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Technical Protocol: Transformation of Biocides in Liquid Manures

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On behalf of the Federal Environment Agency (Germany)

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16. Abstract

The Reference Manure Concept, already developed for laboratory tests on fate and behavior of veterinary medicinal products in liquid manures and manured soils, was successfully applied for biocides used for disinfection purposes and control of insects in animal houses. Since the representative and reproducible sampling of manures from high-volume tanks has been considered impossible, excrement samples of cattle and pigs individually kept at an experimental animal house 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 transformation of the biocides imazalil and cyanamide applied as ^{14}C-labeled radiotracers was investigated in these manure samples.
On the basis of the transformation tests, test manures with 7-day aged biocide residues were prepared and applied in laboratory tests on transformation and sorption in manured soil. By means of this experimental approach, the impacts of aging processes during manure storage and of the manure matrix on the fate of biocides in soils can be assessed already under laboratory conditions. These laboratory tests have been directed as closely as possible to agricultural practice as well as to analytical practicability and quality assurance. Finally, the methodological aspects have been compiled in a Technical Protocol (Draft version).

17. Keywords
Biocides, excrement, reference manure, test manure, soil, transformation, sorption, technical protocol

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15. Zusätzliche Angaben

16. Zusammenfassung

Das Referenzgülle-Konzept, das bereits für Labortests zum Rückstandsverhalten von Veterinärpharmaka in Gülle und güllegedüngtem Boden entwickelt wurde, wurde erfolgreich für in Tierstallungen zur Desinfektion bzw. zur Kontrolle von Insekten applizierten Bioziden angewendet. Da eine repräsentative und reproduzierbare Entnahme von Gülleproben aus großvolumigen Tanks bzw. Silos kaum möglich ist, wurden Exkrementproben von Kühen und Schweinen gesammelt, die einzeln im Versuchsstall gehalten wurden. Diese Proben wurden einer ausführlichen Matrixcharakterisierung unterzogen. Durch die Zugabe von Wasser wurden daraus Referenzgülleproben mit definierten Trockensubstanzgehalten hergestellt, die dann in Langzeittests zum Abbau der ^{14}C-markierten Biozide Imazalil und Cyanamid eingesetzt wurden.
Auf Grundlage dieser Abbautests wurden darüber hinaus Testgülleproben mit 7 Tage gealterten Biozidrückständen hergestellt und für Tests zu Abbau und Sorption in güllegedüngten Böden appliziert. Mittels dieses experimentellen Ansatzes kann bereits unter Laborbedingungen das Rückstandsverhalten von Bioziden in Böden unter Berücksichtigung von Alterungsprozessen während der Güllelagerung und Gülle bedingten Matrixeffekten untersucht werden. Diese Labortests sind damit auf die landwirtschaftliche Praxis sowie die analytische Machbarkeit und Qualitätssicherung ausgelegt. Schließlich wurden die methodischen Aspekte in einem Leitfaden (Technical Protocol; Final Draft Version) zusammengestellt.

17. Schlagwörter
Biozide, Exkrement, Referenzgülle, Testgülle, Boden, Abbau, Sorption, Leitfaden

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Abbreviations

BBA	German Federal Research Center for Agriculture and Forestry
BE	Bovine excrement
BHZP	German Federal Hybrid Breeding Program
BM	Bovine manure
BOD_5	Biological oxygen demand within 5 days
CVMP ERAWP	Committee for Medicinal Products for Veterinary Use, Environmental Risk Assessment Working Party
DT_{50}	Disappearance time in days for 50 % of the initially applied test substance
ds	Dry substance
Eh	Redox potential
EMEA	European Medicines Agency, London, UK
ER	Extractable residues
IR heater	Infrared heater
ISO	International Organization for Standardization
K_d	Soil/water distribution coefficient
K_{OC}	Soil/water distribution coefficient normalized on the organic substance in soil
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
PE	Pig excrement
PM	Pig manure
R_f	Retention factor
RTLC	Radio thin layer chromatography

SIR	Substrate-induced respiration
STD	Standard application
TM_B	Test manure prepared from bovine reference manures
TM_P	Test manure prepared from pig reference manures
TOC	Total organic carbon
UBA	Federal Environmental Agency, Dessau-Roßlau, 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

Definitions

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

Extractable residues (ER) represent compounds occurring in the organic solvent used for extraction of as parent compound or metabolite.

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

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

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

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

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

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

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

Standard application. The manure or soil samples under study are 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 manures define real manure samples that were fortified with the respective test substance and incubated for 7 days to simulate short-term aging processes of the test substance during the manure storage.

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

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

1 Introduction and objectives

Within the Manure Project*, funded by the German Federal Environmental Agency (UBA) and performed by the Institute of Ecological Chemistry and Waste Analysis, Technische Universität Braunschweig (Kreuzig et al., 2007b), a technical protocol for tiered laboratory tests of veterinary medicinal products (VMP) in liquid bovine and pig manures and manured soils has already been developed. This innovative research concept took into special consideration that liquid manures are heterogeneous matrices of high complexity and variability. Since a representative and reproducible sampling in high-volume manure tanks is considered impossible, excrements from cattle and pigs kept in experimental stables and fed under standard nutrition conditions were sampled. This approach definitely reduced the matrix variabilities of tank manures and, furthermore, guaranteed that these samples are free of any contamination by VMP and disinfection agents possibly applied in animal houses of intensive husbandry. By adding tap water, dry substance contents of typical bovine and pig manures were adjusted to prepare reference manures. The thorough matrix characterization of excrements and reference manures clearly showed that, following this concept, reference manures of definitely higher homogeneity than tank manures became available for reproducible laboratory testing. On the basis of the transformation tests of VMP in reference manures, test manures containing short-term aged residues of the test substances under study were used to monitor transformation and sorption in manured soils. By means of this tiered experimental design, 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.

Besides VMP, biocides used in animal houses or directly applied into liquid manures for disinfection purposes or control of insects may also undergo transformation processes under anaerobic conditions. This fact has been already considered relevant for the authorization procedure of biocidal products by the Directive 98/8/EC (European Commission, 1998) complemented by the technical notes for guidance on data requirements for active substances and biocidal products (TNsG, 2000). In accordance to the latter, the OECD Guideline 311 (OECD, 2006) linked to ISO 11734 (International

* Veterinary Medicinal Products in Manures and Manured Soils: Development of a Technical Protocol for Laboratory Tests" (UBA-FKZ 204 67 455; 2005-2007)

Organization for Standardization, 1995) should be applied to assess the transformation of biocides in liquid manures by the investigation of the ultimate biodegradability under anaerobic conditions in digested sludge of a municipal wastewater treatment plant. Within this 60-day screening test under optimized boundary conditions of an anaerobic digester at 35 °C, fermentative and methanogenic bacteria may transform the applied test substances to methane and carbon dioxide measured by the increase of the head-space pressure in the laboratory-test system. Due to significantly diverging conditions, information about fate and behavior of biocides can be hardly transferred from digested sludge to liquid bovine or pig manures under tank storage conditions. This was clearly shown by fate monitoring studies on different ^{14}C-labeled test substances, i.e., ketoprofen, sulfamethoxazole and acetyl-sulfamethoxazole (Kreuzig et al., 2007b, Höltge and Kreuzig, 2007).

Therefore, there is the need for the development of a real simulation test for fate monitoring of biocides in liquid bovine and pig manures as well as in manured soils. In continuation of the research activities performed within the Manure Project, the technical protocol for laboratory tests should be advanced within the frame of the Biocide Project. The detailed steps to be taken were the following:

First, the reproducible sampling of bovine and pig excrements and the reproducible preparation of reference manures should be confirmed. This approach implied tests on the use of reference manures after long-term storage at -20 °C and reconditioning at ambient conditions in order to uncouple laboratory testing from sampling and dispatching fresh excrements, if other laboratories are involved in those studies. In this context, the selection of parameters for matrix characterization should be reduced to the essential ones required by the principles of analytical quality assurance.

Second, transformation tests in liquid bovine and pig manures should be performed using two biocidal test substances of different application patterns, namely ^{14}C-imazalil and ^{14}C-cyanamide. Bovine and pig reference-manure samples of 10 % and 5 % dry substance, respectively, were prepared. Test periods were up to 100 days, exemplarily up to 177 days, simulating long-term manure storage requirements of the German Ordinance Concerning Fertilization (2006). Taking the wide range of dry substance contents of tank manures into special account, i.e., 0.4 % to 12 %, reference-manure samples were also tested at 2.5, 5 and 10 % dry substance contents within 30-day incubation experiments. For this purpose, extraction efficiency tests were conducted first for

both test substances. Besides the direct treatment by organic solvents, e.g., ethyl acetate or acetonitrile, manure suspensions were separated into aqueous and solid phases by means of centrifugation or the aqueous phase was removed by lyophilization prior to solvent extraction, e.g., acetone, acetonitrile, ethyl acetate or methanol, of the solid manure materials.

Third, test manures containing ^{14}C-imazalil and ^{14}C-cyanamide residues were prepared and applied to monitor transformation and sorption in manured soil samples. For this purpose, the manure application amount was reduced to the maximum accepted by the German Ordinance Concerning Fertilization (2006) while this amount was still exceeded by the fourfold during the method development within the Manure Project to avoid any losses during analytical handling of low-weight samples.

Fourth, the working program of the Biocide Project ab initio involved methodological experiences of European experts of research institutions, industry and authorities. Besides the participation at international conferences, e.g., ERAPharm Conference at York, UK, September 2007, an international workshop was held at Technische Universität Braunschweig on 1 and 2 April 2008. The outcomes have then initiated the EMEA Concept Paper "On Fate of VMP in Manure", published in February 2009 (EMEA, 2009).

Fifth, the outcomes of the Biocide Project were incorporated in the revised "Technical Protocol on Transformation of Veterinary Medicinal Products and Biocides in Liquid Bovine and Pig Manures ". This compilation was disseminated as one answer to the EMEA Concept Paper in order to assist the CVMP ERAWP to hold a focus group meeting at EMEA on 23 June 2009 preparing a technical guidance note for harmonization of manure transformation tests. In order to prepare this meeting by disputing the Technical Protocol together with the European experts, the final UBA Expert Meeting was held at Technische Universität Braunschweig on 27 May 2009.

2 Excrements, reference manures and soil samples

Liquid manures are matrices of high complexity and variability affected by numerous factors like animal species, race and age of the animals, and feeding conditions. This fact has been clearly shown by the long-term survey on the composition of more than 2000 bovine and pig manures conducted by LUFA Nord-West, Oldenburg, Germany, from 1997 to 2004 (Merkel, 2005). Selected results of the survey and further studies are listed in **Tab. 2.1** and **2.2**.

Tab. 2.1: Composition of tank-manure samples of cattle from European countries (values on fresh weight basis)

Origin Authors	ds [%]	TOC [g kg^{-1}]	pH	NH_4-N [g kg^{-1}]	N_{total} [g kg^{-1}]	BOD_5 [g kg^{-1}]
Germany Buning (1997)	7.0	50	7.7	1.7	3.2	-
Germany Merkel (2005)	0.4-12.3 [8.7]	-	-	0.01-2.9	0.4-5.7	-
Germany Schuchardt and Hahne (1996)	3	-	-	1.7	3	12-21
	8	-	-	2.2	4	16-27
	10	-	-	2.8	5	20-34
Denmark Nielsen et al. (2004)	5.7±0.1	-	7.3	1.5	2.5	-
	5.5±0.1	-	7.2	1.3	2.1	-
The Netherlands Bouwman and Reus (1994)	9.6±2.1	68±15	8.2	-	4.9±0.8	-
European Community Burton and Turner (2003)	1.5-12.3 [6.5]	-	-	1.0-4.9	2.0-7.0	-

[ ]: average values

Tab. 2.2: Composition of tank-manure samples of pigs from European countries and USA (values on fresh weight basis)

Origin Authors	ds [%]	TOC [g kg^{-1}]	pH	NH_4-N [g kg^{-1}]	N_{total} [g kg^{-1}]	BOD_5 [g kg^{-1}]
Germany Buning (1997)	5.1	37	8.1	5.3	6.5	-
Germany Hahne (2001)	1.4-10.6	10 -77	-	1.9-7.7	2.5-9.9	9-49
Germany Merkel (2005)	0.4-11.6 [4.9]	-	-	0.3-4.9	0.6-8.3	-
Germany Schuchardt and Hahne (1996)	3	-	-	1.8	2.6	9 -23
	6	-	-	3.6	5.1	18-46
	9	-	-	5.4	7.7	27-69
The Netherlands Bouwman and Reus (1994)	7.4±2.5	49±19	8.0	-	6.5±1.4	-
Spain Moral et al. (2005)	2.3±3.1	-	7.4	2.0±1.1	2.6±1.3	14±9
European Community Burton and Turner (2003)	1.5-9.2 [5.1]	-	-	1.9-6.1	1.2-8.2	-
USA Ndegwa et al. (2003)	2.9±0.7	-	7.7	4.1±0.1	4.1±0.1	8±0

[ ]: average values

Additionally, residues of feeding stuff and straw as well as VMP, disinfection and cleaning agents may be released into the storage tanks by farming practices enhancing the heterogeneity of liquid manures (Hoffmann and Hege, 1991, Montforts and Tarazona Lafarga, 2003, Schuchardt and Hahne, 1996). Finally, organic constituents and conta-

minants of manures undergo transformation processes during the storage of manures up to several months (German Ordinance Concerning Fertilization, 2006).
Hence, a representative and reproducible sampling in manure tanks is considered impossible. Since the Manure Project, therefore, excrement samples of cattle and pigs individually kept in experimental stables of the Institute of Animal Nutrition, Friedrich-Löffler-Institut, Braunschweig, Germany, have been sampled in order to reduce the variabilities of tank-manure samples and to guarantee that those excrement samples and the resulting reference-manure samples are free of any VMP or biocide contamination.

2.1 Excrement sampling and conditioning

Within the Biocide Project further sampling activities were performed at these experimental stables in order to confirm this research concept. Thus, excrement samples, i.e., urine and feces, were taken from an individually kept dairy cow in September 2007 and from a group of 8 pigs in November 2007. Sampling was then repeated in August 2008. Animals' characteristics and feeding conditions are listed in **Tab. 2.3**.

Tab. 2.3: Origin of cattle and pig excrements

Excrement	race	age	feeding conditions
Dairy cow	German Holstein (black and white)	4 years	maize and grass silage [66:34] (70 %), pellets (30 %)
Pigs	German Federal Hybrid Breeding Program	< 1 year	wheat (59 %), barley (17 %), soy bean extract (18 %), minerals, vitamins (2.9 %) and amino acids (0.15 %), soy bean oil (3 %)

Directly after sampling, those excrements may not be strictly anaerobic. Furthermore, readily degradable substances, e.g., nitrogen containing compounds as urea, undergo rapid transformation enhancing the matrix heterogeneity (Strauch et al., 1977, Ndegwa et al., 2003). To minimize this effect, the excrement samples were stored in plastic containers (approximately 20 L) at ambient temperature. Within the 21-d conditioning pe-

riod, they were daily homogenized using an electric stirrer. Constant conditions were indicated by dissolved oxygen contents < 0.1 mg kg^{-1}, the redox potential Eh < 0 mV (cf. chapter 2.2) and an ammonium content stable up to ± 0.2 g kg^{-1}. Thereafter, the excrements could be used for the reference-manure preparation or long-term stored at -20 °C up to 360 d. After defrosting, reconditioning and matrix characterization, the excrement samples can be used for further laboratory testing (cf. chapter 2.3).

Tab. 2.4: Matrix-characterization methods of excrement and manure samples

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

2.2 Matrix characterization of excrement samples

Excrement samples underwent a thorough matrix characterization in order to identify the parameter variabilities within the different sample batches of the Manure and Biocide Projects. The list of parameters under study within the Manure Project, however, was streamlined to the essential ones listed in **Tab. 2.4**.
The determination of mineral, phosphate and copper contents as well as of the chemical oxygen demand were cancelled without any loss of relevant information about the variations of excrement compositions and their impact on the transformation of VMP and biocides in reference manures. Hence, the time and cost frames of analytical processing could be effectively reduced.

Dry substance content

For the determination of the dry substance contents (ds), 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 [%].

Total organic carbon

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 ($C_2H_2O_4 \times 2\ H_2O$) 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 7 %.

pH value, dissolved oxygen content and redox potential

The pH value was measured directly in the homogenized excrement or manure samples (50 to 100 g) using a pH electrode. The pH value was 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 decimal place.

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

The redox potential was measured directly in the homogenized excrement or manure samples (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. During the redox electrode measurement in the complex suspensions of excrement and reference-manure samples, interferences may occur. Sometimes, redox potentials of Eh > 0 mV can be found (Kreuzig et al., 2007b). Since permanent aerobic conditions only occur at Eh > 150 mV (Ndegwa et al., 2003, Michels et al., 2000) and the limit of microbial activity in liquid media under aerobic conditions is O_2 = 0.1 mg kg^{-1} (Strauch et al., 1977, Domsch, 1992), those interferences could be neglected. On that account, the transformation tests were especially conducted under nitrogen atmosphere to guarantee strictly anaerobic conditions (cf. chapter 4.3).

Ammonium and total nitrogen content

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 decimal place, 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 98 ± 6 % 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 98 ± 8 %, analyzed for ammonium sulfate standards without matrix.

Biological oxygen demand

For the determination of the biological oxygen demand within 5 days (BOD_5), the excrement samples were diluted by adding tap water in dependency on the dry substance content to 1:2000 or 1:4000. 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, Ger-

many). 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 decimal places. Results between 1 g kg^{-1} and 10 g kg^{-1} were reported to one decimal place. Results ≥ 10 g kg^{-1} were reported without any decimal place.

2.3 Reproducibility of excrement sampling

Within the Manure Project, six bovine and pig excrements each were sampled at the experimental stable as well as at conventional stables. The results of the matrix characterization of the bovine manure samples are listed in **Tab. 2.5**. The variations of the matrix parameters are expressed by minimum and maximum values supplemented by the median. There, it has to be taken into account that excrement samples were consciously collected from test animals of different ages, i.e., 8 months to 5 years, and kept under different feeding conditions typical for herbivores. Within the Biocide Project, sampling activities were repeated in September 2007 and August 2008 at the experimental stable of the Friedrich-Löffler-Institut. The matrix characteristics of these excrement samples definitely met those ranges confirming the reproducibility of this sampling concept (**Tab. 2.5**).

Tab. 2.5: Matrix characterization of bovine excrements

Parameter	ds [%]	pH	Eh [mV]	O_2 [mg L^{-1}]	NH_4-N [g kg^{-1}]	N_{total} [g kg^{-1}]	TOC [g kg^{-1}]	BOD [g kg^{-1}]
Minimum[1]	10	6.2	-100	---	1.2	3.1	40	6
Median[1]	13	6.7	-30	< 0.1	2.0	4.3	49	10
Maximum [1]	13	8.4	40	---	4.5	6.5	57	23
Excrement[2]	13	6.2	-80	< 0.1	1.8	4.7	54	11
Excrement[3]	13	6.6	-120	< 0.1	4.5	8.2	57	18

[1] Sampling of 6 bovine excrements during the Manure Project from 2004 to 2007, matrix characterization after the 21-d conditioning period

[2] Sampling of 1 bovine excrement in September 2007, matrix characterization after the 21-d conditioning period

[3] Sampling of 1 bovine excrement in August 2008, matrix characterization after the 21-d conditioning period

The same tendencies were found for the pig excrements additionally sampled in November 2007 and August 2008 although the feeding conditions of the test animals differed stronger due to the omnivore character of pigs (**Tab 2.6**). Taking into account that one group of fattened pigs was kept in a conventional farm and mainly fed by potato refuse instead of barley or wheat, reproducible excrement sampling should be possible in every stable so far the administration of VMP to the test animals and the application of biocides in the stable could be excluded. Otherwise, defined diets of cattle and pig nutrition may contribute to a minimum parameter variation of excrements.

In order to uncouple laboratory testing from sampling fresh excrements and to facilitate saving reserve samples for analytical quality assurance, conditioned and matrix characterized excrement samples were long-term stored at -20 °C up to approximately 360 days. After defrosting, the excrements are reconditioned at 20 ± 2 °C for 3 d to remobilize the excrements inherent microorganisms. The subsequent matrix characterization clearly showed that the parameters matched the variation ranges of freshly

conditioned excrement samples (**Tab 2.6**). Hence, these results confirmed those of the Manure Project.

Tab. 2.6: Matrix characterization of pig excrements

Parameter	ds [%]	pH	Eh [mV]	O_2 [mg L^{-1}]	NH_4-N [g kg^{-1}]	N_{total} [g kg^{-1}]	TOC [g kg^{-1}]	BOD [g kg^{-1}]
Minimum[1]	13	5.7	-180	---	3.4	6.8	56	21
Median[1]	18	6.6	-100	< 0.1	5.8	9.2	72	24
Maximum[1]	23	7.4	40	---	9.2	13.8	103	28
Excrements[2]	14	6.0	-30	< 0.1	4.4	8.8	67	18
Excrement[3]	15	6.1	50	< 0.1	4.8	8.6	57	21
Excrements[4]	14	6.3	-80	< 0.1	4.5	8.2	57	18

[1] Sampling of 6 pig excrements during the Manure Project from 2004 to 2007, matrix characterization after the 21-d conditioning period

[2] Sampling of 1 pig excrement in November 2007, matrix characterization after the 21-d conditioning period

[3] Sampling of 1 pig excrement in November 2007, frozen after the 21-d conditioning period and first matrix characterization at -20 °C until July 2008, reconditioned at ambient temperature, second matrix characterization

[4] Sampling of 1 pig excrement in August 2008, matrix characterization after the 21-d conditioning period

2.4 Preparation of reference-manure samples

Reference manures were prepared by mixing bovine and pig excrements with tap water to adjust dry substance contents of 10 and 5 %, respectively. These values correspond to the average values given for Europe (Bouwman and Reus, 1994, Buning, 1997, Burton and Turner, 2003, Merkel, 2005, Møller et al., 2004, Schuchardt and

Hahne, 1996) and were thus recommended by Kreuzig et al. (2007b). In order to optionally study the effect of the dry substance content on transformation of the biocides imazalil and cyanamide, reference-manure samples of different dry substance contents, i.e., 2.5, 5, 10 %, were applied in short-term tests, too.

The concept of excrement sampling and reference-manure preparation facilitates laboratory testing on transformation of VMP and biocides in liquid manures in an adequate frame of time and costs. In contrast to this approach, the OECD guideline 307 "Aerobic and Anaerobic Transformation in Soil" followed an alternative procedure (OECD, 2002). There, four soils of definite different physico-chemical properties have to be monitored in order to gather data on the impact of variable boundary conditions in soils. Due to the natural composition of soils, the use of one reference soil is considered impossible. Beyond that, mixing of different soil components would only produce artificial substrates.

The take over of this procedure for transformation tests in 4 different tank manures of one animal species, will quadruplicate the experimental expenditures of the reference-manure concept. Further analytical expenditure will be necessary for additional matrix characterization tests and screening analyses of the tank manures for interfering VMP and biocide contaminants based on complex extraction and clean-up procedures as well as LC/MS/MS analyses. Following the reference-manure concept, those additional screening analyses are not necessary because the excrements to be taken are operationally free of any VMP and biocide contamination. Thus, this concept will save time and costs and, nevertheless, will gather an appropriate data pool for the environmental risk assessment of the authorization procedure of VMP and biocides. Due to those advantages, the reference-manure concept should be more likely acceptable for stakeholders at research institutions, industry and authorities.

2.5 Soil samples

For testing on sorption and transformation of biocides in soil and manured soil, a farmland soil was topsoil sampled. The investigation site Adenstedt, Lower Saxony, Germany, has been already selected for the test-plot and corresponding laboratory ex-

periments within the Runoff Project (Kreuzig et al., 2007a)[†]. Soil characteristics are listed in **Tab. 2.7**.

Tab. 2.7: Soil properties of the farmland soil in Adenstedt, Germany

Investigation site	farmland at Adenstedt
Landscape	Ambergau, Forelands of the Harz Mountains
Vegetation	wheat
Soil type	Luvisol
Soil texture Sand (63 – 2000 µm) Silt (2 – 63 µm) Clay (< 2 µm)	silty clay 5.0 % 55.5 % 38.5 %
pH_{CaCl_2}	6.9
Maximum water holding capacity	58.2 %
Organic carbon	1.3 %

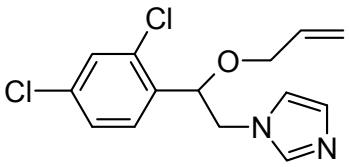
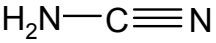
The soil samples were sieved < 2 mm and stored in closed containers at -20 °C because contemporary sampling directly before experimental processing was impossible. Prior to the start of the transformation tests, therefore, the frozen soil samples were reconditioned at room temperature for at least 2 days. In order to check for microbial activity, the substrate induced respiration (SIR) was determined (ISO 17155, 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, independent if sampled field-fresh or reconditioned.

[†] Investigations on Runoff of Veterinary Medicinal Products from Farmland and Grassland after Manure Application and Sprinkler Irrigation (UBA-FKZ 202 67 435; 2002-2004)

3. Test substances

In intensive animal husbandry, biocides are frequently applied in order to control insects or to disinfect animal houses or liquid manures. These measures are performed in order to reduce the pressure of infection diseases for production animals. For this purpose, the total bacterial count should be kept less < 1000 cm^{-2} (aid, 1996). According to both different application patterns, two biocidal test substances were selected, namely imazalil and cyanamide. Both test substances were applied as ^{14}C-labeled radiotracers to allow for the set up of detailed mass balances with special consideration of mineralization and extractable as well as non-extractable residues. The extractable fractions were additionally screened for parent compounds and metabolites by means of radio thin layer chromatography (RTLC).

Tab. 3.1: Chemical structures and physico-chemical properties of imazalil and cyanamide

Common name	Imazalil	Cyanamide
Chemical structure	 $C_{14}H_{14}Cl_2N_2O$	 CH_2N_2
Chemical name	(R,S)-1-[2-allyloxy-2-(2,4-dichloro-phenyl) ethyl] imidazole	amino-methanenitrile
CAS	35554-44-0	420-04-2
Molar mass	297.2 g mol^{-1}	42.0 g mol^{-1}
Vapor pressure	9.3 x 10^{-6} Pa (20 °C)	5.0 x 10^{-1} Pa (20 °C)
Melting point	53 °C	46 °C
Boiling point	347 °C	260 °C (decomposition)
Solubility in water	1.4 g L^{-1}	4590 g L^{-1}

Chemical structures and physico-chemical properties of imazalil and cyanamide are shown in **Tab. 3.1**. The ^{14}C-labeled radiotracers were namely (R,S)-1-[2-allyloxy-2-(2,4-dichloro-U-^{14}C-phenyl) ethyl] imidazole and amino-^{14}C-methanenitrile. The specific radioactivities were 133 MBq mmol^{-1} (0.45 MBq mg^{-1}) and 1850 MBq mmol^{-1} (44 MBq mg^{-1}), respectively. Radiochemical purity of ^{14}C-imazalil, determined by radio thin layer chromatography (RTLC), was 97 %, while that of ^{14}C-cyanamide was limited to approximately 80 % possibly by autoradiolysis (Draganic et al., 1978).

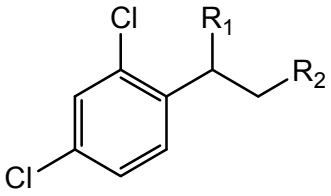
RTLC was furthermore used for metabolite screening of manure and soil extracts. For this purpose, unlabeled reference chemicals of imazalil and cyanamide metabolites were used. The transformation products of cyanamide are also reported to be formed by autoradiolysis of ^{14}C-cyanamide (Draganic et al., 1978). The chemical names and structures are given in **Tab 3.2** and **3.3.**

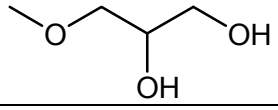
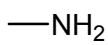
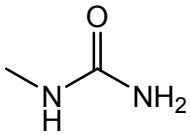
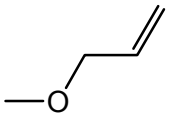
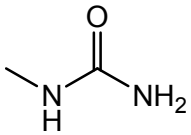
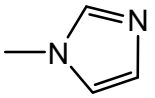
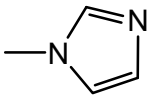
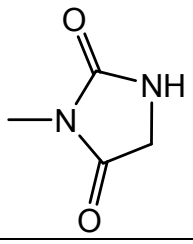
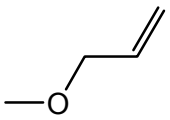
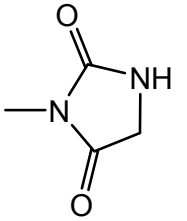
3.1 Imazalil

Imazalil (R,S-1-[2-allyloxy-2-(2,4-dichlorophenyl)ethyl]imidazole) belongs to the group of stable disinfection agents. It is an imidazole derivative with a N-C linkage between the imidazole and the remainder of the molecule. Due to its inhibiting effect of the ergosterol biosynthesis of fungi, it is a highly effective systemic fungicide with a protective and curative mode of action applied in postharvest protection of fruits (pears, apples, banana, citrus), vegetables and ornamental plants. Imazalil is also used for the protection of crop seeds.

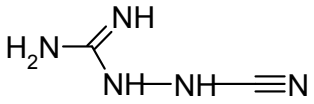
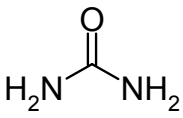
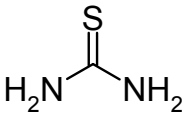
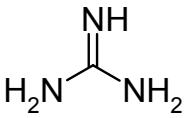
The transformation of imazalil in soil was reported to occur in the prophenyl side chain through epoxidation and hydration. A transformation to 1-[2(2,4-dichlorophenyl)-2-(2,3-dihydroxypropyloxy)ethyl]-1*H*-imidazole comes along with a detoxification because the metabolite is less toxic than the parent compound (Guan et al., 1992). Chu et al. (2007) could enhance the transformation rates of imazalil in soil by long-term irradiation (UV, sunlight) and by a high microbial activity. In a 1-year transformation test on ^{14}C-imazalil in soil, 22 % of the applied substance was mineralized to ^{14}C-carbon dioxide and 33 % was converted to soil-bound residues (Van Leemput et al., 1985). The half-life was 170 days. In contrast, Chu et al. (2007) found a half-life of 31 days. Imazalil and its metabolites were classified as quite immobile in soil. A transport into deeper soil horizons and to the groundwater was not found (Van Leemput et al., 1986).

Tab. 3.2: Reference chemicals for screening on imazalil metabolites in manure and soil samples



Substance	R_1	R_2
3-[2-amino-1-(2,4-dichlorophenyl)ethoxy] propane-1,2-diole		
1-[2-(2,4-dichlorophenyl)-2-hydroxy-ethyl]-urea		
1-[2-(2,4-dichlorophenyl)-2-(prop-2-en-1-yloxy)ethyl]-urea		
1-[2-(2,4-dichlorophenyl)-2-(1H-imidazole-1-yl)-ethanone		
1-(2,4-dichlorophenyl)-2-(1H-imidazole-1-yl)-ethanol		
3-[2-(2,4-dichlorophenyl)-2-hydroxyethyl]-imidazole-idine-2,4-dione		
3-[2-(2,4-dichlorphenyl)-2-(prop-2-en-1-yloxy)ethyl] imidazole-idine-2,4-dione		

Tab. 3.3: Reference chemicals for screening on cyanamide metabolites in manure and soil samples

Substance	Structure
dicyandiamide	
urea	
thiourea	
guanidine	

3.2 Cyanamide

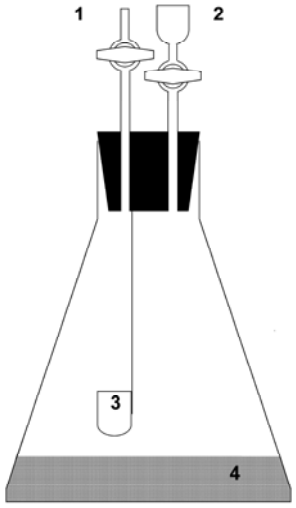
Cyanamide belongs to the stable and manure disinfection as well as insecticidal agents that is applied to control the face fly (*Musca autumnalis*) and pig dysentery (*Brachyspira hyodysenteriae*) (Althaus, 2008). It is a highly reactive molecule with two functional groups directly linked together: The nucleophilic nitrogen atom of the amino group and the electrophilic carbon of the nitrile group. Cyanamide was the first synthetic compound used as a nitrogen fertilizer. The N-fertilization is based on the transformation of urea to ammonium compounds in several steps: First, calcium cyanamide is hydrolyzed to cyanamide. Second, cyanamide is enzymatically or catalytically converted to urea. Third, urea is degraded by the enzyme urease via ammonium carbonate to ammonia. Due to its high reactivity several other products can be released from cyanamide like dicyandiamide or carbodiimide, resulting from dimerization or isomerization processes, respectively (Bart and Mizelli, 1995). Milks and Janes (1956) also reported about by-products of the manufacture of dicyandiamide from cyanamide. They

identified numerous substances by means of paper chromatography, namely cyanourea, biguanidine, melamine, three salts of guanidine, urea, guanylthiourea, thiocyanate, thiourea, dicyanamide and cyanamide. Several enzyme mediated degradation processes were described for cyanamide, like the hydration of cyanamide to urea by the soil fungus *Myrothecium verrucaria* (Stransky und Amberger, 1973) or the degradation to ammonia compounds by urease from jack bean (*Canavalia ensiformis*) (Estermaier et al., 1992). These soil metabolites may be also formed by autoradiolysis of ^{14}C-cyanamide (O´Connel et al., 1991). In addition to its ability to provide nitrogen it acts also as a herbicide, insecticide, fungicide and bactericide (Huang et al., 2006, Klasse, 2002). It is used as a growth inhibitor for bacteria and as a plant growth regulator in halting the dormancy of grape vines and other fruits.

4. Experimental design

4.1 Laboratory-batch system

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), a closed laboratory-batch system traced back to the OECD guideline 304 A (OECD, 1981a, Kreuzig et al., 2003) was applied for transformation tests in liquid manures and terrestrial topsoils (**Fig. 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 or soil sample

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

For this purpose, the system is equipped with an inlet and an outlet valve allowing for a discontinuous gas exchange realizing strictly anaerobic or aerobic conditions. It is additionally equipped with an internal trap filled with potassium hydroxide solution to absorb ^{14}C-carbon dioxide released by mineralization of the applied ^{14}C-labeled test sub-

stance. Furthermore, external traps filled with ethylene glycol, sulfuric acid and a ^{14}C-carbon dioxide absorbing scintillation cocktail may be coupled to the laboratory-batch system in order to absorb volatile metabolites of the test substance stripped out of the incubation flask´s headspace. Due to these technical characteristics, the performance of this laboratory-batch system is adequate to the flow-through system described by the OECD guideline 307 (OECD, 2002) as proven by transformation tests on ^{14}C-sulfamethoxazole in bovine and pig manures as well as on ^{14}C-acetyl-sulfamethoxazole in soil and manured soil (Kreuzig et al., 2007b). The vulnerability of both laboratory-test systems is the direct determination of ^{14}C-methane released out of the ^{14}C-labeled test substance under methanogenic conditions. Hence, indirect measurement is need for both laboratory test-systems. Consequently, the ^{14}C-carbon dioxide free headspaces of the incubations flasks have to be transferred to a combustion apparatus where ^{14}C-methane is oxidized to ^{14}C-carbon dioxide. The latter is to be trapped again in an absorbing scintillation cocktail and then scintillation counted.

4.2 Extraction efficiency tests

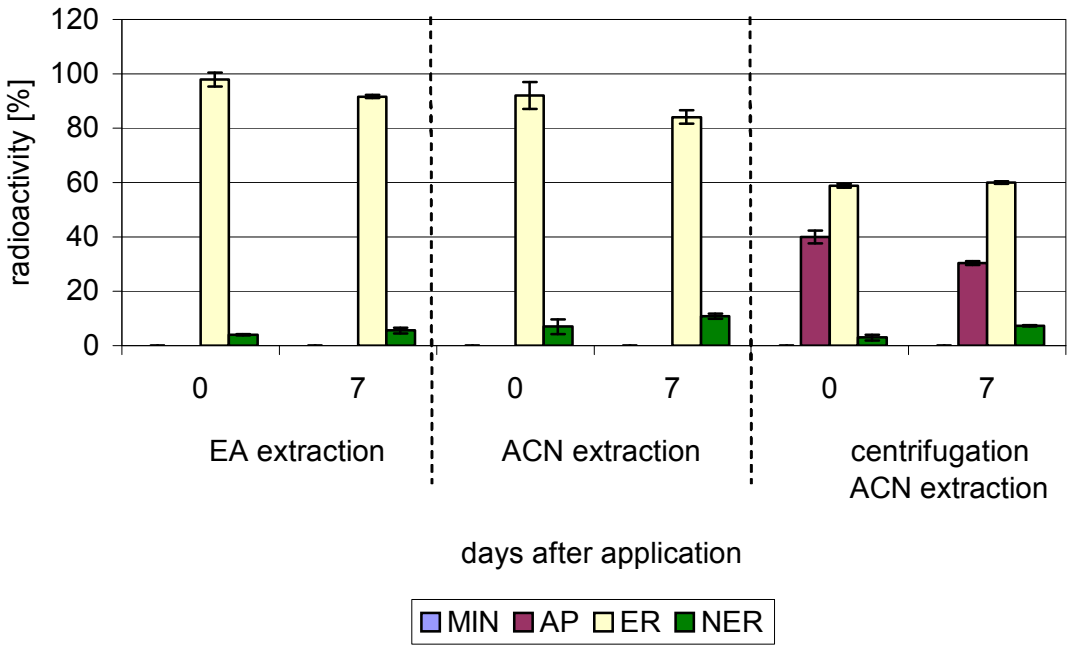
Every extraction procedure is targeted at a quantitative release of chemicals out of the sample matrices under study. Therefore, solvents and extraction procedures have to be carefully selected particularly considering parent compound and metabolites as well as sample matrix specific properties. Those should not be significantly changed during the extraction procedure (IUPAC, 1984) as to differentiate between extractable and non-extractable residues for an appropriate environmental risk assessment.

In order to check for the extraction efficiency of different extraction procedures, 50-g reference manure or soil samples were fortified by 0.05 MBq of ^{14}C-imazalil and ^{14}C-cyanamide, respectively. Samples were prepared without incubation (d 0). For manure samples, the laboratory-batch systems were additionally incubated in the dark under anaerobic conditions at 20 $\pm$ 1°C for 7 days. In order to determine mineralization, 8 mL of 0.1 M potassium hydroxide solution were used for the absorption of ^{14}C-carbon dioxide. After the extraction procedures, extracted manure solids were mixed with quartz sand and cellulose (ratio 4:4:1). For soil samples, only cellulose was added. After drying, the non-extractable residues were then determined by combustion and scintillation counting.

4.2.1 Extractability of imazalil in manure and soil samples

Liquid manure samples

For the extraction of ^{14}C-imazalil out of liquid manure samples, three different procedures were applied. Thus, reference-manure samples were directly treated by means of 100 mL ethyl acetate or acetonitrile. The samples were extracted on a horizontal shaker overnight (200 rpm). The extracts were filtrated, concentrated and used for scintillation counting. The extracts were RTLC metabolite screened. However, only the parent compound initially applied could be found.

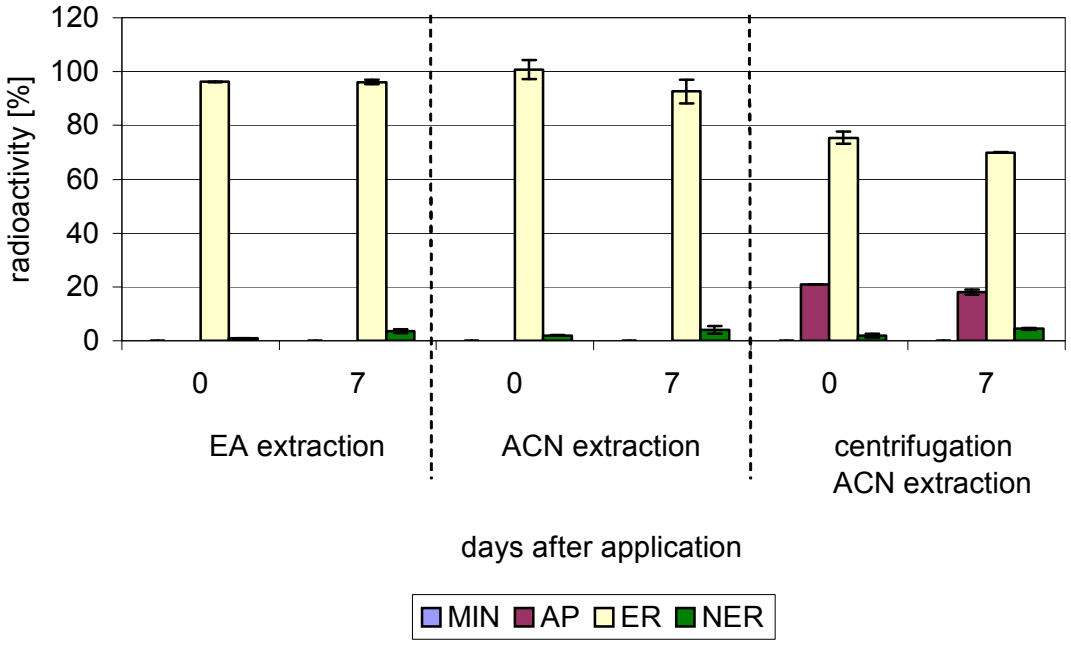


EA: ethyl acetate, **ACN:** acetonitrile, **MIN:** mineralization, **AP:** aqueous phase, **ER:** extractable residues, **NER:** non-extractable residues

Fig. 4.2: Extraction efficiency tests of ^{14}C-imazalil in bovine manure samples (balances: 99 ± 3 %)

Otherwise, the aqueous and the solid phases of manure samples were separated first by means of centrifugation (4000 rpm, 15 min). The radioactivity amounts of the aqueous phases were scintillation counted. Due to the difficult evaporation of aqueous fractions, a metabolite screening could not be directly accomplished out of those solutions.

Solid phases were extracted using 100 mL acetonitrile on a horizontal shaker overnight. The non-extractable residues were determined by combustion and scintillation counting.



EA: ethyl acetate, **ACN:** acetonitrile, **MIN:** mineralization, **AP:** aqueous phase, **ER:** extractable residues, **NER:** non-extractable residues

Fig. 4.3: Extraction efficiency tests of ^{14}C-imazalil in pig manure samples (balances: 98 ± 3 %)

The highest extraction efficiency for imazalil out of bovine manure samples amounted to 98 % by means of ethyl acetate followed by 92 % by means of acetonitrile extraction (**Fig. 4.2**). After the 7-day incubation period the extractable residues dropped to 92 % and 84 %, while the non-extractable ones increased up to 6 % and 11 %, respectively. After centrifugation, however, the less polar imazalil was partly partitioned into the aqueous phase while up to 60 % was subsequently extracted out of the solid manure materials by means of acetonitrile. Non-extractable residues were of minor relevance.
Similar tendencies were found for imazalil in pig manure samples (**Fig. 4.3**). These test series have been already indicated that lower amounts of non-extractable residues are

formed in pig manure. Furthermore, there was a change for the efficiency of the combined centrifugation and acetonitrile extraction procedure. In contrast to bovine manure samples, the amounts of the centrifugates dropped to 20 %, while approximately 60 % of the radioactivity initially applied occurred in the acetonitrile extracts.
In both test series, the highest extraction efficiency of ethyl acetate was accompanied by a rapid sedimentation of the solid manure materials after the extraction procedure. Furthermore, the ethyl acetate phases can be easily separated from the aqueous ones and then easily evaporated in order to increase the concentration of the analytical solutions for metabolite screening purposes. Based on these extraction efficiency tests and on the experiences by monitoring VMP in bovine and pig manures (Kreuzig and Höltge, 2005, Höltge and Kreuzig, 2007, Kreuzig et al., 2007b), ethyl acetate was used for the extraction of ^{14}C-imazalil residues within the transformation tests in reference-manure samples.

Soil samples

Different extraction solvents, e.g., ethyl acetate (Garrido et al., 1997), acetonitrile (Lehotay, 2007) and acetone (Rissato et al., 2005) were applied to release imazalil residues out of soil samples, fruits and vegetables. Those were also checked for the quantitative release of imazalil out of soil and manured soil samples. According to efficiencies of 104 % for ethyl acetate, 83 % for acetone and 80 % for acetonitrile, ethyl acetate was also used for soil analysis. Furthermore, this selection was promoted by the rapid sedimentation of the soil solids, the quantitative phase separation and the rapid evaporation when ethyl acetate was used.

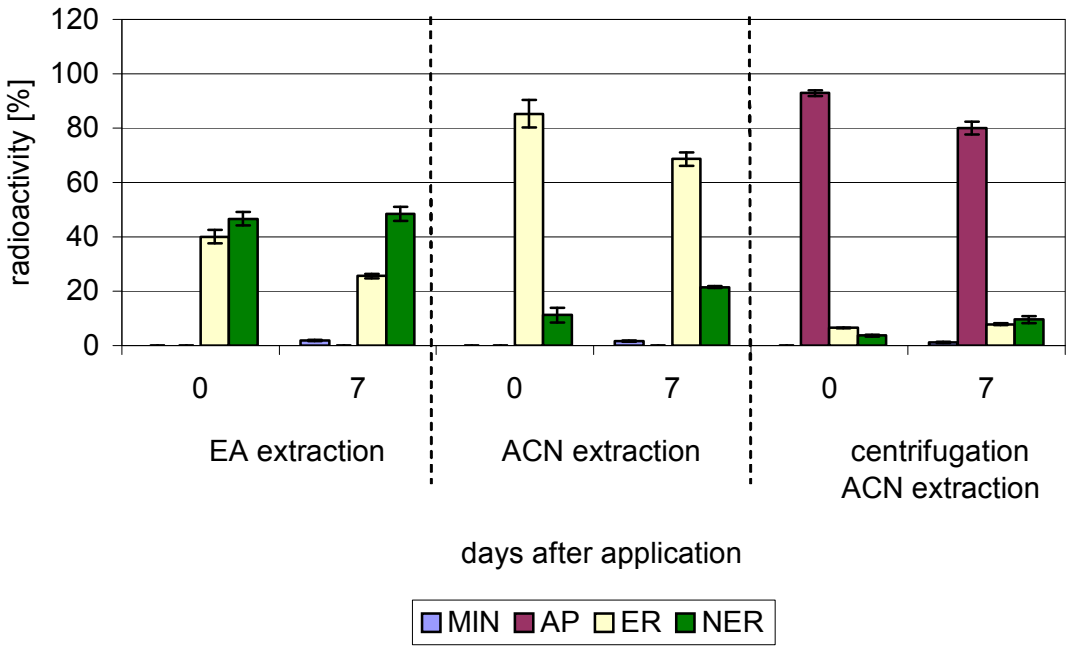
4.2.2 Extractability of cyanamide in manure and soil samples

Liquid manure samples

Due to the high water solubility of cyanamide, the extraction power of ethyl acetate was not exhaustive, neither in bovine nor in pig manure samples (**Fig. 4.4** and **4.5**). Although ethyl acetate has an intermediate polarity ($E^0_{Al_2O_3}$: 0.58), it is not miscible with water and its water solubility is only 79 g L^{-1}. Directly after the fortification of the ^{14}C-cyanamide to bovine and pig reference manures, extractable residues amounted to 40 % and 35 %, respectively. The non-extractable fractions were > 40 %. Within the 7-

day incubation period, the extractability decreased while 2 % and 6 % of ^{14}C-carbon dioxide was released by mineralization.

A higher extraction efficiency was achieved by the direct acetonitrile treatment of bovine and pig manures. In contrast to ethyl acetate, acetonitrile is more polar ($E^0_{Al_2O_3}$: 0.65) and miscible with water in every ratio. Extractables amounted to 85 % and 96 %, while non-extractables were 11 % and 4 %, respectively. Similar tendencies were found when the aqueous phases of the manure samples were first separated by centrifugation and the solids were then extracted by means of acetonitrile. Here, highest radioactivity amounts were determined in the related centrifugates reflecting again the high water solubility of cyanamide.

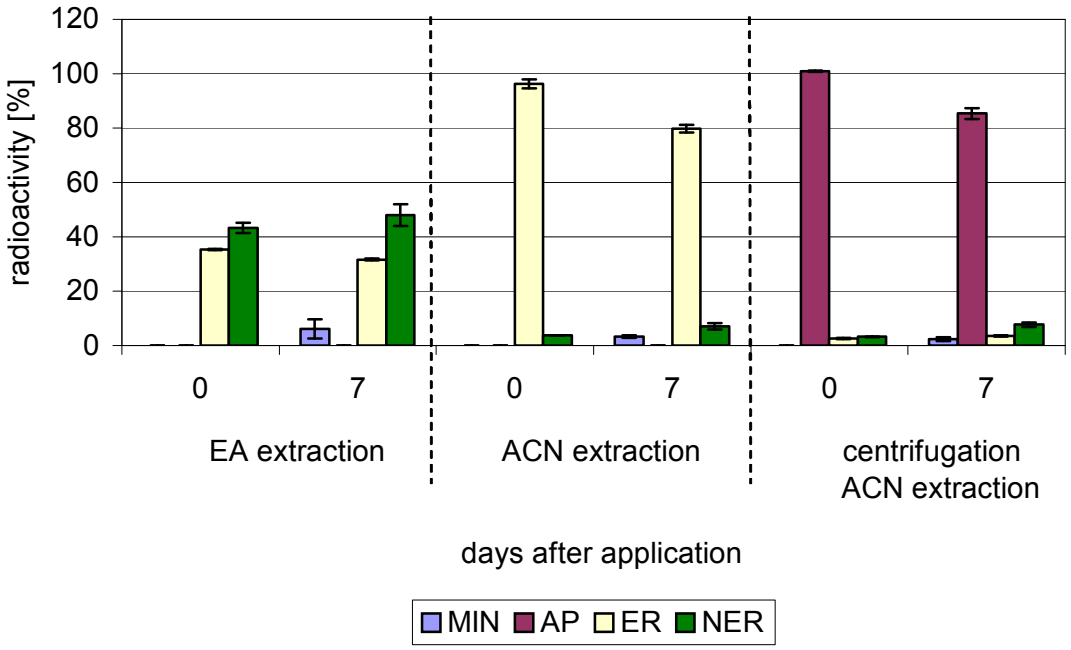


EA: ethyl acetate, **ACN:** acetonitrile, **MIN:** mineralization, **AP:** aqueous phase, **ER:** extractable residues, **NER:** non-extractable residues

Fig. 4.4: Extraction efficiency tests of ^{14}C-cyanamide in bovine manure (balances: 92 ± 10 %)

Despite higher extraction efficiencies of the latter both procedures, it has to be taken into account that, due to the unlimited miscibility of acetonitrile and water, the test sub-

stance under study and corresponding metabolites cannot be quantitatively transferred into the organic solvent. This fact is a definite disadvantage for the metabolite screening by means of radio thin layer chromatography (RTLC) or radio high performance liquid chromatography (RHPLC) when the analytes occur at low concentrations.



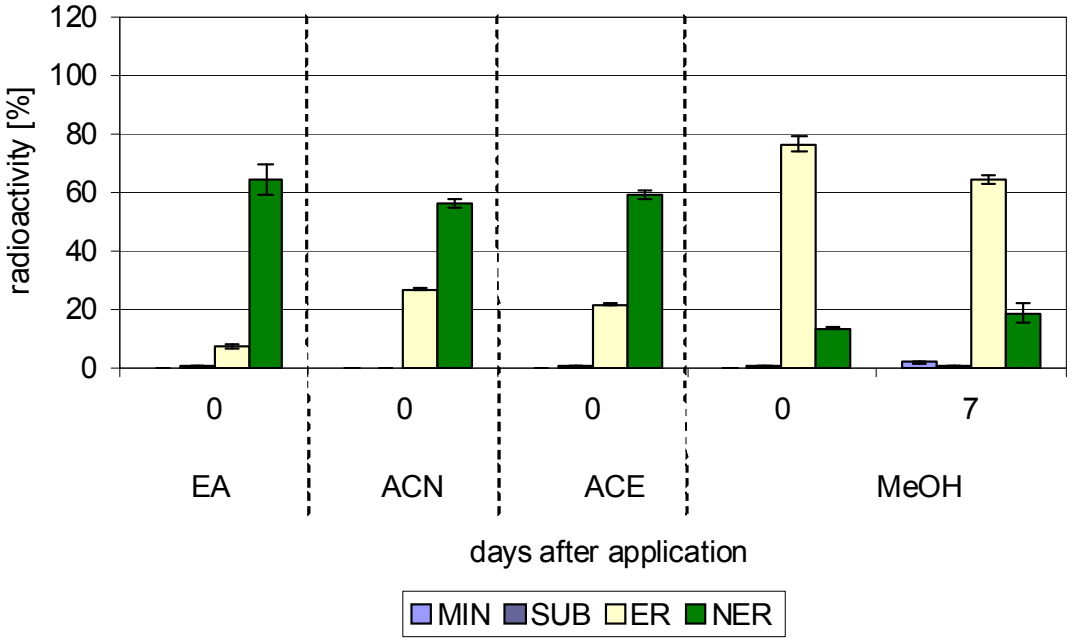
EA: ethyl acetate, **ACN:** acetonitrile, **MIN:** mineralization, **AP:** aqueous phase, **ER:** extractable residues, **NER:** non-extractable residues

Fig. 4.5: Extraction efficiency tests of ^{14}C-cyanamide in pig manure (balances: 93 ± 10 %)

The rotary evaporation process to remove the surplus solvents may become another difficulty because acetonitrile is more volatile than water and evaporates first. By this process aqueous samples are produced often affected by the precipitation of coextractants out of the solid manure matrices. Those analytical suspensions cannot be sufficiently introduced in further analytical processing.

In order to obviate those interfering difficulties, therefore, lyophilization was first applied to remove the aqueous phases of manure samples. In order to check for losses during water elimination, aliquots of the sublimates were also scintillation counted. Then, the

solvent extraction of the dried solids followed. Solvents used were ethyl acetate, acetonitrile, acetone and methanol. As shown in **Fig. 4.6**, the highest extraction efficiency was achieved by the application of methanol to release ^{14}C-cyanamide out of the bovine manure samples under study. Here, 77 % of extractable residues were accompanied by 13 % non-extractables ones. Particularly, the extraction power of ethyl acetate but, also that of acetonitrile and acetone, was definitely lower. Thus, the predominated non-extractable fractions amounted to approximately 60 %.



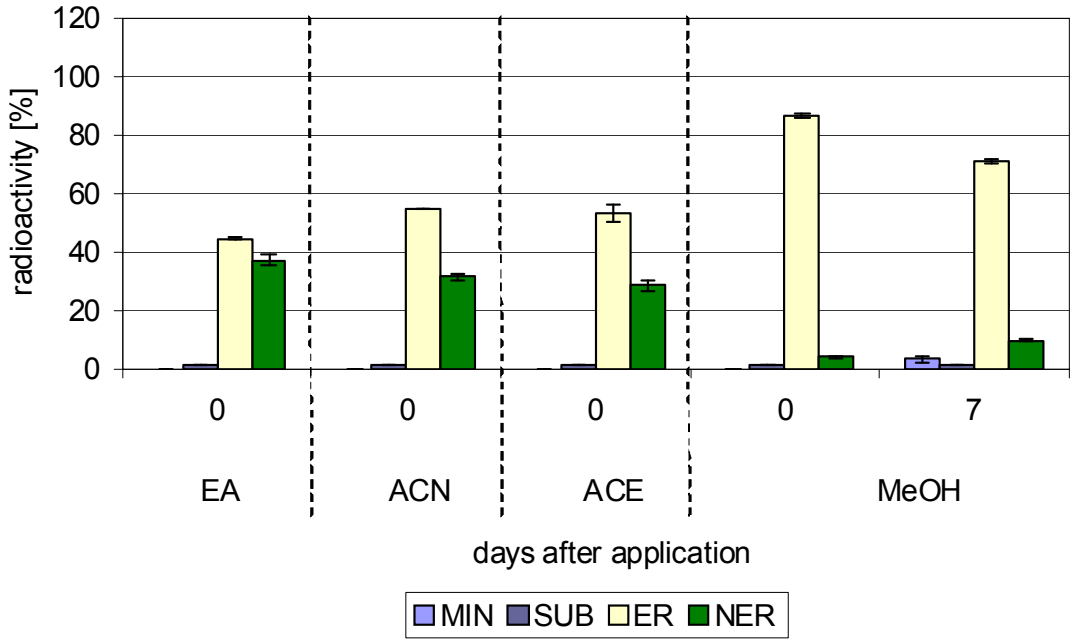
EA: ethyl acetate, **ACN:** acetonitrile, **ACE:** acetone, **MeoH:** methanol, **MIN:** mineralization, **SUB:** sublimate, **ER:** extractable residues, **NER:** non-extractable residues

Fig. 4.6: Extraction efficiency for ^{14}C-cyanamide after lyophilization in bovine manure (balances: 82 ± 7 %)

In every extraction efficiency tests in pig manures after lyophilization, highest amounts of ^{14}C-cyanamide were also extractable by methanol (**Fig. 4.7**). This fraction amounted to approximately 50 % when ethyl acetate, acetonitrile or acetone were used. Contrary to that, the extraction efficiency of methanol was 87 % emphasizing again the advantages of that extraction technique versus others, i.e., direct solvent extraction or cen-

trifugation before solvent extraction.

In addition to the experiences gathered within the Runoff Project and the Manure Project (Kreuzig et al., 2007a, b), the comparison of extraction efficiency tests for ^{14}C-imazalil and ^{14}C-cyanamide clearly showed that a test substance specific extraction technique has to be developed or adapted before running transformation tests in liquid manures.



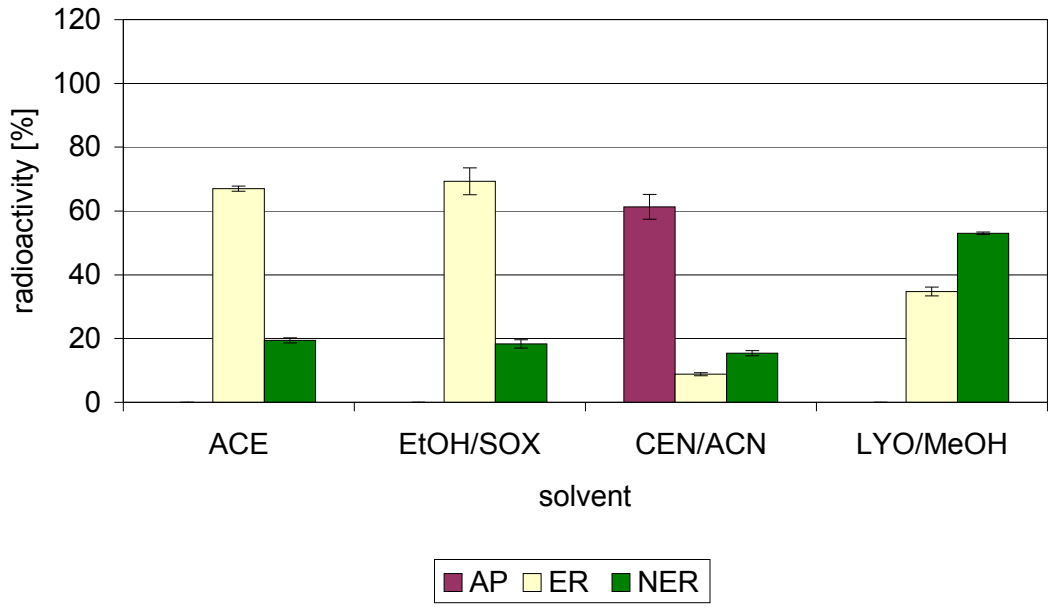
EA: ethyl acetate, **ACN:** acetonitrile, **ACE:** acetone, **MeoH:** methanol, **MIN:** mineralization, **SUB:** sublimate, **ER:** extractable residues, **NER:** non-extractable residues

Fig. 4.7: Extraction efficiency for ^{14}C-cyanamide after lyophilization in pig manure (balances: 86 ± 4 %)

Soil samples

Based on the extraction efficiency tests of ^{14}cyanamide out of bovine and pig reference-manure samples, lyophilization of the soil samples followed by methanol extraction of the solids was tested first. As shown in **Fig. 4.8**, the extractable residues amounted only to 35 % while 53 % remained non-extractable. The analysis of the sublimates did not indicate any losses of the substance. Due to the low efficiency of this procedure, further extraction techniques were tested. Thus, the soil samples under

study were water extracted with respect to the high water solubility of cyanamide. The resulting suspensions were separated by means of centrifugation and the remaining solids were extracted using acetonitrile. Here, 61 % of the radioactivity initially applied was found in the aqueous phase, 9 % was acetonitrile extractable and 15 % remained non-extractable. Besides the sufficient extraction efficiency, it had to be taken into account that the evaporation procedure and the metabolite screening were hindered by the handling of the aqueous phases and the acetonitrile extracts as described before.



ACE: acetone extraction, **EtOH/SOX:** ethanol extraction followed by soxhlet extraction using methanol, **CEN/ACN:** centrifugation followed by acetonitrile extraction of solids, **LYO/MeOH:** lyophilization followed by methanol extraction of solids; **AP:** aqueous phase, **ER:** extractable residues, **NER:** non-extractable residues

Fig. 4.8: Efficiencies of different extraction techniques for ^{14}C-cyanamide out of soil samples after standard application

Next, the soil samples were ethanol extracted on a horizontal shaker overnight followed by a methanol soxhlet extraction (2 × 8 h). In comparison to the single ethanol treatment, this sequential procedure enhanced the extractable fraction from 51 % to 69 %

and non-extractable fraction dropped from 36 % to 18 %, respectively. Hence, this procedure reached a slightly higher extraction efficiency accompanied, however, by a definitely higher analytical expenditure.
Finally, several solvents, i.e., acetone, acetonitrile, ethanol, 2-propanol, water, ethyl acetate and diethyl ether, were tested via soil sample treatment on a horizontal shaker overnight. Within these test series, highest extraction efficiency was achieved by water, disadvantages are discussed before, followed by acetone. For the latter, extractable residues amounted to 67 % while non-extractable residues were 19 %. Due to the most appropriate handling of acetone extracts, i.e., separation of solids by sedimentation and filtration and the easiness of evaporation, acetone was selected for further extraction purposes of soil samples.

4.3 Transformation tests in liquid manures

The transformation tests were conducted in the before mentioned laboratory-batch systems (**Fig. 4.1**). For this purpose, 50-g aliquots of bovine or pig manure with 10 and 5 % dry substance contents, 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 radioactivity amounted to 0.1 to 0.2 MBq. Corresponding concentrations were defined by the specific radioactivity of the radiotracers under study.
In parallel tests, the effects of the test substances and the spiking 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 at selected incubation intervals.

Mineralization

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 $\pm$ 1 °C for 0, 3, 7, 30, 72, 100 and exemplarily 177 days. All transformation tests were conducted in dupli-

cates. 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) (Wallac 1409 DSA, Perkin Elmer, Rodgau, Germany). After the potassium hydroxide exchange, the batch systems were rinsed with nitrogen for 5 min to save anaerobic conditions and incubated again.

Extractable residues

At the end of every incubation interval, the manure samples of the ^{14}C-imazalil transformation tests were directly extracted on a horizontal shaker (Type 3020; Gesellschaft für Labortechnik, Burgwedel, Germany) at 200 rpm overnight. The solvent used was ethyl acetate (150 mL) as described before. For testing ^{14}C-cyanamide, however, liquid manure samples underwent lyophilization followed by the methanol extraction of the solid materials.

Tab. 4.1: Plate materials and solvent systems for the metabolite screening by means of radio thin layer chromatography

Plate material	Solvent system
^{14}C-Imazalil	
silica gel plates 60 F_{254}	ethyl acetate/methanol (90:10) chloroform/methanol (70:30)
^{14}C-Cyanamide	
silica gel plates 60 F_{254}	ethyl acetate ethyl acetate/methanol (3:1)
reversed-phase plates 60 RP-18 F_{254}	acetonitrile/water/acetic acid (50:50:1) butanol/butanone/water/ ammonium hydroxide (4:3:2:1) benzene/ethanol (2:1)

The suspensions were decanted and filtrated. The already extracted manure matrix was threefold rinsed with 50 mL ethyl acetate or methanol. Extracts were pooled and rotary evaporated. Subsequently, 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 compounds and metabolites by means of radio thin layer chromatography (RTLC) (Tracemaster 20 Automatic TLC-linear analyzer B 284; Berthold, München, Germany). The extracts were applied onto silica gel plates (20 × 20 cm^2, 60 F_{254}). For ^{14}C-cyanamide, those silica gel plates were activated at 105 °C overnight prior to analytical processing. Additionally, reversed-phase plates (20 × 20 cm^2, 60 RP-18 F_{254}; both Merck, Darmstadt, Germany) were used. These plates were then developed in closed chambers by using appropriate solvent systems (**Tab. 4.1**).

Substance identification was performed by R_F-value comparison applying two independent chromatographic systems. The parent compounds were allocated in the reference standard solution and the samples by their radiolabels. For the metabolites, non-labeled reference chemicals were used. Due to the fluorescence indicator that covered the RTLC plates, imazalil metabolites were visualized by the substance specific fluorescence quench. Visualization of cyanamide metabolites based on dyeing by ferricyanide-nitroprusside (**Fig. 4.9**) (Milks and Janes, 1956).

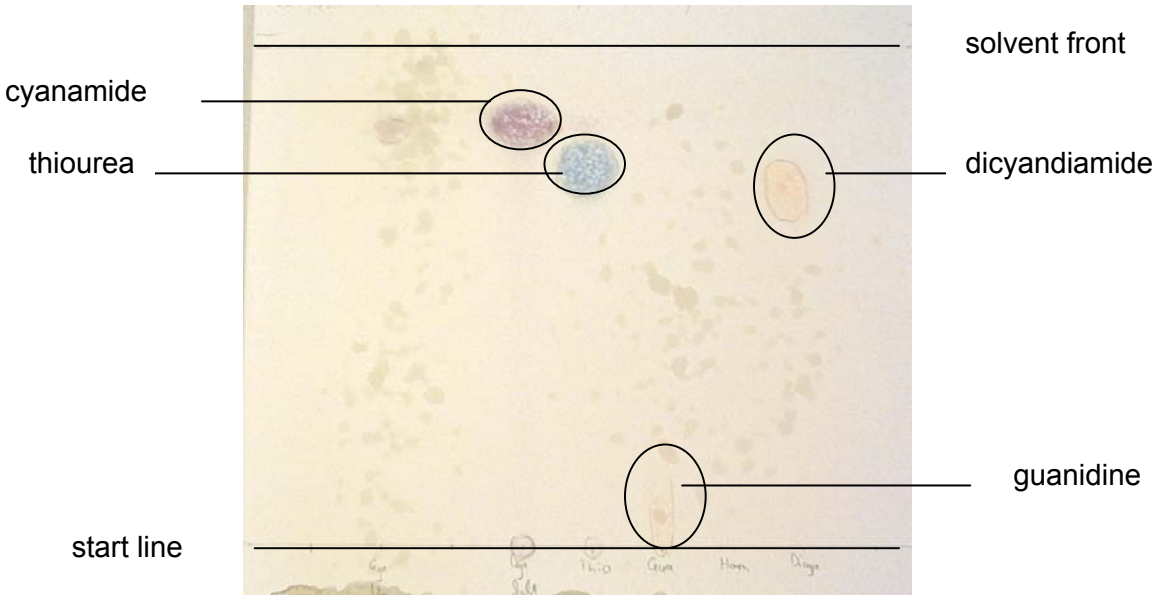


Fig. 4.9: Visualization of RTLC spots of cyanamide and transformation products by ferricyanide-nitroprusside dyeing on a silica gel plate developed using ethyl acetate/methanol (3:1)

Non-extractable residues

After extraction, the wet manure solids were transferred to evaporation dishes and mixed with a mixture of sea sand/cellulose (4:1; Roth, Karlsruhe, Germany) in order to improve the homogenization and the combustion process of the low-weight samples. Depending on the initial dry substance weights (2.5, 5, 10 %) of the liquid bovine and pig manure samples, 1.6, 3.1 and 6.3 g of this mixture were added to the extracted solids, respectively. Those mixed samples were then air dried and finally homogenized by means of a mortar mill (Typ RM-O; Retsch, Haan, Germany). Thereafter, 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. The efficiency of the oxidizer was regularly checked by the combustion of the reference standard ^{14}C-LU-111995 ((+)-exo-{2-[6-(4-fluorophenyl)-3-aza-bicyclo[3.2.0]heptane-3-yl]ethyl}-1,3H-chinazoline-2,4-doin-fumerate; Knoll, Ludwigshafen, Germany).

Modification of the test parameters

The heterogeneity and complexity of bovine and pig manures are still reflected by their dry substance contents ranging between 0.4 and 12 % (Bouwman and Reus, 1994; Burton and Turner, 2003; Lallai et al., 2002; Merkel, 2005; Møller et al., 2004; Schuchhardt and Hahne, 1996; Shah et al., 2004). Therefore, pig reference-manure samples under study were adjusted to 2.5, 5 and 10 % dry substance contents to investigate the impact of the dry substance on the transformation of both test substances during an incubation period up to 30 days.

4.4 Preparation of test manures

In order to mimic the biocide entry route into soils already under laboratory conditions, test manures were prepared. For transformation tests, 10-g reference-manure samples (fresh weight basis; matrix characterized) were filled into incubation flasks and spiked with one test substance as described for the transformation tests in manure. To study the sorption behavior, 5-g manure samples were used. In accordance to the transformation tests in liquid manures, 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 substances by means of RTLC screening.
Mixing soil with test-manure samples as described before resulted in the fourfold of the maximum manure amount accepted by the German Ordinance Concerning Fertilization (2006). The calculation of the manure amount was based 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. In order to check if sorption and transformation of ^{14}C-imazalil in manured soil were affected by the slightly raised manure amounts, test-manure samples of 2 and 1 g initial weight, respectively, were also prepared.

4.5 Transformation tests in manured soil

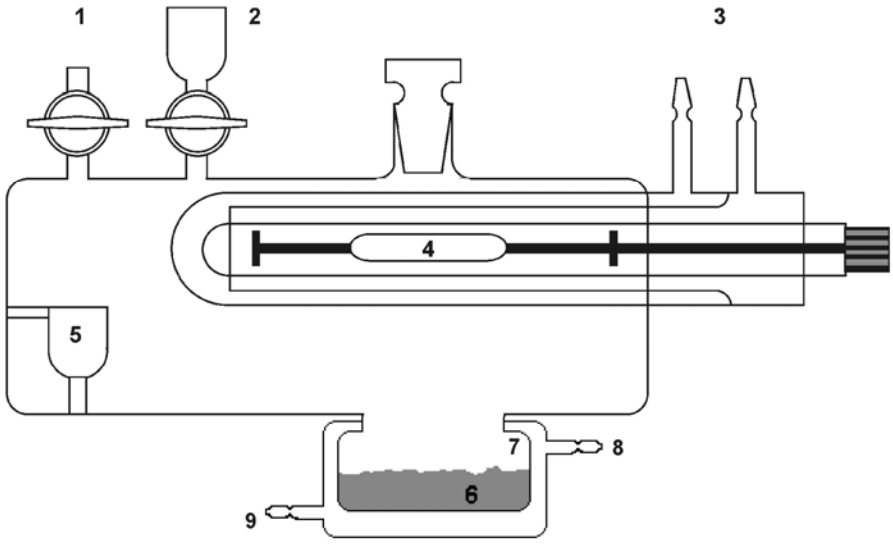
4.5.1 Soil inherent biodegradation

In addition to transformation tests in soils after standard application described in the OECD guidelines 304 A and 307 (OECD 1981a, 2002), test substances under study were introduced into the tests via test-manure application in order to simulate the real entry route already under laboratory conditions and to investigate the impact of manure matrices on the metabolic dynamics. For this purpose, 50 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 have been inevitably occurred when test-manure samples were transferred into flasks containing soil samples. Each batch series was incubated in the dark at 20 ± 1 °C for at least 28 days and up to 100 days. For quality assurance, batch experiments were performed in duplicates and mass balances were set up. In the laboratory batch-systems, potassium hydroxide solutions and air were simultaneously exchanged every 3 to 4 days to conserve aerobic conditions.
At the end of each incubation interval, soil samples containing ^{14}C-imazalil or ^{14}C-cyanamide residues were exhaustively extracted on a horizontal shaker (150 rpm) overnight. The solvents (100 mL) used were ethyl acetate and acetone, respectively. Thereafter, the supernatants were decanted and filtrated. The extracted soil samples were then rinsed three times using the same solvent (2 x 50 mL, 1 x 30 mL).

The extracts were pooled and scintillation counted to determine the radioactivity amounts of the extractable fractions. Those were subsequently rotary evaporated and metabolite screened by means of radio thin layer chromatography as already described for liquid manure samples. The already extracted soil samples were air dried and finally homogenized by means of a mortar mill (Typ RM-O; Retsch, Haan, Germany). Thereafter, 10 mg cellulose were added to 150-mg aliquots that 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.

4.5.2 Photoinduced biodegradation

For pesticides, it is well-known that the inherent biodegradation may be interfered by photoinduced effects on soil surfaces. This was clearly shown for the azole fungicide prochloraz studied under laboratory and field conditions. Thus, the faster disappearance of the parent compound under field conditions was understood when laboratory tests were performed by means of a special irradiation apparatus (Höllrigl-Rosta et al., 1999). After further technical development (Kreuzig et al., 2003, Kreuzig and Höltge, 2005), this laboratory-test system was used to monitor photoinduced effects on the transformation of ^{14}C-imazalil that also belongs to the group of fungicidal imidazole derivatives (**Fig. 4.10**). For this purpose, ^{14}C-imazalil was spiked via standard and test-manure application onto soil surfaces. All batches were incubated at 20 $\pm$ 2 °C with 10-hours light/14-hours dark periods per day. The incubation period was limited to 3 days considering that soil management should contemporary follow manure application. After incorporation into soil, photoinduced effects can be excluded due to the low light penetration of soil. Sample preparation and analysis were conducted as described above.



1: inlet valve, **2:** outlet valve with activated charcoal filter, **3:** Pyrex® water-cooling device, **4:** medium-pressure mercury lamp (cut off: λ = 290 nm), **5:** ^{14}C-carbon dioxide trap (8 mL of 0.1 M potassium hydroxide solution), **6:** soil sample, **7:** exchangeable sample container, **8,9:** water-cooling device

Fig. 4.10: Irradiation apparatus for batch experiments on photoinduced tranformation of biocides in soil

4.6 Sorption tests in manured soils

In addition to sorption tests in soils after standard application (OECD 1981b, 2000), sorption tests after test-manure application were conducted. For this purpose, the 5-g, exemplarily 1-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 1-mL aliquots were added to 10 mL of Quicksafe A (Zinsser, Frankfurt, Germany) and liquid scintillation counted.

Tab. 4.2: 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

On the basis of those batch-equilibrium tests, the soil/water distribution coefficients were calculated and finally normalized to the organic carbon contents. The assessment of mobility tendencies, on the one hand, was based on the trigger value defined by Fichter and Holden (1992). Thus, values < 5 L kg^{-1} indicated an enhanced mobility and leaching tendencies in soil. On the other hand, the mobility classification of pesticides defined by Hollis (1991) was used (**Tab. 4.2**).

5 Transformation of biocides in reference manures

Within the Runoff Project (Kreuzig et al., 2007a), test-plot experiments were performed to study the surface runoff of veterinary medicinal products (VMP) after test-manure application and sprinkler irrigation. This approach, furthermore, facilitated the investigation of fate and behavior of the applied test substances in liquid manures and manured soil under field conditions. In order to improve the understanding of the complex concentration determining processes in liquid manures and soils, those studies were lined by concerted laboratory testing. For this purpose, liquid bovine and pig manure samples were taken from channels in animal houses and from manure tanks. The test substances, namely sulfadiazine, sulfadimidine, sulfamethoxazole, acetyl-sulfamethoxazole, flubendazole and fenbendazole, were applied as ^{14}C-labeled radiotracers. Nevertheless, it was obvious that the properties of those manure samples were highly heterogeneous and variable. Within the Manure Project, therefore, a technical protocol for laboratory testing on transformation of VMP in liquid bovine and pig manures was developed (Kreuzig et al., 2007b). Here, an innovative approach was pursued starting with taking excrement samples of individually kept cattle and pigs in experimental stables and followed by the preparation of reference manures of defined dry substance contents. The transformation tests on sulfamethoxazole, acetyl-sulfamethoxazole, sulfadiazine, erythromycin, ketoprofen and paracetamol, every test substance was applied again as a ^{14}C-labeled radiotracer, supplemented by thorough matrix characterization tests, definitely confirmed that, following this concept, reference manures can be applied for reproducible laboratory testing.

Currently, an ultimate anaerobic biodegradation test in digested sewage sludge (e.g., ISO 11734, 1995; OECD 311, 2006) is proposed to evaluate the biodegradation of biocides in manures based on the Technical Notes for Guidance on Data Requirements, specifying the Directive 98/8/EC (European Commission, 1998). However, the recommended method for digested sludge is not appropriate to reflect biodegradation in manure matrices. Within the Biocide Project, therefore, the reference-manure concept was consequently advanced to allow an adequate investigation of biocides degradation in manures by a higher tiered simulation test and to substitute the recommended screening test in digested sludge. For this purpose, ^{14}C-imazalil and ^{14}C-cyanamide were tested as examples for biocides used for the disinfection and insect control in animal houses or liquid manures.

5.1 Transformation of imazalil in bovine and pig manures

The fate of ^{14}C-imazalil was monitored in bovine and pig reference manures up to 177 days simulating the long-term manure storage up to 6 month. In bovine manure, the extractable residues decreased very slowly from 98 % to 90 % of the radioactivity initially applied (**Fig. 5.1**). That corresponded to decreasing concentrations of the unchanged parent compound from 43.4 to 40.1 mg kg^{-1} dry bovine manure (4.3 to 4.0 mg kg^{-1} fresh bovine manure). The metabolite screening did not offer any indication about the occurrence of any ^{14}C-imazalil metabolite. Hence, the parent compound did not undergo a relevant transformation revealed by a DT_{50} value > 177 days (according to ModelMaker, 2007). Simultaneously, the non-extractable residues, operationally defined by means of the ethyl acetate extraction, slowly increased from 2 % to 15 %. Within the 177-day incubation period, mineralization was of minor relevance. As indicated by a ^{14}C-carbon dioxide release of only 0.1 % and by average balances of 101 ± 4 %, a release of ^{14}C-methane out of the ^{14}C-imazalil could be disregarded.

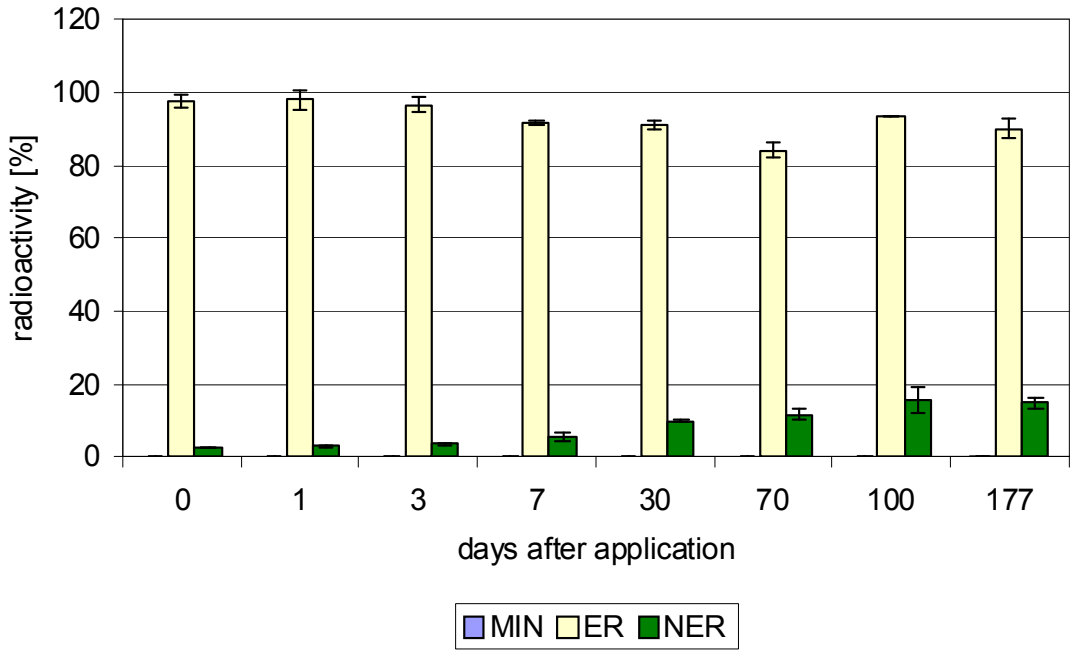


Fig. 5.1: Transformation of ^{14}C-imazalil in bovine reference manure (balances: 101 ± 4 %)

In parallel batch experiments, in which the non-labeled test substance was applied,

selected matrix parameters were determined at the incubation intervals of 0, 30 and 100 days. Dissolved oxygen contents of < 0.1 mg kg^{-1} and redox potentials Eh < -120 mV confirmed strictly anaerobic conditions during this transformation test series. pH values slightly increased from 6.7 to 7.3. The biological oxygen demand dropped from 8.5 g kg^{-1} at the beginning of the incubation period to 0.6 g kg^{-1} after 100 days, indicating the decrease of the activity of aerobic microorganisms. However, the weak dynamics of extractable and non-extractable residues continued. These results showed that the validity of the biological oxygen demand in anaerobic manure samples may be limited. Certainly, there is not any alternative method without any interference. The determination of the dehydrogenase activity, feasible to determine the activities of aerobic and anaerobic microorganisms, is limited by its final photometric measurement of triphenyl formazan at λ = 485 nm or λ = 546 nm because of the deeply colored manure extracts (DIN-ISO-23753-1, 2005). The application of a readily degradable reference substance, e.g., sodium benzoate, in parallel batch experiments causes other inadequacies. In order to check the microbial activity of manure samples at the start of the transformation test series, this test is too time consuming due to its 4-week test period specified by the OECD guideline 311 (OECD, 2006). Due to the different experimental design, the degradability of this test substance is only measured by the gas production, this test is not appropriate to check the microbial activity at longer incubation intervals. So far the application of an external standard substance should be followed in the future, there is the need to identify an appropriate ^{14}C-labeled reference substance that shows a characteristic behavior in bovine and pig manure within incubation intervals up to 100 or 177 days.

A slightly different situation was found in pig reference manure (**Fig. 5.2**). Within 7 days of incubation, the amount of extractable residues remained nearly constant at 96 % of the radioactivity initially applied. This amount subsequently dropped to 77 % after 177 days. That corresponded to decreasing concentrations of the unchanged parent compound from 90.3 to 72.2 mg kg^{-1} dry pig manure (4.5 to 3.6 mg kg^{-1} fresh pig manure). Nevertheless, the DT_{50} value was > 177 days. In these extracts, the RTLC screening did not indicate any metabolite. In accordance to the decrease of the extractable residues, the non-extractable fractions successively increased from 1 % to 21 %. Mineralization was only 0.1 %. Taking average mass balances of 100 ± 5 % into account, a release of ^{14}C-methane could be disregarded.
The parallel matrix characterization tests also showed strictly anaerobic conditions.

Dissolved oxygen contents were < 0.1 mg kg^{-1} and redox potentials Eh < -120 mV. pH values increased from 6.4 to 7.7. In contrast to the bovine manure samples, the biological oxygen demand increased from 7.3 g kg^{-1} to finally 9.4 g kg^{-1}.
The differences of these transformation tests in bovine and pig reference manures clearly showed that a new biocide has to be tested in both manure matrices for regulatory purposes when it is applicable in cattle and pig stables.

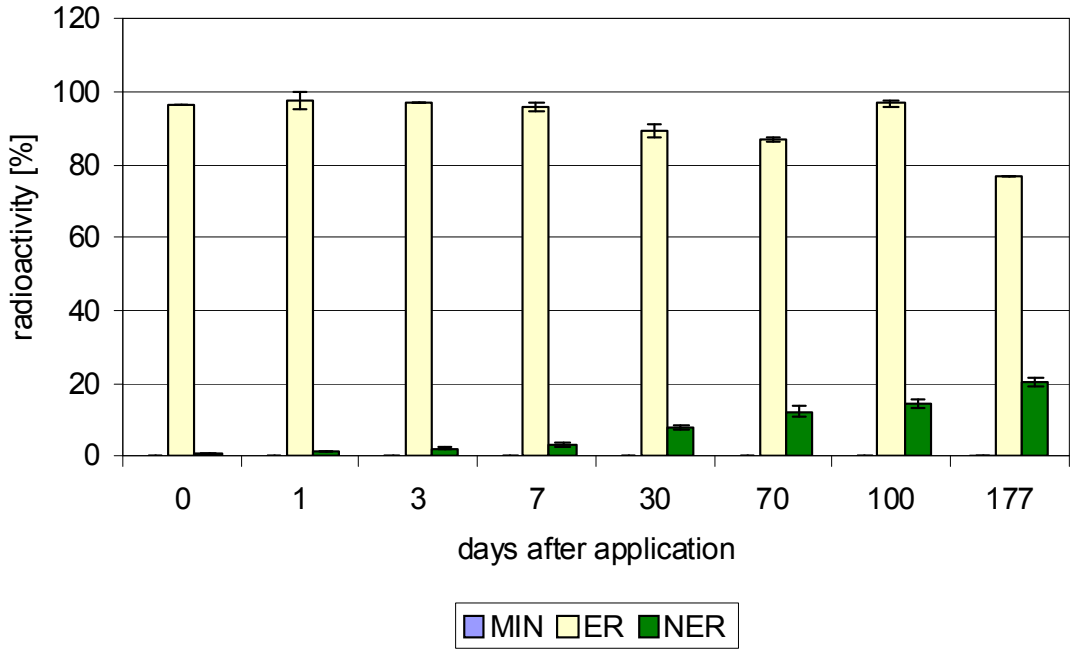


Fig. 5.2: Transformation of ^{14}C-imazalil in pig reference manure (balances: 100 ± 5 %)

As shown by the long-term survey of LUFA Nord-West, Oldenburg, Germany (Merkel, 2005), dry substance contents of pig tank manures may vary between 0.4 to 11.6 %. Similar dry substance contents were found within further studies conducted in Europe and USA (Bouwman and Reus, 1994, Moral et al., 2005, Burton et al., 2003, Ndegwa et al., 2003). Therefore, the transformation of ^{14}C-imazalil in dependency on the dry substance contents of 2.5, 5 and 10 % were additionally studied in 100-day incubation tests. The results are illustrated in **Fig. 5.3**. Due to the weak metabolic dynamics of ^{14}C-imazalil, nearly the same intensity patterns of extractable and non-extractable residues were detected in every test series. The extractable residues slightly decreased within 30 days and then dropped to approximately 80 % of the radioactivity initially ap-

plied. The non-extractables behaved vice versa achieving approximately 20 % in the 100-day samples. Mineralization was of minor relevance.

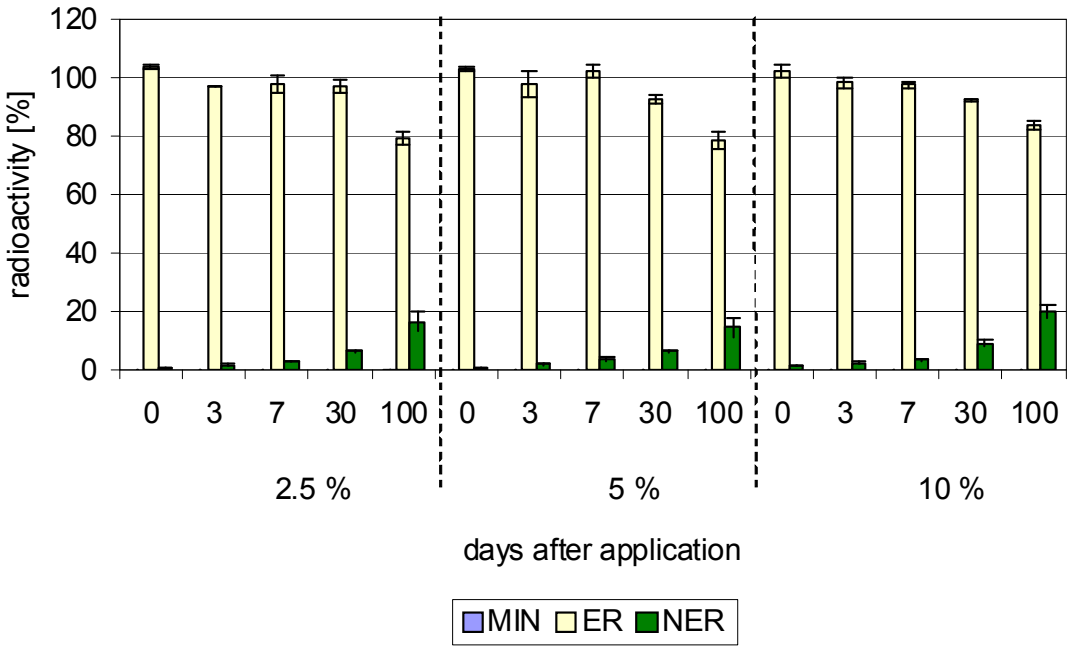


Fig. 5.3: Transformation of ^{14}C-imazalil in pig reference manure at different dry substance contents and at 20 ± 1 °C (balances: 101 ± 3%)

5.2 Transformation of cyanamide in bovine and pig manures

In contrast to ^{14}C-imazalil, the handling of ^{14}C-cyanamide turned out to be more challenging. First, cyanamide itself is a highly reactive molecule with two functional groups. Second, cyanamide shows a different extraction behavior in manure and soil samples. Therefore, different extraction techniques for both matrices had to be checked for extraction efficiency. Third, the ^{14}C-labeling focused on the low-molecular weight compound resulting in a high specific radioactivity of 1850 MBq mmol^{-1} making this radiotracer sensitive for autoradiolysis. Fourth, this process may form the same transformation products as formed by biodegradation in manures and soils. Fifth, every transformation product, 16 ones are reported by Milks and Janes (1956), is of low-molecular weight and similar polarity. Thus, it was demanding to adapt two radio thin layer chromatographic systems of nearly equivalent separation performance.

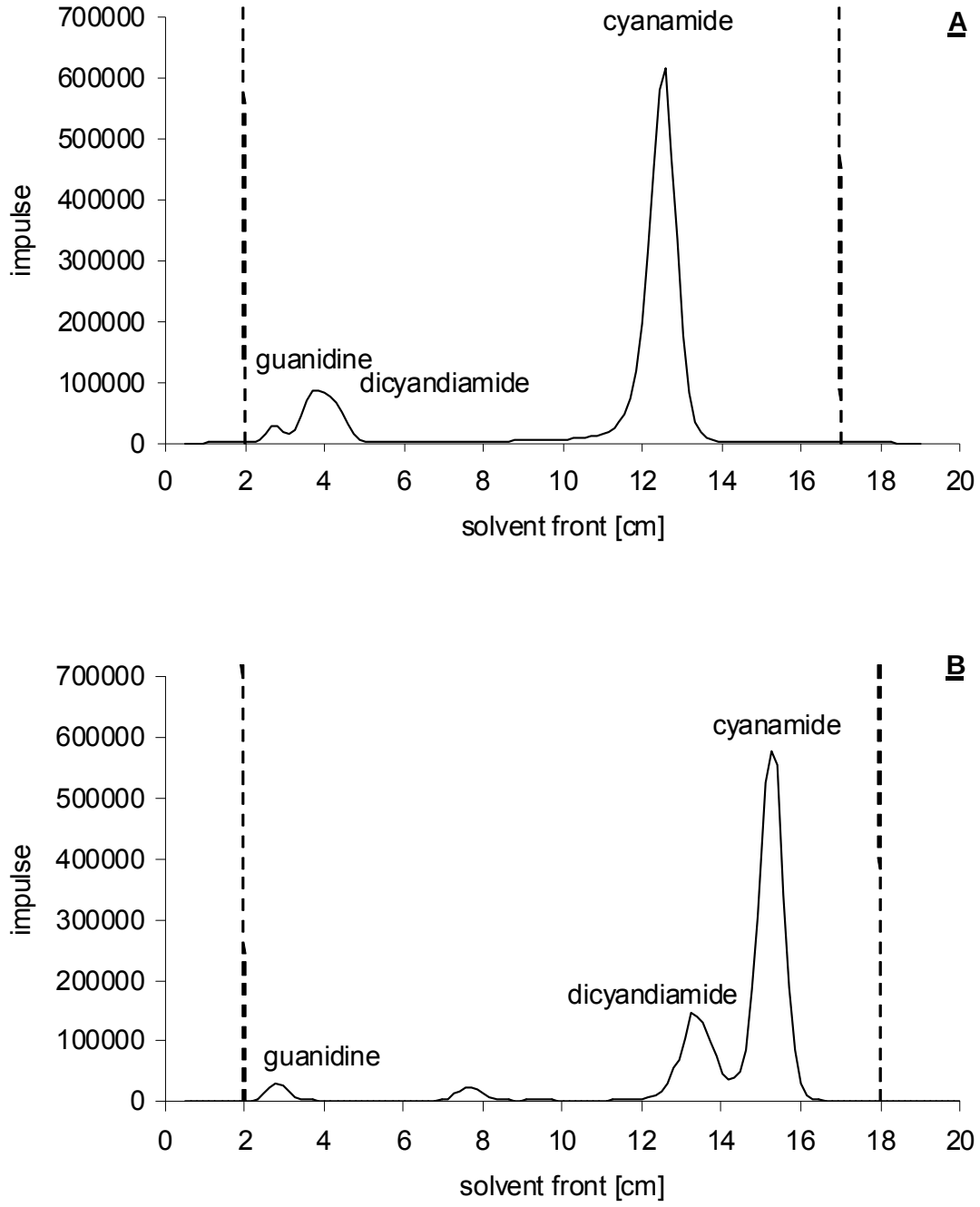


Fig. 5.4: Performance of two radio thin layer chromatographic systems for analyzing the stock-standard solution of ^{14}C-cyanamide dissolved in ethanol. Silica gel plates developed using ethyl acetate (A) and ethyl acetate/methanol (3:1) (B)

In order to obviate the latter inadequacies, normal and reversed-phase thin layer plates combined with numerous solvents or solvent systems were tested. The two most sufficient chromatographic systems were illustrated in **Fig. 5.4**. The corresponding R_f values are listed in **Tab. 5.1**.

Tab. 5.1: R_F values determined by two radio thin layer chromatographic systems for analyzing the stock-standard solution of ^{14}C-cyanamide dissolved in ethanol. Silica gel plates developed using A: ethyl acetate and B: ethyl acetate/methanol (3:1)

Analyte	guanidine	dicyandiamide	cyanamide
A: R_f value	0.02	0.10	0.70
B: R_f value	0.06	0.74	0.85

The analysis of extracts from the transformation tests clearly showed the higher separation performance of ethyl acetate/methanol. Therefore, this solvent system was used for the quantitative determination of the unchanged ^{14}C-cyanamide in order to calculate the respective DT_{50} values.

Within the transformation test in bovine reference manure, ^{14}C-cyanamide underwent dynamic transformation processes (**Fig. 5.5**). In contrast to ^{14}C-imazalil, relevant amounts of the initially applied radioactivity were transformed to ^{14}C-carbon dioxide. Thus, mineralization rose from 0.5 % to 16 % within the 100-day incubation period. Simultaneously, the amounts of extractable residues continuously dropped from 94 % to 30 %. As shown by the RTLC screening, ^{14}C-cyanamide amounts decreased from 73 % to 13 % of the extractable fraction. That corresponded to decreasing concentrations of the unchanged parent compound from 331 to 18 µg kg^{-1} dry bovine manure (33 to 2 µg kg^{-1} fresh manure) resulting in a DT_{50} value of 2 days. Together with 2 unknown metabolites, guanidine and dicyandiamide could also be detected. Since the latter both compounds have already occurred in the initially applied ^{14}C-cyanamide reference standard, reducing the radiochemical purity to approximately 80 %, and their amounts remained nearly constant at the initial values during the incubation period, their contri-

bution to the metabolic fate of ^{14}C-cyanamide could not be definitely shown. The non-extractable residues, operationally defined by lyophilization and subsequent methanol extraction of the solids, increased from 9 % to 33 % within 3 days and then remained nearly constant until the end of the incubation period.

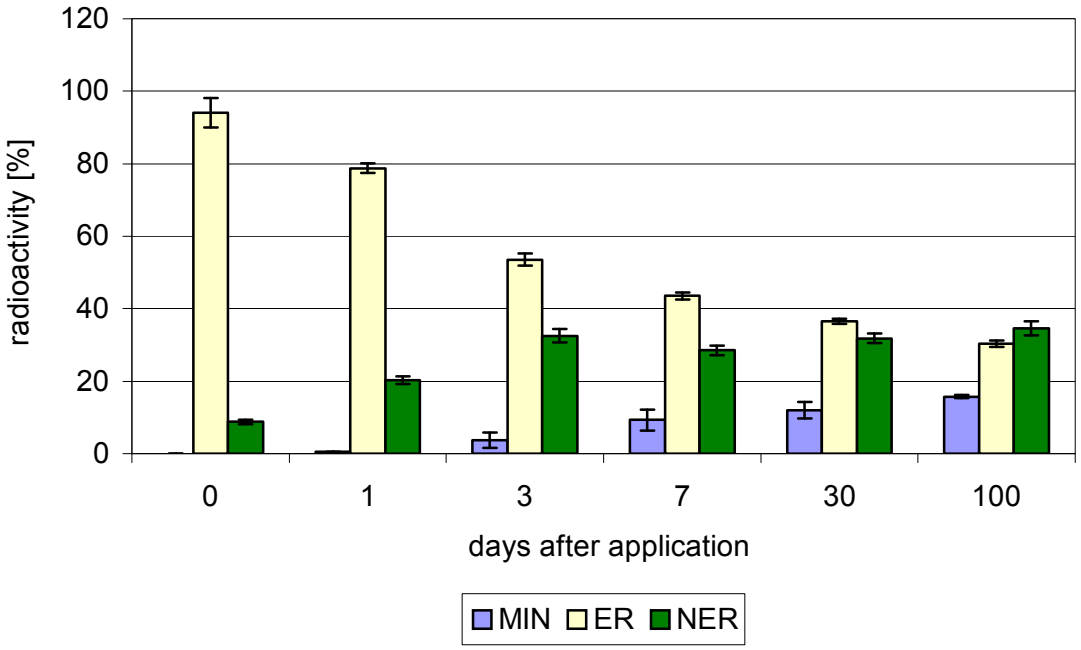


Fig. 5.5: Transformation of ^{14}C-cyanamide in bovine reference manure (balances: 89 ± 10 %)

Studying the mass balances, the decrease of 103 % to 81 % within the 100-day incubation period was obvious. As shown by the corresponding matrix characterization tests, the redox potential ranged between -200 mV to -120 mV indicating strictly anaerobic to methanogenic conditions. Particularly considering the relevant formation of ^{14}C-carbon dioxide, the losses within the mass balances was presumably attributed to the formation of ^{14}C-methane out of the parent compound initially applied.

In pig reference manure, slightly different metabolic dynamics of ^{14}C-cyanamide were found (**Fig. 5.6**). The mineralization increased more slowly and 16 % were still reached at the end of the 175-day incubation period. The extractable residues simultaneously decreased from 82 % to 51 %. Those fractions contained ^{14}C-cyanamide that dropped from 53 % to 11 % of the extractable fraction within 15 days. Thereafter, the amount of

the parent compound remained nearly constant. The cyanamide concentration thus decreased from 387 to 77 µg kg^{-1} dry pig manure (19 to 4 µg kg^{-1} fresh manure) resulting in a DT_{50} value of 4 days. Metabolites were also detected. However, the quantitation was interfered by matrix effects. The non-extractable residues, operationally defined by lyophilization and subsequent methanol extraction of the solids, increased from 7 % to 18 %. A final decrease of the mass balance to 85 % also indicated a release of ^{14}C-methane.

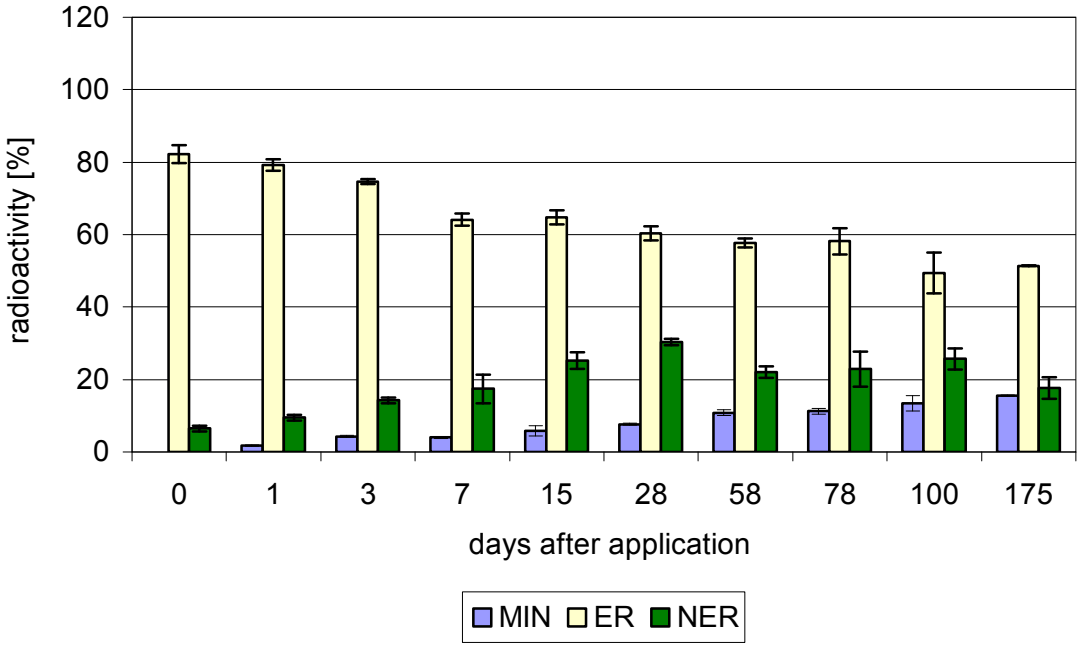


Fig. 5.6: Transformation of ^{14}C-cyanamide in pig reference manure (balances: 91 ± 4 %)

Considering the wide range of dry substance contents in pig tank manures, the transformation tests on ^{14}C-cyanamide were also carried out in reference-manure samples adjusted to 2.5, 5, and 10 %. Results are illustrated in **Fig. 5.7**. The comparison of the results of different test series on different test substances, i.e., cyanamide, imazalil, sulfamethoxazole and erythromycin, the two latter were studied within the Manure Project (Kreuzig et al., 2007b), clearly showed that a definite dependency of fate and behavior of VMP and biocides on the dry substance contents of the manures under study was overlayed by test substance and manure matrix specific properties. At the beginning of the incubation tests, non-extractable sulfamethoxazole and erythromycin resi-

dues were preferably formed at higher dry substance contents of the manure samples. However, this effect was compensated by time. In contrast, the behavior of imazalil did not considerably differ at different boundary conditions. For cyanamide, however, the most pronounced metabolic dynamics were found at 2.5 % dry substance content. Here, highest mineralization and formation of non-extractable residues were found that amounted to 28 and 33 %, respectively, while extractable residues amounted to 40 % in the 100-day samples. These intensity patterns were dominated by the extractable fractions when the reference-manure samples were adjusted to 5 % and 10 % dry substance content. In this sequence, mineralization and non-extractable residues decreased.

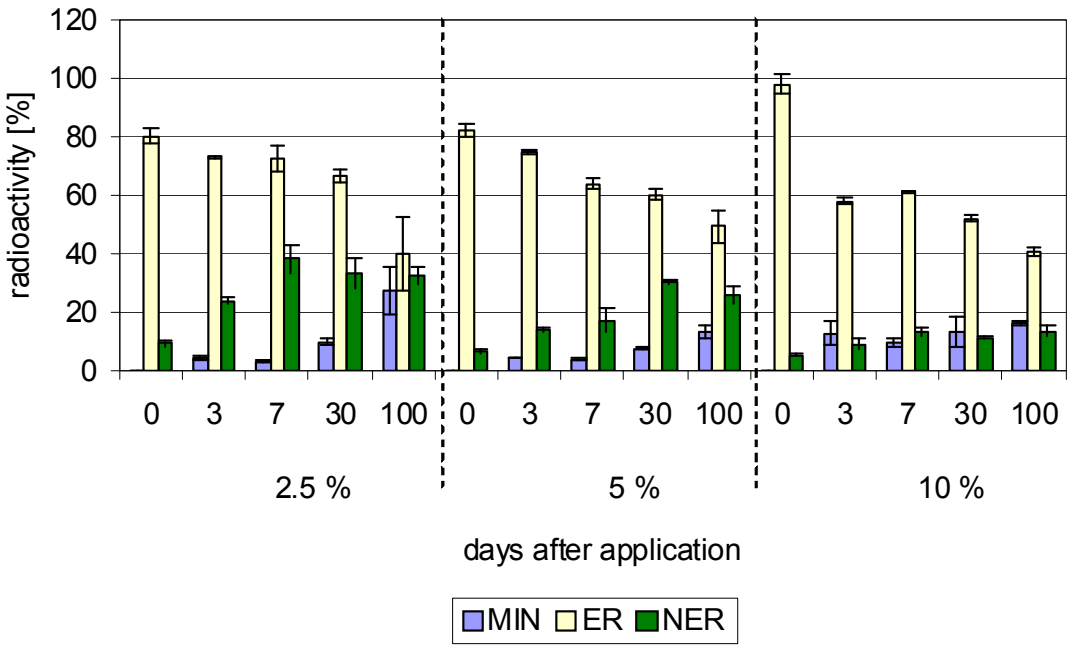


Fig. 5.7: Transformation tests of ^{14}C-cyanamide in pig manure at different dry substance contents and at 20 ± 1 °C (balances: 92 ± 12%)

These tests definitely confirmed the concept of using reference-manure samples adjusted to defined dry substance contents for reproducible laboratory testing. That cannot be guaranteed when tank manure samples were applied with dry substance contents ranging from 0.4 % to 12 % that additionally differ in composition, microbial activity and possible contamination by VMP and biocides.

6. Fate monitoring of biocides in soil

The laboratory and field studies already carried out in the Runoff Project clearly showed that fate and behavior of VMP in manured soils may be considerably affected by the manure application. Within the Manure Project, this fact was confirmed by fate monitoring on ketoprofen, paracetamol and erythromycin after standard and test-manure application. Therefore, sorption and transformation tests on the biocides imazalil and cyanamide were conducted, too. Due to their use as a fungicidal pesticide and a nitrogen fertilizer, respectively, background information about sorption and transformation in soil was available (cf. chapter 3). However, the entry route of biocides via manure application has not been investigated.

6.1 Sorption and transformation of imazalil in manured soil

6.1.1 Mobility in soil

In order to assess mobility tendencies in soil, soil/water distribution coefficients were determined after standard and test-manure application. The K_d and K_{OC} values determined and calculated, respectively, are listed in **Tab. 6.1**.

Tab. 6.1: Sorption of ^{14}C-imazalil in silty clay soil after standard and test-manure application

Application technique	OC [%]	K_d [L kg^{-1}]	K_{OC} [L kg^{-1}]
Standard	1.7	69	4059
Test manure (bovine, 5 g)	2.7	50	1852
Test manure (pig, 5 g)	2.6	36	1385

OC: organic carbon content in soil

The K_d and K_{OC} values of 69 L kg^{-1} and 4059 L kg^{-1} revealed a strong sorption to the silty clay soil under study when ^{14}C-imazalil was applied via standard application. After

the application of bovine and pig test-manures, according to the hitherto concept, 25 g soil were mixed with 5 g test-manure, K_{OC} values dropped to 1852 L kg^{-1} and 1385 L kg^{-1}. Although the manure matrices lowered those values, the mobility tendencies did not considerably change. According to Hollis (1991), K_{OC} values ranging from 500 to 4000 L kg^{-1} are classified as slightly mobile. Hence, a risk of groundwater pollution could not be derived from these values, neither after standard nor after test-manure application.

When the test-manure amount was reduced to the maximum acceptable by the German Ordinance Concerning Fertilization (2006), i.e., 25 g soil were mixed with 1 g test manure, an intermediate K_{OC} value of 3190 L kg^{-1} was calculated. That emphasized two aspects: When exaggerated test-manure amounts are applied, the manure effect on mobility may be overestimated. Nevertheless, impacts of manure should not be disregarded when sorption of VMP and biocides should be assessed for manured soils by laboratory testing.

6.1.2 Transformation after standard application

In order to determine the principal tendencies of the biodegradation of ^{14}C-imazalil in the silty clay soil under study, the ^{14}C-labeled test substance was dissolved in ethanol and spiked to the microbially active soil samples. The results are illustrated in **Fig. 6.1**. Within the 100-day incubation period, the extractable residues successively dropped from 102 % to 47 %. As clearly shown by means of the RTLC screening, only the unchanged parent compound imazalil occurred in the extracts of the first 14 days. Thereafter, the *ortho*-dealkylated transformation product of imazalil namely 1-[2-(2,4-dichlorophenyl)-2-hydroxyethyl]-1*H*-imidazole, already identified by Van Leemput et al. (1985), was also found. After 100 days, that amounted to 8 % of the extractable fraction, corresponding to 4 % of initially applied radioactivity. The concentration of imazalil decreased from 4.7 to 1.9 mg kg^{-1} soil resulting in a DT_{50} value of 83 days. For this calculation, a first order kinetic was considered (ModelMaker, 2007). Simultaneously to the decrease of extractable fractions, the non-extractable residues, operationally defined by the ethyl acetate extraction, increased from 2 % to 36 %. After 7 days, the release of ^{14}C-carbon dioxide amounted to 0.1 % increasing to 10 % after 100 days. Taking these results into account, imazalil did not fulfill the persistence criteria (Annex I exclusion

criteria) for biocides and pesticides that were defined by mineralization < 5 % and formation of non-extractable residues > 70 % within 100 days under laboratory conditions (Biocides: TNsG for Annex I inclusion, 2000; Pesticides: 91/414/EEC Annex VI, 1998, BBA, 1998).

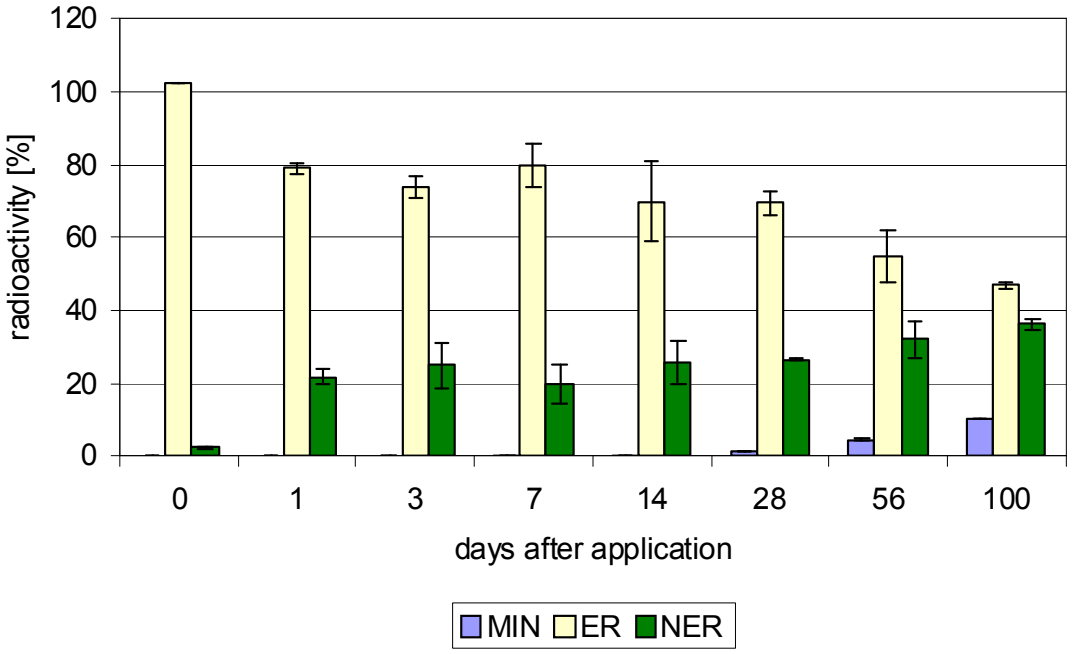


Fig. 6.1: Transformation of ^{14}C-imazalil in silty clay soil after standard application (balances: 98 ± 4%)

In addition to those batch experiments, matrix characterization tests were performed to analyze the boundary test conditions after 0, 28 and 100 days. During this period, the pH values slightly decreased from 7.5 to 6.9. Contrary to this, the redox potentials (Eh > 510 mV) and the substrate-induced respiration (> 2.7 mg O_2 100 g^{-1} ds h^{-1}) remained nearly constant indicating that the transformation tests were carried out under permanently aerobic conditions with microbially active soil samples.

6.1.3 Transformation after test-manure application

Bovine test manure

In order to simulate the manure application with short-term aged biocide residues already under laboratory conditions, bovine and pig reference-manure samples were spiked with ^{14}C-imazalil and incubated for 7 days. As shown by the transformation tests in bovine and pig manure (cf. chapter 5) and by parallel batch tests, those test-manure samples contained extractable residues with exclusively imazalil as unchanged parent compound. Those test-manure samples were then mixed with soil samples and incubated up to 100 days.

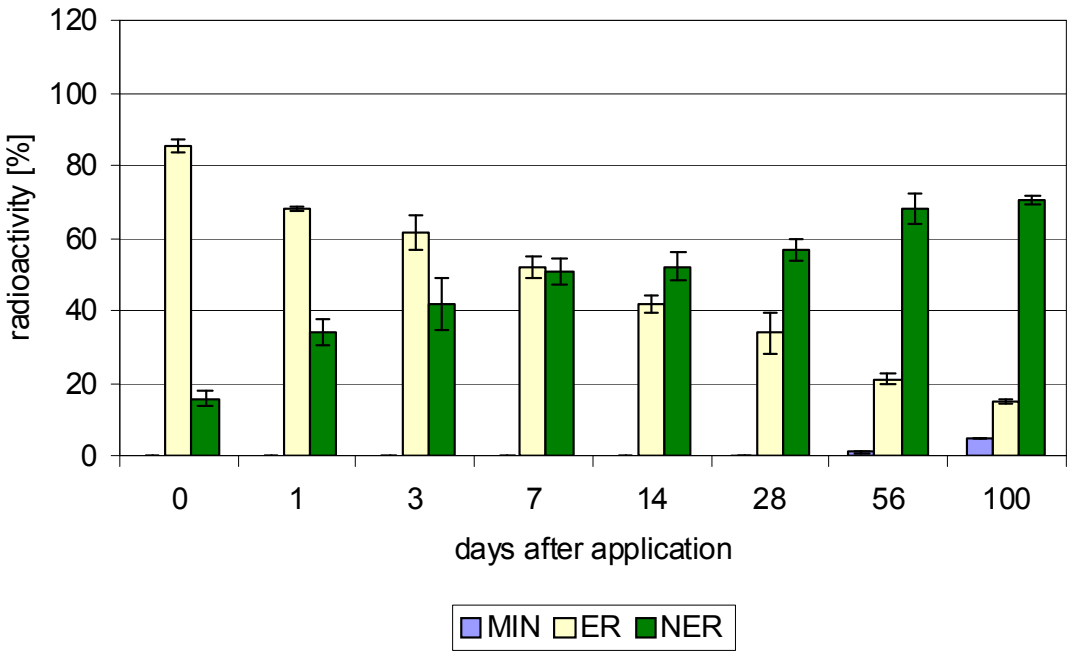


Fig. 6.2: Transformation of ^{14}C-imazalil in silty clay soil (45% of WHC_{max}) after bovine test-manure application (balances: 97 ± 6 %)

The results of the first test series with bovine test-manure application are illustrated in **Fig. 6.2**. Here, the soil samples were initially adjusted to 45 % of the maximum water holding capacity (WHC_{max}), before the test manure was applied. In contrast to the batch experiments after standard application, a continuous decrease of extractable residues from 85 % to 15 % was found. Until 14 days after test-manure application, only the

parent compound could be determined by RTLC analysis. Thereafter, the *ortho*-dealkylated transformation product also occurred. After 100 days, that amounted to 14 % of the extractable fraction, corresponding to 2 % of the initially applied radioactivity. Considering decreasing concentrations of imazalil from 3.8 to 0.5 mg kg^{-1} soil following a first order kinetic again, DT_{50} was 21 days. However, the non-extractable residues are formed more rapidly. Those increased from 16 % to 71 %. ^{14}C-carbon dioxide was released first after 56 days at an amount of only 1 % that finally increased to 5 %.

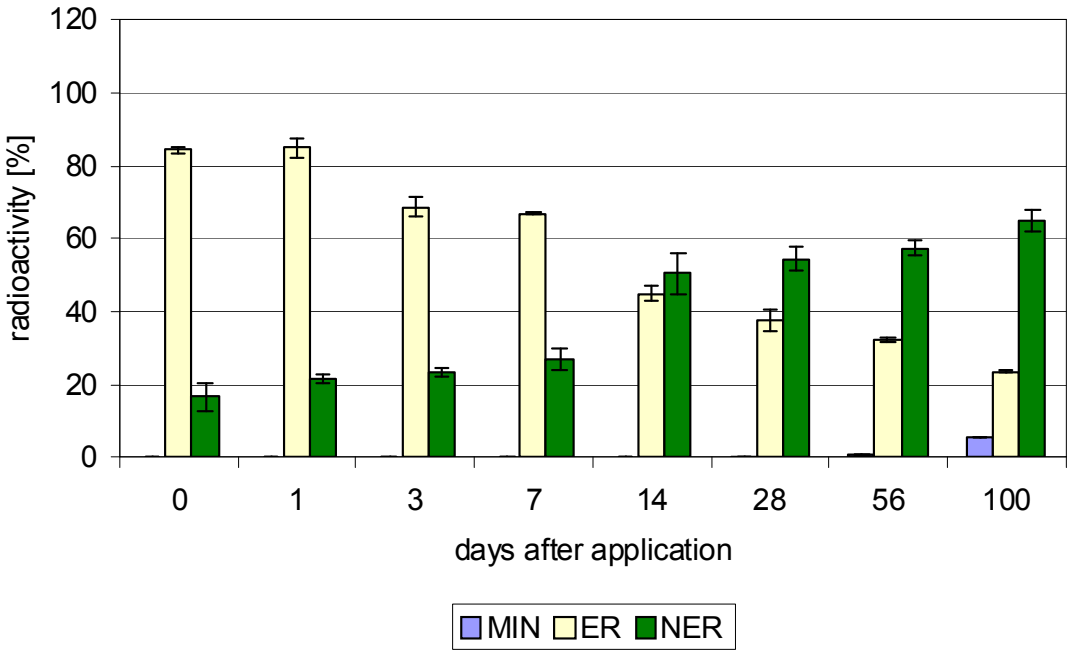


Fig. 6.3: Transformation of ^{14}C-imazalil in silty clay soil (35 % of WHC_{max}) after bovine test-manure application (balances: 96 ± 5 %)

Within the second test series, the soil samples were initially adjusted to only 35 % of the maximum water holding capacity in order to check for the impact of initially different soil moisture contents. The results, illustrated in **Fig. 6.3**, showed slight differences at the beginning that were compensated within the consecutive incubation period. Thus, the extractable residues, containing again only the parent compound within the first 14 days, decreased more slowly. Thereafter, the main metabolite occurred, too. The concentrations of imazalil dropped from 3.5 mg kg^{-1} to 0.8 mg kg^{-1} resulting in a DT_{50} value of 29 days. The formation of non-extractable residues was also decelerated. After

100 days, however, the intensity pattern of mineralization, extractable and non-extractable residues (6 %, 24 % and 65 %) nearly matched those of the first test series (5 %, 15 % and 71 %). More considerable differences would have been found when the soil samples were air dried before experimental processing due to the inhibition of the soil inherent microorganisms. This was shown for transformation tests of ^{14}C-ibuprofen by Kreuzig et al. (2005).

Although the microbial activity was enhanced by the bovine reference-manure application, revealed by an initial substrate-induced respiration of 4.4 mg O_2 100 g^{-1} ds h^{-1} dropped to 1.9 mg O_2 100 g^{-1} ds h^{-1} within 100 days, strictly aerobic conditions were maintained by the discontinous air exchange in the laboratory-batch systems. Redox potential thus increased from 380 to 500 mV. pH values slightly decreased from 7.5 to 7.1.

In comparison to the transformation test series after standard application, these batch experiments clearly showed that the metabolic dynamics of imazalil were influenced by the bovine manure matrix. Since the beginning development of this experimental design within the Runoff Project and continued within in the Manure Project (Kreuzig et al., 2007a, b), the applied soil/manure ratio of 50 g soil to 10 g bovine test-manure corresponded to the fourfold of the maximum manure application amount accepted by the German Ordinance Concerning Fertilization (2006) (cf. chapter 4.4). In order to check the effect of this exaggerated manure amount, these tests were advanced by the reduction of the soil/manure ratio to 50 g soil and 2 g test manure. This approach met the maximum acceptable manure amount. The set up of mass balances of the short-term incubation experiments to prepare test-manure samples proved that this lower manure amount could be reproducibly handled although the ground of the laboratory-batch system was scarcely covered by the reference-manure samples. The comparison of both approaches is illustrated in **Fig. 6.4**.

After the application of the 10-g test-manure samples, the formation of non-extractable residues was particularly promoted within the first incubation intervals. When the 2-g test manure samples were applied, the initial balances were mainly dominated by the extractable fractions. Finally, those differences were nearly compensated. Within the test series after standard application, however, the intensity pattern of mineralization, extractable and non-extractable residues amounted to 10 %, 47 % and 36 %. Due to the considerable differences between standard and test-manure application, this comparison justified the concept of fate monitoring in manured soil already under laboratory

conditions. Thus, impacts of manure should not be disregarded when transformation of VMP and biocides should be assessed for manured soils by laboratory testing.

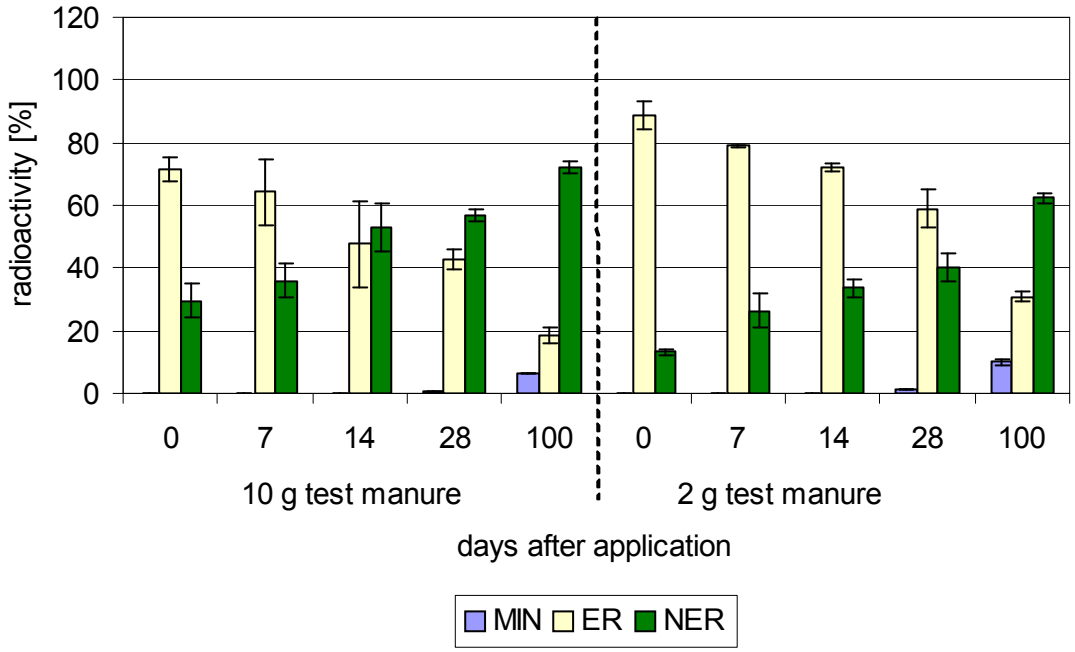


Fig. 6.4: Transformation of ^{14}C-imazalil in silty clay soil (35 % of WHC_{max}) after application of different amounts of bovine test manure (balances: 102 ± 3 %)

Pig test manure

This implication was confirmed when the test-manure samples (10-g level) were prepared on the basis of pig reference manures and then introduced into the transformation tests. The results are illustrated in **Fig. 6.5**. Within the first 14 days, the amounts of extractable residues, containing only the parent compound, remained nearly unchanged. They dropped only from 70 % to 64 % of the radioactivity initially applied. Thereafter, the *ortho*-dealkylated transformation product also occurred. After 100 days, that amounted to 13 % of the extractable fraction, corresponding to 3 % of the initially applied radioactivity. The extractable fractions finally decreased to 26 %. Corresponding to that, the concentration of imazalil decreased from 3.1 to 1.0 mg kg^{-1} within 100 days. DT_{50} was 48 days. The application of pig test-manure first promoted a rapid formation of non-extractable residues. Immediately after the application, those reached 26 %. After 100 days, however, they increased only to 55 %. Mineralization finally

amounted to 9 %.

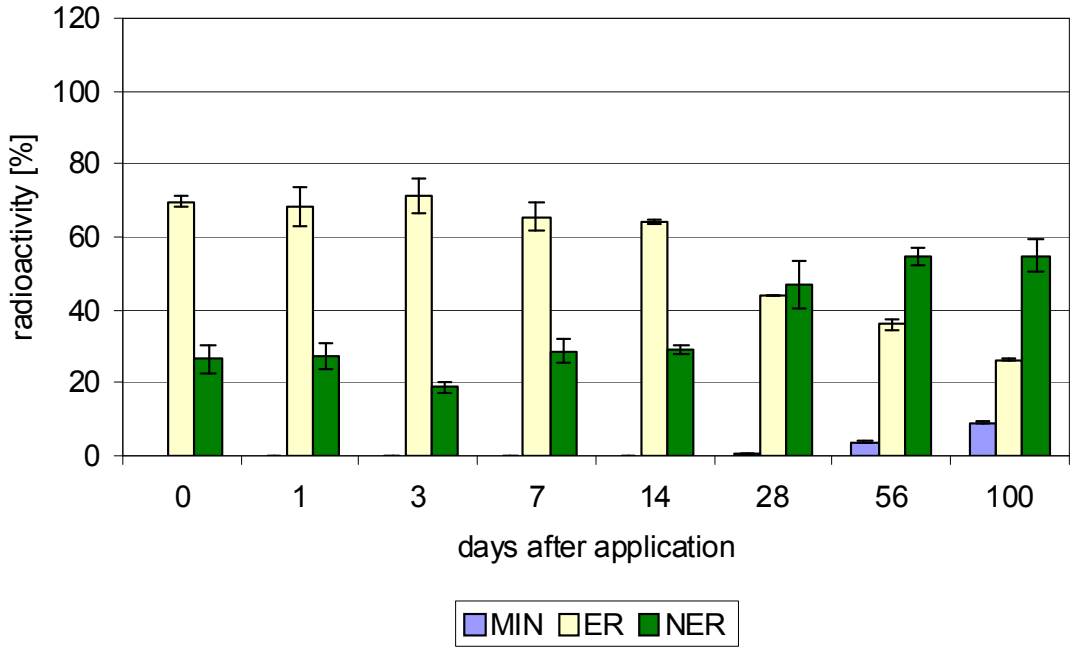


Fig. 6.5: Transformation of ^{14}C-imazalil in silty clay soil (35 % of WHC_{max}) after pig test-manure application (balances: 93 ± 1%)

The corresponding matrix characterization tests confirmed again that the transformation tests were conducted in microbially active soils samples under strictly aerobic conditions. The substrate-induced respiration decreased from 2.7 to 1.5 mg O_2 100 g^{-1} ds h^{-1} and the redox potential incresed from 290 to 510 mV. pH values slightly decreased from 7.4 to 6.9.

6.1.4 Tests on photoinduced effects

Discrepancies of laboratory and field testing on the metabolic fate of the imidazole derivative prochloraz, applied as an azole fungicide to protect crops, could be successfully attributed to photoinduced impacts under field conditions, identified by laboratory testing using a special irradiation apparatus (Höllrigl-Rosta, 1999; cf. chapter 4.5.2). Therefore, the imidazole derivative imazalil was also tested in the dark (MT) and under irradiation (PMT) as well as after standard and after test-manure application. For the

latter, bovine and pig reference manures were used to prepare the corresponding test-manure samples. The results are illustrated in **Fig. 6.6**.

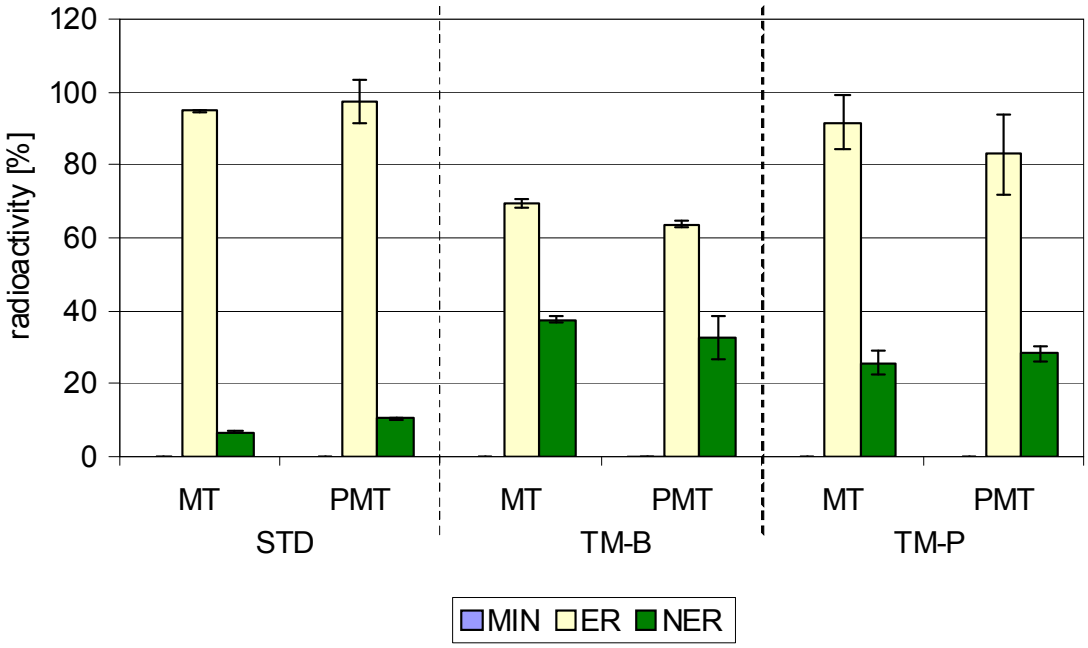


Fig. 6.6: Microbial (MT) and photoinduced microbial transformation (PMT) of ^{14}C-imazalil in silty clay soil after standard (STD) and test-manure application (TM) on the basis of bovine manure (B) and pig manure (P) within 3 days

Although the metabolic fate of prochloraz was accelerated by photoinduced effects, reflected by the faster decrease of the parent compound, the faster formation of the formylurea and the urea metabolite, higher mineralization and the faster formation of non-extractable residues, definite differences were not found for imazalil. Within these 3-day experiments, the extractable residues, containing exclusively the parent compound, were the superior fractions. The formation of non-extractable residues was mainly dependent on the application techniques of the test substance. Lowest amounts were found after standard application while the test-manure applications initiated the formation of higher non-extractable amounts. Here, bovine manure was more effective than pig manure reflecting the same tendencies as found in the related long-term transformation tests.

6.2 Sorption and transformation of cyanamide in manured soil

6.2.1 Mobility in soil

As already shown in the frame of the extraction efficiency tests of manure and soil samples, cyanamide was expected as a potential leacher due to its complete water miscibility. Testing the silty clay soil after standard application, however, the determined K_d value was 4.6 L kg^{-1} resulting in the K_{OC} value of 271 L kg^{-1} (**Tab. 6.2**). ^{14}C-cyanamide was thus classified to be moderately mobile (Hollis, 1991). The same mobility class was achieved when bovine and pig test-manures were applied though K_{OC} values decreased to 96 and 81 L kg^{-1}.

Tab. 6.2: Sorption of ^{14}C-cyanamide in silty clay soil after standard and test-manure application

Application technique	**OC** [%]	**K_d** [L kg^{-1}]	**K_{OC}** [L kg^{-1}]
Standard	1.7	4.6	271
Test manure (bovine, 5 g)	2.7	2.6	96
Test manure (pig, 5 g)	2.6	2.1	81

OC: organic carbon content in soil

6.2.2 Transformation after standard application

In the silty clay soil under study, ^{14}C-cyanamide immediately disappeared. Directly after the standard application, extractable residues amounted to 83 % containing 38 µg cyanamide kg^{-1} soil. Within 28 days, this fraction dropped to 0.1 % (**Fig. 6.7**). Due to those low amounts, a further metabolite screening could not be performed. The non-extractable residues increased within the first days reaching 13 % at maximum. Thereafter, they dropped to 3 % at the end of the incubation period. The fate of ^{14}C-cyanamide was mainly determined by mineralization. Already after one day, 77 % of the initially applied radioactivity was released as ^{14}C-carbon dioxide that reached a maximum of 93 %. Since the non-extractable fractions decreased within the incubation

period, ^{14}C-carbon dioxide was likely released out of this fraction, too.
The matrix characterization tests at the beginning and the end of this test series clearly showed that the boundary test conditions remained nearly constant. The pH values only decreased from 6.8 to 6.5. The redox potential averaged to Eh = 510 mV revealing strictly aerobic conditions in the laboratory-batch systems. The microbial activity was also saved indicated by the measurement of the substrate-induced respiration. The oxygen demand remained at 13 mg 100 g^{-1} ds h^{-1}.

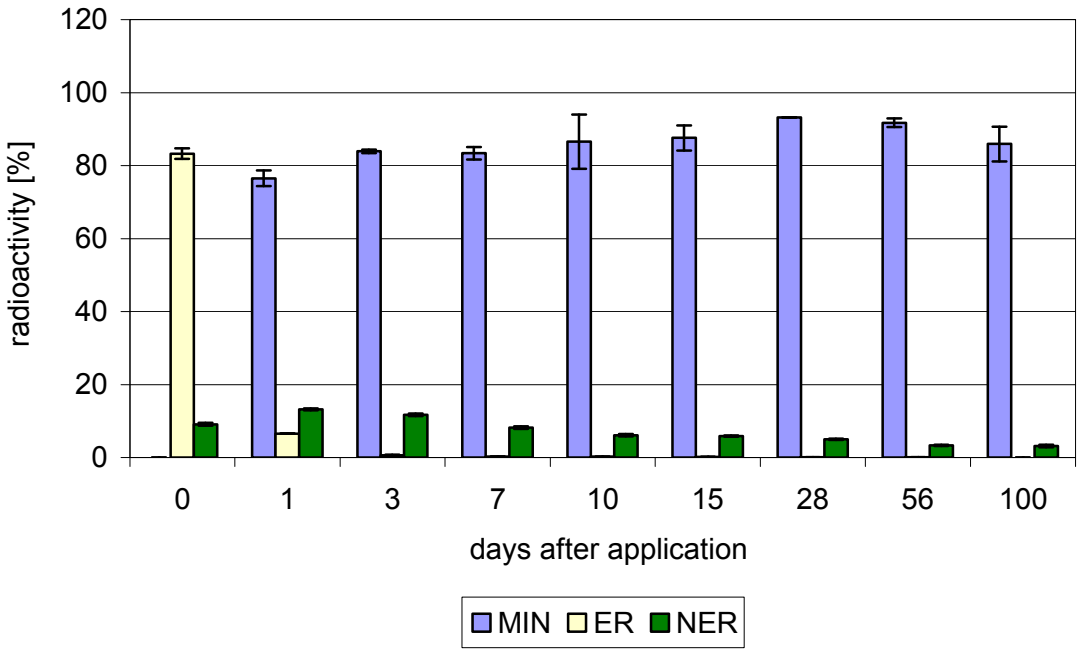


Fig. 6.7: Transformation of ^{14}C-cyanamide in silty clay soil after standard application (balances: 94 ± 3 %)

6.2.3 Transformation after test-manure application

This immediate transformation was considerably interfered when ^{14}C-cyanamide was applied via test-manures. After the 7-day incubation period, the extractable fraction of the bovine test-manure amounted to 42 % containing 11% of ^{14}C-cyanamide as the unchanged parent compound. That corresponded to the concentration of 14 µg cyanamide kg^{-1} fresh bovine manure (140 µg cyanamide kg^{-1} dry manure). The non-extractable residues, operationally defined by the methanol extraction after lyophiliza-

tion, were 41 %. The results of the transformation tests after the application of bovine test-manures are illustrated in **Fig. 6.8**. When 10-g manure amounts exceeding the maximum acceptable application amount (German Ordinance Concerning Fertilization, 2006) were applied, the metabolic fate of ^{14}C-cyanamide was mainly determined by the non-extractable fractions that increased up to 61 % within 3 days and subsequently decreased to 28 %. Since the extractable residues simultaneously dropped from 23 % to 0.3 %, the increasing amounts of ^{14}C-carbon dioxide up to 38 % had to be released mainly out of the non-extractable fraction. The corresponding matrix characterization tests confirmed again that this transformation test series was carried out with microbially active soil samples under strictly aerobic conditions.

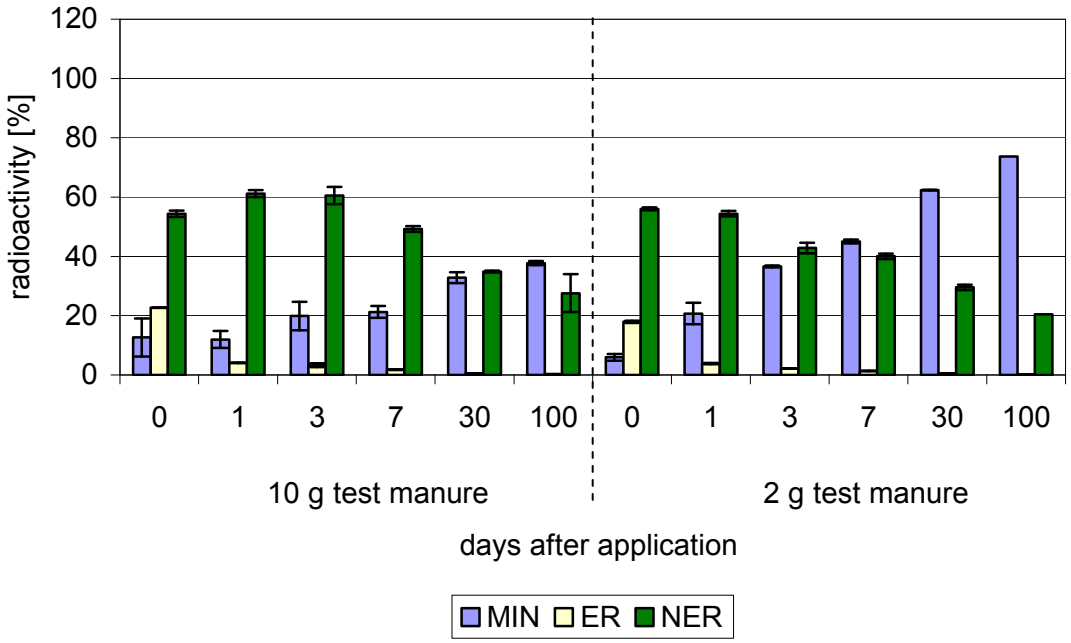


Fig. 6.8: Transformation of ^{14}C-cyanamide in silty clay soil after application of different amounts of bovine test manure (balances: 84 ± 13 %)

When 2-g manure amounts, meeting the maximum acceptable application amount (German Ordinance Concerning Fertilization, 2006), were applied, those non-extractable residues formed during the first incubation intervals dropped more rapidly while mineralization simultaneously increased. The extractable fractions of both test series were of minor relevance.

The extractable fraction of the pig test-manure amounted to 83 % after the 7-day incu-

bation period, containing 12% of ^{14}C-cyanamide as the unchanged parent compound. That corresponded to the concentration of 36 µg cyanamide kg^{-1} fresh manure (724 µg cyanamide kg^{-1} dry manure). The non-extractable residues, operationally defined by the methanol extraction after lyophilization, were 32 %. The results of the long-term transformation test after pig test-manure application resembled rather those after standard application (**Fig. 6.9**).

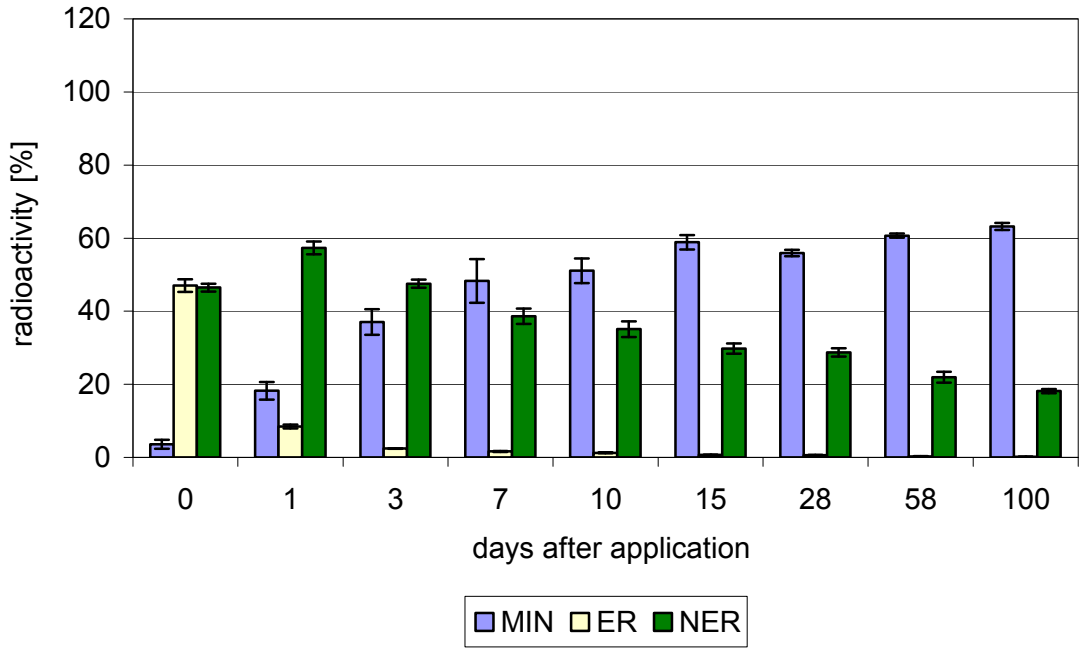


Fig. 6.9: Transformation of ^{14}C-cyanamide in silty clay soil after pig test-manure application (balances: 87 ± 5 %)

The extractable fraction immediately dropped from 47 % to 9 % within the first day. That decrease consecutively continued to 0.2 % within 100 days. Directly after the test-manure application, 47 % of non-extractable residues were found. This fraction increased to 57 % within one day and then decreased to 18 % within 100 days. During this 100-day incubation period, mineralization continuously raised from 4 % up to 63 %. These four test series clearly showed that ^{14}C-cyanamide was readily degradable in soil samples under study. Nevertheless, the transformation was affected by the test-manure applications.

7 Summary and conclusions

Innovative concept of laboratory testing

Within the current authorization procedure "TNsG for data requirements for active substances and biocidal products (2000)", biocides applied for disinfection purposes or insect control in animal houses are to be studied on ultimate biodegradability under anaerobic conditions. Due to the lack of an established simulation test in liquid manures, the biocidal guidance documents recommend a screening test according to OECD 311 using digested sludge of a municipal wastewater treatment plant under optimized boundary conditions of an anaerobic digester. Form the scientific perspective, therefore, fate and behavior of biocides can be hardly transferred from sludge in a digester to liquid bovine or pig manures in a tank because of significantly diverging boundary conditions. Therefore, the Biocide Project has been targeted at the development of a real simulation test on the transformation of biocides in bovine and pig manures as well as on fate in manured soils. Thus, these research activities consequently advanced the technical protocol already developed for veterinary medicinal products (VMP) within the frame of the Manure Project.

Transformation of biocides in reference manures

This technical protocol focused on the reproducible sampling of excrements of cattle and pigs individually kept in experimental stables under standard feeding conditions. These excrements were thoroughly matrix characterized by the following parameters: dry substance content, total organic carbon, pH value, redox potential, dissolved oxygen content, ammonium and total nitrogen content as well as biological oxygen demand. The excrement samples were conditioned for 21 days to reach strictly anaerobic conditions and to transform readily degradable manure inherent substances. Then, reference-manure samples were prepared by adding tap water to adjust typical dry substance contents of 10 % for bovine manure and 5 % for pig manure.

These reference-manure samples were used for transformation tests of two biocides, namely imazalil and cyanamide, applied for disinfection or insect control in animal houses and liquid manures, respectively. Both test substances were spiked as ^{14}C-labeled radiotracers to the reference-manure samples. The batches were then incubated in the dark at 20 $\pm$ 1 °C up to 100 or 177 days. Different extraction techniques, i.e., direct ethyl acetate treatment and lyophilization followed by methanol extraction, were applied for imazalil and cyanamide spiked manure samples. As shown by mass

balances and metabolite screening, the extractable fractions containing only the parent compound dominated the transformation tests on ^{14}C-imazalil in bovine and pig reference-manures revealed by DT_{50} > 177 days. Thus, the concentration of this imidazole biocide did not considerably decrease within the 177-day incubation periods. Mineralization, extractable and non-extractable residues amounted to 0.1 %, 90 % and 15 % in bovine and 0.1 %, 77 % and 21 % in pig reference manure.

Within the 100-day transformation test on ^{14}C-cyanamide in bovine reference manure, mineralization, extractable and non-extractable residues finally reached 16 %, 30 % and 35 %, respectively. DT_{50} values were 2 days. Decreasing mass balances indicated furthermore the release of ^{14}C-methane out of ^{14}C-cyanamide under strictly methanogenic conditions. Corresponding data within the 175-day transformation test in pig reference manure were 16 %, 51 % and 18 %. DT_{50} values were also 2 days.

Fate monitoring in manured soil

On the basis of the long-term transformation tests in reference manures, test-manure samples containing 7-day aged ^{14}C-imazalil or ^{14}C-cyanamide residues were prepared. Those were mixed with soil samples in order to monitor sorption and transformation in manured soils. In comparison to the conventional standard application of the test substance, the impact of the manure matrices could be clearly shown. Thus, K_{OC} values of both test substances were slightly reduced after test-manure application, however, mobility tendencies did not considerably change. Hence, ^{14}C-imazalil was classified as slightly mobile, while ^{14}C-cyanamide was moderately mobile.

The transformation of both test substances in soil was affected by the test-manure application, too. After standard application of ^{14}C-imazalil, mineralization, extractable and non-extractable residues amounted to 10 %, 47 % and 36 %, resuluting in the DT_{50} value was 83 days. In contrast, the metabolic dynamics were enhanced by bovine and pig test-manure application. Mineralization, extractable and non-extractable residues amounted to 6 %, 24 % and 65 % after bovine and 9 %, 26 % and 55 % after pig test-manure application. DT_{50} values were 29 and 48 days, respectively.

The transformation tests on ^{14}C-cyanamide were dominated by mineralization, particularly after standard application. After the 100-day incubation period, mineralization, extractable and non-extractable residues amounted to 86 %, < 0.1 % and 3 %, respectively. After test-manure application, considerable amounts of non-extractable residues were also formed. At the end of the incubation periods, however, ^{14}C-carbon dioxide was released out of these fractions, too. Amounts of mineralization, extractable and

non-extractable residues were 38 %, 0.3 % and 28 % after bovine and 63 %, 0.2 % and 18 % after pig test-manure application.

Evaluation of the reference-manure concept

Following this concept, bovine and pig reference-manure samples can be introduced into reproducible laboratory testing on transformation of VMP and biocides in manures and on sorption and transformation in manured soils. The needed excrement samples are to be preferably taken in experimental stables of animal husbandry. Taking into account that, besides cattle, one group of fattened pigs under study within the Manure Project was kept in a conventional farm and mainly fed by potato refuse instead of barley or wheat, reproducible excrement sampling should be possible in every stable so far the administration of VMP to the test animals and the application of biocides in the stable could be excluded. Furthermore, defined diets of cattle and pig nutrition may contribute to a minimum parameter variation of excrements.

On this basis, the transformation of VMP and biocides can be monitored in reference manures at defined dry substance contents within an appropriate frame of time and costs. This principle of practicability and feasibility cannot be realized when, instead of one reference manure, at least four different tank manures of one animal species were alternatively taken for laboratory testing. The latter approach would quadruplicate the experimental expenditures of the reference-manure concept. Further analytical expenditure would be necessary for additional matrix characterization tests and screening analyses of the tank manures for interfering VMP and biocide contaminants based on complex extraction and clean-up procedures as well as LC/MS/MS analyses. Following the reference-manure concept, those additional screening analyses are not necessary because the excrements to be taken are operationally free of any VMP and biocide contamination. Thus, this concept will save time and costs and, nevertheless, will gather an appropriate data pool for the environmental risk assessment of the authorization procedure of VMP and biocides. Due to those advantages, the reference-manure concept should be more likely acceptable for stakeholders at research institutions, industry and authorities.

Furthermore, the reference manure concept may initiate laboratory testing of sorption and transformation of VMP and biocides in manured soils. Thus, on the basis of transformation tests in reference-manures, test-manure samples, containing short-term

aged residues of VMP or biocides, can be reproducibly prepared and used to simulate the manure application already under laboratory conditions. Numerous laboratory tests on sorption and transformation of VMP and biocides in manured soils emphasized the need of studying fate and behavior of those substances under the impact of the manure matrices in order to understand better complex concentration determining processes in soils under field conditions. Then, those latter aspects are relevant for the prospective evaluation of VMP and biocides in order to advance the environmental risk assessment.

The reference-manure concept has been developed by research activities performed within the Manure Project and the Biocide Project from 2005 to 2009. Due to this limited research period, the compiled data pool is inevitably limited but, it is the most comprehensive one until today resulting in a draft version of the first technical protocol. Since there is an increasing demand for transformation tests in liquid manures, the application of this technical protocol will contribute to gather further experiences and to advance these methodological approaches. There is further need of research because only bovine and pig liquid manures have to be taken into account until today. Thus, there are definite lacks for transformation tests of VMP and biocides in poultry manures due to more different animal husbandry systems and more different manure compositions and storage conditions. There is the need of more harmonization of the experimental and analytical procedures that could be gained by interlaboratory tests resulting in technical guidance documents or guidelines.

8. Zusammenfassung und Schlussfolgerungen

Innovatives Konzept für Labortests

Im Rahmen des aktuellen Zulassungsverfahrens sind Biozide für den Einsatz als Insektizide oder Desinfektionsmittel in Ställen der Tierhaltung gemäß "TNsG for data requirements for active substances and biocidal products (2000)" auf ihre vollständige biologische Abbaubarkeit unter anaeroben Milieubedingungen hin zu untersuchen. Aufgrund des bisherigen Fehlens eines etablierten Simulationstests in Gülle wird in diesem Zulassungsverfahren ein Screeningtest nach OECD Richtlinie 311 in Faulschlamm einer kommunalen Abwasserbehandlungsanlage unter den optimierten, anaeroben Bedingungen eines Faulturmes empfohlen. Doch aufgrund der deutlich abweichenden Testbedingungen ist eine Übertragung des Abbauverhaltens von Bioziden in Faulschlamm auf Rinder- und Schweinegülle aus wissenschaftlicher Sicht nicht sinnvoll. Das Ziel des Biozid-Projektes war deswegen die Entwicklung eines Simulationstests für das Abbauverhalten von Bioziden in Rinder- und Schweinegülle sowie das Abbau- und Sorptionsverhalten in güllegedüngten Böden. Diese Forschungsaktivitäten sollten damit den Leitfaden (Technical Protocol), der bereits für Veterinärpharmaka im Rahmen des Gülle-Projektes erarbeitet wurde, weiterentwickeln.

Abbau von Bioziden in Referenzgülle

Dieser Leitfaden stützte sich dabei auf die reproduzierbare Sammlung der Exkremente von Rindern und Schweinen, die im Versuchsstall unter Standardfütterungsbedingungen gehalten wurden. Nach der Probenahme wurden diese Exkremente einer umfassenden Matrixcharakterisierung unterzogen, bei der folgende Parameter gemessen wurden: Trockensubstanz, gesamter organischer Kohlenstoff, Ammonium- und Gesamtstickstoff, pH, Redoxpotential, gelöster Sauerstoff und biologischer Sauerstoffverbrauch. Die Exkrementproben wurden für 21 Tage konditioniert, um zum einen strikt anaerobe Milieubedingungen und die Umwandlung leicht abbaubarer gülleinhärenter Substanzen zu erzielen. Danach wurden Referenzgülleproben gezielt hergestellt, indem definierte Trockensubstanzgehalte von 10 % für Rindergülle und 5 % für Schweinegüllle durch Wasserzugabe eingestellt wurden.

Diese Referenzgülleproben wurden in den Abbautests für die Biozide Imazalil und Cyanamid eingesetzt, die zur Desinfektion oder Insektenbekämpfung in Ställen Anwendung finden. Beide Testsubstanzen wurden zu den Referenzgülleproben als ^{14}C-

markierte Radiotracer zugegeben und die dotierten Proben dann im Dunkeln bei 20 ± 1 °C bis zu 100 bzw. 177 Tage inkubiert. Für die Probenanalyse wurden unterschiedliche Extraktionstechniken angewendet. So wurde Imazalil mittels einer Schüttelextraktion unter Verwendung von Ethylacetat freigesetzt, während Cyanamid erst nach Gefriertrocknung der Gülleproben mit Methanol extrahiert wurde. Wie aus den Massenbilanzen und dem Metaboliten-Screening abgeleitet werden konnte, lag in den Extrakten des Imazalil-Abbautests dieses als unveränderte Ausgangsverbindung vor, so dass sich hieraus DT_{50} > 177 Tage errechnete. Damit konnte für dieses Imidazol-Biozid während der 177-tägigen Inkubationszeit keine wesentliche Konzentrationsabnahme festgestellt werden. Mineralisation, extrahierbare und nicht-extrahierbare Rückstände erreichten 0.1 %, 90 % and 15 % in Rinder- und 0.1 %, 77 % und 21 % in Schweinereferenzgülle.

Im 100-tägigen Abbautest für Cyanamid nahmen dagegen die extrahierbaren Rückstände kontinuierlich ab, während die nicht-extrahierbare Rückstände und die Mineralisation gleichzeitig anstiegen. Nach 100 Tagen erreichten Mineralisation, extrahierbare und nicht-extrahierbare Rückstände schließlich 16 %, 30 % bzw. 35 %. Hieraus resultierte ein DT_{50}-Wert von 2 Tagen. Abnehmende Massenbilanzen zeigten ferner eine Freisetzung von ^{14}C-Methan aus ^{14}C-Cyanamid an. Im 175-tägigen Test in Schweinereferenzgülle ergaben sich für Mineralisation, extrahierbare und nicht-extrahierbare Rückstände Werte von 16 %, 51 % bzw. 18 %. Der DT_{50}-Wert war wiederum 2 Tage.

Rückstandsverhalten in güllegedüngtem Boden

Auf Grundlage der Langzeitabbautests in Referenzgülle wurden gezielt Testgülleproben hergestellt, die 7 Tage gealterte ^{14}C-Imazalil oder ^{14}C-Cyanamid-Rückstände enthielten. Diese wurden mit Bodenproben gemischt, um Sorption und Abbau in güllegedüngtem Boden zu untersuchen. Im Vergleich zur konventionellen Standard-Applikation der Testsubstanzen konnte der Einfluss der Güllematrizes deutlich gezeigt werden. So wurden die K_{OC}-Werte beider Testsubstanzen geringfügig erniedrigt, ohne dass allerdings die Mobilitätstendenzen grundlegend verändert wurden. ^{14}C-Imazalil war danach als leicht mobil, ^{14}C-Cyanamid als mäßig mobil einzustufen.

Der Abbau beider Substanzen im Boden wurde ebenfalls durch die Testgülle-Applikation beeinflusst. Nach Standard-Applikation von ^{14}C-Imazalil lagen für Mineralisation, extrahierbare und nicht-extrahierbare Rückstände 10 %, 47 % and 36 % vor woraus ein ein DT_{50}-Wert von 83 Tagen resultierte. Die Abbauprozesse wurden dage-

gen durch die Testgülle-Applikation beschleunigt. Mineralisation, extrahierbare und nicht-extrahierbare Rückstände erreichten 6 %, 24 % und 65 % nach Rinder- bzw. 9 %, 26 % und 55 % nach Schweinetestgülle-Applikation. So errechneten sich DT_{50}-Werte von 29 Tagen bzw. 48 Tagen.
Insbesondere nach Standard-Applikation unterlag ^{14}C-Cyanamid einer sehr raschen Mineralisation. Damit lagen nach 100-tägiger Inkubation Anteile von 86 %, < 0.1 % bzw. 3 % für Mineralisation, extrahierbare und nicht-extrahierbare Rückstände vor. Nach Testgülle-Applikation wurden gleichzeitig nicht-extrahierbare Rückstände gebildet, aus denen dann allerdings zum Ende der Inkubationsperiode ebenfalls ^{14}C-Kohlendioxid freigesetzt wurde. Mineralisation, extrahierbare und nicht-extrahierbare Rückstände erreichten schließlich 38 %, 0.3 % bzw. 28 % nach Rinder- und 63 %, 0.2 % bzw. 18 % nach Schweinetestgülle-Applikation.

Bewertung des Referenzgülle-Konzeptes

Entsprechend dieses Konzeptes ist es möglich, Rinder- und Schweinereferenzgülleproben reproduzierbar in Labortests zum Abbau von Veterinärpharmaka und Bioziden in Gülle sowie zu Sorption und Abbau in güllegedüngtem Boden einzusetzen. Dafür sind die erforderlichen Exkrementproben bevorzugt in einem Versuchsstall zu entnehmen. Da aber bereits im Gülle-Projekt neben Rindern auch Schweine beprobt wurden, die in einem konventionellen Stall unter deutlich abweichenden Fütterungsbedingungen (überwiegend Kartoffelabfälle anstatt Gerste oder Weizen) gehalten wurden, sollte eine reproduzierbare Exkremententnahme in jedem Stall möglich sein, sofern die Verabreichung von Veterinärpharmaka sowie der Einsatz von Bioziden sicher ausgeschlossen werden können. Andererseits können definiertere Fütterungsbedingungen zu einer Minimierung der Parametervariation der Exkremente gezielt beitragen.

Auf dieser Basis kann nun der Abbau von Veterinärpharmaka und Bioziden in Referenzgüllematrizes definierter Trockensubstanzgehalte in einem definierten Zeit- und Kostenrahmen untersucht werden. Diesem Prinzip der Praktikabilität und Machbarkeit kann so nicht gefolgt werden, wenn anstatt einer Referenzgülle 4 verschiedene Tankgülleproben einer Tierart für diese Labortests eingesetzt werden. Letztgenannter Ansatz würde den experimentellen Aufwand der Untersuchungen vervierfachen. Das beträfe auch den analytischen Aufwand für zusätzliche Matrixcharakterisierungstests sowie umfangreiche Screeninganalysen, einschließlich komplexer Extraktions- und Cle-

an-up-Verfahren sowie LC/MS/MS-Analysen auf Kontaminationen mit Veterinärpharmaka- und Biozid-Rückständen in den Tankgülleproben. Dem Referenzgülle-Konzept folgend sind diese umfangreichen Screeninganalysen nicht erforderlich, da der Einsatz von Veterinärpharmaka und Bioziden im Versuchstall definiert ausgeschlossen werden kann. Damit erspart das Referenzgülle-Konzept zeitaufwendige und kostenintensive Untersuchungen und sichert die Erfassung eines umfassenden Datensatzes für die im Rahmen des Zulassungsverfahrens von Veterinärpharmaka und Bioziden vorzunehmende Risikoabschätzung. Aufgrund der Vorteile dieses Konzeptes sollten entsprechende Abbautests in Gülle leichter die Akzeptanz von Forschungseinrichtungen, Industrie und Behörden finden.
Darüber hinaus kann das Referenzgülle-Konzept Labortests zu Sorption und Abbau von Veterinärpharmaka und Bioziden in güllegedüngten Böden initiieren. Denn auf der Grundlage der Abbautests in Referenzgülle ist es ebenfalls möglich, Testgülleproben, die kurzfristig gealterte Rückstände von Veterinärpharmaka oder Bioziden enthalten, gezielt herzustellen und zur Simulation der Gülleausbringung bereits unter Laborbedingungen einzusetzen. Denn zahlreiche Untersuchungen zum Rückstandsverhalten dieser Substanzen in güllegedüngten Böden betonen die Notwendigkeit von Untersuchungen, die den Einfluss der Gülle berücksichtigen, um die komplexen konzentrationsbestimmenden Prozesse in Böden unter Freilandbedingungen besser zu verstehen und die Verfahren der Risikoabschätzung weiterzuentwickeln.

Das Referenzgülle-Konzept wurde durch Forschungsaktivitäten entwickelt, die im Rahmen des Gülle-Projektes und des Biozid-Projektes von 2005 bis 2009 durchgeführt wurden. Aufgrund dieses begrenzten Forschungszeitraumes ist der erarbeitete Datensatz zwangsläufig begrenzt, doch auch augenblicklich der umfassendste, aus dem der Entwurf des ersten Leitfadens hervorgeht. Da augenblicklich eine steigende Nachfrage nach Abbautests in Gülle zu verzeichnen ist, würde die Anwendung dieses Leitfadens zu weiteren Erfahrungen und zur Weiterentwicklung der methodischen Ansätze führen. Somit besteht die Notwendigkeit weiterer Forschungsaktivitäten, zumal bisher nur Rinder- und Schweinegülle Berücksichtigung fanden. Beträchtliche Lücken bestehen für Abbautests von Veterinärpharmaka und Bioziden in Geflügelgülle, die infolge sehr unterschiedlicher Haltungssysteme und Güllelagerungsbedingungen deutlich schwieriger zu handhaben sein wird. Es besteht die Notwendigkeit der weiteren Harmonisierung der experimentellen und analytischen Ansätze, die durch Ringversuche erzielt werden

könnte und somit dann Leitfäden oder Richtlinien erarbeitet werden könnten.

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11. Data of ^{14}C-imazalil and ^{14}C-cyanamide transformation tests

Tab. 11.1: Transformation of ^{14}C-imazalil in bovine reference manure

Radioactive	**incubation [days]**							
fraction [%]	**0**	**1**	**3**	**7**	**30**	**70**	**100**	**177**
$^{14}CO_2$	**---**	**< 0.1**	**< 0.1**	**< 0.1**	**< 0.1**	**< 0.1**	**< 0.1**	**0.1**
extractable	**98**	**98**	**97**	**92**	**91**	**84**	**93**	**90**
imazalil	98	98	97	92	91	84	93	90
M1	nd	nd	nd	nd	nd	nd	nd	nd
non-extractable	**2**	**3**	**4**	**6**	**10**	**12**	**15**	**15**
other volatiles	na	na	na	na	na	na	na	na
total	**100**	**101**	**101**	**98**	**101**	**96**	**108**	**105**

nd: not detected, **na:** not analyzed;

M1: 1-[2-(2.4-dichlorphenyl)-2-hydroxyethyl]-1*H*-imidazol

Tab. 11.2: Transformation of ^{14}C-imazalil in pig reference manure

Radioactive	**incubation [days]**							
fraction [%]	**0**	**1**	**3**	**7**	**30**	**70**	**100**	**177**
$^{14}CO_2$	**---**	**< 0.1**	**< 0.1**	**< 0.1**	**< 0.1**	**< 0.1**	**< 0.1**	**0.1**
extractable	**96**	**98**	**97**	**96**	**89**	**87**	**97**	**77**
imazalil	96	98	97	96	89	87	97	77
M1	nd	nd	nd	nd	nd	nd	nd	nd
non-extractable	**1**	**2**	**2**	**3**	**8**	**12**	**14**	**21**
other volatiles	na	na	na	na	na	na	na	na
total	**97**	**100**	**99**	**99**	**97**	**99**	**111**	**98**

nd: not detected ; **na:** not analyzed

M1: 1-[2-(2.4-dichlorphenyl)-2-hydroxyethyl]-1*H*-imidazol

Tab. 11.3: Transformation of ^{14}C-imazalil in pig reference manure at different dry substance contants and at 20 ± 1 °C

2.5 % dry substance

Radioactive fraction [%]	**incubation [days]**				
	0	**3**	**7**	**30**	**100**
$^{14}CO_2$	**---**	**< 0.1**	**< 0.1**	**< 0.1**	**0.1**
extractable	**104**	**97**	**98**	**97**	**80**
imazalil	na	na	na	na	na
M1	na	na	na	na	na
non-extractable	**1**	**2**	**3**	**7**	**17**
other volatiles	na	na	na	na	na
total	**105**	**99**	**101**	**104**	**97**

nd: not detected ; **na:** not analyzed

M1: 1-[2-(2.4-dichlorphenyl)-2-hydroxyethyl]-1*H*-imidazol

5 % dry substance

Radioactive fraction [%]	**incubation [days]**				
	0	**3**	**7**	**30**	**100**
$^{14}CO_2$	**---**	**< 0.1**	**< 0.1**	**< 0.1**	**< 0.1**
extractable	**103**	**98**	**103**	**93**	**79**
imazalil	na	na	na	na	na
M1	na	na	na	na	na
non-extractable	**1**	**2**	**4**	**7**	**15**
other volatiles	na	na	na	na	na
total	**104**	**100**	**107**	**100**	**94**

nd: not detected ; **na:** not analyzed

M1: 1-[2-(2.4-dichlorphenyl)-2-hydroxyethyl]-1*H*-imidazol

Tab. 11.3: (continued)

10 % dry substance

Radioactive fraction [%]	incubation [days]				
	0	**3**	**7**	**30**	**100**
$^{14}CO_2$	**---**	**< 0.1**	**< 0.1**	**< 0.1**	**< 0.1**
extractable	**103**	**99**	**98**	**93**	**84**
imazalil	na	na	na	na	na
M1	na	na	na	na	na
non-extractable	**2**	**3**	**4**	**9**	**20**
other volatiles	na	na	na	na	na
total	**105**	**102**	**102**	**102**	**104**

nd: not detected ; **na:** not analyzed

M1: 1-[2-(2.4-dichlorphenyl)-2-hydroxyethyl]-1*H*-imidazol

Tab. 11.4: Transformation of ^{14}C-imazalil in silty clay soil after standard application

Radioactive fraction [%]	incubation [days]							
	0	**1**	**3**	**7**	**14**	**28**	**56**	**100**
$^{14}CO_2$	**---**	**< 0.1**	**< 0.1**	**0.1**	**0.3**	**1**	**5**	**10**
extractable	**102**	**79**	**74**	**80**	**70**	**70**	**55**	**47**
imazalil	102	79	74	80	70	66	52	41
M1	nd	nd	nd	nd	nd	4	3	6
non-extractable	**2**	**22**	**25**	**20**	**26**	**27**	**32**	**36**
other volatiles	na	na	na	na	na	na	na	na
total	**104**	**101**	**99**	**100**	**96**	**98**	**92**	**93**

nd: not detected; **na:** not analyzed

M1: 1-[2-(2.4-dichlorphenyl)-2-hydroxyethyl]-1*H*-imidazol

Tab. 11.5: Transformation of ^{14}C-imazalil in silty clay soil (45 % of WHC) after bovine test-manure application

Radioactive fraction [%]	incubation [days]							
	0	**1**	**3**	**7**	**14**	**28**	**56**	**100**
$^{14}CO_2$	**---**	**< 0.1**	**< 0.1**	**< 0.1**	**< 0.1**	**< 0.1**	**1**	**5**
extractable	**85**	**68**	**62**	**52**	**42**	**34**	**21**	**15**
imazalil	85	68	62	52	42	30	17	12
M1	nd	nd	nd	nd	nd	4	4	3
non-extractable	**16**	**34**	**42**	**51**	**52**	**57**	**68**	**71**
other volatiles	na	na	na	na	na	na	na	na
total	**101**	**102**	**104**	**103**	**94**	**91**	**90**	**91**

nd: not detected; **na:** not analyzed

M1: 1-[2-(2.4-dichlorphenyl)-2-hydroxyethyl]-1*H*-imidazol

Tab. 11.6: Transformation of ^{14}C-imazalil in silty clay soil (35 % of WHC_{max}) after bovine test-manure application

Radioactive fraction [%]	incubation [days]							
	0	**1**	**3**	**7**	**14**	**28**	**56**	**100**
$^{14}CO_2$	**---**	**< 0.1**	**< 0.1**	**< 0.1**	**< 0.1**	**0.2**	**0.8**	**6**
extractable	**84**	**85**	**69**	**67**	**45**	**38**	**33**	**24**
imazalil	84	85	69	67	45	32	26	19
M1	nd	nd	nd	nd	nd	4	7	5
non-extractables	**17**	**22**	**24**	**27**	**51**	**54**	**57**	**65**
other volatiles	na	na	na	na	na	na	na	na
total	**101**	**107**	**93**	**94**	**96**	**92**	**91**	**95**

nd : not detected; **na:** not analyzed

M1: 1-[2-(2.4-dichlorphenyl)-2-hydroxyethyl]-1*H*-imidazol

Tab. 11.7: Transformation of ^{14}C-imazalil in silty clay soil (35 % of WHC_{max}) after application of different amounts of bovine test manure

10 g bovine test manure + 50 g soil

Radioactive fraction [%]	**incubation [days]**				
	0	**7**	**14**	**28**	**100**
$^{14}CO_2$	**---**	**< 0.1**	**< 0.1**	**0.3**	**7**
extractable	**72**	**64**	**48**	**43**	**18**
imazalil	na	na	na	na	na
M1	na	na	na	na	na
non-extractable	**29**	**36**	**53**	**57**	**72**
other volatiles	na	na	na	na	na
total	**101**	**100**	**101**	**100**	**97**

nd: not detected ; **na:** not analyzed

M1: 1-[2-(2.4-dichlorphenyl)-2-hydroxyethyl]-1*H*-imidazol

2 g bovine test manure + 50 g soil

Radioactive fraction [%]	**incubation [days]**				
	0	**7**	**14**	**28**	**100**
$^{14}CO_2$	**---**	**< 0.1**	**0.1**	**1**	**10**
extractable	**89**	**79**	**72**	**59**	**31**
imazalil	na	na	na	na	na
M1	na	na	na	na	na
non-extractable	**13**	**27**	**34**	**40**	**62**
other volatiles	na	na	na	na	na
total	**102**	**106**	**106**	**100**	**103**

nd: not detected ; **na:** not analyzed

M1: 1-[2-(2.4-dichlorphenyl)-2-hydroxyethyl]-1*H*-imidazol

Tab. 11.8: Transformation of ^{14}C-imazalil in silty clay soil (35 % of WHC_{max}) after pig test-manure application

Radioactive fraction [%]	Incubation Time [days]							
	0	**1**	**3**	**7**	**14**	**28**	**56**	**100**
$^{14}CO_2$	**---**	**< 0.1**	**< 0.1**	**< 0.1**	**< 0.1**	**0.3**	**4**	**9**
extractable	**70**	**68**	**72**	**66**	**64**	**44**	**36**	**26**
imazalil	70	68	72	66	64	38	31	22
M1	nd	nd	nd	nd	nd	6	5	4
non-extractable	**26**	**27**	**19**	**29**	**29**	**47**	**55**	**55**
other volatiles	na	na	na	na	na	na	na	na
total	**96**	**95**	**91**	**95**	**93**	**91**	**95**	**90**

nd : not detected; **na:** not analysed

M1: 1-[2-(2.4-Dichlorphenyl)-2-hydroxyethyl]-1H-imidazol

Tab. 11.9: Microbial (MT) and photoinduced microbial transformation (PMT) of ^{14}C-imazalil in silty clay soil after standard (STD) and test-manure application (TM) on the basis of bovine manure (B) and pig manure (P) within 3 days

Radioactive fraction [%]	STD		TM-B		TM-P	
	MT	**PMT**	**MT**	**PMT**	**MT**	**PMT**
$^{14}CO_2$	**< 0.1**	**< 0.1**	**< 0.1**	**0.1**	**< 0.1**	**< 0.1**
extractable	**95**	**97**	**70**	**64**	**92**	**83**
imazalil	na	na	na	na	na	na
M1	na	na	na	na	na	na
non-extractable	**7**	**10**	**38**	**33**	**26**	**28**
other volatiles	na	na	na	na	na	na
total	**102**	**107**	**108**	**97**	**118**	**111**

nd: not detected ; **na:** not analyzed

M1: 1-[2-(2.4-dichlorphenyl)-2-hydroxyethyl]-1*H*-imidazol

Tab. 11.10: Transformation of ^{14}C-cyanamide in bovine reference manure

Radioactive fraction [%]	incubation [days]					
	0	**1**	**3**	**7**	**30**	**100**
$^{14}CO_2$	**---**	**1**	**4**	**9**	**12**	**16**
extractable	**94**	**79**	**54**	**44**	**37**	**30**
cyanamide[#]	69	48	20	4	3	4
non-extractable	**9**	**20**	**33**	**29**	**32**	**35**
other volatiles	na	na	na	na	na	na
total	**103**	**100**	**91**	**82**	**81**	**81**

nd: not detected, **na:** not analyzed

Tab. 11.11: Transformation of ^{14}C-cyanamide in pig reference manure

Radioactive fraction [%]	incubation [days]									
	0	**1**	**3**	**7**	**15**	**28**	**58**	**78**	**100**	**175**
$^{14}CO_2$	**---**	**2**	**4**	**4**	**6**	**8**	**11**	**11**	**14**	**16**
extractable	**82**	**79**	**75**	**64**	**65**	**60**	**58**	**58**	**49**	**51**
cyanamide[#]	44	32	23	12	7	7	5	8	6	8
non-extractable	**7**	**10**	**14**	**17**	**25**	**30**	**22**	**23**	**26**	**18**
other volatiles	na	na	na	na	na	na	na	na	na	na
total	**89**	**91**	**93**	**85**	**96**	**98**	**91**	**92**	**89**	**85**

nd: not detected ; **na:** not analyzed

[#] Together with 2 unknown metabolites, guanidine and dicyandiamide could be also detected. Since the latter both compounds have already occurred in the initially applied ^{14}C-cyanamide reference standard, reducing the radiochemical purity to approximately 80 %, and their amounts remained nearly constant at those values during the incubation period, their contribution to the metabolic fate of ^{14}C-cyanamide could not be definitely shown.

Tab. 11.11: Transformation of ^{14}C-cyanamide in pig reference manure at different dry substance contants and at 20 ± 1 °C

2.5 % dry substance

Radioactive fraction [%]	**incubation [days]**				
	0	**3**	**7**	**30**	**100**
$^{14}CO_2$	**---**	**5**	**3**	**10**	**28**
extractable	**80**	**73**	**73**	**67**	**40**
cyanamide	na	na	na	na	na
non-extractable	**9**	**24**	**38**	**33**	**33**
other volatiles	na	na	na	na	na
total	**89**	**102**	**114**	**110**	**101**

nd: not detected ; **na:** not analyzed

5 % dry substance

Radioactive fraction [%]	**incubation [days]**				
	0	**3**	**7**	**30**	**100**
$^{14}CO_2$	**---**	**4**	**4**	**8**	**13**
extractable	**82**	**75**	**64**	**60**	**49**
cyanamide	na	na	na	na	na
non-extractable	**6**	**14**	**17**	**30**	**26**
other volatiles	na	na	na	na	na
total	**88**	**93**	**85**	**98**	**88**

nd: not detected ; **na:** not analyzed

Tab. 11.11: (continued)

10 % dry substance

Radioactive fraction [%]	incubation [days]				
	0	**3**	**7**	**30**	**100**
$^{14}CO_2$	**---**	**13**	**10**	**13**	**16**
extractable	**98**	**58**	**61**	**52**	**41**
cyanamide	na	na	na	na	na
non-extractable	**6**	**9**	**13**	**11**	**14**
other volatiles	na	na	na	na	na
total	**104**	**80**	**84**	**76**	**71**

nd: not detected ; **na:** not analyzed

Tab. 11.12: Transformation of ^{14}C-cyanamide in silty clay soil after standard application

Radioactive fraction [%]	incubation [days]								
	0	**1**	**3**	**7**	**10**	**15**	**28**	**56**	**100**
$^{14}CO_2$	**---**	**77**	**84**	**83**	**87**	**88**	**93**	**92**	**86**
extractable	**83**	**7**	**1**	**0.3**	**0.2**	**0.1**	**0.1**	**nd**	**nd**
cyanamide[#]	43	nd	na	na	na	na	na	na	na
non-extractable	**9**	**13**	**12**	**8**	**6**	**6**	**5**	**3**	**3**
other volatiles	na	na	na	na	na	na	na	na	na
total	**92**	**97**	**97**	**91**	**93**	**94**	**98**	**95**	**89**

nd: not detected; **na:** not analyzed

Tab. 11.13: Transformation of ^{14}C-cyanamide in silty clay soil after application of different amounts of bovine test manure

10 g bovine test manure + 50 g soil

Radioactive fraction [%]	**incubation [days]**					
	0	**1**	**3**	**7**	**30**	**100**
$^{14}CO_2$	**13**	**12**	**20**	**21**	**33**	**38**
extractable	**23**	**4**	**3**	**2**	**1**	**0.3**
cyanamide#	3	1	na	na	na	na
non-extractable	**54**	**61**	**61**	**49**	**35**	**28**
other volatiles	na	na	na	na	na	na
total	**90**	**77**	**84**	**72**	**69**	**66**

nd: not detected ; **na:** not analyzed

2 g bovine test manure + 50 g soil

Radioactive fraction [%]	**incubation [days]**					
	0	**1**	**3**	**7**	**30**	**100**
$^{14}CO_2$	**6**	**21**	**37**	**45**	**62**	**74**
extractable	**18**	**4**	**2**	**1**	**1**	**0.2**
cyanamide#	1	nd	na	na	na	na
non-extractable	**56**	**54**	**43**	**40**	**30**	**21**
other volatiles	na	na	na	na	na	na
total	**80**	**79**	**82**	**86**	**93**	**95**

nd: not detected ; **na:** not analyzed

Tab. 11.14: Transformation of ^{14}C-cyanamide in silty clay soil after pig test-manure application

Radioactive	**incubation [days]**								
fraction [%]	**0**	**1**	**3**	**7**	**10**	**15**	**28**	**58**	**100**
$^{14}CO_2$	**4**	**18**	**37**	**48**	**51**	**59**	**56**	**61**	**63**
extractable	**47**	**9**	**2**	**2**	**1**	**1**	**1**	**0.3**	**0.2**
cyanamide[#]	11	1	na	na	na	na	na	na	na
non-extractable	**47**	**57**	**48**	**39**	**30**	**29**	**29**	**22**	**18**
other volatiles	na	na	na	na	na	na	na	na	na
total	**98**	**84**	**87**	**89**	**82**	**89**	**86**	**83**	**81**

nd : not detected; **na:** not analyzed

12. Proposal for a Technical Protocol (Draft Version)

Anaerobic Transformation in Liquid Bovine and Pig Manures

INTRODUCTION

1. This technical protocol describes a laboratory test method to evaluate the transformation of chemicals in liquid bovine and pig manures under anaerobic conditions and is primarily designed for veterinary medicinal products (VMP) and biocides. The environmentally relevant entry routes into liquid manures occur via urine and feces of cattle and pigs in stable housings after excretion of VMP (as parent compounds or metabolites) and after the application of biocides in animal housings (e.g., as disinfectants or insecticides). In loose housing stables with slatted floors, the excrements are discharged into manure aboveground silos or underground pits. After the storage of liquid manures up to several months, they are applied to farmland and grassland soils as organic fertilizers. Via this route, VMP and biocides may enter soil environments. Thus, the persistence of the chemicals during manure storage under anaerobic conditions decides on the environmental relevance of this entry route.

Further entry routes, i.e., solid dung application and direct dung pat deposition by production animals on pasture, are not considered by this technical protocol. Solid dung of poultry is also not in the scope of this technical protocol due to its mainly aerobic storage conditions.

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

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

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

II. Testing of anaerobic transformation of chemicals in reference manures.

PRINCIPLE OF THE TESTS

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

APPLICABILITY OF THE TESTS

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

INFORMATION ON THE TEST SUBSTANCE

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

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

– Solubility in water according to OECD Guideline 105 [2].
– Solubility in organic solvents.
– Vapor pressure according to OECD Guideline 104 [3] and Henry´s law constant.
– n-Octanol/water partition coefficient according to OECD Guidelines 107 or 117 [4,5].
– Chemical stability in dark (hydrolysis) according to OECD Guideline 111 [6].
– pK_a if a molecule is liable to protonation or deprotonation according to OECD Guideline 112 [7].

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

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

DEFINITIONS

10. See Annex A.

QUALITY CRITERIA

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

Recovery

12. Recoveries for the experiments are given by the respective mass balances that should range from 90 % to 110 % for radio-labeled test substances. When residue ana-

lytical methods are applied, recoveries are considered acceptable from 70 to 110 %.

Repeatability

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

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

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

Accuracy of transformation data

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

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

CHARACTERIZATION OF THE EXCREMENT AND MANURE SAMPLES

Equipment and chemicals

17. Standard laboratory equipment is required:

- Plastic containers with lids, 20 L, 2 L and 1 L
- Mechanical mixing device (e.g., electric stirrer).
- Drying oven (105 $\pm$ 5 °C).
- Muffle furnace.
- Infrared heater.
- Incubator (5 to 20 $\pm$ 2 °C).
- Apparatus for determination of the total carbon content.
- Analytical balances (accuracy $\leq$ 1 mg).
- pH-meter with pH electrode and appropriate test solution.
- Millivoltmeter with redox electrode and appropriate test solution.
- Oxygen meter with measuring probe.
- Distillation and digestion systems with distillation flasks or tubes.
- Incubation bottles (Karlsruher bottles).
- Spectral photometer, digestion stand with digestion flasks or tubes.

18. Chemicals used include, for example:

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

Sampling and feeding conditions

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

Conditioning, storage and matrix characterization of excrement samples

20. Directly after excretion, readily degradable organic compounds of the excrements undergo rapid decomposition enhancing the matrix heterogeneity [8,9]. To minimize this effect, conditioning of excrement samples is necessary. For this purpose, the excrement samples are kept in plastic containers (approximately 20 L) at ambient temperature. Within a 21-d period, they are daily homogenized using an electric stirrer. Thereafter, the excrements samples are to be matrix characterized by the parameters as follows: dry substance content, total organic carbon, pH, redox potential, dissolved oxygen, ammonium and total nitrogen, biological oxygen demand. Constant conditions are indicated by the dissolved oxygen contents < 0.1 mg kg^{-1}, the redox potential Eh < 0 mV[‡] and an ammonium content stable at $\pm$ 0.2 g kg^{-1}, are established [11-16]. The excrements can be directly used for the reference-manure preparation or long-term stored up to 360 d at -20 °C until analytical processing. After defrosting, the excrements are stored at 20 $\pm$ 2 °C for 3 d to remobilize the excrements inherent microorganisms. Then, matrix characterization has to be repeated.

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

Reference-manure preparation

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

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

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

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

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

22. The dry substance contents of bovine and pig manures of 10 % and 5 %, respectively, are adjusted by the addition of tap water to the corresponding excrement samples. These dry substance contents correspond to values given for Europe in a range between 0.9 and 12 % [17-25]. In order to additionally study the effect of the dry substance content on the transformation of test substance, different dry substance contents (e.g., 2.5, 5 and 10 %) can be optionally tested.
Subsequently, the required quantity of the excrement is weighed into a sample bottle or directly into the flask of the laboratory-test system. After adding the tap water, the manure sample is to be homogenized and the matrix characterization is to be exemplarily carried out again. The prepared manure samples can be stored in closed sample bottles at 4 °C for 7 d, without any relevant change of the matrix parameters.

Dry substance content (ds)

23. Excrements and manure samples (1 to 6 g) are dried in a drying oven to constant mass at 105 ± 5 °C [26]. Alternatively, an infrared heater can be used to drive out the water. The difference of an amount of excrement or manure before and after the drying procedure is used to calculate the dry substance content. The dry substance content is expressed in percentage with an accuracy of ± 1 % (*w*/*w*).

Total organic carbon (TOC)

24. The carbon present in excrement or manure samples is oxidized to carbon dioxide by heating up at least 900 °C in a flow of oxygen-containing gas that is free from carbon dioxide [27]. Prior to combustion, carbonates are to be removed from the dried samples (50-100 mg) by an excess of hydrochloric acid (4 mol L^{-1}). Subsequently, the samples are to be dried at 105 °C, homogenized and mixed with aluminium oxide (1:20). The mixed samples are combusted and the released amount of carbon dioxide is measured by appropriate methods. The total organic carbon is expressed in % dry substance or in g kg^{-1} fresh weight with an accuracy of 1 g kg^{-1}.

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

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

pH value

25. The pH value is measured directly in the stirred and homogenized excrement or manure sample (50 to 100 g) using a pH electrode [28].

Redox potential (E_h)

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

Dissolved oxygen content (O_2)

27. The dissolved oxygen content is measured directly in the homogenized excrement or manure sample (50 to 100 g) using an electrochemical cell which is isolated from the sample by a gas permeable membrane [30].

Ammonium nitrogen (NH_4-N)

28. Under mildly alkaline conditions [31], a distillation of the homogenized excrement or manure sample (1 to 4 g) is performed. The released ammonia is trapped in a receiving flask containing 50 mL boric acid solution (20 g L^{-1}) and an indicator solution (e.g., 200 µL mixed indicator No. 5). Titration of the ammonium in the distillate is conducted with standard volumetric hydrochloric acid solution (0.1 mol L^{-1}). The ammonium nitrogen concentration (NH_4-N) expressed in g NH_4-N kg^{-1} and rounded to one

significant figure is calculated using the formula:

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

V_1: volume of hydrochloric acid used in the titration of the sample [mL]
V_0 : volume of hydrochloric acid used in the blank test [mL]
m: mass of the excrement or manure sample [g]
c: concentration of hydrochloric acid [0.1 mol L^{-1}]
M_N: molar mass of nitrogen [14.01 g mol^{-1}].

Total nitrogen (N_{total})

29. The total nitrogen content of homogenized excrement and manure samples (1 to 4 g) is determined by Kjeldahl digestion that transfers the nitrogen containing compounds (proteins, amines, etc.) into ammonium compounds [32]. After the addition of bases, ammonia is released by distillation and titrated. The reaction is accelerated by Kjeldahl tablets (5 g) that contains sulfates and metallic salts. The sulfates increase the boiling point of the concentrated sulfuric acid (10 mL). The selenium, copper or titanium salts shorten the time of digestion. After a boiling period of at least 3 h, the distillation of the released ammonia follows. The distillate finally trapped in 50 mL boric acid (20 g L^{-1}) is titrated using a standard volumetric hydrochloric acid solution (0.1 mol L^{-1}) as well as an indicator solution (e.g., 200 µL mixed indicator No. 5). The total content of nitrogen expressed in g N kg^{-1} and rounded to one significant figure is calculated using the formula:

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

V_1: volume of hydrochloric acid used in the titration of the sample [mL]
V_0: volume of hydrochloric acid used in the blank test [mL]
m: mass of the excrement or manure sample [g]
c: concentration of hydrochloric acid [0.1 mol L^{-1}]
M_N: molar mass of nitrogen [14.01 g mol^{-1}].

Biological oxygen demand in 5 d (BOD_5)

30. The microbial activity of excrement and manure samples has to be checked before transformation tests in reference manures are conducted. For this purpose, the biological oxygen demand[§] can be determined [33]. Excrement and manure samples are diluted with varying volumes (Table 1) of tap water nearly saturated with oxygen and containing allylthiourea (2 mg L^{-1}) to suppress nitrification.

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

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

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

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

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

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

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

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

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

V_t : total volume [mL]

V_e : sample volume [mL]

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

PART II: ANAEROBIC TRANSFORMATION TEST IN REFERENCE MANURES

Equipment, instruments and chemicals

31. For transformation tests of chemicals in manure, the application of ^{14}C-labeled test substances is strongly recommended to set up mass balances differentiating between mineralization and the formation of extractable and non-extractable residues. A closed batch apparatus allowing for a discontinuous gas exchange can be used. This batch apparatus shown in Figure 1A (see Annex C), traces back to the biometer-type flask already mentioned in the OECD Guideline 304 [35] that has been slightly modified by the installation of an internal ^{14}C-carbon dioxide trap and additionally equipped with an external stripping device (Figure 1B; see Annex C). The latter allows for the gas analysis of the incubation flask´s headspace to check for the release of volatile metabolites.

32. Alternatively, the flow-through system mentioned in the OECD Guideline 307 [36] (Figure 2; see Annex C) can be used. Here, nitrogen is to be used as stripping gas introduced in stop-flow mode only because there is not any necessity for a continuous gas exchange in the transformation tests in manures under anaerobic conditions.

33. The vulnerability of both laboratory-test systems is the direct determination of ^{14}C-methane released out of the ^{14}C-labeled test substance under methanogenic conditions. In both laboratory test-systems, the ^{14}C-carbon dioxide free headspaces of the incubations flasks have to be transferred into a combustion apparatus where ^{14}C-methane is oxidized to ^{14}C-carbon dioxide. The latter is to be trapped again in an absorbing scintillation cocktail and then scintillation counted.

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

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

following:

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

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

Test conditions

37. Anaerobic conditions are to be ensured permanently. In the closed laboratory-batch system, nitrogen is rinsed directly after the test-substance application and directly before incubation for at least 5 min. This procedure is to be repeated for every gas exchange (for trapping of stripping gases see paragraph 31). Using the flow-through system, nitrogen is discontinuously introduced in stop-flow mode to maintain anaerobic conditions.

38. The temperature is to be maintained constant at 20 ± 2 °C to study the transformation of VMP and biocides in manure under standard laboratory conditions. Since the temperature in manure tanks is dependent on ambient conditions, transformation tests in manure can be optionally carried out at lower temperature, too (e.g., 10 °C, 5 °C).

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

Test substance application

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

solved in water or, when necessary, in minimum amounts of organic solvents in which the test substance is sufficiently soluble and stable. However, the amount of the selected solvent should not have any relevant effect on manure inherent microorganisms. In order to ensure an even active substance distribution in the samples, the solvent volume should be 40 to 75 µL per sample [36].
The use of solvents which inhibit the microbial activity, such as dimethyl sulfoxide, chloroform, dichloromethane and other halogenated solvents, should be avoided. If this is not possible, the test substance can also be added as a solid, e.g., mixed in quartz sand. If the test substance is added using a solvent, the solvent should be allowed to evaporate before the spiked carrier is added to the sample [36].

41. For the adjustment of the applied amounts of manure and radiotracer, the substance specific exposure assessment as well as the analytical feasibility mainly defined by the specific radioactivity of the radiotracer under study is to be taken into account. The applied concentration should be based on the substance specific exposure assessment of the chemical under study [37]. If the corresponding detection limit is not achievable, the concentration may be enlarged up to a factor of 10.

Performance of the transformation tests in manure

42. About 50 to 100 g manure (fresh weight basis) are placed into each incubation flask of the laboratory-test systems illustrated in Figure 1 or 2 (see Annex C) and the test substance is applied as described in paragraph 39. For each sampling time point separate sample flasks and additional control flasks are prepared in at least triplicate (duplicate samples for analytical purposes of the test compound, one sample for matrix characterization). The minimal set of flasks should allow sampling on days 0, 1, 3, 7, 30, 72 and 100. Optionally, the test can be continued up to day 180.

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

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

44. The manure samples are to be extracted exhaustively. In preliminary tests, therefore, the extraction efficiency of solvents of different polarity (sequential extraction technique) and of different extraction procedures[**] has to be investigated for every test substance.

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

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

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

DATA AND REPORTING

Treatment of results

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

For ^{14}C-labeled test substances, the mass balance should differentiate between the formation of carbon dioxide or methane and other volatile compounds as well as the formation of extractable and non-extractable residues.

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

TEST REPORT

47. The report must include:

Test substance:

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

Excrements and manure:

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

Test conditions:

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

Results of excrement and manure characterization:

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

Excrements: dry substance.

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

Results of transformation experiments:

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

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

- tables of results expressed as % of applied initial dose and, if appropriate, as mg kg^{-1} manure (on a fresh weight basis),
- mass balances until the end of the studies,
- characterization of non-extractable radioactivity or residues in manures,
- quantification of released $^{14}CO_2$, gaseous and volatile transformation products,
- plots of the concentrations for the test substance and, where appropriate, for relevant transformation products in manure versus time,
- half-life or DT_{50} (DT_{90}, if possible) for the test substance and, where appropriate, for relevant transformation products (> 10%) including confidence limits and parameters on goodness of fit of the kinetic models (i.e., error level chi^2, t-test, r^2),
- proposed pathways of transformation, where appropriate,
- discussion and interpretation of results.

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Annex A

Definitions

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

Extractable residues (ER) represent compounds occurring in the organic solvent used for extraction of as parent compound or metabolite.

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

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

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

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

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

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

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

Reference manures are excrement samples to that water is added to adjust defined

dry substance contents typical for bovine or pig manures.

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

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

Annex B

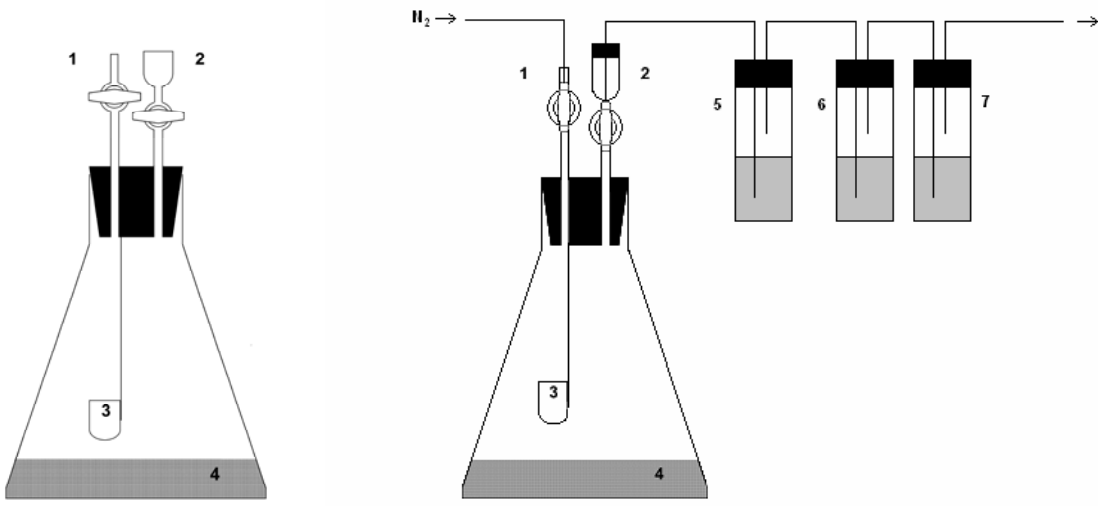
Methods of the matrix characterization of excrement and manure samples [26-33]

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

Annex C

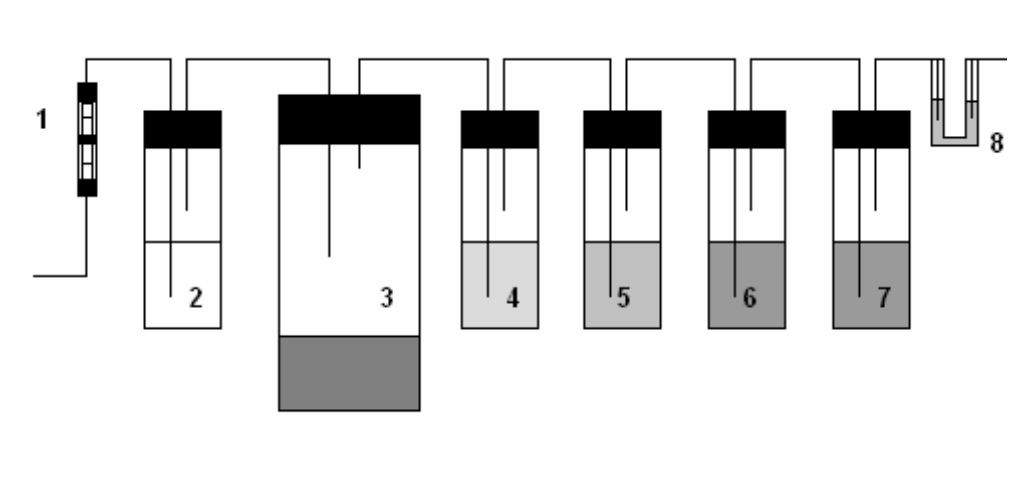
Laboratory-test systems

A B



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

Figure 1: Laboratory-batch system for transformation tests of ^{14}C-labeled test substance in liquid manures. A: without and B: with additional stripping device [39, modified in accordance to 35]



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

Figure 2: Flow-through system for transformation tests of ^{14}C-labeled test substance in liquid manures [36]