)	[30]	Oxi 340i with OxiCell 325 oxygen-electrode (Fa. WTW, Weilheim, Germany), Karlsruher bottles, 250 mL (Schott, Mainz, Germany)

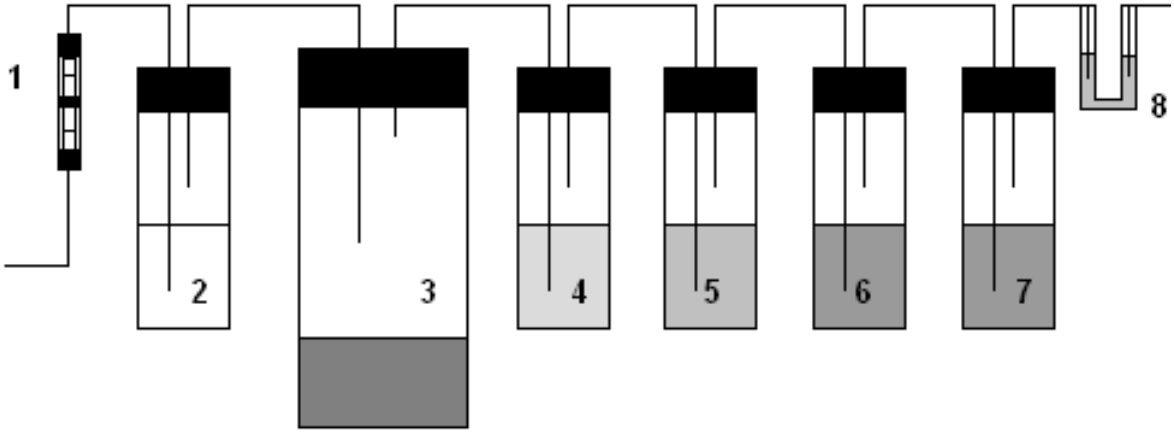
Annex C

Laboratory-test systems



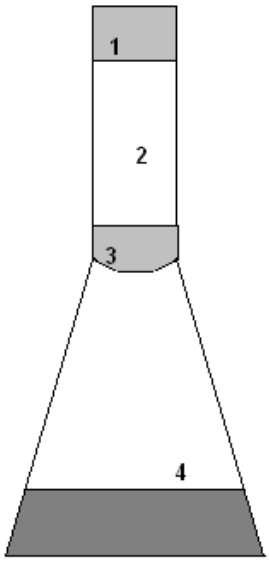
1: inlet valve, 2: outlet valve with activated charcoal filter, 3: internal ^{14}C-carbon dioxide trap, 4: manure or manured soils sample, 5: external trap for ^{14}C-methane with ethylene glycol (10 mL), 6: external trap with sulfuric acid (10 mL, 0.05 M), 7: external ^{14}C-carbon dioxide trap with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

Figure 1: Laboratory-batch system for degradation tests of ^{14}C-labeled VMP in manure and degradation tests in manured soils without (A) and with additional stripping device (B) [4, modified in accordance to 30]



1: flow meter, 2: gas moistening flask, 3: incubation flask with the 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 degradation tests of ^{14}C-labeled VMP in manure and degradation tests in manured soils [1]



1: glass wool, 2: soda-lime trap (15 g), 3: oil treated glass wool, 4: soil sample

Figure 3: Biometric flask with soda-lime trap for degradation tests in manured soils [1]