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Development of a test protocol to study the transformation of veterinary pharmaceuticals and biocides in liquid manure

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Development of a test protocol to study the transformation of veterinary pharmaceuticals and biocides in liquid manure

by

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Kurzbeschreibung

Ein Haupteintragspfad von Tierarzneimitteln in die Umwelt ist die Ausbringung von Gülle behandelter Tiere auf landwirtschaftliche Nutzflächen. Dieser Eintragspfad spielt daher auch in der Umweltbewertung von Tierarzneimitteln im Rahmen des Zulassungsverfahrens eine Rolle. Biozide, die zur Stalldesinfektion genutzt werden, gelangen ebenfalls über diesen Weg in die Umwelt. Für die Transformation von Stoffen in Gülle gibt es jedoch bisher keine experimentelle Anleitung zur Durchführung von Studien im Rahmen einer Bewertung von Risiken und Gefahren für die Umwelt. Ein Leitfaden der Europäischen Arzneimittelagentur (EMA), die „Guideline on determining the fate of veterinary medicinal products in manure“ (EMA/CVMP/ERA/ 430327/2009 (revised in 2011)) gibt Hinweise zur regulatorischen Auswertung der Studien und einigen Rahmenbedingungen, eine experimentelle Anleitung, wie sie z.B. für Böden oder Wasser-Sedimentsysteme im Rahmen der OECD Prüfrichtlinien existieren, fehlt jedoch für Studien zur Transformation in Gülle.

Dieses Vorhaben hatte zum Ziel, eine Methodik zur Erfassung der Transformation von Stoffen in Gülle zu entwickeln. Dabei wurde in einem ersten Schritt die Heterogenität der Gülle verschiedener Herkunft untersucht und die Möglichkeit homogene Proben aus einem Gülletank zu entnehmen. Eine repräsentative Probennahme in Gülletanks erwies sich als möglich, wenn in den Tanks oder auf den Höfen verfügbare Mischeinrichtungen zur Homogenisierung vor Probenahme genutzt wurden. Um einen Überblick über die Variabilität möglichst unterschiedlicher Güllen (Schweine-/Rindergülle, Winter-/Sommergülle, unterschiedliche Herkunftsregionen und Tierhaltungsbedingungen) zu bekommen, wurden 30 verschiedene Güllen beprobt und einer Matrixcharakterisierung unterzogen. Es wurde ebenfalls eine Temperaturmessung in den Gülletanks vorgenommen um die Wahl einer möglichst realitätsnahen Testtemperatur abzusichern. Sechs verschiedene Güllen wurden ausgewählt, um mit drei verschiedenen Testsubstanzen (Salizylsäure und Paracetamol als Tierarzneimittelwirkstoffe sowie ein Biozidwirkstoff) vergleichende Transformationsstudien durchzuführen. Die entwickelte Methode wurde einem ersten Interlaborvergleich unterzogen, an dem fünf Labore teilnahmen. Die Erfahrungen der Teilnehmer wurden auf einem Workshop vorgestellt und diskutiert und die entwickelte, detaillierte Testanleitung anschließend anhand der Vorschläge angepasst.

Abstract

For veterinary medicinal products (VMP) manure application onto agriculturally used soils is their main path of entry into the environment. Consequently, this path is considered as part of the environment risk assessment within the VMP marketing authorization process. Biocides which are used for stable disinfection treatments might also enter the environment via liquid manure. So far, only regulatory guidance (Guideline on determining the fate of veterinary medicinal products in manure (EMA/CVMP/ERA/ 430327/2009 (revised in 2011)) addresses this issue. However, an experimental guidance is still missing as it is available in form of OECD test guidelines for testing fate in soils and water-/sediment systems.

It was the aim of the project to develop a method to determine the transformation of substances in liquid manure. In a first step, the heterogeneity of manure of different type and origin was tested. Furthermore, a procedure was tested which is capable to take samples homogeneously from a manure storage tank. Obviously, representative sampling is possible in case mixing devices which are routinely applied by the farmers are used prior to sampling.

In order to get an overview on the variability of liquid manure of different type and origin (pig-/cattle manure; manure sampled in summer and winter, from different regions and livestock breeding) 30 manures were sampled and characterized. Furthermore, temperature (including a temperature profile) in manure tanks was reported in order to select a realistic test temperature.

Six manures were selected to perform transformation studies of two veterinary medicinal products (Salicylic acid, Paracetamol) and a biocide. The experimental methodology which was developed in the project was used in a first inter-laboratory comparison (ring test) with 5 laboratories. Experiences of the participants were presented and discussed on a workshop and based on the results the developed test protocol was adapted.

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Abkürzungen / Abbreviations

aR	applied radioactivity
BAY	Bayern (Bavaria)
cpm	counts per minute
d	day
DFOP	double first order in parallel kinetics
dm	dry matter
DT_{50}	time [d] needed for the disappearance of 50 % of the parent compound, disappearance time
DT_{90}	time [d] needed for the disappearance of 90 % of the parent compound, disappearance time
dpm	decays per minute
EMA	European Medicines Agency
ER	extractable residues
FOMC	first order multi compartment kinetics
Fw	fresh weight
HPLC	high performance liquid chromatography
HS	hockey stick kinetics
LSC	liquid scintillation counting
MIN	mineralization
NDS	Niedersachsen (Lower Saxony)
NER	non-extractable residues
NRW	Northrhine-Westphalia
PEC	predicted environmental concentration
SFO	single first order kinetics
SOP	standard operating procedure
TC	total carbon
TFA	trifluoroacetic acid
TLC	thin layer chromatography
TP	transformation product
VFA	volatile fatty acid
VMP	veterinary medicinal products

Summary

Background

For veterinary medicinal products (VMP) and biocides manure application onto agriculturally used soils is the main path of entry into the environment. Thus, respective guidance documents – such as Guideline on determining the fate of veterinary medicinal products in manure (EMA/CVMP/ERA/ 430327/2009 (revised in 2011)) – foresee experimental tests on the transformation of these substances in manure. The transformation studies will be used in registration processes and therefore it is crucial to have a harmonized design to be mutually acceptable and have to yield reliable, comparable results.

In 2011, the guideline EMA/CVMP/ERA/ 430327/2009 (revised in 2011) came into effect. This guideline was established by the European Medicines Agency (EMA) and provides a regulatory frame to assess the transformation of VMPs in manure. The term "manure" is used throughout this document to describe a mixture with high water content of about 90%- 95 % comprised of urine, faeces produced by animals housed in stables and water to clean the stables and potentially bedding material. The manure usually is stored before being spread on land. This report does not consider dung which is produced by pasture animals. Only manure from pigs and cattle housed in stables was examined in this project.

Technical details on how to measure endpoints such as mineralization, amount of non-extractable residues (NER), and disappearance time for half of the parent (DT_{50}-values) are not given in the EMA Guideline EMA/CVMP/ERA/ 430327/2009 (revised in 2011). Several aspects which might influence the outcome of transformation tests in manure are insufficiently known. These are among others: The influence of storage conditions, storage duration, origin of manure, and type of manure (pig, cattle) on manure parameters, and thus on the transformation capacity.

Questions addressed in the project

It was the aim of this project to develop a method to study the transformation of chemicals (veterinary pharmaceuticals and biocides) in manure (cattle and pig manure) and to start validation of the method.

In detail the following questions were addressed:

- Identification of manure parameter needed to comprehensively describe and characterize the respective manure.
- Influence of storage conditions and origin (regional origin and species) on manure parameters
- Development of a method to homogenize manure both in the manure storage tank and in the laboratory prior to performing the transformation study.
- Variability of transformation between manure of the same species but of different regional origin as well as between manure of different species.
- Experimental variability between a series of studies using different test substances and cattle and pig manure, which are performed in the same laboratory (intra-laboratory-variability)
- Experimental variability between different laboratories (inter-laboratory- variability) using the same test substance.

Manure parameter for a comprehensive manure characterization

The "Guideline on determining the fate of veterinary medicinal products in manure" (EMA/CVMP/ERA/ 430327/2009 (revised in 2011)) requires the determination of the parameters: pH-value, dry matter content, organic matter content (% OM, total organic carbon (TOC) can additionally be measured), nitrogen content (total nitrogen and ammonium nitrogen), redox potential, temperature, and microbial activity.

To fulfill the requirements, dry matter content, organic matter content and nitrogen content were measured prior to the start of a transformation study. pH value, redox potential and temperature were measured on-site or – if this was not possible – directly after the sampled manure arrived in the laboratory, at start of manure acclimation, at the beginning and at the end of a transformation study. The microbial activity was routinely measured at start and end of a transformation study. As far as possible, standardized tests were used. In detail these were:

pH-value:	ISO 10390 (2005)
Dry matter (dm) content:	DIN EN 12880 (2001)
Loss of ignition / organic dry matter content:	DIN EN 12 879 (2001)
Total carbon (TC) content:	DIN EN 13137
Total nitrogen content (Kjeldahl):	DIN ISO 11261 (1997)
Ammonium nitrogen content:	ISO 5664 (1984)
Redox potential:	ISO 11271 (2002)
Microbial activity:	a) Reduction of DMSO to DMS (Griebler & Slezak (2001) b) Measuring the mineralization of a readily degradable ^{14}C-labelled compound (e.g. ^{14}C-glucose) under anaerobic conditions. The method has been adapted to measurements under anaerobic conditions and is based on the commonly used method to determine the biological activity by substrate induced respiration (SIR) (DIN / ISO 14240 – (2011)). c) Determination of microbial biomass (no microbial activity!) by the fumigation method (DIN ISO 14240 – 2 (1999). The active and the inactive biomass are determined, but false positive or false negative results are obtained quite often.

Influence of storage conditions and origin (regional origin and species) on manure parameters

Manure sampling campaign

As scarce information was available on regional and seasonal variability of manure parameters, a comprehensive manure sampling campaign was performed. Sampled manure was characterized with regard to the above mentioned matrix parameters. Manures for testing purposes were chosen such as to include a most diverse set of manures representing a wide range of different origins, farming conditions, and matrix parameters to obtain the worst-case variability in the transformation studies.

First, cattle manure was sampled as for cattle highest regional and seasonal variability was expected. Those regions which are of highest importance for cattle rearing in Germany were covered. In order to consider any possible seasonal influence due to differences in the feeding regime the manure sampling was performed at two different times: So called "summer manure" (sampled in April to November) and "winter manure" (sampled in December to March) was collected. By sampling in summer and in winter, also manure of

various storage periods (summer manure: up to two months, winter manure: up to six months) was investigated. Thus, seasonal influences and influences by different storage periods in the tank on the manure parameters were analyzed simultaneously. In total, summer manure and winter manure obtained from 10 different sites in Germany were subjected to characterization. Thereof, 2 sites were located in Bavaria, 2 sites in Lower-Saxony, 5 sites in North-Rhine-Westphalia, and 1 site was located in Hessen. For an anonymized presentation sampling sites were encoded by the state and a number, e.g. NRW_1, BAY_2 etc. If needed the suffix “c” or “p” further characterizes the manure as cattle or pig manure.

Secondly, pig manure was sampled. Though – compared to cattle manure – a regional and seasonal variability of the pig manure parameters should be less pronounced (due to perennial indoor housing of the pigs) it was attempted to collect pig manure from regions in Germany which are important for pig rearing. Three sites each in Bavaria and North-Rhine-Westphalia were selected. In order to obtain ten different samples for the variance analyses, some of the manure storage tanks were sampled on two or three occasions.

The samples were analyzed for the parameters dry matter content [%], organic matter content [% fw], ammonia nitrogen [mg/kg fw], total nitrogen [mg/kg fw], organic carbon content [% fw], pH-value, redox-potential [mV] and temperature [°C]. PH-value, redox-potential and temperature were measured on-site at the location of sampling whereas the other parameters were analyzed off-site, i.e. in the laboratory. Ten data points per parameter were measured in order to have sufficient data for the statistical evaluation

Storage of manure in the laboratory

Once the manure has been sampled from the storage tank it is transferred to the laboratory. There, a further storage might be necessary until the manure is used for a transformation study. The influences of storage duration and storage temperature on the microbial activity of the manure were examined. This was achieved by storing cattle manure for various time periods and at several temperatures followed by determining the mineralization/microbial activity using ^{14}C-glucose as an easily degradable substance after an incubation period of 7 days.

Statistical evaluation

Differences of several manure parameters, namely dry matter content, organic matter content, ammonia-nitrogen, total nitrogen and organic carbon content with respect to origin, duration of storage in the tank, and storage conditions (duration and temperature) in the laboratory were analyzed for homogeneity of variances and equality of the central moments. Testing on homogeneity of variances was by application of Levene's test (Levene (1960)). Levene´s test allows to test whether variances for two or more groups are equal (homogeneity of variance or homoscedasticity). If the p-value of Levene's test is less than the critical value, the null hypothesis of homogeneity of variances has to be rejected (i.e. there is a statistical significant differences in the variances). A significance level of 5 % was used (p-value < 0.05 considered significant). Testing on equal central moments was by applying the U-test. The U-test is a non-parametric test based on ranks. The U-test can only be applied to compare two populations. A significance level of 5 % was used (p-value < 0.05 considered significant).

Conclusions: Influence of storage conditions and origin (regional origin and species) on manure parameters

→ Manure sampling at any season is possible and the influence of storage time in the manure tank at the farm at the time point of sampling and temperature on manure parameters is negligible.

→ A comparison of the microbial activity (^{14}C-glucose mineralization for different storage temperatures of -20°C, + 4 °C and +20 °C) showed differences in mineralization. Preferably, the storage in the laboratory prior to use for a transformation study should be at test temperature.

→ A comparison of ^{14}C-glucose mineralization for different storage periods after sampling of 28, 63 and 105 d showed that the storage period after sampling has an influence on mineralization. Therefore, it is necessary to establish a maximum storage period to ensure comparable testing conditions. If sampling is not possible prior to the start of the study, a maximum storage period of 2 months in the laboratory prior to use for a transformation study is recommended.

→ A comparison of ^{14}C-glucose mineralization for the tested acclimation periods of 3 days and 21 days showed that the length of the acclimation (or pre-incubation) period influences the mineralization. Therefore it is recommended to use an acclimation period of 21 days in order to ensure comparable testing conditions.

Homogeneity of sampling

Scope

The methodology of sampling manure for testing purposes has to be a reliable and reproducible method. Otherwise, any further step of a transformation study might be questionable. In order to prove the homogeneity of the sampling procedure, 10 replicates of cattle manure each were sampled at the sites NRW_1c and NRW_2c, both in winter and in summer.

Sampling manure from tanks for testing purposes

Prior to collection, the liquid manure was homogenized by mixing in the respective manure storage tank. For mixing, the devices either installed in the tank or external devices (e.g. Kreutzkämper, mixing device Type E 102, SUMA Zapfwellen mixing device, Rührgigant Z3) were used. Mixing for one hour was done by the farmers themselves.

Liquid pig manure and cattle manure have to be handled differently: Pig manure should be stirred immediately before sampling as separation into liquid and solid phase easily occurs. This recommendation is based on experience communicated to us by the farmers. The fact of phase separation also can be seen when having a look at the tanks before mixing, immediately after mixing and one hour after mixing. Cattle manure can be stirred up to one day before sampling.

After mixing, the subsamples are collected from the tank by appropriate equipment, namely a ladle with a large beaker or a large beaker on a rope. The ladle with the beaker is put into the manure storage tank and turned slightly into various directions. Thereafter, the equipment is withdrawn from the tank and the manure filled into containers. The manure parameter temperature, redox-potential and pH-value are recorded. Thereafter, the containers are sealed with an outlet to allow for gas expansion and transferred to the laboratory.

The sampled replicates were analyzed for the parameters dry matter content [%], organic matter content [% fw], ammonium nitrogen [mg/kg fw], total nitrogen [mg/ kg fw] and organic carbon content [% fw].

Statistical evaluation

As described above, results were analyzed for homogeneity of variances and equality of the central moments.

Conclusion: homogeneity of sampling

→ A comparison of the matrix parameters for 10 independent replicates sampled from the same cattle manure tank showed excellent homogeneity. Coefficients of variation for dry matter content, organic matter content, ammonium and total nitrogen are well below 10% for the site NRW_2 and equal or below 15% for NRW_1. In comparison to the variability observed for 10 different manures (compared to 10 independently sampled replicates from one tank), coefficients of variation are considerably lower for the 10 replicates from the same tank. Thus, it can be concluded that the sampling methodology applied in this project is a suitable methodology to sample manure from tanks for testing purposes.

Experimental variability between a series of studies using different test substances and cattle and pig manure, which are performed in the same laboratory (intra-laboratory-variability)

Selection of manure for transformation studies

Out of the ten sites (cattle manure) and six sites (pig manure), three sites each were selected for manure sampling to be used in the transformation studies. Prior to the choice of the manures for transformation testing, matrix parameters dry matter content, organic matter content, ammonium nitrogen content, total nitrogen content, and organic carbon content were determined. Manures were chosen to represent a most diverse set for transformation testing purposes (different origins, different size/type of farms, different feed of the animals, etc.). Furthermore, the following was important for sampling site selection: Easy accessibility of the manure storage tank (short distance to the laboratory, excellent personal contact to the farmer) and regional distribution of sampling sites, consideration of regions of importance for cattle and pig rearing.

Based on all criteria, the sites NRW_1c, NRW_2c, BAY_2c (cattle manure), NRW_1p, NRW_2p, and BAY_2p (pig manure) were selected.

Set-up for performance of transformation tests

Adjustment of dry matter content

Before performing the transformation test, the manure was acclimatized to test conditions. Prior to the start of the acclimation period, the dry matter content of the manure was determined and adjusted to standardized values. The recommended dry matter content in cattle and pig manure is 10% ± 1% and 5% ± 1%, respectively (EMA, 2011). If the dry matter content of the original manure was below the recommended value, it was concentrated by careful centrifugation for 10 minutes at 740 x g. However, centrifugation does not only mean a removal of water but also of DOC and micro-organisms. Thus, the initial dry matter content should not be below 8% (cattle) or 3% (pig). If this is not the case, fresh manure should be collected. If dry matter content is too high, water (de-ionized water, bubbled with nitrogen for 30 min) should be added as needed.

Acclimation of manure

After the adjustment of the dry matter content, cattle manure was homogenized by gently mixing using a glass bar. Subsamples of 50 – 100 g (wet weight) each were directly filled into the incubation vessels which were used for the acclimation and transformation study. No additional measures to prevent introduction of oxygen were used during both processes, homogenization and filling of incubation vessels.

Flow through apparatus

Transformation tests may be conducted in a static or a flow-through system. For all transformation studies in this phase of the project a flow through apparatus was used.

The flow through apparatus is a gas tight system of incubation vessels and traps set in sequence. Humidified nitrogen is gently passed over the liquid manure sub-samples. At the gas inlet nitrogen is given with a slight excess. By having a T-junction, excessive gas can escape via a washing flask whereas the needed nitrogen is passed over the manure samples. By such a design back-flush can be avoided.

The nitrogen is passed over the samples at a flow rate of 50 – 200 mL/min. First, the nitrogen is bubbled through water in order to humidify the gas. Thereafter, the humidified gas is passed over the manure subsamples. Six replicates per sampling point are set in sequence. The vessels for the individual sampling points are set in parallel. Once the gas has passed over the sixth replicate it is bubbled through two adsorption traps in sequence containing 2 M NaOH. Traps are for sorbing the evolving $^{14}CO_2$ and other possibly occurring volatiles. Since the formation of $^{14}CH_4$ is expected in such an anaerobic system the gas is furthermore passed through an oven at 850°C. $^{14}CH_4$ is catalytically (CuO-catalyst + O_2 feeding to the tube) converted to $^{14}CO_2$ which again is trapped in a third NaOH trap.

Test temperature and light conditions

During the whole test period the manure samples were incubated in the dark at 20°C.

Anaerobic/methanogenic incubation conditions

Test on microbial activity of cattle and pig manure was performed under anaerobic conditions. It is not possible to relate a certain redox potential directly to anaerobic/methanogenic conditions. However, as a quality criterion Eh < -100 mV should never be exceeded. The redox potential was measured and recorded at termination of the test on biological activity.

Abiotic controls (sterile samples)

For information on the abiotic transformation of the test substance, sterile controls were included. Sampling of sterile controls should be according to the sampling schedule or at least once during and at the end of the study. However, in the present research project sterile controls were sampled at the end of the test only.

Manure was sterilized by autoclaving twice (15 min, 121 °C, 100 bar). In order to avoid foaming, vessels were pre-heated overnight at 100°C. The application solution was sterilized by passing over a sterile filter. Application of the test substance was under sterile conditions using a clean-bench. Thereafter, flasks were closed and kept closed carefully. Applied samples were incubated without connecting to the flow through. As no evolution of $^{14}CO_2$ or $^{14}CH_4$ was expected, samples were locked gastight till sampling.

Application of test substance

The test substance should be dosed into the manure at a concentration that reflects the maximum expected manure concentration. If this concentration is not sufficient for detection and identification of transformation products, the test may be conducted at increased substance start concentrations. However, excessively high concentrations potentially toxic to microorganisms should be avoided.

The test substances were dissolved in an appropriate solvent and added into the acclimated manure in the respective incubation vessels by thoroughly mixing while maintaining anaerobic conditions. This was

achieved by maintaining to pass the nitrogen stream over the samples during the application procedure. The volume of the solvent used for application should not exceed 1% v/v of the manure volume and – if possible – be water miscible. The suggested value of 1% v/v is in analogy to the value given in the OECD guideline 308 (Aerobic and anaerobic transformation in aquatic sediment systems, OECD (2002a)).

The required volume of stock solution was pipetted into the manure under simultaneous stirring using the pipette tip. As soon as the solution was evenly distributed in the manure the pipette remained in the manure. The pipette tip was left in the manure sample in order to avoid any losses since manure always sticks to the tip. Rinsing with water would decrease the dry matter content of the sample.

Test duration and sampling

Test duration did depend on the rate of transformation of the parent compound and transformation products. There should be enough sampling time points to unambiguously derive all required (kinetic) parameters for parent substance and transformation products. If the study is further prolonged, e.g. because increasing amounts of transformation products have been observed a test for microbial activity may be conducted at the beginning and end of the prolongation period. It might therefore be useful to have a further spare incubation vessel for this purpose.

In the course of the present research project six replicate incubation flasks were sacrificed per sampling point. This was needed to obtain a reliable data base for statistical analyses. In transformation studies for registration purposes at least duplicates should be sacrificed per sampling. Besides sampling directly after application, at 9 additional sampling points were included.

Measurement of $^{14}CO_2$, $^{14}CH_4$ and ^{14}C volatile fatty acids (VFA)

CO_2, CH_4 and volatile fatty acids, as a precursor of CH_4, are major volatile final transformation products which are expected from transformation under anaerobic conditions.

Quantification of trapped volatiles was by radio-counting (liquid scintillation counting, LSC) of aliquots of the trapping solutions. Furthermore, it was proven whether evolved $^{14}CO_2$ is purged quantitatively when passing the humidified nitrogen over the manure samples. This was verified by addition of HCl to the manure sub-samples in order to strip CO_2 (or HCO_3^- / CO_3^{2-}) being potentially dissolved in the manure matrix. Purging by addition of HCl was applied in case the amount of $^{14}CO_2$ exceeded the level of 10 % of the total radioactivity (TRR).

Humidified nitrogen was passed over the manure sub-samples at a rate in the range of approximately 50 – 200 mL/min. By such a constant N_2-stream evolved $^{14}CO_2$ is purged from the manure samples, transported and captured in traps 1 and 2 containing 2 M NaOH. Potentially formed $^{14}CH_4$ passes the NaOH traps. After the addition of oxygen it is catalytically (= CuO) oxidized in an oven at 850°C to form $^{14}CO_2$. The formed $^{14}CO_2$ is trapped in the NaOH-filled trap situated at the outlet of the oven. To verify that the radioactivity captured in the NaOH traps 1 and 2 is $^{14}CO_2$ and not from potentially also formed volatile fatty acids (VFA), $Ba^{14}CO_2$ precipitation of the radioactivity was conducted. The radioactive content in the supernatant after precipitation can be attributed to VFAs whereas the difference of radioactive content before precipitation minus radioactive content after precipitation can be attributed to evolved $^{14}CO_2$.

Clean-up of manure samples

At a specific time point, the manure samples were sacrificed by removing the incubation vessels from the flow-through system and cleaned-up directly after sampling without storage of the samples prior to clean-up

e.g., at -20°C. Thereafter, extraction was done by the use of an appropriate solvent or solvents of different polarity depending on the properties of the substance. The extraction was achieved by the addition of a sub-portion of the first extraction solvent to the incubation vessel, gently shaking the mixture and transferring it into a centrifuge tube. This procedure was repeated until the complete manure was removed from the incubation vessel. The manure-solvent mixture was shaken for 30 minutes. Thereafter, the mixture was centrifuged for 10 minutes at 739 x g. The supernatant, i.e. the first extract, was collected. Further extraction solvent was added to the pellet. The whole process was repeated twice. Extracts 1 – 3 were combined, counted for their radioactivity content and subjected to substance specific analysis.

The pellet was dried at room temperature. Aliquots were quantified by combustion and radio-assaying the evolved $^{14}CO_2$. In case non-extractable residues (NER) exceeding 10 [% aR] were observed, exhaustive extraction methods were applied additionally. These methods comprise e.g. pressurized liquid extraction (e.g. ASE®), reflux, soxhlet etc. with appropriate solvents. Usually, these solvents are identical to those used in the first extraction steps. According to experiments performed prior to the main tests it is advisable to apply the harsh extraction twice in order to exhaustively extract the residues. Extracts are not combined but radio-counted. In case the amount of radioactivity exceeds 5 [% aR] the extract should be subjected to substance specific analysis. Residues remaining after the last extraction step (non-extractable residues, NER) were quantified by combustion and radio-assaying the evolved $^{14}CO_2$.

Establishment of mass balance

A mass balance was determined and calculated for each sampling interval. This was done by radiocounting the phases and summing-up the amount of radioactivity given in [% of applied radioactivity; % aR] in the aqueous/organic extracts plus ASE® plus volatiles other than $^{14}CO_2$ plus + $^{14}CO_2$ + non-extractable residues (NER).

Measurements of test substance and transformation products

Amounts of the test substance and the transformation products at every sampling time were determined. In general, transformation products detected at ≥ 10% of the applied radioactivity at any sampling time should be identified. Transformation products occurring at ≥ 10 [% aR] are seen as major transformation products whose identification is one of the endpoint parameters in a transformation study.

However, in the course of the research project no substance identification was performed but a chromatographic characterization by co-chromatography of known possible transformation products (reference substances). In this project TLC only was applied since matrix interferences did not lead to interpretable results when using HPLC without further sample preparation.

Kinetic Modeling

Compounds were analyzed based on the recommendation of the Forum for the Co-ordination of pesticide fate models and their Use (FOCUS) degradation kinetics (FOCUS (2006)). In addition to the standard kinetics (SFO = Single First Order) FOCUS recommends three biphasic kinetics, which are often more suitable to describe the fate of substances than the traditional single first order degradation. For all transformation studies performed in the course of this R&D-project, chi^2-values of all kinetic models were compared and furthermore, a visual check of the graphs of all models was performed.

Statistical evaluation

Statistical evaluations were performed in order to obtain information on the influence of storage conditions (temperature and duration of storage in the tank) and origin of manure on substance transformation. The following endpoints were recorded from the tests: DT_{50}-values to describe the kinetics of the disappearance of the test substance, percentage of transformation products (TP), non-extractable residues (NER), and mineralization products (MIN; i.e. $^{14}CO_2$ plus $^{14}CH_4$) at the end of the study. Differences of the endpoints with respect to the type and origin of manure, and the duration of storage in the tank were analyzed for homogeneity of variances and equality of central moments.

Conclusions: Experimental variability between a series of studies using different test substances and cattle and pig manure, which are performed in the same laboratory (intra-laboratory-variability)

→ Comprehensive statistical analyses were applied testing whether manure of different type and origin yields significantly different results for the transformation studies. Several parameters were selected for these analyses. From the results it is obvious, that in nearly all cases significant differences were observed. That means, that manure of the same type (cattle or pig manure, respectively) sampled from different sites resulted in significant differences in NER, extractables, sum of transformation products and DTx-values.

→ A further analysis of original data of NER, ER, and mass balances over the incubation period showed that trends are comparable and also coefficients of variation are in the same range for the same parameter and stage of the incubation period. For cattle manure, COV were in the range of 0.4 – 100 % (all test compounds considered), and for pig manure the respective range was 0.6 – 158%. Transformation of Salicylic acid showed COV in the range of 0.4 – 100% (cattle and pig manure considered), for Paracetamol the values were between 0.8 – 100 %, and for Biocide B between 0.6 – 158.6 %.

→ High coefficients of variation mostly were observed for NER occurring in small quantities. COV of 100 % and 158.6 % were obtained for NER with arithmetic means between 1.8 – 7.8 [% aR]. This might be due to methodological difficulties when analyzing NER of small quantities. From this observation the conclusion is drawn, that NER in quantities below 10 [% aR] can be determined with a fairly high uncertainty only.

→ The comparison of single time point results for ER, NER and mass balance was only done for informative purposes, as the regulatory guideline (EMA, 2011) requires taking into account the levels of parent, TP and NER at the half maximal manure storage time (as a single time point value). It is in general not advisable to use single time point values to evaluate the variability but instead use parameters derived from fitting a kinetic model to the data and then compare the derived kinetic parameter (e.g. the SFO DT50 values). These values are more robust, because they integrate the different measurements during the time course of the experiment.

→ COV observed for DT50 values range from 0.04 – 0.968 for salicylic acid, 0.08 – 0.269 for acetaminophen, and 0.057 – 0.181 for Biocide B.

Experimental variability between different laboratories (inter-laboratory- variability, ring test) using the same test substance

In order to test the applicability in other laboratories and the clarity of the draft test method that has been developed, an international inter-laboratory comparison (pre-validation ring test) was organised.

For that purpose, twenty-five institutes (20 from Europe, 3 from Northern America and 2 from Asia) have been invited in January 2012 to take part in the ring test. In addition, an informative meeting has been organised in Berlin in the framework of the 6th SETAC World Congress in May 2012. Five institutes (4 from Europe, 1 from Northern America) registered for the ring-test.

Test method and test performance

As basis for the performance of the pre-validation ring test all participating institutes were provided with the then current draft test method and an evaluation sheet for documentation of the results. In addition, details for the performance of the pre-validation ringtest (e.g. test duration, sampling time points, test concentrations, radioactivity to be applied per test vessel, sterile controls, extraction procedures) were given prior to the start of the experiments.

Evaluation of ring test results

The ring test was evaluated on basis of ^{14}C-mass balance, NER-formation, and degradation kinetics (based on the recommendations of FOCUS degradation kinetics (FOCUS (2006) using SFO (Single First Order)-kinetics).

Statistical evaluation

DT_{50}-values were determined to describe the kinetics of the disappearance of the test substance. For DT_{50}-values arithmetic means, standard deviation and coefficients of variation (COV) were calculated. In addition, arithmetic means, standard deviation and coefficients of variation were determined for NER, mineralization and mass balances at termination of the studies.

Workshop

A two-day workshop on the pre-validation ring test was held at ECT Oekotoxikologie GmbH, Flörsheim, Germany on April 18th/19th 2013. At the workshop results from method development were presented. One main focus was the discussion of results and experiences of the participants with the pre-validation ring test together with international experts. Furthermore, the current draft test method as well as necessary changes or problems were discussed. Hands-on experiments during the workshop were performed by technicians of several participants to ensure the use of the same techniques amongst all the participants, e.g. for test substance application. Every step of the method was discussed in detail to identify crucial points and to update the draft guideline accordingly to obtain higher reproducibility.

In particular, the following topics were identified as crucial points which might have an influence on the reproducibility and thus need further in-depth consideration and harmonisation:

- Duration of the test.
- Adjustment of dry matter content: centrifugation does not only mean a removal of water but also a removal of microorganisms and dissolved substances.
- Meaning of results from ^{14}C-glucose mineralization experiments.
- Suitability of the test design: flow-through versus static system.
- Homogeneity of manure samples before dividing into subs-samples for incubation.
- Replicates to be set in series or in parallel.

Conclusions: Experimental variability between different laboratories (inter-laboratory- variability, ring test) using the same test substance

→ Variability of the test results seems to be mainly caused by differences in the test design and test procedures at the different laboratories. For that reason, a more precise description of manure handling has been added to the protocol.

→ A prolongation of the test duration (e.g. up to 90 days) is required and already considered for further studies.

→ Adjusting the dry matter content seems to be a crucial point: Centrifugation does not only mean a removal of water but also of DOC and micro-organisms. Hence, a limit of minimum dry matter content was included in the draft test protocol which states: "If the dry matter content is below the recommended value, it can be concentrated by careful centrifugation (e.g. for 10 minutes at 740 x g). However, the initial dry matter content should not be below 8% (cattle) or 3% (pig). If dry matter content is too high, water (de-ionized water, bubbled with nitrogen for 30 min) should be added as needed".

→ In further studies the influence of different dry matter contents on the parameters mineralization, DT_{50}-values, and NER will be examined.

→ The outcome of ^{14}C-glucose mineralization as a measure of microbial activity of the manure seems not to be predictive for the test results with the active ingredient.

→ No final conclusion could be drawn on the suitability of the test design (static/flow-through systems). There are concerns about a too fast stripping of H_2 and CO_2 in a flow-through system. A static system does rather represent the real conditions in a manure storage tank and can be handled more easily by laboratories. It is recommended to prevent air entering the system during removal of single replicates. This might be done by using valves or a special set-up design (replicates for one sampling time point in series instead of in parallel). In further tests static systems will be compared to the flow-through system.

Overall conclusions

→ A homogeneity check of manure parameters determined for 10 replicates showed excellent homogeneity. Thus it can be concluded that it is feasible to use manures sampled directly from a tank for testing purposes and that the sampling methodology developed and applied in the project is suitable for this purpose.

→ A comparison of ^{14}C-glucose mineralization for different storage temperatures of -20°C, +4 °C and +20 °C showed that differences in mineralization cannot be neglected. Therefore storage in the laboratory prior to use for a transformation study should be at test temperature.

→ A comparison of ^{14}C-glucose mineralization for different storage periods after sampling of 28, 63 and 105 d showed that the storage period after sampling influences the mineralization. Therefore it is necessary to establish a maximum storage period to ensure comparable testing conditions. If sampling is not possible prior to the start of the study, a maximum storage period of 2 months in the laboratory prior to use for a transformation study is recommended.

→ A comparison of ^{14}C-glucose mineralization for the tested acclimation periods of 3 days and 21 days showed that the length of the acclimation (or pre-incubation) period influences the mineralization. Therefore it is recommended to use an acclimation period of 21 days in order to ensure comparable testing conditions.

→ The suggested and tested study design is applicable and can be handled for routine measurements of the transformation of veterinary medicines and biocides in manure.

→ Standard deviations for six replicates are low. Thus, the obtained results per sampling point are reliable. It is necessary to test manure species specific. A transfer of results from pig to cattle manure or vice versa is not possible.

→ Output from the pre-validation ringtest: variability of the test results seems to be mainly caused by differences in the test design and test procedures at the different laboratories. For that reason, a precise description of manure handling, especially for critical steps, is needed and the test protocol has been adapted with regard to further ring testing exercises.

→ A prolongation of the test duration (e.g. up to 90 days) is required and already considered for further ring test in the framework of the follow-up project.

→ Adjusting the dry matter content seems to be a crucial point: Centrifugation does not only mean a removal of water but also of DOM and microorganisms. If the dry matter content is below the recommended value of 10 ± 1% (cattle) and 5 ± 1% (pig), it can be concentrated by careful centrifugation (e.g. for 10 minutes at 740 x g). However, the initial dry matter content should not be below 8% (cattle) or 3% (pig). If this threshold is not reached, fresh manure should be collected. If dry matter content is too high, water (de-ionized water, bubbled with nitrogen for 30 min) should be added as needed. In further studies the influence of different dry matter contents on the parameters mineralization, DT_{50}-values, and NER will be examined.

→ The outcome of ^{14}C-glucose mineralization as a measure of microbial activity of the manure seems not to be predictive for the test results with the active ingredient.

→ No final conclusion could be drawn on the suitability of the test design (static/flow-through systems). There are concerns about a too fast stripping of H_2 and CO_2 in a flow-through system. A static system does rather represent the real conditions in a manure storage tank and can be handled more easily by laboratories. Maintaining anaerobic/methanogenic conditions is a prerequisite for an acceptable study. Therefore great care has to be taken to prevent air entering the system.

Zusammenfassung

Hintergrund

Für Tierarzneimittel und Biozide, die in der Tierhaltung eingesetzt werden, ist die Gülleausbringung auf landwirtschaftlich genutzte Flächen der Haupteintragspfad in die Umwelt. Von daher sehen entsprechende Leit- und Richtlinien (zum Beispiel: “Guideline on determining the fate of veterinary medicinal products in manure“ (EMA/CVMP/ERA/ 430327/2009 (revised in 2011) experimentelle Untersuchungen zur Transformation dieser Substanzen in Gülle vor. Die Transformationsstudien werden im Rahmen des Zulassungsprozesses benötigt. Von daher ist es notwendig, dass das entsprechende Testdesign allgemein akzeptabel ist und vergleichbare und verlässliche Ergebnisse liefert.

In 2011 trat die Richtlinie EMA/CVMP/ERA/ 430327/2009 (revised in 2011) in Kraft, die einen regulatorischen Rahmen zur Untersuchung der Transformation von Tierarzneimitteln in Gülle gibt. Der Begriff “Gülle” bezeichnet in diesem Dokument eine Mischung, mit 90-95% Wassergehalt, die aus Urin und Faeces von Stalltieren, sowie dem Wasser zum Reinigen der Ställe besteht. Die Gülle wird in der Regel in Tanks gelagert bevor sie auf die landwirtschaftlich genutzte Fläche aufgebracht wird. Der hier vorliegende Bericht betrachtet keinen Dung, der durch Weidetiere produziert wird, sondern ausschließlich Rinder- und Schweinegülle.

In der EMA-Richtlinie EMA/CVMP/ERA/ 430327/2009 (revised in 2011) werden technische Details wie beispielsweise die Durchführung der Messung der Endpunkte Mineralisierung, Menge nicht-extrahierbarer Rückstände (NER) und die Halbwertszeit für das Verschwinden der Ausgangssubstanz (DT_{50}-Werte) nicht beschrieben. Auch verschiedene Aspekte, die möglicherweise die Ergebnisse von Transformationsstudien beeinflussen können, sind nicht hinreichend bekannt. Dabei handelt es sich beispielsweise um: Einfluss der Lagerbedingungen, der Lagerdauer, des Ursprungs der Gülle und der Tierart auf die Gülleparameter und von daher auf die Transformationskapazität der Gülle.

Im Projekt behandelte Fragestellungen

Es war das Ziel des Projektes, eine Methode zu entwickeln, mit der die Transformation von Chemikalien (Tierarzneimittel und Biozide) in Rinder- und Schweinegülle untersucht werden kann. Im Einzelnen wurden folgende Fragestellungen betrachtet:

- Identifizierung von Gülleparametern, die benötigt werden, um Güllen umfassend zu beschreiben und zu charakterisieren.
- Einfluss von Lagerbedingungen und Ursprung (regionaler Ursprung und Tierart) auf Gülleparameter.
- Entwicklung einer Methode zur Homogenisierung der Gülle im Gülletank und im Labor vor Durchführung der Transformationsstudie.
- Variabilität der Transformationskapazität zwischen Güllen derselben Tierart aber aus unterschiedlichen Regionen und zwischen Güllen unterschiedlicher Tierarten.
- Experimentelle Variabilität von einer Serie von Studien mit verschiedenen Testsubstanzen in Rinder- und Schweinegülle, die im selben Labor durchgeführt werden (“intra-laboratory-variability”).
- Experimentelle Variabilität zwischen Studien mit derselben Testsubstanz, die in verschiedenen Testlaboren durchgeführt werden (“inter-laboratory- variability”).

Parameter für die Charakterisierung von Gülle

Die Richtlinie “Guideline on determining the fate of veterinary medicinal products in manure” (EMA/CVMP/ERA/ 430327/2009 (revised in 2011)) fordert die Bestimmung der Parameter: pH-Wert, Trockenmassegehalt, Gehalt an organischem Material (% OM), Stickstoffgehalt (Gesamtstickstoff und Ammoniumstickstoff), Redox-Potential, Temperatur und mikrobielle Aktivität. Um diese Anforderungen zu erfüllen, wurden im Rahmen des Projektes der Trockenmassegehalt, der Gehalt an organischem Material, und der Stickstoffgehalt zu Beginn der Transformationsstudie bestimmt. pH-Wert, Redox-Potential und die Temperatur wurden on-site gemessen oder – falls dies nicht möglich war – unmittelbar nach Eintreffen der Proben im Labor, sowie zu Beginn der Akklimatisierung, und zu Beginn und am Ende der Transformationsstudie. Die mikrobielle Aktivität wurde zu Beginn und am Ende einer Transformationsstudie bestimmt. Soweit möglich wurden standardisierte Testverfahren eingesetzt. Im Einzelnen handelte es sich um:

pH-Wert:	ISO 10390 (2005)
Trockenmassegehalt (dm):	DIN EN 12880 (2001)
Glühverlust (Gehalt an organischem Material):	DIN EN 12 879 (2001)
Gesamkohlenstoffgehalt (TC)	DIN EN 13137
Gesamtstickstoffgehalt (Kjeldahl):	DIN ISO 11261 (1997)
Ammoniumstickstoffgehalt:	ISO 5664 (1984)
Redox-Potential:	ISO 11271 (2002)
Mikrobielle Aktivität:	a) Reduzierung von DMSO zu DMS (Griebler & Slezak (2001) b) Messung der Mineralisierung einer leicht abbaubaren, ^{14}C-markierten Substanz (z.B. ^{14}C-Glukose) unter anaeroben Bedingungen. Diese Methode wurde an anaerobe Bedingungen angepasst und basiert auf der Methode der Messung der biologischen Aktivität durch Substrat-induzierte Atmung (SIR) (DIN / ISO 14240 – (2011)). c) Bestimmung der mikrobiellen Biomasse (nicht der mikrobiellen Aktivität!) durch die Fumigationsmethode (DIN ISO 14240 – 2 (1999). Bei dieser Methode werden die aktive und die inaktive Biomasse bestimmt, wodurch es zu falsch-positiven und falsch-negativen Ergebnissen kommen kann.

Einfluss von Lagerbedingungen und Ursprung (Region und Tierart) auf Gülleparameter

<u>Probenahme-Kampagne</u>

Da nur begrenzte Informationen zur regionalen und saisonalen Variabilität von Gülleparametern vorlagen, wurde eine umfassende Probenahme-Kampagne durchgeführt. Die Gülleproben wurden mithilfe der oben genannten Parameter charakterisiert. Die für anschließende Testzwecke eingesetzten Güllen wurde so ausgewählt, dass sie ein möglichst breites Spektrum an Ursprung, Haltungsbedingungen der Tiere (konventionelle oder biologische Landwirtschaft) und Gülleparametern abdeckten.

Zunächst wurde Rindergülle beprobt, da für Rinder die höchsten regionalen und saisonalen Unterschiede erwartet wurden. Dabei wurden die Regionen in Deutschland abgedeckt, in denen Milchviehhaltung eine Rolle spielt. Um mögliche saisonale Unterschiede aufgrund unterschiedlicher Fütterungen zu erfassen, wurden Probenahme-Kampagnen zu zwei verschiedenen Zeiten durchgeführt: es wurde sogenannte Sommergülle (Probenahmezeitraum April bis November) und Wintergülle (Probenahmezeitraum Dezember

bis März) beprobt. Indem sowohl im Sommer als auch im Winter Probenahmen stattfanden, wurden gleichzeitig unterschiedliche Lagerdauern (Sommergülle: bis zu zwei Monate, Wintergülle: bis zu sechs Monate) berücksichtigt. Von daher wurden gleichzeitig saisonale Einflüsse und Einflüsse durch unterschiedliche Lagerdauern auf die Gülleparameter berücksichtigt. Sommer- und Wintergüllen von zehn verschiedenen Standorten in Deutschland wurden einer Charakterisierung unterzogen. Zwei Standorte befanden sich in Bayern, zwei in Niedersachsen, fünf in Nordrhein-Westfalen und einer in Hessen. Zur anonymisierten Darstellung wurden die Probenahmestellen durch das Bundesland und eine Ziffer kodiert, zum Beispiel: NRW_1, BAY_2 etc. Falls notwendig wurde das Suffix “c” bzw. “p” angefügt, um die Gülle als Rindergülle (“c”, cattle) bzw. Schweinegülle (“p”, pig) zu bezeichnen.

Außer der Rindergülle wurde Schweinegülle beprobt. Obwohl im Vergleich zur Rindergülle aufgrund der ganzjährigen Stallhaltung der Schweine regionale und saisonale Unterschiede geringer sein sollten, wurden Güllen aus verschiedenen Regionen in Deutschland gesammelt, in denen Schweinezucht eine Rolle spielt. Es wurden jeweils 3 Stellen in Bayern und Nordrhein-Westfalen ausgewählt. Um wiederum 10 verschiedene Proben für die Varianzanalysen zur Verfügung zu haben, wurden die Gülletanks zu unterschiedlichen Zeiten beprobt.

In den Proben wurden die Parameter Trockenmassegehalt [%], Gehalt an organischem Material [% fw], Ammoniumstickstoff [mg/kg fw], Gesamtstickstoff [mg/kg fw], organischer Kohlenstoffgehalt [% fw], pH-Wert, Redox-Potential und Temperatur bestimmt. Dabei wurden die Parameter pH-Wert, Redox-Potential und Temperatur vor Ort erhoben, während alle anderen Parameter im Labor analysiert wurden. Um ausreichenden Datenumfang für die statistische Varianzanalyse zur Verfügung zu haben, wurden die Gülletanks jeweils 10-fach beprobt.

Lagerung der Gülle im Labor

Nach Entnahme aus dem Tank wird die Gülle-Probe in das Prüflabor gebracht. Dort ist bis zur Durchführung der Transformationsstudie möglicherweise eine weitere Lagerung notwendig. Deshalb wurde im Projekt der Einfluss von Lagerdauer und Lagertemperatur auf die mikrobielle Aktivität der Güllen untersucht. Dies erfolgte durch Lagerung von Rindergülle bei unterschiedlichen Temperaturen und über unterschiedliche Zeiträume und anschließende Erfassung der mikrobiellen Aktivität durch Testung der Mineralisierung von ^{14}C-Glukose nach 7-tägiger Inkubation.

Statistische Analysen

Unterschiede von Parametern (Trockenmasse, Gehalt an organischem Material, Ammonium- und Gesamtstickstoff, organischer Kohlenstoffgehalt) bei Güllen unterschiedlichen Ursprungs und Lagerungsbedingungen im Tank und im Labor wurden auf Signifikanz hin analysiert. Die statistischen Analysen umfassten einen Test auf Varianzhomogenität und auf Gleichheit der Mittelwerte. Der Test auf Varianzhomogenität erfolgte mithilfe des Levene´s Tests (Levene (1960)). Der Levene´s Test ermöglicht die Testung, ob Varianzen von zwei oder mehreren Gruppen gleich sind (Varianzhomogenität). Falls der p-Wert des Levene´s Test kleiner als ein kritischer Wert ist, muss die Nullhypothese der Varianzhomogenität zurückgewiesen werden, das bedeutet, es gibt statistisch signifikante Unterschiede in den Varianzen. Im Vorhaben wurde ein Signifikanz-Level von 5 % genutzt, d.h. p-Werte $<$ 0.05 wurden als signifikant betrachtet. Der Test auf Gleichheit der Mittelwerte erfolgte mittels U-Test. Der U-Test ist ein parameterfreier Test, der auf Rängen basiert. Er kann nur angewandt werden, um zwei Populationen miteinander zu vergleichen. Auch hier wurde ein Signifikanz-Level von 5 % gewählt.

Schlussfolgerungen: Einfluss von Lagerbedingungen und Ursprung (regional und Tierart) auf Gülleparameter

→ Die Probenahme von Gülle ist zu jeder Zeit möglich. Der Einfluss von Lagerdauer im Tank und Temperatur im Tank auf die Gülleparameter ist vernachlässigbar.

→ Ein Vergleich der mikrobiellen Aktivität (^{14}C-Glukose-Mineralisierung) von Gülle, die bei -20°C, +4°C und +20°C gelagert war, zeigte Unterschiede in der Mineralisierung. Vorzugsweise sollte die Gülle im Labor vor Durchführung einer Transformationsstudie bei der Testtemperatur gelagert werden.

→ Ein Vergleich der mikrobiellen Aktivität (^{14}C-Glukose-Mineralisierung) nach Lagerung über einen Zeitraum von 28 d, 63 d und 105 d zeigte einen Einfluss der Lagerdauer auf die Mineralisierungsrate. Von daher ist es notwendig, eine maximale Lagerdauer festzulegen, um vergleichbare Testbedingungen zu erzielen. Sollte vor Durchführung der Transformationsstudie eine Lagerung im Labor notwendig sein, wird eine maximale Lagerdauer bis zu zwei Monaten empfohlen.

→ Ein Vergleich der mikrobiellen Aktivität (^{14}C-Glukose-Mineralisierung) nach Akklimatisierungsphasen von 3 Tagen und 21 Tagen zeigte, dass die Dauer der Akklimatisierungsphase einen Einfluss auf die Mineralisierungsrate hat. Von daher wird es empfohlen, eine Akklimatisierungsphase von 21 Tagen einzuhalten, um vergleichbare Testbedingungen zu erzielen.

Homogenität der Probenahme

Zielsetzung

Die Methode der Probenahme von Güllen zu Testzwecken muss verlässlich und reproduzierbar sein. Andernfalls wäre jeder weitere Schritt im Rahmen einer Transformationsstudie zu hinterfragen. Um die Homogenität der Probenahme-Prozedur zu überprüfen, wurden jeweils 10 Replikate von Sommer- und Wintergüllen (Rindergülle) an den Probenahmestellen NRW_1c und NRW_2c entnommen.

Tank-Entnahme von Gülle zu Testzwecken

Vor der Gülle-Entnahme wird die Gülle im betreffenden Tank homogenisiert. Dazu werden entweder bereits im Tank installierte oder externe Rührwerke eingesetzt (Beispiele: Kreutzkämper, Mischer Type E 102, SUMA Zapfwellen Mischer, Rührgigant Z3). Das Rühren erfolgte über einen Zeitraum von einer Stunde und wurde durch die Landwirte selbst durchgeführt.

Rinder- und Schweinegülle müssen unterschiedlich behandelt werden: da bei Schweinegülle leicht eine Phasentrennung auftritt, muss sie unmittelbar vor Probenahme gerührt werden, während es bei Rindergülle ausreicht, die Homogenisierung einen Tag vor Probenahme durchzuführen. Diese Aussage basiert auf Erfahrungen der Landwirte und wurde von uns so übernommen. Die Tatsache der Phasentrennung kann leicht beobachtet werden, wenn man vor, unmittelbar nach und eine Stunde nach der Durchmischung einen Blick in den Gülletank wirft.

Nach Durchmischung wurden die Replikate durch eine geeignete Vorrichtung, nämlich einen Eimer an einer Stange oder einer Kette, entnommen. Die Stange mit Eimer wurde in den Gülletank getaucht, leicht in verschiedene Richtungen bewegt und anschließend nach oben gezogen. Dann wurde die Gülleprobe in einen Vorratseimer gefüllt und die Parameter pH-Wert, Redox-Potential und Temperatur bestimmt. Anschließend wurde der Eimer verschlossen, mit einem Entlüftungsrohr versehen, damit entstehende Gase entweichen können, und in das Prüflabor gebracht. Die Replikate wurde auf folgende Parameter hin analysiert:

Trockenmasse [%], Gehalt an organischem Material [% fw], Ammonium-Stickstoff [mg/kg fw], Gesamt-Stickstoff [mg/ kg fw] und organischer Kohlenstoffgehalt [% fw].

Statistische Auswertung

Wie bereits oben beschrieben wurden die Ergebnisse in Hinblick auf Varianzhomogenität und Gleichheit der Mittelwerte getestet.

Schlussfolgerung: Homogenität der Probenahme

→ Ein Vergleich der Matrix-Parameter für 10 unabhängige Replikate aus demselben Rindergüllen-Tank zeigte ausgezeichnete Homogenität. Die Variationskoeffizienten für Trockenmasse, Gehalt an organischem Material, Ammonium-Stickstoff, Gesamt-Stickstoff und organischer Kohlenstoffgehalt lagen deutlich unter 10 % für die Probenahmestelle NRW_2c, und bei oder unter 15% für die Probenahmestelle NRW_1. Im Vergleich zu den Variationskoeffizienten, der für Güllen von 10 verschiedenen Probenahmestellen erhalten wurden, liegen die Variationskoeffizienten der 10 Replikate aus einem Tank deutlich niedriger. Von daher kann die Schlussfolgerung gezogen werden, dass die beschriebene Methode zur Probenahme für Testzwecke geeignet ist.

Experimentelle Variabilität zwischen Transformationsstudien mit verschiedenen Testsubstanzen und Güllen (Rind und Schwein), die in einem Labor durchgeführt wurden ("Intra-Labor-Variabilität")

Auswahl der Güllen für Transformationsstudien

Von den 10 Probenahmestellen (Rindergülle) bzw. 6 Probenahmestellen (Schweinegülle) wurden jeweils 3 zur Durchführung der Transformationsstudien ausgesucht. Zuvor waren die Güllen in Hinblick auf die Matrix-Parameter (Trockenmasse [%], Gehalt an organischem Material [% fw], Ammonium-Stickstoff [mg/kg fw], Gesamt-Stickstoff [mg/ kg fw] und organischer Kohlenstoffgehalt) untersucht worden. Die betreffenden Probenahmestellen wurden dahingehend ausgewählt, dass die Güllen ein möglichst repräsentatives Spektrum abdecken (Ursprung der Gülle, Größe / Typ der Farm, Fütterung etc.). Darüber hinaus waren wichtig: leichte Zugänglichkeit des Gülletanks, kurze Entfernung zum Testlabor, persönliche Kontakte zum Landwirt, Auswahl von Regionen, die für Rinder- bzw. Schweinehaltung typisch sind.

Basierend auf diesen Kriterien wurden ausgewählt: Probenahmestellen NRW_1c, NRW_2c, BAY_2c (Rindergüllen), NRW_1p, NRW_2p, und BAY_2p (Schweinegüllen).

Vorgehensweise zur Durchführung der Transformationsstudie

Einstellung des Trockengewichts

Vor Durchführung einer Transformationsstudie wird die Gülle an die Testbedingungen akklimatisiert. Vor Beginn der Akklimatisierungsphase wird das Trockengewicht bestimmt und gegebenenfalls an die Standardwerte angepasst. Die empfohlenen Werte liegen für Rindergülle bei 10% ± 1% und für Schweinegülle bei 5% ± 1% (EMA, 2011). Falls der Trockenmasse-Gehalt der Original-Gülle unterhalb des empfohlenen Wertes liegt, wird die Gülle durch vorsichtiges Zentrifugieren (10 Minuten, 740 x g) aufkonzentriert. Es ist zu beachten, dass mit dem Zentrifugieren nicht nur Wasser, sondern auch DOC und Mikroorganismen entfernt werden. Von daher sollte der ursprüngliche Trockenmasse-Gehalt nicht unter 8 % (Rind) bzw. 3 % (Schwein) liegen. Ist das nicht der Fall, sollte neue Gülle genommen werden. Ist der Trockenmasse-Gehalt zu hoch, kann die benötigte Menge Wasser (de-ionisiert, für 30 Minuten mit Stickstoff begast) zugefügt werden.

Akklimatisierung der Gülle

Nach Einstellung des Trockenmasse-Gehaltes wird die Rindergülle durch vorsichtiges Mischen homogenisiert. Das erfolgt mithilfe eines Glasstabes. Proben (50 – 100 g Feuchtgewicht) werden direkt in die Inkubationsgefäße gefüllt, die sowohl für die Akklimatisierung als auch die eigentliche Transformationsstudie genutzt werden. Keinerlei weitere Maßnahmen zum Ausschluss von Sauerstoff werden ergriffen.

Schweinegülle wird mithilfe eines Küchenmixers homogenisiert. Dabei wird durch Überleiten von Stickstoff gewährleistet, dass die anaeroben Bedingungen erhalten bleiben.

Durchfluss-Anlage

Transformationsstudien können in einem (semi)-statischen oder Durchfluss-System durchgeführt werden. Im vorliegenden Vorhaben wurden alle Tests in einer Durchfluss-Anlage durchgeführt.

Bei der Durchfluss-Anlage handelt es sich um ein gasdichtes System von Inkubationsgefäßen und Fallen, die hintereinander angebracht sind. Angefeuchteter Stickstoff wird vorsichtig über die Gülleproben geleitet. Am Gaseingang wird der Stickstoff mit einem leichten Überdruck aufgegeben. Durch Anbringen eines T-Stücks kann überschüssiges Gas über eine Waschflasche entweichen, während der benötigte Stickstoff über die Proben geleitet wird. Durch dieses Design kann ein Rückschlagen verhindert werden.

Der Stickstoff wird mit einer Flussrate von 50 – 200 mL/min über die Proben geleitet. Zunächst wird das Gas durch Wasser geführt, um es anzufeuchten. Danach wird es über die Gülleproben geleitet. Sechs Replikate sind in Serie geschaltet. Sobald das Gas über das sechste Replikat geleitet ist, wird es durch zwei Adsorptionsfallen geleitet, die 2 M NaOH enthalten. Auf diese Weise können $^{14}CO_2$ und mögliche andere flüchtige Verbindungen sorbiert werden. Da die Bildung von 14CH4 unter anaeroben Bedingungen nicht ausgeschlossen werden kann, wird das Gas bei 850 °C durch einen Ofen geleitet. Dabei wird $^{14}CH_4$ katalytisch (CuO-Katalysator + zugeführter Sauerstoff) zu $^{14}CO_2$ oxidiert, das wiederum in einer dritten NaOH-Falle sorbiert wird.

Testtemperatur und Lichtbedingungen

Über den gesamten Testzeitraum werden die Proben bei 20°C im Dunkeln gehalten.

Anaerobe / methanogene Inkubationsbedingungen

Die Tests in Rinder- und Schweinegülle werden unter anaeroben Bedingungen durchgeführt. Es ist nicht möglich, ein bestimmtes Redoxpotential unmittelbar anaeroben / methanogenen Bedingungen zuzuordnen. Jedoch sollte – als Qualitätskriterium – der Wert von Eh ≤ 100 mV nicht überschritten werden. Das Redoxpotential wird zu Beginn der Akklimatisierungsphase, nach Applikation und bei Beendigung des Tests gemessen und dokumentiert.

Abiotische Kontrollen (Sterilproben)

Um Informationen über abiotische Transformationsprozesse zu erhalten, werden Sterilproben angesetzt. Die Beprobung der Sterilproben sollte entweder entsprechend der Beprobung der nicht sterilen Proben oder einmal jeweils während und am Ende der Inkubationsphase erfolgen. Im vorliegenden Forschungsvorhaben wurden Sterilproben lediglich am Ende des Tests genommen.

Die Gülle wurde durch zweimaliges Autoklavieren (15 min, 121 °C, 100 bar) sterilisiert. Um ein Schäumen zu verhindern, wurden die Gefäße zuvor über Nacht bei 100 °C gehalten. Die Applikationslösungen wurden durch Filtrieren über ein Sterilfilter sterilisiert, die entsprechenden Substanz-Applikationen erfolgten unter einer Sterilbank. Anschließend wurden die Gefäße sorgfältig verschlossen aufbewahrt, ohne sie an die Durchfluss-Anlage anzuschließen. Da nicht mit einer $^{14}CO_2$ – oder $^{14}CH_4$ – Bildung zu rechnen war, wurden die Behälter bis zur Probenahme gasdicht verschlossen aufbewahrt.

Applikation der Testsubstanz

Die Testsubstanz sollte in der Menge appliziert werden, die der maximal zu erwartenden Konzentration in der Gülle entspricht. Falls diese Konzentration in Hinblick auf die Nachweisgrenze und die Identifizierung von Transformationsprodukten zu gering ist, kann der Test mit höheren Startkonzentrationen durchgeführt werden. In jedem Fall sind Konzentrationen in der Höhe zu vermeiden, die toxisch für Mikroorganismen sind.

Die Testsubstanz wird in einem geeigneten Lösungsmittel gelöst und unter sorgfältigem Rühren unter Erhalt der anaeroben Bedingungen zur akklimatisierten Gülle gegeben. Das kann dadurch erreicht werden, dass die Stickstoff-Überleitung während der Appliationsprozedur aufrecht erhalten bleibt. Das Volumen des Lösungsmittels sollte 1% v/v des Güllevolumens nicht überschreiten und das Lösungsmittel sollte – falls möglich – mit Wasser mischbar sein. Der vorgeschlagene Wert von 1% v/v orientiert sich an dem Wert, der in der OECD-Richtlinie 308 (Aerobic and anaerobic transformation in aquatic sediment systems, OECD (2002a)) angegeben ist.

Das erforderliche Volumen der Applikationslösung wird unter Rühren mit der Pipettenspitze in die Gülle gegeben. Sobald die Lösung gleichmäßig verteilt ist, verbleibt die Pipettenspitze in der Gülle, um durch nachträgliches Entfernen der Pipettenspitze das damit verbundene Entfernen von Feststoff zu vermeiden. Ein Spülen der Pipettenspitze mit Wasser würde den Trockenmasse-Anteil ebenfalls verändern.

Testdauer und Probenahme

Die Testdauer hing von der Transformationsrate der Ausgangssubstanz und der Transformationsprodukte ab. Es sollten ausreichend Probenahme-Zeitpunkte vorliegen, um alle erforderlichen (kinetischen) Parameter mit ausreichender Sicherheit zu erfassen. Sollte gegenüber der ursprünglichen Planung eine Testverlängerung notwendig sein – zum Beispiel aufgrund des Konzentrationsanstiegs eines Transformationsproduktes – ist die Testung der mikrobiologischen Aktivität zu Beginn und Ende der Verlängerungsphase angebracht. Es ist von daher zweckmäßig, Zusatz-Proben zur Bestimmung der mikrobiologischen Aktivität einzuplanen.

Im Rahmen des vorliegenden Forschungsvorhabens wurden 6 Replikate pro Probenahmezeitpunkt aufgearbeitet. Dies war notwendig, um eine verlässliche Datenbasis für die statistische Auswertung zu haben. In Transformationsstudien für Zulassungszwecke sollten mindestens zwei Replikate aufgearbeitet werden. Neben Probenahme unmittelbar nach der Applikation wurden 9 weitere Probenahmezeitpunkte eingeschlossen.

Messung von $^{14}CO_2$, $^{14}CH_4$ und ^{14}C flüchtigen Fettsäuren (VFA)

CO_2, CH_4 und flüchtige Fettsäuren als Vorläuferprodukte von CH_4 sind in anaeroben Transformationsstudien üblicherweise zu erwartende flüchtige Verbindungen. Die Quantifizierung nach Sorption in der betreffenden Falle erfolgt durch Auszählen von Aliquots am Flüssigszintillationszähler. Weiterhin wurde nachgewiesen, ob $^{14}CO_2$ quantitativ ausgetrieben wird, wenn Stickstoff über die inkubierten Proben geleitet wird. Dieser Nachweis wird dadurch erbracht, dass HCl zu den inkubierten Proben gegeben wird, wodurch CO_2 (oder HCO_3^- / CO_3^{2-}), das möglicherweise in der Güllematrix gelöst ist, ausgetrieben wird. Dieses Verfahren wurde immer dann angewandt, wenn die Menge an $^{14}CO_2$ den Wert von 10 % der applizierten Radioaktivität (% aR) überschritt.

Befeuchteter Stickstoff wurde mit einer Geschwindigkeit von etwa 50 – 200 mL/min über die Proben geleitet. Bei diesem konstanten N_2-Strom wurde gebildetes $^{14}CO_2$ aus den Gülleproben entfernt, transportiert und in den NaOH-enthaltenden Fallen 1 und 2 sorbiert. Möglicherweise gebildetes $^{14}CH_4$ wird nicht sorbiert, sondern – nach Zudosierung von O_2 – in einem Ofen bei 850 °C katalytisch (CuO-Katalysator) zu CO_2 oxidiert. Das gebildete ^{14}CO2 wird in einer NaOH-enthaltenden Falle am Ausgang des Ofens sorbiert. Um zu bestätigen, dass es sich bei der sorbierten Radioaktivität um 14CO2 handelt und nicht um flüchtige Fettsäuren, wird eine $Ba^{14}CO_2$-Fällung durchgeführt. Die nach Fällung im Überstand verbleibende Radioaktivität kann den flüchtigen Fettsäuren zugeordnet werden, während die Differenz zwischen Radioaktivität vor und nach der Fällung dem entstandenen $^{14}CO_2$ zugeordnet werden kann.

Aufarbeitung der Proben

Zu den Probenahmezeitpunkten wurden die Proben aus der Durchflussanlage entfernt und ohne weitere Zwischenlagerung sofort aufgearbeitet. Die Aufarbeitung erfolgte – in Abhängigkeit von der Identität der Testsubstanz und Transformationsprodukten – durch Anwendung eines geeigneten Extraktionsmittels oder einer Reihe von Extraktionsmitteln, die sich hinsichtlich ihrer Polarität unterscheiden. Die Extraktionen erfolgten durch Zugabe des ersten Extraktionsmittels, vorsichtiges Schütteln der Mischung und Überführung in ein Zentrifugengefäß. Die Prozedur wird solange wiederholt, bis die gesamte Gülle aus dem Inkubationsgefäß entfernt ist. Das Gülle-Lösungsmittel-Gemisch wird für 30 Minuten geschüttelt. Danach wird für 10 Minuten bei 739 x g zentrifugiert. Der Überstand, d.h. der erste Extrakt, wird entfernt und weiteres Extraktionsmittel zum Pellet gegeben. Die Prozedur wird zweimal wiederholt. Extrakte 1 – 3 werden vereinigt, die Gesamtmenge an Radioaktivität wird ausgezählt, und es wird eine substanz-spezifische Analytik durchgeführt.

Das Pellet wird nach dem letzten Extraktionsschritt bei Raumtemperatur getrocknet. Der Gehalt an Radioaktivität im Pellet wird bestimmt, indem Aliquots verbrannt werden und das entstehende $^{14}CO_2$ gemessen wird. Liegen nicht-extrahierbare Rückstände (NER) im Mengen von mehr als 10 [% aR] vor, wird zusätzlich eine erschöpfende Extraktion durchgeführt. Entsprechende Methoden sind zum Beispiel Flüssigextraktion unter erhöhtem Druck (ASE, accelerated solvent extraction), Kochen unter Rückfluss oder Soxhlet-Extraktion unter Nutzung eines geeigneten Lösungsmittels. Üblicherweise ist das Lösungsmittel identisch zum dem, das im vorangegangenen Extraktionsschritt eingesetzt wurde. Erfahrungsgemäß ist es angebracht, die erschöpfende Extraktion zweimal durchzuführen, um zu einer möglichst weitgehenden Extraktion zu gelangen. Die Extrakte werden nicht kombiniert, sondern einzeln ausgezählt. Falls die Menge an Radioaktivität 5 [% aR] überschreitet, sollten die Extrakte einer substanz-spezifischen Analytik unterzogen werden. Feste Rückstände nach der erschöpfenden Extraktion (nicht-extrahierbare Rückstände, NER) werden getrocknet und durch Verbrennung mit anschließendem Radioassay des entstandenen $^{14}CO_2$ quantifiziert.

Erstellung einer Massenbilanz

Eine Massenbilanz für jeden Probenahmezeitpunkt erstellt. Das erfolgt durch Auszählung der verschiedenen Phasen und anschließende Addition der Radioaktivität (angegeben als % aR) in den organisch / wässrigen Extrakten einschließlich ASE®-Extrakten plus flüchtige organische Verbindungen plus $^{14}CO_2$ plus $^{14}CH_4$ plus NER.

Messung von Testsubstanz und Transformationsprodukten

Zu jedem Probenahmezeitpunkt wurde die Gesamtmenge an Testsubstanz und Transformationsprodukten bestimmt. Grundsätzlich sollte jedes Transformationsprodukt, das zu irgendeinem Probenahmezeitpunkt in Mengen ≥ 10% der applizierten Radioaktivität auftritt, identifiziert werden. Transformationsprodukte mit ≥ 10 [% aR] sind als „Haupt-Transformationsprodukte“ anzusehen, deren Identität einer der Endpunkte einer Transformationsstudie ist.

Im Rahmen des vorliegenden Forschungsvorhabens wurden jedoch keine Substanzidentifizierungen durchgeführt. Vielmehr wurden durch Co-Chromatographie mit bekannten Transformationsprodukten (Referenzsubstanzen) Substanzcharakterisierungen durchgeführt. Weiterhin wurden ausschließlich dünnschichtchromatographische Analysen durchgeführt, da die Matrix Gülle ohne weitere Probenvorbereitung bei der HPLC zu nicht-interpretierbaren Ergebnissen führte.

Kinetische Modellierung

Die getesteten Verbindungen wurden auf Basis der Empfehlungen des „Forum for the Co-ordination of pesticide fate models and their Use“ (FOCUS) degradation kinetics (FOCUS (2006)) analysiert. Zusätzlich zur Standardkinetik (SFO = Single First Order) empfiehlt FOCUS den Einsatz von drei bi-phasischen Kinetik-Modellen, die häufig eher als das traditionelle SFO-Modell geeignet sind, das Verhalten einer Substanz zu beschreiben. Für alle Transformatiosstudien, die Laufe des Forschungsvorhabens durchgeführt wurden, wurden die chi^2-Werte aller kinetischen Modelle miteinander verglichen. Darüber hinaus wurden visuelle Checks der Graphen aller Modelle durchgeführt.

Statistische Auswertung

Statistische Auswertungen wurden durchgeführt, um Informationen über den Einfluss von Lagerbedingungen (Temperatur, Lagerdauer im Tank) und Ursprung der Gülle auf die Transformation einer Testsubstanz zu erhalten. Folgende Endpunkte wurden in die statistische Auswertung einbezogen: DT50-Werte zur Beschreibung der Kinetik des Verschwindens der Ausgangssubstanz, prozentuale Bildung [% der applizierten Radioaktivität] von Transformationsprodukten, nicht-extrahierbaren Rückständen und Mineralisierung (CO2 + CH4) am Ende der Studie. Unterschiede in den Endpunkten bei Güllen verschiedener Tierarten, unterschiedlichen Ursprungs und Lagerdauer wurden hinsichtlich Varianzhomogenität und Gleichheit der Mittelwerte getestet.

<u>Schlussfolgerungen: Experimentelle Variabilität zwischen einer Reihe von Studien, in denen verschiedene Testsubstanzen und Rinder- und Schweinegülle in einem Labor getestet worden sind (“intra-laboratory-variability”)</u>

→ Umfassende statistische Analysen wurden durchgeführt, um zu testen, ob Güllen unterschiedlichen Typs und Ursprungs signifikant unterschiedliche Ergebnisse von Transformationsstudien liefern. Verschiedene Parameter wurden für diese statistischen Analysen ausgewählt. Es ist offensichtlich, dass in nahezu allen

Fällen signifikante Unterschiede beobachtet wurden. Das bedeutet, dass Güllen von derselben Tierart (Rinder- oder Schweinegülle), die von verschiedenen Probenahmestellen stammt, signifikant unterschiedliche Ergebnisse für DT_{50}-Werte, Bildung von Transformationsprodukten, NER und extrahierbaren Rückstände (ER) am Ende der Studie liefern.

→ Eine weitere Analyse der Originaldaten von NER, ER und Massenbilanzen über den gesamten Untersuchungszeitraum zeigen, dass Trends vergleichbar sind und auch die Variationskoeffizienten (COV) für alle Parameter und Probenahmezeitpunkte im selben Bereich liegen. Für Rindergülle lagen die COV im Bereich von 0.4 – 100 % (alle Testsubstanzen), für Schweinegüllen im Bereich von 0.6 – 158%. Die Transformation von Salizylsäure zeigte COV im Bereich von 0.4 – 100% (Rinder- und Schweinegülle), von Paracetamol im Bereich von 0.8 – 100 %, und von Biozid B im Bereich von 0.6 – 158.6 %.

→ Hohe Variationskoeffizienten wurden vornehmlich für NER beobachtet, die in geringen Mengen auftraten. So wurden COV von 100 % - 158.6 % für die NER gefunden, deren arithmetische Mittelwerte zwischen 1.8 – 7.8 [% aR] lagen. Das ist möglicherweise auf methodische Schwierigkeiten bei der Quantifizierung von NER unter 10 [% aR] zurückzuführen.

Experimentelle Variabilität zwischen Laboren (Inter-Labor-Variabilität, "Vorvalidierungs-Ringtest"), die dieselbe Testsubstanz nutzen

Um die Anwendbarkeit der entwickelten Methode durch andere Testlabore und die Genauigkeit der Verfahrensbeschreibung zu überprüfen, wurde ein internationaler, sog. "Vorvalidierungs-Ringtest" organisiert. Der Ringtest wurde als "Vorvalidierungs-Ringtest" bezeichnet, da lediglich eine Testsubstanz untersucht wurde und darüber hinaus abgesehen von einem Labor alle dieselbe Rindergülle genutzt haben.

25 Institute (20 in Europa, 3 in Nordamerika und 2 in Asien) wurden im Januar 2012 eingeladen, am Vorvalidierungs-Ringtest teilzunehmen. Darüber hinaus fand ein Informations-Treffen am Rande des 6. SETAC World Congress im Mair 2012 in Berlin in May 2012 statt. Fünf Institute (4 in Europa, eines in Nordamerika) meldeten sich für den Vorvalidierungs-Ringtest an.

Testmethode und Testdurchführung

Als Grundlage für die Durchführung des Vorvalidierungs-Ringtest erhielten alle teilnehmenden Institute einen Entwurf der Testmethode sowie ein Excel-Sheet zur Dokumentation der Ergebnisse. Zusätzlich wurden weitere Informationen gegeben wie beispielsweise: Testdauer, Probenahmezeitpunkte, Testkonzentration, Menge an Radioaktivität pro Testgefäß, Sterilkontrollen und Extraktionsverfahren.

Auswertung des Vorvalidierungs-Ringtest

Der Ringtest wurde auf der Basis von 14C-Massenbilanz, NER-Bildung und Abbau-Kinetik (SFO-Kinetik basierend auf den Empfehlungen der FOCUS degradation kinetics group (FOCUS (2006)) ausgewertet.

Statistische Auswertung

DT_{50}-Werte wurden bestimmt, um die Kinetik des Verschwindens der Testsubstanz zu beschreiben. Für die DT_{50}-Werte wurden Mittelwerte, Standardabweichungen und COV berechnet. Desgleichen wurden

Mittelwerte, Standardabweichung und COV für NER, Mineralisierung und Massenbilanzen am Studienende berechnet.

Workshop

Zum "Vorvalidierungs-Ringtest" wurde am 18. / 19. April 2013 bei der ECT Oekotoxikologie GmbH in Flörsheim ein zweitägiger Workshop durchgeführt. Dabei wurden die Ergebnisse der Methodenentwicklung vorgestellt. Ein weiterer wichtiger Focus lag auf der Diskussion der Ergebnisse und Erfahrungen der Ringtest-Teilnehmer mit weiteren international anerkannten Experten. Darüber hinaus wurden die vorliegende Methodenbeschreibung sowie notwendige Änderungen der Methode oder Probleme bei ihrer Umsetzung besprochen. TechnikerInnen einiger Teilnehmer demonstrierten vor Ort ihre Vorgehensweise, um sicherzustellen, dass alle Teilnehmer dieselben Techniken einsetzen. Jeder Einzelschritt der Methode wurde ausführlich diskutiert, um kritische Punkte zu identifizieren und die vorliegende Methodenbeschreibung so zu modifizieren, dass eine höhere Reproduzierbarkeit erreicht wird.

Insbesondere die folgenden Aspekte wurden als kritische Punkte identifiziert, die möglicherweise Einfluss auf die Reproduzierbarkeit der Ergebnisse haben und von daher einer vertieften Betrachtung und verbesserten Harmonisierung bedürfen:

- Testdauer.
- Einstellung des Trockenmasse-Anteils: Zentrifugation hat nicht nur ein Entfernen des Wassers zur Folge, sondern bedeutet auch ein Entfernen von Mikroorganismen und gelösten Stoffen.
- Aussagekraft der Ergebnisse zur Bestimmung der mikrobiellen Aktivität durch Mineralisierung von ^{14}C-Glukose.
- Eignung des Testdesigns: Durchfluss-System gegenüber (semi)statischen Systemen
- Homogenität der Gülleprobe vor Aufteilung in Aliquots für die Inkubation.
- Serielle oder parallele Anordnung der Replikate.

Schlussfolgerungen: Experimentelle Variabilität zwischen Laboren ("Inter-laboratory-variability; "Vorvalidierungs-Ringtest") bei Nutzung derselben Testsubstanz

→ Die Variabilität der Testergebnisse scheint hauptsächlich auf Unterschiede im Testdesign und in der Testdurchführung durch die verschiedenen Labore zurückgeführt werden zu können. Aus diesem Grund ist eine präzisere Beschreibung des Umgangs mit der Gülle notwendig.

→ Eine Verlängerung der Testdauer (z.B. auf 90 Tage) ist gefordert und wird bereits in weiteren Studien angewandt.

→ Die Einstellung des Trockenmasse-Anteils scheint ein kritischer Punkt zu sein: durch Zentrifugation wird nicht nur Wasser, sondern auch DOC und Mikroorganismen entfernt. Von daher wurde eine Untergrenze für den Trockenmasse-Anteil in der Methodenbeschreibung folgendermaßen formuliert: "If the dry matter content is below the recommended value, it can be concentrated by careful centrifugation (e.g. for 10 minutes at 740 x g). However, the initial dry matter content should not be below 8% (cattle) or 3% (pig). If dry matter content is too high, water (de-ionized water, bubbled with nitrogen for 30 min) should be added as needed".

→ In weiteren Untersuchungen soll der Einfluss des Trockenmasse-Anteils auf die Mineralisierung, DT_{50}-Werte und NER überprüft werden.

→ Die Ergebnisse der ^{14}C-Glukose-Mineralisierung scheinen als Messgröße für die mikrobielle Aktivität nicht geeignet zu sein.

→ Es konnte keine abschließende Schlussfolgerung hinsichtlich der Eignung des Testdesigns (Durchfluss-System bzw. (semi)-statisches System) gezogen werden. Es wurden Bedenken dahingehend geäußert, dass ein zu schnelles Austreiben von H_2 und CO_2 im Durchfluss-System auftritt. Das (semi)-statische System spiegelt eher die tatsächlichen Bedingungen im Gülletank wider und sollte darüber hinaus leichter handhabbar sein. Es wird empfohlen, den Luftzutritt während der Entfernung von Replikaten zu vermeiden. Dies kann durch den Einsatz von Hähnen oder durch eine parallele anstelle der seriellen Anordnung der Replikate erfolgen. In weiteren Tests soll das statische System mit dem Durchfluss-System verglichen werden.

Gesamtschlussfolgerungen

→ Ein Homogenitäts-Check von Gülleparametern, die für 10 Replikate bestimmt worden waren, zeigte eine ausgezeichnete Homogenität. Daraus konnte die Schlussfolgerung werden, dass es möglich ist, Gülle für Testzwecke direkt aus einem Gülletank zu sammeln. Weiterhin wurde deutlich, dass die im Forschungsvorhaben entwickelte und angewandte Probenahme-Methode für den Zweck der Durchführung von Transformationsstudien geeignet ist.

→ Ein Vergleich der ^{14}C-Glukose-Mineralisierung durch Gülleproben, die bei Temperaturen von -20°C, +4 °C und +20 °C im Labor gelagert worden waren, zeigte, dass die Unterschiede nicht vernachlässigbar sind. Es wird empfohlen, die Gülle vor Durchführung einer Transformationsstudie bereits bei Testtemperatur zu lagern.

→ Ein Vergleich der ^{14}C-Glukose-Mineralisierung durch Gülleproben, die über Zeiträume von 28, 63 und 105 d gelagert worden waren, zeigt, dass die Unterschiede nicht vernachlässigbar sind. Es ist von daher notwendig, eine maximale Lagerdauer im Labor vor Durchführung einer Transformationsstudie festzulegen. Sollte die Durchführung des Tests unmittelbar nach Probenahme der Gülle nicht möglich sein, wird eine maximale Lagerdauer von einem Monat empfohlen.

→ Ein Vergleich der ^{14}C-Glukose-Mineralisierung durch Gülleproben, die über einen Zeitraum von 3 Tagen und 21 Tagen akklimatisiert worden sind, zeigt, dass die Akklimatisierungsdauer die Mineralisierung beeinflusst. Es wird von daher empfohlen, eine Akklimatisierungsdauer von 21 Tagen einzuhalten, um vergleichbare Testergebnisse bei der Transformationsstudie zu erzielen.

→ Das vorgeschlagene und getestete Studiendesign ist anwendbar und kann routinemäßig zur Testung der Transformation von Tierarzneimitteln und Bioziden in Gülle eingesetzt werden.

→ Standardabweichungen und Variationskoeffizienten für 6 Replikate pro Probenahmezeitpunkt sind niedrig. Das bedeutet, dass die erzielten Ergebnisse belastbar und verlässlich sind.

→ Es ist notwendig, Spezies-spezifisch zu testen. Eine Übertragung der Ergebnisse von Schweinegülle auf Rindergülle oder umgekehrt ist nicht möglich.

→ Ergebnis aus dem Vorvalidierungs-Ringtest: die Variabilität der Testergebnisse scheint hauptsächlich auf Unterschiede im Testdesign und in der Testdurchführung in den verschiedenen Laboren zurück zu führen sein. Aus diesem Grund ist eine präzise Beschreibung der Handhabung der Gülle und der kritischen Schritte notwendig. Das vorliegende Testprotokoll muss entsprechend der Erfahrungen in einem kommenden Ringtest angepasst werden.

→ Eine Verlängerung der Testdauer auf beispielsweise maximal 90 Tage ist erforderlich und wird bereits im Ringtest des Folgevorhabens umgesetzt.

→ Die Einstellung des Trockenmasse-Anteils scheint ein kritischer Punkt zu sein: durch Zentrifugation wird nicht nur Wasser, sondern auch DOM und Mikroorganismen entfernt. Falls der Trockenmasse-Anteil unterhalb der empfohlenen Werte von 10 ± 1% (Rindergülle) bzw. 5 ± 1% (Schweinegülle) liegt, kann durch vorsichtige Zentrifugation (z.B. für 10 Minuten bei 740 x g) aufkonzentriert werden. Allerdings sollte der Trockenmasse-Anteil nicht unter 8% (Rindergülle) bzw. 3% (Schweinegülle) liegen. Werden diese Richtwerte unterschritten, sollte frische Gülle geholt werden. Liegt der Trockenmasse-Anteil zu hoch, kann die entsprechende Menge Wasser (de-ionisiertes Wasser, für 30 Minuten Stickstoff durch leiten) zugegeben werden. In weiteren Untersuchungen wird der Einfluss des Trockenmasse-Anteils auf die Parameter Mineralisierung, DT_{50}-Werte und NER überprüft.

→ Die Testung der Mineralisierung von ^{14}C-Glukose scheint nicht geeignet zu sein, um als Maß für die mikrobiologische Aktivität einer Gülle, die in einer Transformationsstudie eingesetzt wird, zu gelten.

→ Es konnte keine abschließende Schlussfolgerung hinsichtlich der Eignung des Testdesigns (Durchfluss-System bzw. (semi)-statisches System) gezogen werden. Es wurden Bedenken dahingehend geäußert, dass ein zu schnelles Austreiben von H_2 und CO_2 im Durchfluss-System auftritt. Das (semi)-statische System spiegelt eher die tatsächlichen Bedingungen im Gülletank wider und sollte darüber hinaus leichter handhabbar sein. Die Einhaltung von anaeroben / methanogenen Bedingungen ist Voraussetzung für eine akzeptable Studie. Von daher sollte große Sorgfalt darauf verwendet werden, den Luftzutritt zu vermeiden.

1 Introduction

For veterinary medicinal products (VMP) and biocides manure application onto agriculturally used soils is the main path of entry into the environment. Thus, respective guidance documents – such as Guideline on determining the fate of veterinary medicinal products in manure (EMA, 2011) – foresee experimental tests on the transformation of these substances in manure. The transformation studies will be used in registration processes and therefore it is crucial to have a harmonized design to be mutually acceptable and have to yield reliable, comparable results.

In 2011, the guideline (EMA, 2011) came into effect. This guideline was established by the European medicines Agency (EMA) and provides a regulatory frame to assess the transformation of VMPs in manure. The term "manure" is used throughout this document to describe a mixture with high water content of about 90%- 95 % comprised of urine, faeces produced by animals housed in stables and water to clean the stables and potentially bedding material, which is stored before being spread on land. This report does not consider dung which is produced by pasture animals. Only manure from pigs and cattle was examined in this project.

Technical details on how to measure endpoints such as mineralization, amount of non-extractable residues (NER), and disappearance time for half of the parent (DT_{50}-values) are not given in EMA, 2011. Several aspects which might influence the outcome of transformation tests in manure are insufficiently known. These are among others:

- Influence of storage conditions, storage duration, and origin of manure on manure parameters, and thus on the transformation capacity,
- Influence of type of manure (pig, cattle) on transformation capacities,
- Adaption of technical and methodological details to the specifics of the matrix manure. These are for example: determination of microbial activity, determination of CO_2 and CH_4, determination of other typical volatile products such as volatile fatty acids.

When designing a test system for transformation of chemical substances in manure, specifics of the matrix have to be taken into account. Weinfurtner (2010) carried out a literature study to acquire information on the variability of the composition of different types of manure with regard to species and production type, and thus to gather information on the conditions that prevail in manure storage tanks. It was obvious from the literature study that the availability of data is highly variable. Information is sparse concerning housing systems and storage systems. From the available information, it was concluded that in the EU at least 50 % of produced manure is a (semi)-liquid manure (slurry) with a large regional variation. The storage time ranges between 1 and 2 months in summer and 3-6 months in winter and is mostly influenced by agricultural and regulatory requirements. Matrix parameters such as dry matter content, total N and NH_4-N are different for cattle and pig manure but do not differ within a species with exception of calf production. In cattle manure, a higher dry matter content and a lower total N and NH_4-N concentration as in pig manure was observed. The data indicate differences in matrix parameters for pig manure between northern and southern Europe because manures of southern Europe show lower dry matter contents and lower total N and NH_4-N concentrations. Seasonal influences on matrix parameters were pointed out by several authors, however, the main influence of season was observed on storage temperature. During storage a reduction of dry matter content and total nitrogen was observed by the most studies. For pig fattening an influence of diet on matrix parameters such as dry matter content, pH value and total N was observed. This information was taken into

account when designing the different approaches to investigate the influences of manure parameters on the transformation of a test substance.

It was the aim of this project to develop a method to study the transformation of chemicals (veterinary pharmaceuticals and biocides) in manure (cattle and pig manure) and to start validation of the method. The method was to be laid down in a detailed description including standard operation procedures (SOP). It furthermore was the aim to conduct a pre-validation ringtest (intra-laboratory comparison).

In detail the following questions were addressed:

- Variability of results (MIN, NER, DT50, TP) between manure of the same species but of different regional origin.
- Variability of results (MIN, NER, DT50, TP) between manure of different species (cattle and pig)
- Development of a method to homogenize manure both in the manure storage tank and in the laboratory prior to performing the transformation study. Conditions (redox-potential, pH-value) in the storage tank and in the incubation system should be as identical as possible. A Standard-Operation-Procedure (SOP) for manure sampling and preparation is needed.
- Development of an assay to determine the microbial activity; selection of an appropriate, easily degradable model substrate (e.g. ^{14}C-glucose, ^{14}C-salicylic acid). A Standard-Operation-Procedure (SOP) for such an assay is needed.
- Development of a method to determine the formation of $^{14}CO_2$ independent from pH-value of the manure and thus being independent from the possibility of trapping CO_2 in the matrix manure. A Standard-Operation-Procedure (SOP) for the quantification of CO_2-formation in manure is needed.
- Development of a method to quantify the formation of methane and of volatile fatty acids (VFA). A Standard-Operation-Procedure (SOP) for the quantification mineralization including CH_4-formation is needed.

2 Materials and Methods

2.1 Manure sampling site selection

Selection of ten sites for variance analyses of parameters

First, in order to answer the question whether obtaining homogenous manure samples from a tank is possible and to get an overview of the variability of manure matrix parameters like dry matter content, pH or redox conditions, cattle manure was sampled as for cattle highest regional and seasonal variability was expected. Those regions which are of highest importance for cattle rearing in Germany were covered. In order to consider any possible seasonal influence due to differences in the feeding regime the manure sampling was performed at two different times, in summer and in winter. In total, summer manure and winter manure obtained from 10 different sites in Germany were subjected to characterization. Thereof, 2 sites were located in Bavaria, 2 sites in Lower-Saxony, 5 sites in Northrhine-Westphalia, and 1 site was located in Hesse.

Selection of the individual farms was by personal contact to the farmers. Contacts either already existed (as the farmers were personally known to staff members of Fh-IME and Fh-UMSICHT) or were made first by telephone calls and thereafter by visits. Farmers were informed comprehensively about the aims of the research project. As most of the farmers were personally known no flyer was needed. However, for further sampling campaigns it might by advisable to have either a flyer or another hand-out. To convince them of cooperation it was most important to ensure that no veterinary pharmaceutical screening was made, and that the farms were anonymized by referring to the postal code and the Federal State only. Few farmers were interested in the results of their manure characterization, in particular in the nitrogen content. The selected sampling sites are coded in Table 1:

Table 1: Codes of the selected sampling sites for cattle manure collection

Coded area	Code used in the project
Southern Northrhine-Westphalian hill land	NRW_1c
Lower Rhine (right side)	NRW_2c
Southern Northrhine-Westphalian hill land	NRW_3c
Lower Rhine (left side)	NRW_4c
Munster land	NRW_5c
Southern Lower Saxony	NDS_1c
Southern Lower Saxony	NDS_2c
Vogelsberg-Kreis	He_1c
Upper Bavarian tertiary hill land	BAY_1c
Upper Bavarian tertiary hill land	BAY_2c

In order to obtain more background information on the sampling sites, which might be helpful to interpret the results of manure characterization, a questionnaire was developed. It is shown in annex 1 of the report. Detailed information regarding the administration of veterinary pharmaceuticals was not obtained, but general information on the time period since the last treatment was given. Also, the exact time period since last manure spraying onto the arable land as well as the estimate on how long it takes for excrements to reach the manure storage tank was either not or only roughly communicated. Mostly, excrements need 1 – 2 days

to reach the manure storage tank via the collection system. Last manure spraying had been in the range of 2 weeks to 6 months before sampling depending on the time point of our sampling: storage of winter manure was up to 6 months; storage of summer manure was in the range of 2 – 6 weeks (see also Table 4 for details). Apart from that information, Table 4 summarizes the information on stock, manure storage tank, last treatment (general information) and feeding. Results of on-site measurements of manure temperatures, pH-values and redox-potentials (the latter not given on the questionnaire) are presented in chapter 2.3 (manure characterization).

Secondly, pig manure was sampled. Though – compared to cattle manure – a regional and seasonal variability of the pig manure parameters should be less pronounced (due to perennial indoor housing of the pigs) it was attempted to collect pig manure from regions in Germany which are important for pig rearing. Three sites each in Bavaria and Northrhine-Westphalia were selected. In case no personal contact to the farmers already existed, it was hardly possible to obtain permission for manure sampling. This might be due to the fact that intensive animal husbandry – as it is the case for pig breeding and fattening – is a sensible topic of public concern. In order to obtain ten samples for the variance analyses, some of the manure storage tanks were sampled twice or three times. These were BAY_1p (collected twice at two subsequent days in 11/2011), BAY_2p (collected twice at two subsequent days in 11/2011), and NRW_2p (collected three times namely in 01/2012, 02/2012 and 06/2012). The sampling sites are coded in Table 2.

Table 2: Codes of the selected sampling sites for pig manure collection

Coded area	Code used in the project
Southern Northrhine-Westphalian hill land	NRW_1p
Lower Rhine (left side)	NRW_2p
Lower Rhine (left side)	NRW_3p
Upper Bavarian tertiary hill land *)	BAY_1p*)
Upper Bavarian tertiary hill land *)	BAY_2p*)
Upper Bavarian tertiary hill land	BAY_3p

*) two farms in the same region with identical postal codes

Apart from manure temperature, redox-potential and pH-values no further detailed information on the sites is given.

2.2 Collection, handling and storage of manure

Collection of manure

Prior to collection, the liquid manure was homogenized by mixing in the respective manure storage tank. For mixing, the devices either installed in the tank or external devices were used. Mixing for one hour proved sufficient for homogenization of manure in the tanks independent from tank volume. This was proven by the results of the homogeneity testing (see chapter 3.4). Mixing was done by the farmers themselves, not by the staff of Fraunhofer. Mixing devices and procedures were:

NRW_1c:

- Volume of manure tank: 100 m^3
- Mixing device: Kreutzkämper, mixing device Type E 102
- Duration of mixing: 1 hour
- Radius of mixing device: 56 cm
- Rotation speed: not known, mixing device is for agricultural tractors, 80 PS

NRW_2c:

- Volume of manure tank: 100 m^3
- Mixing device: SUMA Zapfwellen mixing device, Rührgigant Z3
- Duration of mixing: 1 hour
- Diameter of mixing device: 52 cm
- Rotation speed: not known, mixing device is for agricultural tractors, 60 PS

Figure 1 shows the mixing device and the mixing procedure; pictures were taken at sampling site NRW_1c.

Liquid pig manure and cattle manure have to be handled differently: Pig manure should be stirred immediately before sampling as separation into liquid and solid phase easily occurs. This recommendation is based on experience communicated to us by the farmers. The fact of phase separation also can be seen when having a look at the tanks before mixing, immediately after mixing and one hour after mixing. Cattle manure can be stirred up to one day before sampling.

After mixing, the subsamples were collected from the tank by appropriate equipment, namely a ladle with a large beaker or a large beaker on a rope. The ladle with the beaker was put into the manure storage tank and turned slightly into various directions. Thereafter, the equipment was withdrawn from the tank and the manure filled into containers.

*Figure 2*Figure 2 gives an impression on the sampling procedure. On-site, also the temperature, pH-value and redox-potential were measured. Figure 3 shows the sampling of liquid cattle manure at sampling site NRW_1c using a ladle with a beaker. Figure 4 shows the sampling of pig manure at sampling site NRW_1c using a beaker on a chain.

After filling the small containers with liquid manure were closed carefully but not air tight in order to allow evolving gas to expand, and directly transferred to the laboratory.

In case an immediate manure characterization was not possible, the sub-samples were stored at – 20°C.

Figure 1: Mixing of the manure on site by a farm specific mixer

Figure 2: manure sampling from tanks

Figure 3: Sampling of cattle manure

Figure 4: Sampling of pig manure

Filling up to approximately ¾ of maximum container volume was proven to be appropriate as the volume of the manure expands due to microbial activity. The containers were closed tightly allowing for expansion of gas, which is generated by continuous microbial activity. This was be achieved by connecting a tube with a fermentation air lock to an outlet in the container (see Figure 5).This also prevents odors from escaping from the containers.

On-site, ambient air temperature, manure temperature, redox-potential and pH-value of the liquid manure were measured and recorded.

Figure 5: Manure storage in the lab

Storage of liquid manure for testing purposes

Prior to further processing, manure might be stored. It was one of the aims of the research project to test the optimal storage conditions, namely duration and temperature. This was achieved by storing liquid cattle manure under various conditions and testing the microbial activity at certain sampling dates. For determination of the microbial activity see chapter 2.3. The results for microbial activity (expressed as % mineralization of ^{14}C-glucose after 7 days) were subjected to statistical significance analyses. Different storage periods (14 d, 28 d, 42 d, 56 d) and temperatures (-20°C , + 4°C and +20 °C) were compared.

Storage had to ensure anaerobic/methanogenic conditions. This was achieved by first passing nitrogen over the liquid manure and thereafter closing the container. Care had to be taken to allow gas, generated by biological activity during storage, to expand to avoid explosion of the container. This was achieved by connecting a tube with a fermentation air lock to an outlet in the container as described above (see Figure 5).

In Figure 6 several photographs are presented showing liquid cattle manure stored for 14 days, 2 months, 3 months and 6 months at 20°C.

Figure 6: Cattle manure at different storage times (2 weeks, top left side; 2 months, top right side; 3 months, down, right side; 6 months, down left side)

2.3 Manure characterization

The "Guideline on determining the fate of veterinary medicinal products in manure" (EMA, 2011) requires the determination of the parameters: pH-value, dry matter content, organic matter content (% OM, total organic carbon (TOC) can additionally be measured), nitrogen content (total nitrogen and ammonium nitrogen), redox potential, temperature, and microbial activity.

Dry matter content, organic matter content and nitrogen content were measured prior to the start of a transformation study. pH value, redox potential and temperature were measured on-site or – if this was not possible – directly after the sampled manure arrived in the laboratory, at start of manure acclimation, at the beginning and at the end of a transformation study. The microbial activity was routinely measured at start and end of a transformation study.

The parameters were measured as follows:

pH-value

The pH value was measured both, directly in manure after sampling at the sampling location (on-site) and in the laboratory (off-site). Respective tables later in the report show the on-site measured values. A standard equipment consisting of, e.g., pH meter (pH 320, WTW), pH-electrode (SenTix® 41, WTW) and integrated temperature probe was used. The pH value can be considered as stable when pH measured over a period of 5 seconds varies by not more than 0.02 units. The pH values are expressed with accuracy of 0.1 units (ISO 10390 (2005)).

Dry matter (dm) content

Manure samples (50 – 80 g) were dried in an oven at 105 ±5 °C to constant mass. The mass difference of a sample before and after the drying procedure was used to calculate the dry mass content that is expressed in percentage of the fresh weight with accuracy of ±0.1 % (DIN EN 12880 (2001)).

Loss of ignition / organic matter content

The dried manure samples were incinerated in a muffle furnace at 550 ±25 °C. The mass difference of a sample before and after incineration was used to calculate the organic dry mass content that is expressed in % dm with an accuracy of ±0.1 % (DIN EN 12 879 (2001)). The dry matter content is used to re-calculate the data to % fresh weight (fw). Results are given in % fw in the report.

Total carbon (TC) content

Total carbon content (as sum of total inorganic carbon, TIC, and total organic carbon, TOC) were analyzed using a CHN-analyzer (vario max CHN, elementar). The carbon present in dried manure is oxidized to carbon dioxide by heating up to at least 900 °C in an oxygen atmosphere. The released amount of carbon dioxide is measured by infrared detection. The TC is expressed in % dm with an accuracy of ±0.1 % (DIN EN 13137). The dry matter content is used to re-calculate the data to % fresh weight (fw). Results are given in % fw in the report.

Total nitrogen content

The total nitrogen content of wet manure samples (up to 6 g) was determined by Kjeldahl digestion (Turbotherm, Gerhardt) that transfers the nitrogen containing compounds (amines, proteins) into ammonium. The digestion process was accelerated by addition of concentrated sulphuric acid and 1 Kjeldahl tablet (Kjeltabs CX, Thompson & Capper Ltd.). After the digestion process, sodium hydroxide (30 % solution) was added and ammonia was released by distillation (Vapodest, Gerhardt). Ammonium was trapped in 50 mL boric acid (20 g/L) and was titrated using standard volumetric sulphuric acid solution (0.1 mol/L) and some drops of an indicator solution (mixed indicator 5, Merck). The total nitrogen content is expressed in mg/kg dm with accuracy of 1 mg/kg (DIN ISO 11261 (1997)). The dry matter content is used to re-calculate the data to % fresh weight (fw). Results are given in % fw in the report.

Ammonium nitrogen content

A wet manure sample (up to 5 g) was alkalized by adding of sodium hydroxide (30 % solution) and was distillated using an automatic distillation apparatus (Vapodest, Gerhardt). The released ammonia was trapped in 50 mL boric acid (20 g/L). Ammonium was titrated using standard volumetric sulphuric acid solution (0.1 mol/L) and some drops of an indicator solution (mixed indicator 5, Merck). The ammonium nitrogen

content is expressed in mg/kg with accuracy of 1 mg/kg (ISO 5664 (1984)). The dry matter content is used to re-calculate the data to % fresh weight (fw). Results are given in % fw in the report.

Redox potential

The redox potential is directly measured in manure sample after sampling on site. A standard equipment consisting of millivoltmeter (pH 320, WTW), redox-electrode (Pt4805-S7/120, Ingold) and integrated temperature sensor was used). The value of redox potential is expressed in mV with accuracy of 1 mV (ISO 11271 (2002)).

Microbial activity

The Guideline (EMA, 2011) mentions two different methods for the determination of the microbial activity, namely:

First:
"Reduction of DMSO to DMS can be used as measurement of anaerobic microbial activity without interference (Griebler & Slezak (2001)): Microbial activity in aquatic environments measured by dimethyl sulfoxide reduction and intercomparison with commonly used methods".

And secondly:
"The microbial activity can be determined by measuring the mineralization of a readily degradable ^{14}C-labelled compound (e.g. ^{14}C-glucose) under anaerobic conditions."

The latter method, which has been adapted to measurements under anaerobic conditions, is based on the commonly used method to determine the biological activity by substrate induced respiration (SIR) (DIN / ISO 14240 – (2011)). SIR has been developed for measurements under aerobic conditions and determines either the oxygen-consumption or CO_2-formation whenever an easily degradable substrate has been added.

A third method to determine the microbial biomass (no microbial activity!) is the fumigation method (DIN ISO 14240 – 2 (1999). The active and the inactive biomass are determined, but false positive or false negative results are obtained quite often. The fumigation method can – in principle – be used to determine the microbial biomass under anaerobic conditions. However, preliminary tests, carried out by Fraunhofer IME outside this study, showed that they are not applicable to the matrix manure due to very high background values.

In the course of a diploma thesis (Bickert, 2012), it was tested which easily degradable substance and which experimental setup is appropriate to be used in a predictive assay for microbial activity. Radioactively labeled acetic acid, a mixture of L-amino acids, benzoic acid, propionic acid and glucose were subjected to anaerobic mineralization in cattle and pig manure which had been stored under different conditions. The use of ^{14}C-glucose in a flow-through system and measuring the formation of $^{14}CO_2$ and $^{14}CH_4$ over a period of 7 days proved to be the best method for assaying the microbial activity.

On the basis of these results, the following experimental setup was finally selected to be used: The microbial activity of manure at different stages of the transformation test procedure was characterized by the addition of ^{14}C-glucose and determination of the mineralization after 7 days of ^{14}C-glucose incubation. Manure subsamples for the microbial activity test were treated in analogy to the samples used for the transformation study apart from test substance application. At the respective stage of test procedure, ^{14}C-glucose was added. The formation of $^{14}CO_2$ under anaerobic conditions over a time period of 7 days was followed and

quantified. $^{14}CO_2$-formation given as [% of the applied radioactivity] was used as a measure for the biological activity of the manure at the respective stage of test.

Acclimation of manure

Subsamples of 50 g (wet weight; dry matter content: 10 % ± 1 % for cattle manure, 5 % ±1 % for pig manure) were directly filled into the incubation vessels which were used for the acclimation period and the subsequent bioactivity tests. 3 x 3 parallels were set up as the microbial activity of the manure was tested at the start and the end of the test substance transformation study and three further parallels were kept as spare vessels in case the transformation study needed to be prolonged.

The same flow through apparatus (see annex 2) as for the transformation studies was used for the determination of microbial activity. The incubation-apparatus was closed and a constant, water saturated stream of nitrogen was passed over the manure at a rate in the range of approximately 50 - 200 mL/min. The acclimation was carried out for 21 days, and thus again was identical to that of the test substance transformation study.

Test temperature and light conditions

During the whole test period the manure samples were incubated in the dark at a test temperature of 20°C.

Anaerobic/methanogenic incubation conditions

Test on microbial activity of cattle and pig manure was performed under anaerobic conditions. It is not possible to relate a certain redox potential directly to anaerobic/methanogenic conditions, but it can be taken as an indication. As a quality criterion, a redox potential of -100 mV should not be exceeded, although typical redox potentials in manure tanks are well below that value. Redox potentials measured in pig and cattle manure from the different sampling campaigns in this study (n = 30) range from – 440 to – 241 mV. The redox potential was measured and recorded at termination of the test on biological activity.

Treatment and application of ^{14}C-glucose

As reference substance for testing the biological activity ^{14}C-glucose was used. An appropriate stock solution of ^{14}C-glucose dissolved in water was added to the manure subsamples in the respective incubation vessels. Approximately 800 µL solution containing 0.8 µg ^{14}C-glucose (concentration in stock solution: 1 µg/mL) were added corresponding to the addition of approximately 50 kBq. Addition of ^{14}C-glucose was followed by thoroughly mixing while maintaining anaerobic conditions. This was achieved as described in chapter 2.4.

Test duration and sampling regime

The procedure and sampling regime for the test on biological activity of manure at the respective stages of the transformation study of the tested chemical can be described as follows:

- At start of acclimation, nine further subsamples for testing the microbial activity are acclimatized.
- At start of the transformation study, to three of the nine acclimated manure subsamples ^{14}C-glucose is added (see also chapter 2.4). The incubation period of manure fortified with ^{14}C-glucose is 7 days. After 7 days, traps to measure mineralization are removed and analyzed for trapped $^{14}CO_2$. The removed traps are replaced by freshly filled traps. Thereafter, the manure subsamples are treated by addition of 10 mL 10% HCl in order to strip potentially dissolved $^{14}CO_2$ (or HCO_3^- / CO_3^{2-}). After adding of 10 mL 10 % HCl the incubation flasks are closed again and nitrogen is passed over for 3 hours. Samples are not

stirred in order to avoid foaming as much as possible. For stirring and foaming see Figure 7. Abandonment of stirring does not influence the results, but $^{14}CO_2$ is stripped quantitatively. After 3 hours NaOH-traps are removed and radio-counted for additionally trapped $^{14}CO_2$.

- Until the end of the transformation study the remaining six subsamples are kept under anaerobic conditions. This is achieved by closing the incubation apparatus closed tightly again and passing a constant, water saturated stream of nitro gen over the manure at a rate of approximately 50 - 200 mL/min.
- At the end of the transformation study to three of the six manure subsamples ^{14}C-glucose is added. These fortified samples are subjected to anaerobic incubation for 7 days at 20°C. Formed $^{14}CO_2$ is quantified after 7 days.
- In case the transformation study has to be prolonged the remaining three subsamples serve as spare samples. At the end of the prolongation to these three sub-samples ^{14}C-glucose is added. These fortified samples are subjected to anaerobic incubation for 7 days at 20°C. Formed $^{14}CO_2$ is quantified after 7 days.

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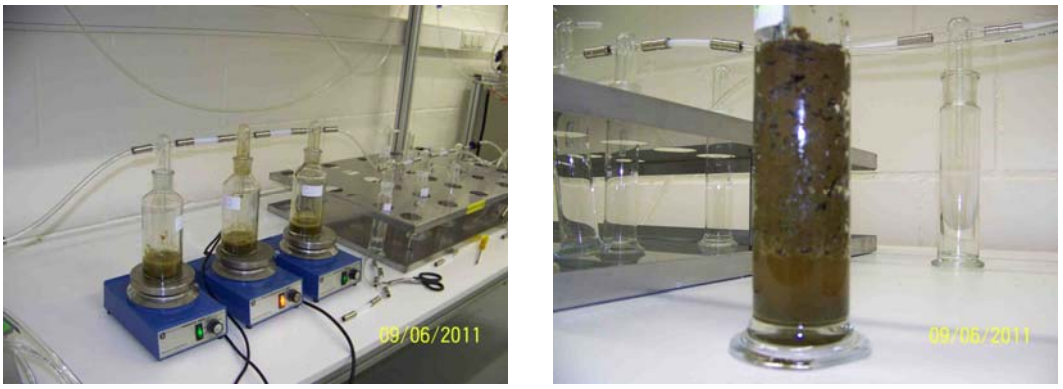


Figure 7: Sample foaming after acidification to strip dissolved $^{14}CO_2$

2.4 Set-up for performance of transformation tests

Once the liquid manure has been sampled from the tank it either was stored under anaerobic conditions preferably at the test temperature for a maximum of two months (see chapter 2.2) or directly used for testing purposes.

Adjustment of dry matter content

Before performing the transformation test, the manure was acclimatized to test conditions. Prior to the start of the acclimation period, the dry matter content of the manure was determined as described in chapter 2.3. To get comparable conditions it was adjusted to standardized values. The recommended dry matter content in cattle and pig manure is 10% ± 1% and 5% ± 1%, respectively (EMA/CVMP/ERA/ 430327/2009 (revised in 2011)). If the dry matter content of the original manure was below the recommended value, it was concentrated by careful centrifugation for 10 minutes at 740 x g. However, extensive increase of dry matter content by centrifugation was avoided since microorganisms are removed together with the supernatant. The option to increase the dry matter content by the addition of the lowermost layer of settled manure was not followed.

If dry matter content was too high, water (de-ionized water, bubbled with nitrogen for 30 min) was added.

Acclimation of manure

After the adjustment of the dry matter content, cattle manure was homogenized by gently mixing using a glass bar. Subsamples of 50 – 100 g (wet weight) each were directly filled into the incubation vessels which are used for the acclimation and transformation study (see Figure 8). No additional measures to prevent introduction of oxygen were used during both processes, homogenization and filling of incubation vessels.

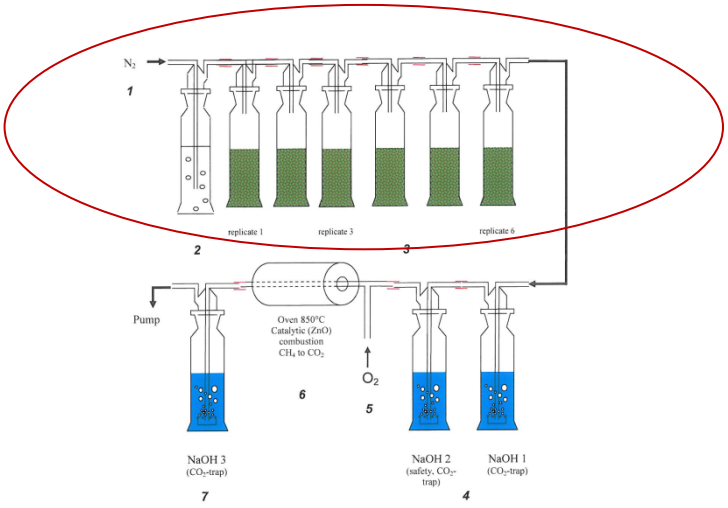
Figure 8: preparation of subsamples

Pig manure was homogenized under anaerobic conditions by a knifetec mill (or similar apparatus) in order to obtain a fairly stable phase. This was achieved by filling the manure into a container or beaker, putting the knifetec mill into the manure, sealing with parafilm and gently passing a nitrogen stream over the manure while mixing for 1 minute (see Figure 9). Thereafter, the dry matter content was adjusted. After a repeated homogenization under anaerobic conditions by thoroughly mixing (set up as above) subsamples of 50 – 100 g (fresh weight) were filled into the incubation vessels. Then the incubation apparatus (for details see next paragraph) was closed and a constant, water saturated stream of nitrogen was passed over the manure at a rate in the range of approximately 50 - 200 mL/min. As pre-tests have shown that an acclimation period of 21 days is appropriate (see chapter 3.3 for results) the acclimation was routinely carried out for 21 days at 20°C.

Figure 9: manure homogenization procedure while keeping anaerobic conditions

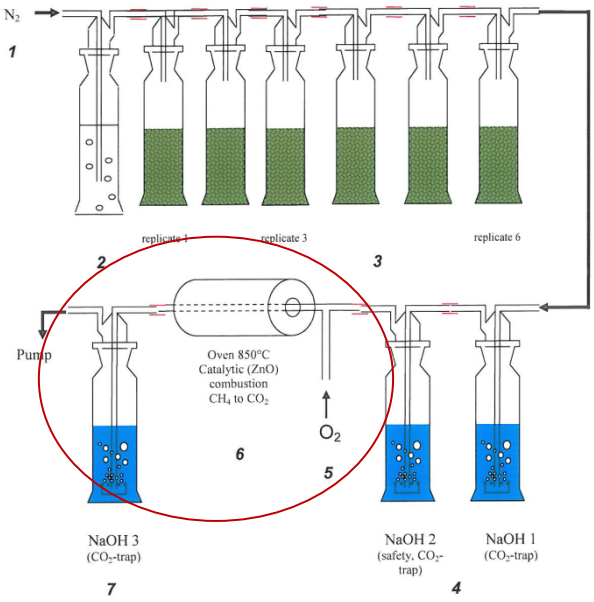
Flow through apparatus

Transformation tests may be conducted in a static or a flow-through system. For all transformation studies a flow through apparatus was used. A schematic presentation of the system is shown in annex 2. Photographs below give an even more precise impression.



1: nitrogen is gently passed over the manure samples (20 – 500 mL/h)
2: gas washing bottle containing water
3: manure transformation flasks filled with at least 50 – 100 g manure (fresh weight)
4: for anaerobic transformation two NaOH-traps in sequence are needed to trap evolving CO_2.
5: addition of oxygen for subsequent catalytic combustion of CH4
6: oven for combustion of CH_4 to form CO_2
7: NaOH filled trap for CO_2 formed from CH_4

Figure 10: Photographs of the flow through apparatus



1: nitrogen is gently passed over the manure samples (20 – 500 mL/h)
2: gas washing bottle containing water
3: manure transformation flasks filled with at least 50 – 100 g manure (fresh weight)
4: for anaerobic transformation two NaOH-traps in sequence are needed to trap evolving CO_2.
5: addition of oxygen for subsequent catalytic combustion of CH4
6: oven for combustion of CH_4 to form CO_2
7: NaOH filled trap for CO_2 formed from CH_4

Figure 11: tube furnaces for combustion of methane to carbon dioxide and subsequent trapping in NaOH-solution

The flow through apparatus is a gas tight system of incubation vessels and traps set in sequence. Humidified nitrogen is gently passed over the liquid manure sub-samples. At the gas inlet nitrogen is given with a slight excess. By having a T-junction Figure 12, left side, excessive gas can escape via a washing flask Figure 12, right side whereas the needed nitrogen is passed over the manure samples. By such a design back-flush can be avoided.

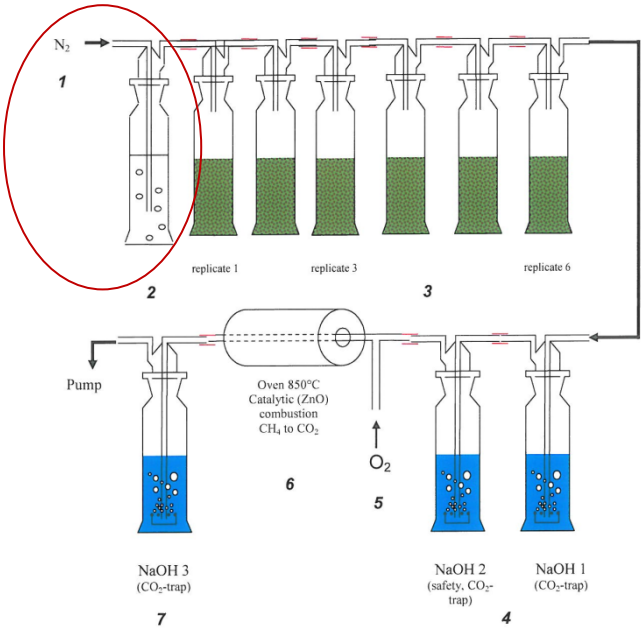


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Figure 12: integral parts of the gas flow through maintainance

The nitrogen is passed over the samples at a flow rate of 50 – 200 mL/min. First, the nitrogen is bubbled through water in order to humidify the gas. Thereafter, the humidified gas is passed over the manure subsamples. Six replicates per sampling point are set in sequence. The vessels for the individual sampling points are set in parallel. Once the gas has passed over the sixth replicate it is bubbled through two adsorption traps in sequence containing 2 M NaOH. Traps are for sorbing the evolving $^{14}CO_2$ and other possibly occurring volatiles. Since the formation of $^{14}CH_4$ is expected in such an anaerobic system the gas is furthermore passed through an oven at 850°C. $^{14}CH_4$ is catalytically (CuO-catalyst + O_2 feeding to the tube) converted to $^{14}CO_2$ which again is trapped in a third NaOH trap.

Test temperature and light conditions

During the whole test period the manure samples were incubated in the dark at 20°C.

Anaerobic/methanogenic incubation conditions

Test on microbial activity of cattle and pig manure was performed under anaerobic conditions. It is not possible to relate a certain redox potential directly to anaerobic/methanogenic conditions. However, as a quality criterion Eh < -100 mV should never be exceeded. Redox potentials measured in pig and cattle manure from the different sampling campaigns in this study are in the range of -240 to -440 mV (see tables A3_1 to A3_3). The redox potential was measured and recorded at termination of the test on biological activity.

Abiotic controls (sterile samples)

For information on the abiotic transformation of the test substance, sterile controls were included. Sampling of sterile controls should be according to the sampling schedule or at least once during and at the end of the study. However, in the present research project sterile controls were sampled at the end of the test only.

Manure was sterilized by autoclaving twice (15 min, 121 °C, 100 bar). In order to avoid foaming, vessels were pre-heated overnight at 100°C (see Figure 13, bottles in the middle of the photo). The application solution was sterilized by passing over a sterile filter. Application of the test substance was under sterile conditions using a clean-bench. Thereafter, flasks were closed and kept closed carefully. Applied samples were incubated without connecting to the flow through. As no evolution of $^{14}CO_2$ or $^{14}CH_4$ was expected, samples were locked gastight till sampling.

Figure 13: manure samples after autoclavation at 121°C and different pre-treatment

Application of test substance

The test substance should be dosed into the manure at a concentration that reflects the maximum expected manure concentration. If this concentration is not sufficient for detection and identification of transformation products, the test may be conducted at increased substance start concentrations. However, excessively high concentrations potentially toxic to microorganisms should be avoided.

The test substances were dissolved in an appropriate solvent and added into the acclimated manure in the respective incubation vessels by thoroughly mixing while maintaining anaerobic conditions. This was achieved by maintaining to pass the nitrogen stream over the samples during the application procedure. The volume of the solvent used for application should not exceed 1% v/v of the manure volume and – if possible – be water miscible. The suggested value of 1% v/v is in analogy to the value given in the OECD guideline 308 (Aerobic and anaerobic transformation in aquatic sediment systems, OECD (2002a)).

The required volume of stock solution was pipetted into the manure under simultaneous stirring using the pipette tip. As soon as the solution was evenly distributed in the manure the pipette remained in the manure (see Figure 14). The pipette tip was left in the manure sample in order to avoid any losses since manure always sticks to the tip. Rinsing with water would decrease the dry matter content of the sample.

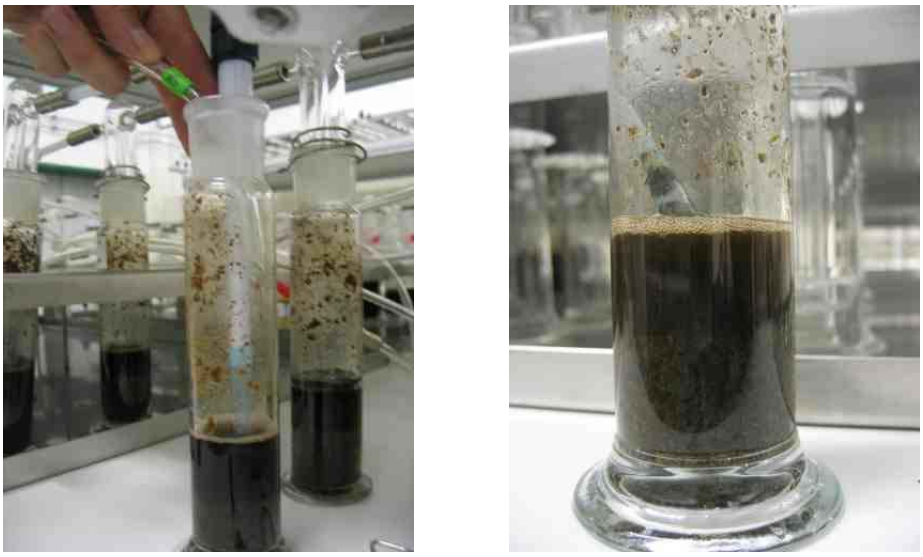

Figure 14: application under anaerobic conditions, stirring with the pipette tip, which remains in the sample

Test duration and sampling

Test duration did depend on the rate of transformation of the parent compound and transformation products. The maximum study duration was 105 days for the substance biocide B. There should be enough sampling time points to unambiguously derive all required (kinetic) parameters for parent substance and transformation products. If the study is further prolonged, e.g. because increasing amounts of transformation products have been observed a test for microbial activity may be conducted at the beginning and end of the prolongation period. It might therefore be useful to have a further spare incubation vessel for this purpose.

In the course of the present research project six replicate incubation flasks were sacrificed per sampling point. This was needed to obtain a reliable data base for statistical analyses. In transformation studies for registration purposes at least duplicates should be sacrificed per sampling.

Besides sampling directly after application, at 9 additional sampling points were included. For exact sampling points for the three substances tested in the course of the present research projects see chapter 2.5.

Measurement of $^{14}CO_2$, $^{14}CH_4$ and ^{14}C volatile fatty acids (VFA)

CO_2, CH_4 and volatile fatty acids, as a precursor of CH_4, are major volatile final transformation products which are expected from transformation under anaerobic conditions. The experimental set-up was such, that the following requirements are fulfilled:

- Quantitative capturing to avoid any losses of volatiles and enable establishment of a mass balance,
- Differentiation between formed CO_2, CH_4 and VFAs.

Quantification of volatiles

Quantification of trapped volatiles was by radio-counting (liquid scintillation counting, LSC) of aliquots of the trapping solutions. Furthermore, it was proven whether evolved $^{14}CO_2$ is purged quantitatively when passing the humidified nitrogen over the manure samples. This was verified by addition of HCl to the manure sub-samples in order to strip CO_2 (or HCO_3^- / CO_3^{2-}) being potentially dissolved in the manure matrix. Purging by addition of HCl was applied in case the amount of $^{14}CO_2$ exceeded the level of 10 % of the total radioactivity (TRR). Purging was not done for all cases as it is a time consuming methodology. NaOH traps 1 and 2 (see annex 2) were removed at the particular sampling point and analyzed for trapped $^{14}CO_2$ and other evolved gases, respectively, by liquid scintillation counting of an aliquot of the trapping solution. The removed traps are replaced by freshly filled ones. Thereafter, the manure incubation flasks to be removed at that particular sampling point are treated by addition of 10 mL 10% HCl in order to strip potentially dissolved CO_2 (or HCO_3^- / CO_3^{2-}). After adding of 10 mL 10 % HCl the incubation flasks were closed again and nitrogen was passed over for 3 hours. Samples were not stirred in order to avoid foaming. Thereafter, manure incubation flasks were removed and manure was subjected to the substance specific clean-up, extraction procedures and analyses. NaOH-traps were also removed and radio-counted for additionally trapped CO_2. In order to avoid interferences and cross-contaminations by evolving gases sampling should start with samples being next to the outlet (e.g. samples 5 and 6 in annex 2). Prior to the addition of 10 % HCl to the manure sub-samples it was checked whether the test substance and transformation products are stable under acidic conditions. If this was not the case further replicates were incubated.

Differentiation between CO_2, CH_4 and VFAs

Humidified nitrogen was passed over the manure sub-samples at a rate in the range of approximately 50 – 200 mL/min. By such a constant N_2-stream evolved $^{14}CO_2$ is purged from the manure samples, transported and captured in traps 1 and 2 (safety trap; see annex 2) containing 2 M NaOH. Potentially formed $^{14}CH_4$ passes the NaOH traps. After the addition of oxygen it is catalytically (= CuO) oxidized in an oven at 850°C to form $^{14}CO_2$. The formed $^{14}CO_2$ is trapped in the NaOH-filled trap situated at the outlet of the oven (NaOH filled trap 3 see Figure 12).

Such a set-up enabled the differentiation between evolved $^{14}CO_2$ (captured in NaOH filled traps 1 and 2; annex 2) and $^{14}CH_4$ that was further oxidized to $^{14}CO2$ (captured in NaOH filled trap 3; Figure 12).

To verify that the radioactivity captured in the NaOH traps 1 and 2 is $^{14}CO_2$ and not from potentially also formed volatile fatty acids (VFA), $BaCl_2$ precipitation of the radioactivity was conducted. This was by first radio-counting the solutions in NaOH traps 1 and 2. Thereafter, 20 mL 0.25 M $BaCl_2$ was added to 10 mL

aliquots of NaOH trapping solution from traps 1 and 2 each. Precipitation of $Ba^{14}CO_2$ occurred. The supernatant was radio-counted again. The radioactive content in the supernatant after precipitation can be attributed to VFAs whereas the difference of radioactive content before precipitation minus radioactive content after precipitation can be attributed to evolved $^{14}CO_2$.

Clean-up of manure samples

At a specific time point, the manure samples were sacrificed by removing the incubation vessels from the flow-through system and cleaned-up directly after sampling without storage of the samples prior to clean-up e.g., at -20°C. Thereafter, extraction was done by the use of an appropriate solvent or solvents of different polarity depending on the properties of the substance. The extraction was achieved by the addition of a sub-portion of the first extraction solvent to the incubation vessel, gently shaking the mixture and transferring it into a centrifuge tube. This procedure was repeated until the complete manure was removed from the incubation vessel. The manure-solvent mixture was shaken for 30 minutes. Thereafter, the mixture was centrifuged for 10 minutes at 739 x g. The supernatant, i.e. the first extract, was collected. Further extraction solvent was added to the pellet. The whole process was repeated twice. Extracts 1 – 3 were combined, counted for their radioactivity content (by liquid scintillation counting of an aliquot) and subjected to substance specific analysis.

The pellet was dried at room temperature. Aliquots were quantified by combustion and radio-assaying the evolved $^{14}CO_2$. In case non-extractable residues (NER) exceeding 10 [% aR] were observed, exhaustive extraction methods were applied additionally. These methods comprise e.g. pressurized liquid extraction (e.g. ASE®), reflux, soxhlet etc. with appropriate solvents. Usually, these solvents are identical to those used in the first extraction steps. According to experiments performed prior to the main tests it is advisible to apply the harsh extraction twice in order to exhaustively extract the residues. Extracts are not combined but radiocounted. In case the amount of radioactivity exceeds 5 [% aR] the extract should be subjected to substance specific analysis. However, in the course of the research project ASE® was applied occasionally only in order to check the potency of the extraction procedure. Residues remaining after the last extraction step (non-extractable residues, NER) were quantified by combustion and radio-assaying the evolved $^{14}CO_2$.

Establishment of mass balance

A mass balance was determined and calculated for each sampling interval. This was done by radiocounting the phases and summing-up the amount of radioactivity given in [% of applied radioactivity; % aR] in the aqueous/organic extracts plus ASE® plus volatiles other than $^{14}CO_2$ plus + $^{14}CO_2$ + non-extractable residues (NER):

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

Quality check of clean-up and extraction procedure

Prior to performing the transformation study the potency of the foreseen clean-up procedure was checked. This was done by the addition of the test substance to 50 g manure (fresh weight) and a subsequent incubation for 15 minutes. Thereafter, the foreseen extraction steps were performed. The amount of radioactivity in each of the phases (see under “establishment of mass balance”) was quantified and summed-up. Recovery had to be between 90 [% aR] and 110 [% aR]. The extracts were subjected to either radio-HPLC or radio-TLC to check whether the analytes were altered by the extraction method. This was demonstrated by co-chromatography of the known substances.

Liquid scintillation counting (LSC)

After mixing an aliquot of the solution of interest with an aliquot of a suitable liquid scintillation cocktail (for example: Ultima Gold LLT, Hionic Flour for aqueous samples and ethylenglycol, and Ultima Gold for organic samples) LSC measurements were performed using a Packard Tri-Carb liquid scintillation analyzer. Each sample was measured for 5 minutes in duplicate in order to increase the sensitivity of the analytical method and to ensure reproducibility. Computer-constructed quench curves, derived from a commercially available series of sealed quenched standards, automatically converted counts per minute (cpm) to decays per minute (dpm).

Levels of detection (LOD) and quantification (LOQ), respectively, for the extracts, the trapping solutions, and NERs were determined as follows:

Organic-/aqueous extracts

- 0.1 Bq can be counted by LSC (instrument specification)
- 0.25 mL were counted by LSC
- Volume of combined extracts = 240 mL
- 96 Bq in 240 mL
- 75860 Bq applied to the manure subsample
- LOD: 0.13 % aR are detectable
- LOQ: 0.4 % aR are quantifiable (LOQ is defined herein as 3 x LOD)

Trapped volatiles

- 0.1 Bq can be counted by LSC (instrument specification)
- 1 mL trapping solution was counted by LSC
- Volume of trapping solution = 50 mL
- 5 Bq in 50 mL
- 75860 Bq applied to the manure
- LOD: 0.007 % aR are detectable
- LOQ: 0.021 % aR are quantifiable (LOQ is defined herein as 3 x LOD).

Non-extractable residues (NER)

Non-extractable residues from manure (NERs) are quantified by combustion the residual solid manure using an oxidizer, trapping of evolving ^{14}C-CO_2 and subsequent analysis by LSC. Levels of detection and quantification, respectively, were:

- 0.1 Bq can be counted by LSC (instrument specification)
- 300 mg extracted and air dried manure are combusted (this is an upper limit for the capacity of the Oxidizer)
- 75860 Bq applied to the manure
- 0.1 Bq/300 mg soil
- 17 Bq/50 g soil
- LOD: 0.02 % aR are detectable
- LOQ: 0.06 % aR are quantifiable (LOQ is defined herein as 3 x LOD).

Measurements of test substance and transformation products

Concentrations of the test substance and the transformation products at every sampling time were determined. In general, transformation products detected at ≥ 10% of the applied radioactivity at any sampling time should be identified. Transformation products occurring at ≥ 10 [% aR] are seen as major transformation products whose identification is one of the endpoint parameters in a transformation study (see also chapter 2.7).

However, in the course of the research project no substance identification was performed but a chromatographic characterization by co-chromatography of known possible transformation products (reference substances). Chromatographic methods which are generally used are high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC). However, in this project TLC only was applied since matrix interferences did not lead to interpretable results when using HPLC without further sample preparation.

In case peaks on the TLC-plates were not annotated to any of the reference substances they were characterized by transformation product T (1), T (2) etc.

Radio High-Performance Liquid Chromatography (radio HPLC)

The parent compound and possible transformation products in the manure extracts can be determined by radio-HPLC. This enables their quantification and characterization by their chromatographic behavior. Reference standards (parent compound and possibly occurring transformation products) are co-chromatographed with the extract samples, e.g., by chromatography prior to a series of extract samples. By comparison of retention times a characterization of the test item and transformation products is possible. Further peaks, which cannot be annotated to any of the reference compounds are described by their retention times and are named as “transformation product 1” etc.

Radio thin layer chromatography (radio TLC)

Due to matrix interferences – that means broad peaks and shifts in HPLC-retention times – it was more appropriate analyse the extract samples by radio-TLC. A further advantage of this chromatographic method is the high sensitivity; samples of low radioactivity content can be analysed easily. The high sensitivity for ^{14}C-labelled compounds is due to the free selectable exposure duration.

Radioactive areas on developed TLC plates were located using a Bio-imager, and the relative proportion of each radioactive area was determined by 2D densitometry (scanner). Radioactively labeled parent compound and unlabeled possible transformation products (if available) were also spotted onto the same TLC-plate. By Rf-value-comparison a characterization of the test item and transformation products was possible.

Levels of detection (LOD) and quantification (LOQ) were calculated as shown in the following example:

- 4 Bq in 250 µL extract are applicable per lane (4 Bq are a minimum with respect to a feasible exposure duration which is one week, and 250 µL are a maximum to be applied onto the plate by reasons of practicability and experience)
- 10% peak area (= 0.4 Bq) can be quantified unequivocally by peak integration
- 240 mL extract were obtained per manure subsample
- 0.4 Bq/250 µL extract = 0.38 kBq/240 mL extract
- 75.86 kBq were applied per manure subsample

- 0.38 kBq = 0.5 % aR = 0.016 mg/kg manure fresh weight = limit of quantification (LOQ defined as 3 times LOD)
- 0.13 kBq = 0.17 %aR = 0.004 mg/kg manure fresh weight = limit of detection (LOD)

Thus, the LOD of the analytical method for the test substance and the transformation products was at least 0.01 mg/kg or 1 % of applied dose (whichever is lower).

2.5 Test substances

Three substances – two veterinary pharmaceuticals and one biocide – namely Salicylic acid, Paracetamol and biocide B were used as test substances in the course of the research project.

Substance characterization

Salicylic acid

Name:	Salicylic acid
Chemical name:	2-Hydroxybenzoic acid
CAS-Number:	69-72-7
Formula:	$C_7H_6O_3$
Molecular weight:	138.1 g/mol
Purity:	99.0 % (radiochemical purity)
State of matter and appearance:	white crystalline powder
Water solubility:	2 g/L (20°)
Origin:	American Radiolabeled Chemicals, Inc.
Specific radioactivity:	15 mCi/mmol = 0.11 mCi/mg = 4.0 MBq/mg
Lot no.:	IO1112

Salicyluric acid (possible transformation product of Salicylic acid)

Name:	Salicyluric acid
Chemical name:	N-(2-hydroxybenzoyl)glycine
CAS-Number:	487-54-7
Formula:	$C_9H_9NO_4$
Molecular weight:	195.2 g/mol
Purity:	pestanal (> 98%)
Melting point	160 °C
State of matter and appearance:	white crystalline powder
Origin:	Sigma-Aldrich fine chemicals

Gentisic acid (possible transformation product of Salicylic acid)

Name:	Gentisic acid
Chemical name:	2,5-dihydroxybenzoic acid
CAS-Number:	490-79-9
Formula:	$C_7H_6O_4$
Molecular weight:	154.12 g/mol
Purity:	pestanal (> 98%)
State of matter and appearance:	white crystalline powder
Melting point:	200 – 205 °C (sublime)
Origin:	Sigma-Aldrich fine chemicals

Paracetamol

Name:	Paracetamol
Chemical name:	4-Hydroxyacetanilide
CAS-Number:	103-90-2
Formula:	$C_8H_9NO_2$
Purity:	99.2 % (radiochemical purity)
State of matter and appearance:	crystalline solid
Melting point:	172.4 ºC
Origin:	American Radiolabeled Chemicals, Inc.
Specific radioactivity:	18.8 MBq/mg
Lot no.:	153-064-077-A-20080611-DR

Paracetamol glucuronide (possible transformation product of Paracetamol)

Name	Paracetamol glucuronide
Chemical name:	p-Acetamidophenyl ß-D-glucuronide
CAS-Number:	120595-80-4
Formula:	$C_{14}H_{16}NNaO_8$
Molecular weight:	349.3 g/mol
Purity:	98.0 %
State of matter and appearance:	off-white powder
Origin:	Sigma-Aldrich fine chemicals
Lot no.:	082K5051

4-Aminophenol (possible transformation product of Paracetamol)

Name:	4-aminophenol
Chemical name:	4-aminophenol
CAS-Number:	123-30-8
Formula:	C_6H_7NO
Molecular weight:	109.13 g/mol
Purity:	99.0 %
State of matter and appearance:	white powder
Origin:	Sigma-Aldrich fine chemicals
Lot no.:	SZB9057XV1

Biocide B

Name:	biocide B
Purity:	≥ 99.0 % (radiochemical purity)
State of matter and appearance:	solid
Specific radioactivity:	4.44 MBq/mg

TP1 of biocide B

Name:	TP1 of biocide B
Purity:	≥ 99.0 %
State of matter and appearance:	yellow cristalline
Origin:	Select Lab Chemicals

TP2 of biocide B

Name:	TP2 of biocide B
Purity:	≥ 98.0 %
State of matter and appearance:	wet, contains 25% water
Origin:	Sigma-Aldrich fine chemicals

TP3 of biocide B

Name:	TP3 of biocide B
Purity:	99.0 %
State of matter and appearance:	wet, contains 25% water
Origin:	Sigma-Aldrich fine chemicals

Glucose (for testing microbial activity)

Name	Glucose
Chemical name:	(2R, 3S, 4R, 5R)-2,3,4,5,6-Pentahydroxyhexanal (as aldehyde)
CAS-Number:	50-99-7
Formula:	$C_6H_{12}O_6$
Molecular weight:	180.2 g/mol
Purity:	99.0 %
State of matter and appearance:	wet, contains 25% water
Origin:	American Radiolabeled Chemicals, Inc.
Specific radioactivity:	300 mCi/mmol = 61.6 MBq/mg
Lot no.:	II0105

Test substance application, incubation and sampling

Salicylic acid

0.02 mg ^{14}C-Salicylic acid (75 kBq) plus 1.18 mg unlabeled Salicylic acid were dissolved in 3 mL ultrapure water containing 0.05% methanol and applied to 50 g manure fresh weight. Thus, the final concentration was 1.2 mg Salicylic acid/50 g manure fresh weight corresponding to 24 mg Salicylic acid/kg manure fresh weight.

Samples were incubated for 0 h, 3 d, 7 d, 14 d, 21 d, 28 d, and 35 d at 20 °C in the flow through apparatus as described in chapter 2.4. Abiotic controls (sterile samples; see chapter 2.4 for handling) were also incubated and taken at the end of the study that is after 35 d. 6 replicates were sacrified per sampling point.

The application procedure, flow-through-system used for incubation, handling, and the sampling is described in detail in chapter 2.4

Paracetamol

0.004 mg ^{14}C-Paracetamol (75 kBq) plus 1.196 mg unlabeled Paracetamol were dissolved in 5 mL ultrapure water containing 0.05% acetonitrile and applied to 50 g manure fresh weight. Thus, the final concentration was 1.2 mg Paracetamol /50 g manure fresh weight corresponding to 24 mg Paracetamol /kg manure fresh weight.

Samples were incubated for 0 h, 7 h, 1 d, 3 d, 7 d, 14 d, and 28 d (for cattle manure and pig manure, BAY_2c and BAY_2p) at 20 °C in the flow through apparatus as described in chapter 2.4. Pig manure samples from

the sites NRW_1p and NRW_2p were incubated for 0 h, 1 d, 3 d, 7 d, 14 d, 28 d, and 41 d. Abiotic controls (sterile samples; see chapter 2.4 for handling) were also incubated and taken at the end of the study that is after 28 d and 41 d, respectively. 6 replicates were sacrified per sampling point.

The application procedure, flow-through-system used for incubation, handling, and the sampling is described in detail in chapter 2.4

Biocide B

0.02 mg ^{14}C-biocide B (75 kBq) plus 0.03 mg unlabeled biocide B were dissolved in 7 mL ultrapure water containing 0.05% acetonitrile and applied to 50 g manure fresh weight. Thus, the final concentration was 0.05 mg Biocide B /50 g manure fresh weight corresponding to 1 mg Biocide B /kg manure fresh weight.

Samples were incubated for 0 h, 7 d, 21 d, 42 d, 63 d, 84 d, and 105 d at 20 °C in the flow through apparatus as described in chapter 2.4. Abiotic controls (sterile samples; see chapter 2.4 for handling) were also incubated and taken after 86 days. 6 replicates were sacrified per sampling point.

The application procedure, flow-through-system used for incubation, handling, and the sampling is described in detail in chapter 2.4

Clean-up and analyses of test substance and transformation products

Salicylic acid

50 g manure sample were extracted once by 80 mL methanol + 1% trifluoroacetic acid (TFA), and thereafter twice by 50 mL methanol + 1 % TFA. For extraction the samples were shaken for 30 minutes on a horizontal shaker and centrifuged for 10 minutes at 739 x g. After centrifugation the supernatant extract was collected and the pellet was subjected to the next extraction step. Further extraction solvent was added to the pellet. The whole process was repeated twice. Extracts were combined, and further analyzed by radio TLC. After the last extraction step the pellet was air dried and aliquots were subjected to combustion and radioassaying to give the information on the amount of non-extractable residues (NER).

For a few samples in addition to the described extraction a further extraction step using ASE® was performed exemplarily. The accelerated solvent extraction (ASE®), i.e. extraction under high pressure and temperature (100°C, 12000 kPa, heat up was for 5 minutes, followed by a static time of 10 minutes) used the same solvent mixture as for the first extraction steps (methanol + 1 % TFA). Extraction was performed twice but extracts were not combined.

As using the extracts without further cleanup influenced HPLC resulting in broad peaks, radio-TLC was preferred over HPLC. The following TLC-system was used:

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

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

R_f-values were:
Salicylic acid: $R_f = 0.50 – 0.55$
Salicyluric acid: $R_f = 0.31 – 0.36$

Gentisinic acid: $R_f = 0.42 – 0.47$

In addition, peaks were observed which were not allocated to any of the used reference substances. They were described by their Rf-values and named as transformation product T1 and T2. Respective Rf-values were:
T1: $R_f = 0.00 – 0.1$
T2: $R_f = 0.60 – 0.64$

Paracetamol

50 g manure sample were extracted once by 80 mL methanol, and thereafter twice by 50 mL methanol. For extraction the samples were shaken for 30 minutes on a horizontal shaker and centrifuged for 10 minutes at 739 x g. After centrifugation the supernatant was decanted, and the pellet was subjected to the next extraction step. Further extraction solvent was added to the pellet. The whole process was repeated twice. Extracts were combined, and further analyzed by radio TLC. After the last extraction step the pellet was air dried at room temperature and aliquots were subjected to combustion and radioassaying to give the information on the amount of non-extractable residues (NER).

For a few samples in addition to the described extraction a further extraction step using ASE® was performed exemplarily. The accelerated solvent extraction (ASE®), i.e. extraction under high pressure and temperature (100°C, 12000 kPa, heat up was for 5 minutes, followed by a static time of 10 minutes) used the same solvent as for the first extraction steps (methanol). Extraction was performed twice but extracts were not combined.

As using the extracts without further cleanup influenced HPLC, radio-TLC was preferred over HPLC. The following TLC-system was used:

- stationary phase: silica gel KG60
- mobile phase: methanol / xylol / ethylacetate / acetic acid; 38/44/15 /3 (v/v/v/v)

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

R_f-values were:
Paracetamol: $R_f = 0.63 – 0.71$
4-Aminophenol: $R_f = 0.43 – 0.49$
Paracetamol glucuronide: $R_f = 0.11 – 0.18$

In addition, peaks were observed which were not allocated to any of the used reference substances. They were described by their Rf-values and named as transformation product T1. The respective Rf-values was:
T1: $R_f = 0.00 – 0.1$

Biocide B

50 g manure sample are extracted once by 80 mL acetonitrile, and thereafter twice by 50 mL acetonitrile. For extraction the samples are shaken for 30 minutes on a horizontal shaker and centrifuged for 10 minutes at 739 x g. After centrifugation the supernatant was decanted, and the pellet was subjected to the next extraction step. Further extraction solvent was added to the pellet. The whole process was repeated twice. Extracts were combined, and further analyzed by radio TLC. After the last extraction step the pellet was air dried and aliquots were subjected to combustion and radioassaying to give the information on the amount of non-extractable residues (NER).

For a few samples in addition to the described extraction a further extraction step using ASE® was performed exemplarily. The accelerated solvent extraction (ASE®), i.e. extraction under high pressure and temperature (100°C, 12000 kPa, heat up was for 5 minutes, followed by a static time of 10 minutes) used the same solvent mixture as for the first extraction stes (acetonitrile). Extraction was performed twice but extracts were not combined.

As the extracts without further cleanup influenced HPLC, radio-TLC was preferred over HPLC. The following TLC-system was used:

- stationary phase: silica gel KG60
- mobile phase: ethylacetate / 2-propanol / water; 65/23/12 (v/v/v)

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

R_f-values were:
Biocide B: $R_f = 0.58 – 0.64$
Nitroguanidine: $R_f = 0.70 – 0.74$
N-Nitrosoguanidine: $R_f = 0.65 – 0.70$
6-Chloronicotinic acid: $R_f = 0.35 – 0.39$

In addition, peaks were observed which were not allocated to any of the used reference substances. They were described by their Rf-values and named as transformation product T1 and T2. Respective Rf-values were:
T1: $R_f = 0.00$
T2: $R_f = 0.03 – 0.1$

2.6 Evaluation of kinetics

Transformation kinetics

Compounds were analysed based on the recommendation of the Forum for the Co-ordination of pesticide fate models and their Use (FOCUS) degradation kinetics (FOCUS (2006)). In addition to the standard kinetics (SFO = Single First Order) FOCUS recommends three bi-phasic kinetics, which are often more suitable to describe the fate of substances than the traditional single first order degradation. The additional kinetics are: HS (Hockey stick), DFOP (Double first order in parallel), and FOMC (First order multi compartment). The use of all kinetic models basically results in information on the time needed for disappearance of 50% (DT50-value) and 90 % (DT90-value) of the parent and transformation products (the latter if a decrease is observed). Furthermore, the so-called chi2-value indicates the robustness of the calculation. In case chi^2-values are above 15% FOCUS, 2006 recommends no use of the results.

By use of the KinGUI-software tool (Mikolasch et al., 2006), DT_{50}-values and DT_{90}-values were calculated for the parent compound and for transformation products (TP) both in cattle and pig manure. All kinetic models, namely “single first order” (SFO), “first order multi compartment” (FOMC), “hockey stick” (HS), and “double first order in parallel” (DFOP) were calculated. Chi^2-values of all kinetic models were compared, and furthermore, a visual check of the graphs of all models was performed. From both comparisons it was obvious, that none of the kinetic models was better compared to the SFO-model for the entire set of all transformation studies. Though from a mechanistic point of view the DFOP-model might be more appropriate, the SFO-model was used in the course of project in order to have a uniform basis for further comparisions and conclusions.

For reasons of completeness, so called "trigger endpoints" (DT_{50}- and DT_{90}-values obtained from the SFO kinetic model) and the "modeling endpoints" were calculated for the transformation of Salicylic acid, Paracetamol and biocide B. The modeling endpoints were calculated by the use of the longer DT_{50}-values of HS and DFOP. This was by applying the equation: DT50 = ln (2) / k_{deg2} with k_{deg2} (1/d) being the rate constant for the second compartment and the slower phase of the degradation process, respectively.

2.7 Statistical evaluation

Statistical evaluations were performed in order to obtain information on the variability of the results of a transformation study.

1. Influence of storage conditions (temperature and duration of storage in the laboratory) and duration of manure acclimation period on microbial activity of manure

Differences of the microbial activity of the manure (expressed as mineralization of the easily degradable substance ^{14}C-glucose) with respect to temperature and duration of storage in the laboratory prior to a transformation study and duration of manure acclimation period were analysed.

2. Variability of manure matrix parameters

Differences of several manure parameters, namely dry matter content, organic matter content, ammonia-nitrogen, total nitrogen and organic carbon content with respect to origin and duration of storage in the tank were analysed for homogeneity of variances and equality of the central moments.

3. Influence of the origin of manure on substance transformation

The following endpoints were considered for manures of different origin:

- DT_{50}-values and DT_{90}-values (not used in the evaluation, as the SFO kinetic model used to determine DT_{50} -values does not give reliable DT_{90} -values) to describe the kinetics of the disappearance of the test substance.
- Percentage of transformation products (TP), non-extractable residues (NER), and mineralization products (MIN; i.e. $^{14}CO_2$ plus $^{14}CH_4$) at the end of the study.

The calculation of the DT_{50}-values and DT_{90}-values is described in chapter 2.5.

Differences of the endpoints with respect to the type and origin of manure, and the duration of storage in the tank were analysed for homogeneity of variances and equality of central moments. Note that for mineralization only one value was available per population and thus, no tests were conducted.

Transformations

DTx values were ln-transformed for calculation of means, standard deviations and the test on homogeneity of variances.

Because data on transformation products (TP), mineralization (MIN), and non-extractable residues (NER) are compositional data bounded within 0 and 100 % they had to be treated differently than the DTx values. The isometric log ratio transformation (ilr-transformation, Egozcue et al. (2003)) was used for testing.

For calculation of the standard deviations per population and the Levene-test, the transformation was done using the following formula using the % found for TP or NER and the difference to 100 %:

$x' = \sqrt{0.5} \ln(x/(100-x))$ with x= % of TP or NER and x' = ilr-transformed value

For calculation of the means the values for TP, NER, MIN, the difference of their sum to 100 % was considered in order to construct a multivariate compositional data point which could be ilr transformed.

Descriptive statistics

For DTx values the following parameters were calculated:

- Re-transformed arithmetic mean of the ln-transformed data
- Standard deviation of the ln-transformed data
- Coefficient of variation as $\sqrt{e^{\sigma^2} - 1}$ with σ^2 = variance of the ln-transformed data

For compositional data the means, standard deviations and coefficients of variation are based on original data.

Testing on homogeneity of variances

Levene's test (Levene (1960)) allows to test whether variances for two or more groups are equal (homogeneity of variance or homoscedasticity). If the p-value of Levene's test is less than the significance level α=5%, the null hypothesis of homogeneity of variances has to be rejected (i.e. there is a statistical significant differences in the variances).

Testing on equal central moments

The U-test is a non-parametric test based on ranks. Because ln-transformation of DT50 and DT90 as well as ilr-transformation of NER data does not mix up ranks, no transformation was necessary. The U-test can only be applied to compare two populations. A significance level of α=5 % was used (i.e. a *p*-value < 0.05 was considered significant).

2.8 Inter-laboratory comparison - Ring test on transformation of veterinary medicinal products and biocides in (liquid) manure

In order to test the applicability in other laboratories and the clarity of the draft test method that has been developed, an international inter-laboratory comparison (pre-validation ring test) was organised.

For that purpose, twenty-five institutes (20 from Europe, 3 from Northern America and 2 from Asia) have been invited in January 2012 to take part in the ring test (see Annex 5 for the invitation). In addition, an informative meeting has been organised in Berlin in the framework of the 6th SETAC World Congress in May 2012. Until September 2012 five institutes (4 from Europe, 1 from Northern America) registered for the ring-test.

The following five institutes took part in the pre-validation ring test:

- Fraunhofer Institut für Molekularbiologie und Angewandte Ökologie (IME), Schmallenberg, Germany
- ECT Oekotoxikologie GmbH, Flörsheim, Germany
- IBACON GmbH, Rossdorf, Germany
- Innovative Environmental Services (IES) Ltd., Witterswil, Switzerland
- Agriculture and Agri-Food Canada (AAFC), London, Canada

Whereas IME, ECT and AAFC started the experiments in October 2012, IBACON and IES started in February/March 2013.

Test method and test performance

As basis for the performance of the pre-validation ring test all participating institutes were provided with the then current draft test method (see chapter 2.4 for the method) and an evaluation sheet for documentation of the results. In addition, details for the performance of the pre-validation ringtest (e.g. test duration, sampling time points, test concentrations, radioactivity to be applied per test vessel, sterile controls, extraction procedures) were given prior to the start of the experiments.

The following extraction procedure for salicylic acid from pig and cattle manure was recommended (all amounts given refer to 50 g manure wet weight per test vessel) and provided to the participants:

> "At the sampling times the test vessels containing the respective spiked manure samples and the corresponding absorption traps were removed from the incubation system. The content of each glass-flask was transferred to a centrifuge tube, extracted once by 80 mL methanol + 1% trifluoroacetic acid (TFA; shaking for 30 minutes), and twice by 50 mL methanol + 1% TFA (shaking for 30 minutes). After each shaking step the samples were centrifuged at 2100 rpm for 10 minutes. After centrifugation the extract was separated from the extracted manure and subjected to further analyses. Extracts were quantified by liquid scintillation counting (LSC) and analysed for the test item e.g. by TLC-analysis or HPLC-analysis. The remaining manure after extraction was analysed for non-extractable residues by combustion with subsequent LSC of the formed $^{14}CO_2$. The volume of the absorption solutions was measured and radioactivity in each solution was determined by LSC at each sampling, and analysed for evolved $^{14}CO_2$ and – if possible - ^{14}C-methane. Evolved $^{14}CO_2$ and ^{14}C-methane could be quantified and the rate of mineralization could be determined."

One cattle manure and one pig manure were tested. The cattle manure was provided by the Co-ordinator, whereas sampling of the pig manure was performed by each participant. Cattle manure was sampled on September 19th, 2012 by Fraunhofer IME in Schmallenberg and was subsequently distributed to the other participants in Europe by ECT. Since an export of manure to Canada was not possible, the Canadian participant collected manure by its own. Tests were run using ^{14}C-radiolabelled substances. The compounds ^{14}C-salicylic acid (test compound, Batch-No. ARC 0287) and ^{14}C-glucose (to determine the microbial activity of the manure, Batch-No. MC 144) have been shipped to all participants by the manufacturer Hartmann Analytic GmbH on September 26th, 2012 (see chapter 2.5 for information on test substances).

A summary of the test method parameters is shown in Table 3.

Table 3: Summary of test method parameters for international the pre-validation ring test

Parameter	
Test compound (radiolabelled)	^{14}C-salicylic acid (CAS No. 69-72-7) (provided by Co-ordinator)
Test matrix	- anaerobic cattle manure (liquid manure sampled from a manure tank or lagoon adjusted to 10% dry matter content, provided by Co-ordinator) - anaerobic pig manure (liquid manure sampled from a tank or lagoon adjusted to 5% dry matter content, sampled individually by each participant)
Pre-treatment	homogenisation
Storage of manure	maximum recommended storage period: 2 months at 20°C (anaerobic)
Manure matrix characterisation (mimimum, ref. to EMA, 2011)	- pH - temperature - organic matter [%] - redox potential - dry matter content - nitrogen content (NH_4-N and N_{tot}) - microbial activity [1]
Amount of manure	50 g (recommended) - 100 g wet weight per incubation vessel
Pre-incubation	21 days at 20°C, anaerobic
Test duration	35 d
Test design	anaerobic, static or flow-through
Temperature	20 ± 2°C
Lighting	complete darkness
Redox conditions	anaerobic (redox potential always below -100 mV); moistened nitrogen is passed above the manure
Number of sampling time points	7 (0, 3, 7, 14±1, 21±1, 28±1, 35±1 days after application; because of practical reasons it was not possible in all laboratories and in all cases to sample at exactly day 14, 21, 28 and 35, but sampling was at day 13, 14 or 15 etc.)
Number of test concentrations	1 (recommended: 75 kBq salicylic acid, corresponding to 18.66 µg salicylic acid per 50 g manure wet weight per test vessel)
Number of replicates	3 (per sampling and type of manure)
Number of sterile controls	2 (per type of manure; without gas trapping; sampling at termination of incubation)
Endpoints / parameters	- mineralization (CO_2 + CH_4) - formation of non-extractable residues (NER) - identification of major transformation products (> 10 % aR) - DT_{50} parent and major transformation products (if applicable) - mass balance
Evaluation of ring test results	by the Co-ordinator (excel file for data reporting was provided to each participant)

[1)] Mineralization of ^{14}C-labeled glucose under anaerobic conditions (see Annex 2, draft test method)

Evaluation of ring test results

^{14}C-mass balance

A mass balance was determined for each smpling time point as described in chapter 2.4.

Degradation kinetics

The test results were analysed based on the recommendations of FOCUS degradation kinetics (FOCUS (2006) using SFO (Single First Order)-kinetics (see chapter 2.6 for details).

Statistical evaluation

DT_{50}-values and DT_{90}-values were determined to describe the kinetics of the disappearance of the test substance. DT_x-values were ln-transformed for calculation of mean and standard deviations. The calculation of DT_{50}-values and DT_{90}-values is described in chapter 2.6. Furthermore, mean and standard deviations for non-extractable residues (NER) at the end of the study were determined. Because NER data are bounded within 0% and 100% they had to be treated differently than the DT_x-values. The isometric log ratio transformation (ilr-transformation) was used for calculation of mean and standard deviations (see chapter 2.6 for details).

Workshop

A two-day workshop on the pre-validation ring test was held at ECT Oekotoxikologie GmbH, Flörsheim, Germany on April 18th/19th 2013. At the workshop results from method development were presented. One main focus was the discussion of results and experiences of the participants with the pre-validation ring test together with international experts. Furthermore, the current draft test method as well as necessary changes or problems were discussed. Hands-on experiments during the workshop were performed by technicians of several participants to ensure the use of the same techniques amongst all the participants, e.g. for test substance application. Every step of the method was discussed in detail to identify crucial points and to update the draft guidline accordingly to obtain higher reproducibility.

3 Results of manure characterization

3.1 Manure characterization

At ten sites all over Germany which are typical for cattle rearing cattle manure samples were taken in winter 2010 / 2011 (sampled in December to January), and in summer 2011 (sampled in April to July). Descriptions of the sites as obtained from the questionnaire and parameters measured on-site are given in the table on the next page. All parameters, i.e. parameters measured on-site and off-site in the laboratory, are shown in the subsequent figure.

Furthermore, at six sites, pig manure samples were taken in the period from November 2011 to September 2012. In order to obtain ten samples for the statistical variance analyses, some of the pig manure storage tanks were sampled on two or three occasions. These were BAY_1p (collected twice at two subsequent days in 11/2011), BAY_2p (collected twice at two subsequent days in 11/2011), and NRW_2p (collected three times namely in 01/2012, 02/2012 and 06/2012).

Sampling was performed as described in detail in chapter 2.1. The samples were analysed for the parameters dry matter content [%], organic matter content [% fw], ammonia nitrogen [mg/kg fw], total nitrogen [mg/kg

fw], organic carbon content [% fw], pH-value, redox-potential [mV] and temperature [°C]. pH-value, redox-potential and temperature were measured on-site at the location of sampling whereas the other parameters were analysed off-site, i.e. in the laboratory.

In order to obtain a general overview on the variability of the measured data for each parameter, the results are presented as figures. Figure 15 and Figure 16 show the individual data (10 data points per parameter) as well as mean values for the parameters dry matter content, organic matter content, organic carbon content, pH-value, temperature, ammonia nitrogen, and total nitrogen. Results presented are for cattle manure sampled in winter, cattle manure sampled in summer and pig manure sampled from November 2011 to September 2012.

The mode of results presentation in the figures and table below the figures is as follows:

- Measured values, i.e. 10 replicates per parameter, are presented in the figures. These are measured values ("original data").
- Mean values of the 10 replicates each are given as arithmetic means. These means are shown in the figures as open squares, and are additionally presented in the table below the figure for reasons of completeness and transparency.
- Furthermore, standard deviations and coefficients of variation are given.

Table 4: Description of cattle manure sampling sites as obtained from the questionnaire.

Postal code	Code	manure storage tank			stock			Last treatment before x months	feeding
		above ground	below ground	volume [m³]	beef cattle	dairy cow	young cattle		
57392	NRW_1c		X	100	-	100	45		pasture, silage (pasture)
57392	NRW_2c		X	100	-	80	30	3 - 6	pasture, silage (pasture), mash
46282	NRW_3c		X	100		80		>6	silage (pasture, corn), shred
47533	NRW_4c		X	80	-	900		>6	silage (corn)
48249	NRW_5c		X	100	200	-	-	>6	silage (corn), food for fattening (e.g. rape shred)
37139	NDS_1c		X	70	-	50	50#	3 - 6	silage (pasture, corn)
37181	NDS_2c		X	100	-	170	170#	>6	silage (pasture), straw
35315	He_1c		X	70	-	90	45	3 - 6	pasture, silage (corn)
85276	BAY_1c		X*)	250	-	65	30	>6	cereals, silage (corn)
85298	BAY_2c		X	2 x 250	-	40	30	>6	silage (pasture, corn), hay

*) open storage tank
calves
NRW: Northrhine-Westphalia (Nordrhein-Westfalen)
NDS: Lower Saxonia (Niedersachsen)
BAY: Bavaria (Bayern)
He: Hesse (Hessen)

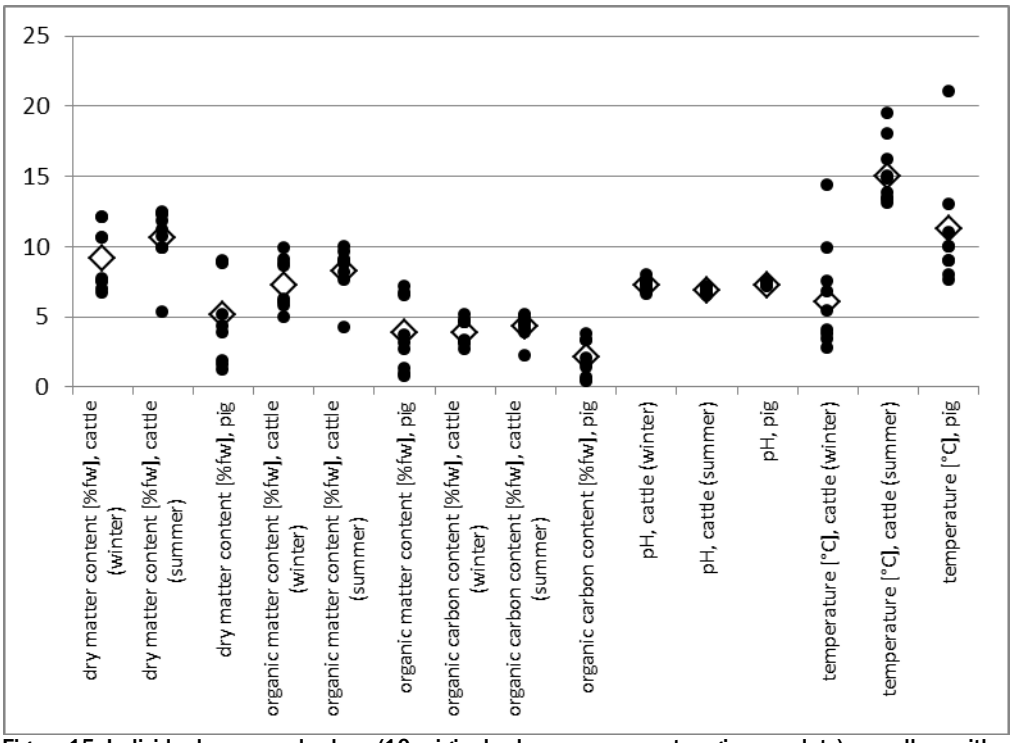

Figure 15: Individual measured values (10 original values per parameter; given as dots) as well as arithmetic mean values (given as open squares) for the parameters dry matter content [%fw], organic matter content [% fw], organic carbon content [% fw], pH-value, temperature [°C] for cattle manure sampled in winter 2010 / 2011, cattle manure sampled in summer 2011, and pig manure sampled in the period November 2011 to September 2012 at ten different sampling sites in Germany are shown. The units of the y- axis are: [% fw] for the parameter dry matter content, organic matter content, and organic carbon content; [°C] for the parameter temperature, and without dimension for the pH-value.

Table 5: Mean-values (arithmetic means), standard deviation (SD) and coefficient of variation (COV) for the parameters dry matter content [%], organic matter content [% fw], organic carbon content [% fw], pH-value, temperature [°C] for cattle manure sampled in winter 2010 / 2011, cattle manure sampled in summer 2011, and pig manure sampled in the period November 2011 to September 2012 at ten different sampling sites in Germany.

	Dry matter content [%]			**organic matter content [% fw]**			**Organic carbon content [% fw]**			**pH-value (on-site)**			**Temperature (on-site)**			**Redox-potential [mV]**		
	Cattle (win-ter)	Cattle (sum-mer)	Pig	Cattle (win-ter)	Cattle (sum-mer)	Pig	Cattle (win-ter)	Cattle (sum-mer)	Pig	Cattle (win-ter)	Cattle (sum-mer)	Pig	Cattle (win-ter)	Cattle (sum-mer)	Pig	Cattle (win-ter)	Cattle (sum-mer)	Pig
Arithmetic mean	9,2	10,6	5,2	7,3	8,3	3,9	3,9	4,3	2,1	7,3	6,9	7,3	6,1	15,0	11,3	-356,6	-394,6	-417,4
SD	2.10	2.12	3.15	1.70	1.61	2.50	0.87	0.85	1.31	0.38	0.20	0.13	3.71	2.28	3.98	71.23	26.06	17.14
COV	0.23	0.20	0.60	0.23	0.19	0.64	0.22	0.20	0.63	0.05	0.03	0.02	0.61	0.15	0.35	0.20	0.07	0.04

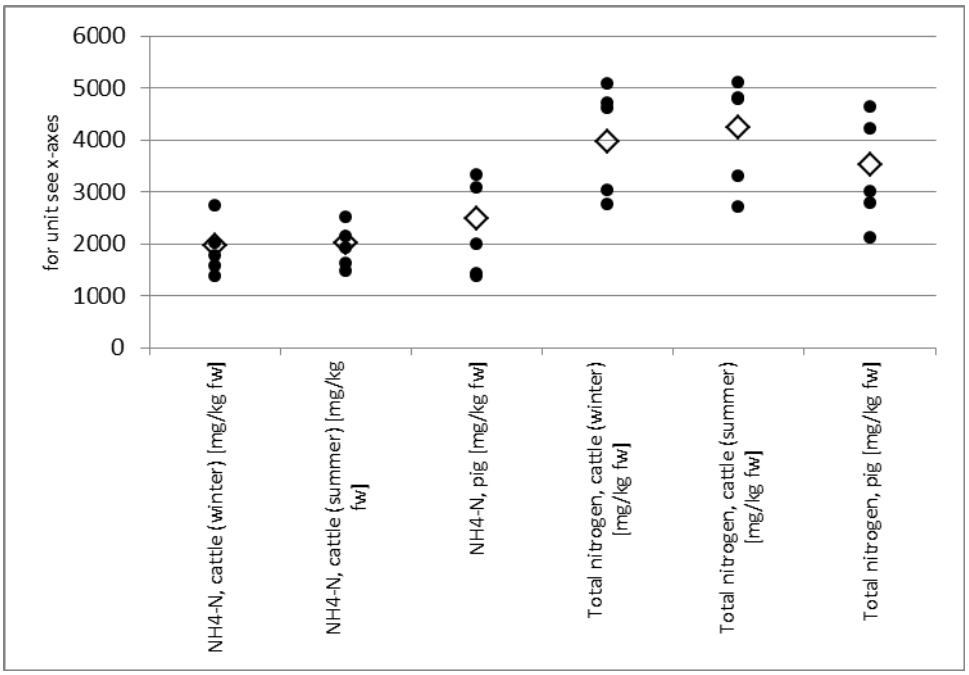

Figure 16: Individual values (10 original values per parameter; given as dots) as well as arithmetic mean values (given as open squares) for the parameters ammonia nitrogen [mg/kg fw] and total nitrogen [mg/kg fw] for cattle manure sampled in winter 2010 / 2011, cattle manure sampled in summer 2011, and pig manure sampled in the period November 2011 to September 2012 at ten different sampling sites in Germany are shown. The units of the y- axis are [mg/kg fw]

Table 6: Mean-values (arithmethic means), standard deviation (SD), and coefficient of variation (COV) for the parameters ammonia nitrogen [mg/kg fw] and total nitrogen [mg/kg fw] for cattle manure sampled in winter 2010 / 2011, cattle manure sampled in summer 2011, and pig manure sampled in the period November 2011 to September 2012 at ten different sampling sites in Germany.

	ammonia nitrogen [mg/kg fw]			**total nitrogen [mg/kg fw]**		
	Cattle (winter)	Cattle (summer)	Pig	Cattle (winter)	Cattle (summer)	Pig
Arithmetic mean	2024.9	2100.0	2654.2	4068.1	4404.7	3749.0
SD	507.88	406.78	995.47	867.91	823.84	1330.24
COV	0.25	0.19	0.38	0.21	0.19	0.35

Furthermore, detailed data as well as all results from the statistical treatment are presented in tables A3_1 to A3_3. Tables A3_1 and A3_2 in annex 3 present the results for cattle manure sampled in winter 2010 / 2011 and in summer 2011. Results correspond to individual samples apart from those for the sites NRW_1c and NRW_2c. At these sampling sites, 10 replicate samples each were taken for homogeneity check of the sampling procedure (see chapter 3.2 for results of the homogeneity check); data represent mean values of the 10 replicate samples. Table A3_3 in annex 3 shows the results for pig manure. As no homogeneity check for the sampling procedure has been done for pig manure, all data are from individual samples.

Cattle manure sampled in winter 2010 / 2011:

Table A3_1 shows all data including results of the statistical treatment. Two aspects in that table A3_1, cattle manure sampled in winter 2010 / 2011, are worth mentioning: Manure from sampling site NRW_5c has not been mixed in the storage tank prior to sampling because of a misunderstanding in communication with the farmer, and at sampling site BAY_1c a thick layer on top of the manure has been intermixed. Thus, the samples might not be as homogenous as the samples in general (as derived from the 10 times sampling).

Highest variation is observed for the temperature measured in the manure storage tank. This is 0.594 for the manure sampled in winter. Measured values for the temperature range between 2.8 to 14.4 °C with a mean of 5.3 °C. Ammonia nitrogen, total nitrogen, dry matter content, organic carbon content, organic matter content, and the redox-potential measured on-site show lower standard deviation (SD). These are: 0.26 (ammonia nitrogen), 0.238 (organic matter content), 0.23 (total nitrogen), 0.232 (Organic carbon content), 0.231 (dry matter content), and 0.223 (redox-potential).

Temperature profile in winter 2010 / 2011:

In addition to the presented characterization in winter 2011/2012 a depth dependent profile of temperatures in the storage tank (cattle manure) was measured before mixing. Results are shown in the following Table 7.

Table 7: Temperature profile in a cattle manure tank (volume: 100 m^3) in winter (surrounding temperature: 10.0 °C). The tank is a below ground tank, the temperature profile was measured before mixing. Region: Hochsauerlandkreis, southern North-Rhine-Westphalia

Depth [cm]	Temperature [°C]
10	8.5
25	9.0
50	8.0
75	8.0
100	7.5
125	6.5
150	6.0
175	6.5

Cattle manure sampled in summer 2011 (table A3_2):

In particular the coefficient of variation (COV) for manure temperature is lower compared to manure sampled in winter. For manure sampled in summer it is 0.15 only (0.61 for winter manure). The high COV of 0.61 seems to be attributed to the value of 14.4 °C measured for winter manure sampled at site NRW_3c. Also, the COV obtained for the redox-potential is lower for the manure sampled in summer (0.07 for summer manure compared to 0.2 for winter manure). The COV of all other manure parameters are comparable for manure sampled in winter and in summer.

Pig manure sampled in the period November 2011 to September 2012 (table A3_3):

The dry matter content (0.756), organic matter content (0.843) and organic carbon content content (0.84) are the parameters with the highest standard deviation. Differences in the dry matter content, for example, are pronounced for the different sampling sites and for the same sampling site when manure is collected in different months of the year (NRW_2p, collected three times namely in 01/2012, 02/2012 and 06/2012). It is less pronounced for the same sampling site when manure is collected twice at two subsequent days (BAY_1p and BAY_2p). The parameters ammonium nitrogen, total nitrogen and on-site temperature show coefficients of variation (COV) in the range of 0.35 – 0.38. COV for the pH-value and redox-potential are much lower and are 0.02 and 0.04, respectively.

3.2 Sampling site selection

Out of the ten sites (cattle manure) and six sites (pig manure), three sites each were selected for manure sampling to be used in the transformation studies. Prior to the choice of the manures for transformation testing, the matrix parameters dry matter content, organic matter content, ammonium nitrogen content, total nitrogen content, and organic carbon content were determined (for methods of parameter characterization see chapter 2.3, for statistical analyses see chapter 2.7). Manures were chosen to represent a most diverse set for transformation testing purposes (different origins, different size/type of farms, different feed of the animals, etc.).

Besides these results (see tables A3_1 to A3_3) the following criteria were also used for sampling site selection:

- Easy accessibility of the manure storage tank (short distance to the laboratory, excellent personal contact to the farmer)
- Regional distribution of sampling sites, consideration of regions of importance for cattle and pig rearing.

Based on all criteria, the following sites were selected. However, the criteria mentioned in the bullet points were the most important ones for the decision.
Cattle manure: NRW_1c, NRW_2c, and BAY_2c.
Pig manure: NRW_1p, NRW_2p, and BAY_2p.

3.3 Characterisation of manure used for transformation studies

Parameters of the manures used in all transformation studies are presented in tables A3_4 to A3_5. Furthermore, figures A4_1 to A4_6 in annex 4 exemplary show the manure parameters for the transformation studies using Salicylic acid as test substance. Beside the parameters discussed in the previous chapter 3.1, the microbial activity measured at the start and the end of the transformation studies is given.

The dry matter content of cattle manures varied between 7.7 and 12.4 [% fw], that of pig manure was in the range of 5.2 and 8.9 [%]. Prior to performing the transformation studies the dry matter content was adjusted to 10 % (for cattle manure) and 5 % (pig manure). The organic matter content was in the range of 3.73 to 10.03 [% fw], values for ammonia nitrogen ranged between 1436 and 3945 [mg/kg], total nitrogen (Kjeldahl) was between 3015 and 5329 [mg/kg], and the Organic carbon content content ranged between 2.06 and 5.2 [% fw].

At the beginning of the transformation studies, the medians of glucose mineralization (cattle manure) were in the range of 54.3 – 99.9 [% aR]. For pig manure, respective medians were in the range of 37.7 – 57.3 [% aR]. For the mineralization at the end of the transformation studies, no unique trend was observed. However, this was depended on the duration of the studies. For transformation studies with duration of 28 – 41 days, the mineralization at the end were comparable to those measured at the beginning. For the transformation of Biocide B in cattle manure with a study duration of 105 days, mineralization at the end of the studies were about half of those at the beginning. Pig manure from site BAY_2p showed a comparable behavior, whereas the mineralization of pig manure from the sites NRW_1p and NRW_2p was comparable at the beginning and end of the studies.

3.4 Homogeneity of sampling: 10 replicates per sampling site

The methodology of sampling manure for testing purposes has to be a reliable and reproducible method. Otherwise, any further step of a transformation study might be questionable. In order to prove the homogeneity of the sampling procedure, 10 replicates of cattle manure each were sampled at the sites NRW_1c and NRW_2c, both in winter and in summer. The collection procedure for the homogeneity check is described in detail in chapter 2.2. The replicates were analysed for the parameters dry matter content [%], organic matter content [% fw], ammonium nitrogen [mg/kg fw], total nitrogen [mg/ kg fw] and organic carbon content [% fw]. The individual data and statistics (mean value, standard deviation) are listed in Table 8 (cattle manure sampled in winter, NRW_1c), Table 9 (cattle manure sampled in summer, NRW_1c), Table 10 (cattle manure sampled in winter, NRW_2), and Table 11 (cattle manure sampled in summer, NRW_2c).

Table 8: Characterization of manure for homogeneity check: cattle manure sampled in winter, site: NRW_1c

sampling	Code sampling site	Dry matter [%]	Organic matter content [% fw]	NH4-N [mg/kg fw]	Total nitrogen (Kjeldahl) [mg/kg fw]	Organic carbon content [% fw]
01 /2011	NRW_1c	9.66	7.81	2726	5125	4.16
		9.49	7.66	2764	5036	4.10
		9.76	7.89	2710	5010	4.25
		9.39	7.54	2741	5081	4.08
		9.48	7.65	2726	4971	4.14
		9.48	7.65	2757	5144	4.12
		9.56	7.70	2772	5156	4.13
		9.66	7.80	2671	5014	4.20
		9.51	7.66	2757	5190	4.12
		9.55	7.71	2788	4986	4.12
Statistical treatment:						
n		10	10	10	10	10
Standard deviation		0.1	0.1	34.2	78.3	0.1
Coefficient of variation (COV, %)		1.15	1.31	1.25	1.54	1.21
Mean		9.6	7.7	2741	5071	4.1

Table 9: Characterization of manure for homogeneity check: cattle manure sampled in summer, site: NRW_1c

sampling	Code sampling site	Dry matter [%]	Organic matter content [% fw]	NH4-N [mg/kg fw]	Total nitrogen (Kjeldahl) [mg/kg fw]	Organic carbon content [% fw]
04 / 2011	NRW_1c	9.6	7.35	2910	5049	4.01
		9.63	7.22	2811	4859	3.99
		10.19	7.63	2545	5148	4.22
		9.94	7.53	2755	5377	4.14
		9.79	7.48	2460	4636	4.05
		15.19	11.98	2694	4877	6.39
		9.92	7.55	2424	4487	4.13
		10.24	7.70	2311	4750	4.23
		9.62	7.28	2162	4502	3.98
		9.84	7.39	2215	4530	4.06
Statistical treatment:						
n		10	10	10	10	10
Standard deviation		1.7	1.4	258.2	300.0	0.7
Coefficient of variation (COV, %)		16.34	18.17	10.21	6.22	16.96
Mean		10.4	7.9	2528.7	4821.5	4.3

Table 10: Characterization of manure for homogeneity check: cattle manure sampled in winter, site: NRW_2c

sampling	Code sampling site	Dry matter [%]	Organic matter content [% fw]	NH4-N [mg/kg fw]	Total nitrogen (Kjeldahl) [mg/kg fw]	Organic carbon content [% fw]
01 /2011	NRW_2c	11.98	9.79	1697	4462	5.11
		11.68	9.53	1641	4223	4.93
		12.18	10.01	1780	4569	5.21
		11.47	9.24	1708	4449	4.87
		11.85	9.64	1775	4453	5.10
		13.01	10.66	1835	4797	5.56
		12.11	9.96	1845	4612	5.20
		12.32	10.11	1900	4641	5.20
		11.86	9.67	1832	4571	5.04
		12.15	9.83	1736	5292	5.16
Statistical treatment:						
n		10	10	10	10	10
Standard deviation		0.4	0.4	79.9	284.1	0.2
Coefficient of variation (COV, %)		3.47	3.88	4.50	6.17	3.66
Mean		12.1	9.8	1774.9	4606.9	5.1

Table 11: Characterization of manure for homogeneity check: cattle manure sampled in summer, site: NRW_2c

sampling	Code sampling site	Dry matter [%]	Organic matter content [% fw]	NH4-N [mg/kg fw]	Total nitrogen (Kjeldahl) [mg/kg fw]	Organic carbon content [% fw]
05 / 2011	NRW_2c	12.57	10.19	1898	4803	5.28
		11.98	9.62	1913	4904	4.98
		12.35	10.03	1919	4802	5.15
		13.22	10.73	1879	4880	5.63
		12.76	10.31	1880	4696	5.31
		12.36	10.02	1870	4792	5.12
		12.88	10.43	1929	4713	5.40
		11.71	9.41	1998	4617	4.89
		12.21	9.89	1926	4771	5.15
		12.28	9.97	1933	4806	5.16
Statistical treatment:						
n		10	10	10	10	10
Standard deviation		0.4	0.4	37.1	85.2	0.2
Coefficient of variation (COV, %)		3.56	3.81	1.94	1.78	4.05
Mean		12.4	10.1	1914.5	4778.4	5.2

As the standard deviation (SD) give information on the homogeneity of data, they are listed as summary in Table 12.

Table 12: Standard deviation (SD) for manure parameters, 10 replicates per sampling site

Sampling	Code sampling site	Standard deviation (SD)				
		Dry matter [%]	Organic matter content [% fw]	NH4-N [mg/kg]	Total nitrogen (Kjeldahl) [mg/kg]	Organic carbon content [% fw]
Winter 2011	NRW_1c	0.1	0.1	34.2	78.3	0.1
Summer 2011		1.7	1.4	258.2	300.0	0.7
Winter 2011	NRW_2c	0.4	0.4	79.9	284.1	0.2
Summer 2011		0.4	0.4	37.1	85.2	0.2

Furthermore, Table 13 summarises coefficients of variation (COV) for all parameters and samplings which also give information on the homogeneity of data.

COV for all parameters of 10 replicates sampled in winter at sampling site NRW_1c are very low and are between 1.1 – 1.5 %. For that sampling site variations are much higher for manure sampled in summer. COV are in the range of 6.2 – 15.2 %. This indicates a more heterogeneous distribution of summer manure within the storage tank compared to winter manure.

In contrast, variations between the 10 replicates sampled at site NRW_2c are somewhat higher compared to winter manure at site NRW_1c. On the other side, differences in variation coefficients between winter and summer manure at site NRW_2c are low. COVs for winter manure are in the range of 3.4 – 6.0 %, and for summer manure are in the range of 1.8 – 4.0 %. This indicates an even distribution of manure in the storage tank both, for manure sampled in summer and in winter.

In total, COV between 10 replicates of sampling are low. Therefore, it can be concluded that the sampling methodology described in chapter 2.2 yields homogeneous manure samples, and thus is a suitable methodology to sample manure for testing purposes.

In order to investigate differences in parameter of manure of different origin results for 10 sites were also subjected to COV-calculation. Coefficients of variation are much higher as for the above comparison. COV both for manure sampled in winter and in summer are in the range of 18.7 – 26.0 %. It can be concluded, that manure for testing purposes should be taken from more than one sampling site.

Table 13: Comparision of coefficients of variation (COV, %) for 10 replicates from NRW_1 and NRW2 sites in summer and winter and 10 different tanks in summer and winter for cattle manure

Manure parameter	Coefficients of variation [%]					
	Site NRW_1c, 10 replicates		Site NRW_2c, 10 replicates		10 sites, 1 replicate	
	Winter	Summer	Winter	Summer	Winter	Summer
Dry matter [%]	1.1	13.9	3.4	3.5	23.1	25.7
Organic matter content [% dm]	1.3	15.2	3.8	3.8	23.8	24.8
NH_4-N [mg/kg]	1.3	10.3	4.5	1.9	26.0	18.7
Total N [mg/kg]	1.5	6.2	6.0	1.8	23.0	21.3
Organic carbon content [% dm]	1.2	14.4	3.6	4.0	23.2	24.7

In order to further investigate the influence of conditions in the storage tank and origin of manure on the variation of manure parameters, the results of manure characterization were subjected to comprehensive statistical analyses (Levene´s test, U-test; see tables A3_6 to A3_10).

In summary, it can be concluded that

- for most – but not all – parameters there are significant differences when manure is sampled at the same site in summer and in winter,
- there are significant differences for all parameters for manure of different origin.

Conclusions:

Manure for testing purposes should be taken from more than one sampling site. Furthermore, for reasons of practicability it is recommended – though slight differences might be observed – that manure can be sampled at any time of the year.

3.5 Influence of storage duration and temperature in the laboratory on microbial activity of manure

Once the manure has been sampled from the storage tank it is transferred to the laboratory. There, a further storage might be necessary until the manure is used for a transformation study. The influences of storage duration and storage temperature on the microbial activity of the manure were examined.

This was achieved by storing cattle manure for various time periods and at several temperatures followed by determining the mineralization/microbial activity using ^{14}C-glucose as an easily degradable substance after an incubation period of 7 days. (see chapter 2.5 for details on the tests).

Table 14: Influence of storage duration and temperature on microbial activity of cattle manure after 3 days of manure acclimation

Storage period (d)	replicate	% ^{14}C-glucose mineralization after 7d		
		+ 20°C	+ 4°C	- 20°C
14	1	41.54	35.50	
	2	1)	1)	
	3	42.63	65.30	
28	1	37.13	41.56	
	2	1)	51.65	
	3	35.06	58.15	
42	1	50.05	58.67	
	2	62.99	57.04	
	3	49.09	40.19	
56	1	54.73	40.04	39.41 2)
	2	56.83	1)	42.02 2)
	3	61.24	41.97	31.15 2)

1) Sample destroyed, not considered
2) Values for storage at -20 °C are listed for further information but not considered for the statistical treatment

^{14}C-glucose mineralization differed for the tested storage periods (table 14). The mineralization for a "short-term period" (storage period of 14 and 28 days) is smaller as for a "long-term period (storage period of 42 and 56 days) at a storage temperature of 20° C but no difference can be observed for a storage temperature of 4°C.

^{14}C-glucose mineralization differed for the tested storage periods of 28 d, 63 d and 105 d. Thus, a maximum storage period should be recommended on the basis of individual data on ^{14}C-glucose mineralization. Data in table 15 show ^{14}C-glucose mineralization 42- 58 % for the storage period of 105 d, between 47 - 87 % for 63 d storage and between 88-107% for 28 d storage and 63-70% for 0 days storage. ^{14}C-glucose mineralization first increases till day 28, while it decreases when manure is stored for longer time periods, indicating a loss of microbial activity.

Table 15: Influence of storage period and temperature on microbial activity of manure after 21 days of manure acclimation

Storage conditions	Data set	% mineralization (21d acclimation)
0d		70.5
		67.4
		63.0
28d, 4°C	Data set 1 28d	89.7
		87.5
		101.6
28d, 20°C		107.1
		105.6
		101.9
63d, 4°C	Data set 2 63d	46.7
		81.5
		72.6
63d, 20°C		87.6
		83.8
		71.4
105d, 4°C	Data set 3 105d	42.4
		44.8
		51.4
105d, 20°C		58.0
		52.9
		49.7

3.6 Influence of duration of manure acclimation period on microbial activity of manure

Prior to use for testing purposes, the stored manure has to be acclimatized to the conditions under which the transformation study is performed. This means, the stored manure is filled into the incubation vessels, which are built into the flow-through system and incubated anaerobically at 20 °C for a certain time period (see chapter 2.4 for details). By acclimation for 3 days and 21 days and a subsequent determination of the microbial activity of the manure, the influence of the duration of the acclimation period was investigated. Previous manure storage temperatures were +4 °C, +20 °C and -20 °C (the latter for one sample only). As above, the test on microbial activity was performed by determining the mineralization of an easily degradable substance, namely ^{14}C-glucose after an incubation period of 7 days (see chapter 2.3 for details).

As there are obvious differences in the ^{14}C-glucose mineralization rates for both acclimation periods (table 16) it is recommended to have an acclimation period of 21 days in order to ensure comparable biological activity.

Table 16: Influence of acclimation period on microbial activity of cattle manure

Sample	Parallel	Mineralization rate [%] after 8d of ^{14}C-glucose degradation with acclimation period of	
		3 d (data set 1)	21 d (data set 2)
0 days	_1	35.5	56.3
	_2	35.2	62.7
	_3	37.8	47.7
56 d storage at - 20°C	_1	54.7	74.6
	_2	56.8	73.9
	_3	61.2	72.6
56 d storage at + 4°C	_1	40.0	59.4
	_2	27.8	65.5
	_3	42.0	72.1
56 d storage at - 20°C	_1	39.4	64.0
	_2	n.a.	n.a.
	_3	n.a.	n.a.

3.7 Conclusions: Influence of storage conditions and origin on manure parameters

- A comparison of the matrix parameters for 10 independent replicates sampled from the same cattle manure tank showed excellent homogeneity. The coefficients of variation for dry matter content, organic matter content, ammonium and total nitrogen are well below 10% for the site NRW_2 and equal or below 15% for NRW_1. In comparison to the variability observed for 10 different manures (compared to 10 independently sampled replicates from one tank), coefficients of variation are considerably lower for the 10 replicates (Tables A3_1 and A3_2 compared to A3_6 and A3_7). Thus, it can be concluded that the sampling methodology applied in this project and described in chapter 2.2 is a suitable methodology to sample manure from tanks for testing purposes.
- Manure sampling at any season is possible and the influence of storage time in the manure tank at the farm at the time point of sampling and temperature on manure parameters is negligible.
- A comparison of the microbial activity (^{14}C-glucose mineralization for different storage temperatures of -20°C, + 4 °C and +20 °C) showed differences in mineralization. Preferably, the storage in the laboratory prior to use for a transformation study should be at test temperature (Table A3_11).
- A comparison of ^{14}C-glucose mineralization for different storage periods after sampling of 28, 63 and 105 d showed that the storage period after sampling seems to have an influence on mineralization. Therefore it is necessary to establish a maximum storage period to ensure comparable testing conditions. If sampling is not possible prior to the start of the study, a maximum storage period of one month in the laboratory prior to use for a transformation study is recommended.
- A comparison of ^{14}C-glucose mineralization for the tested acclimation periods of 3 days and 21 days showed that the length of the acclimation (or pre-incubation) period seems to influence the mineralization. Therefore it is recommended to use an acclimation period of 21 days in order to ensure comparable testing conditions.

4 Results of studies on transformation with ^{14}C-Salicylic acid, ^{14}C-Paracetamol and ^{14}C-Biocide B in cattle and pig manure

Two veterinary pharmaceutical products and one biocide were used to test the feasibility of the suggested test design. To cover the most diverse set of different manures concerning origin, period stored in the tank and matrix parameters, the manure sampling sites where chosen to represent different geographic locations, different matrix parameters and summer and winter manures.

Mass balances were established by quantification of extractable radioactivity (ER), non-extractable residues (NER), and mineralization (formation of CO_2 + CH_4).

Besides radioactivity distribution and mass balances, the disappearance time for 50 % (DT50) of the parent compound were also determined.

The following chapters 4.1 and 4.2 present the measured data obtained for mass balances, NER, extractables, mineralization and DT_{50}-values. Chapter 4.3 comprises a statistical analysis as described in 2.7, i.e. tests whether manure of different type and origin yields significantly different results for the transformation studies. Chapter 4.4 gives overall conclusions.

4.1 Extractable residues, NER, mineralization and mass balance

A mass balance was determined and calculated for each sampling interval. This was done by summing-up the amount of radioactivity given in [% of applied radioactivity; % aR] in the aqueous/organic extracts plus ASE® (ASE ® performed in exceptional cases only) plus volatiles other than $^{14}CO_2$ plus + $^{14}CO_2$ + non-extractable residues (NER). To determine the formation of extractable residues, NER and mineralization, manure was processed as described in chapter 2.3.

Results for mass balances and radioactivity distribution are presented on the basis of figures and tables. Tables show the arithmetic means of 6 replicates, standard deviation (SD), and coefficient of variation (COV). An exception is the presentation of mineralization: in this case no replicates could be determined due to the experimental set-up (see chapter 2.4) and thus no mean values, SD, and COV are presented. In order to calculate a mass balance of each replicate the identical value for the mineralization was used.

General remark concerning poor mass balances:

In the course of all transformation studies with Salicylic acid, 378 individual values for the parameter “mass balance” were obtained. These values were in the range of 52.3 – 124.8 [% aR]. 145 values out of 378 were below 90 [% aR] and in the range of 52.3 – 89.6 [% aR]. 29 values out of 378 were above 110 [% aR] and in the range of 110.4 – 124.8 [% aR]. Thus, for a significant portion mass balances are quite poor. They are observed in particular in those cases where mineralization is high. Even slight leakages in the incubation system – which in particular occur when handling the incubation flasks and sampling – might result in measurable losses. Such losses can be avoided by the use of stand-alone samples. Therefore changes in the experimental setup will be considered for follow-up experiments. These observation indicate that a mass balanced based criterion might not be advisable as a strict yes or no criterion for determining validity as this is described e.g. in the OECD guidelines for other simulation type studies [OECD (2002a), OECD 2002b)].

^{14}C-Salicylic acid

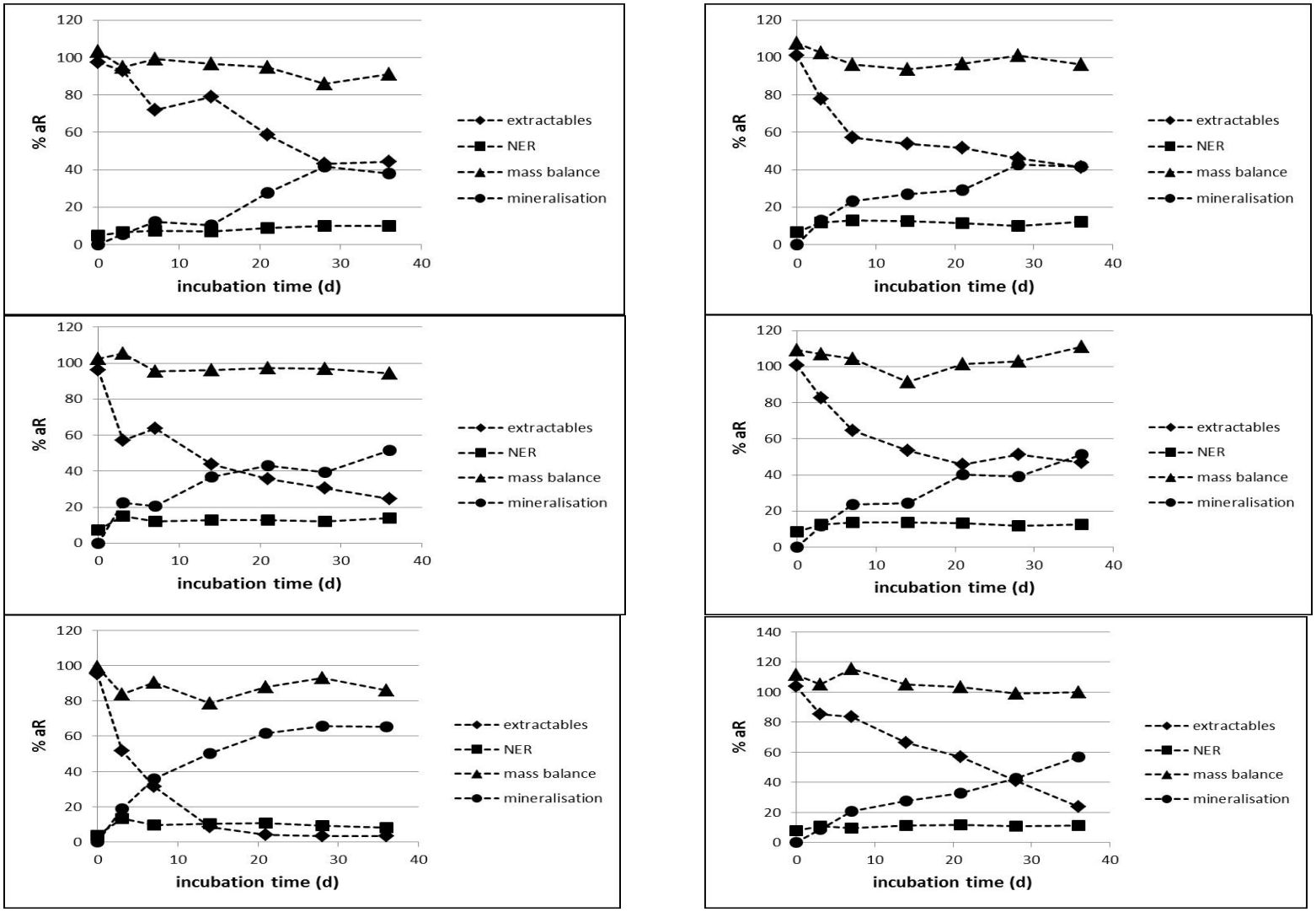

Figure 17: Radioactivity distribution for the transformation of ^{14}C-Salicylic acid in cattle manure. Sites and sampling times are: left side, top to bottom = NRW_1c, winter; NRW_2c, winter; BAY_2c, winter. Right side, top t bottom = NRW_1c, summer; NRW_2c, summer; BAY_2c, summer

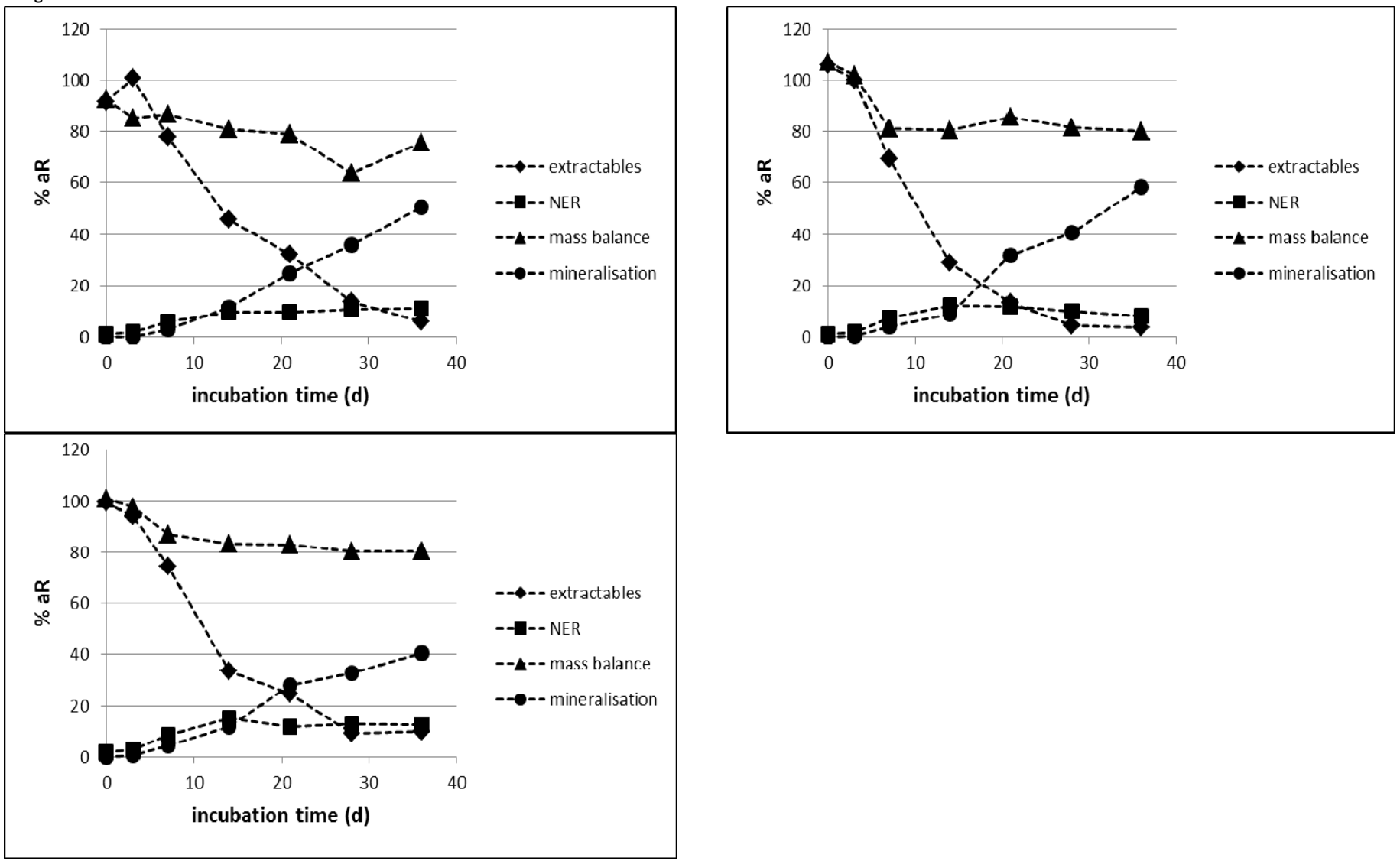

Figure 18: Radioactivity distribution for the transformation of ^{14}C-Salicylic acid in pig manure; sites: NRW_1p, NRW_2p, BAY_2p (from left to right)

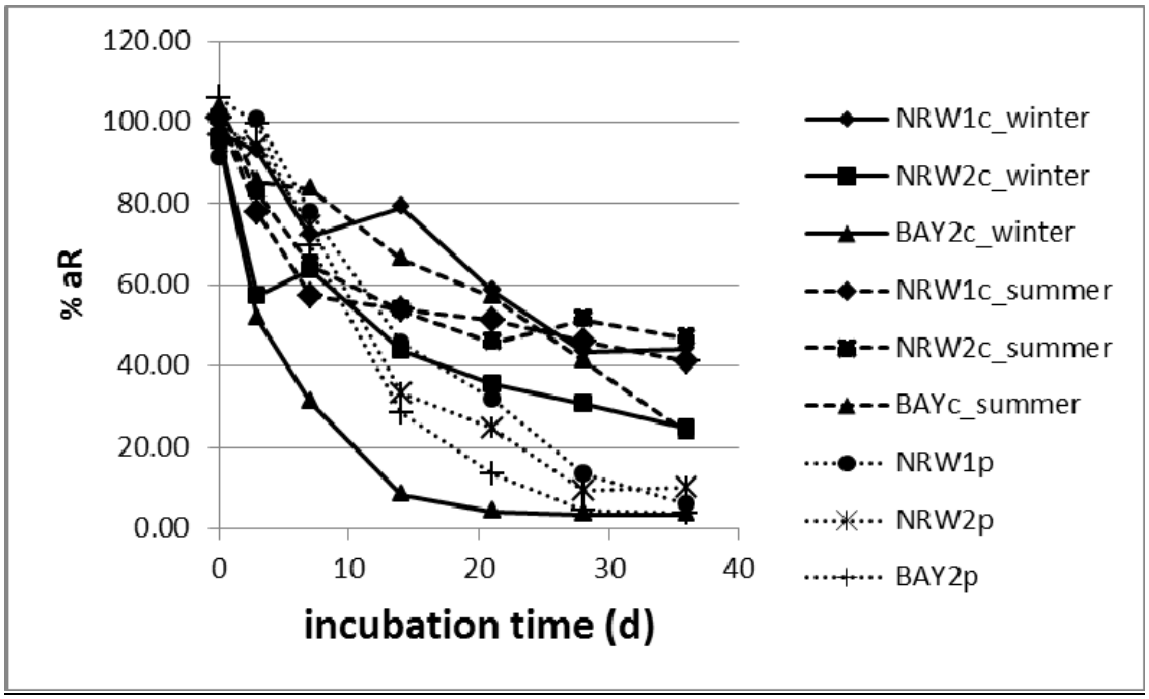

Figure 19: Extractable residues: Salicylic acid in winter and summer manure from cattle and from pig manure

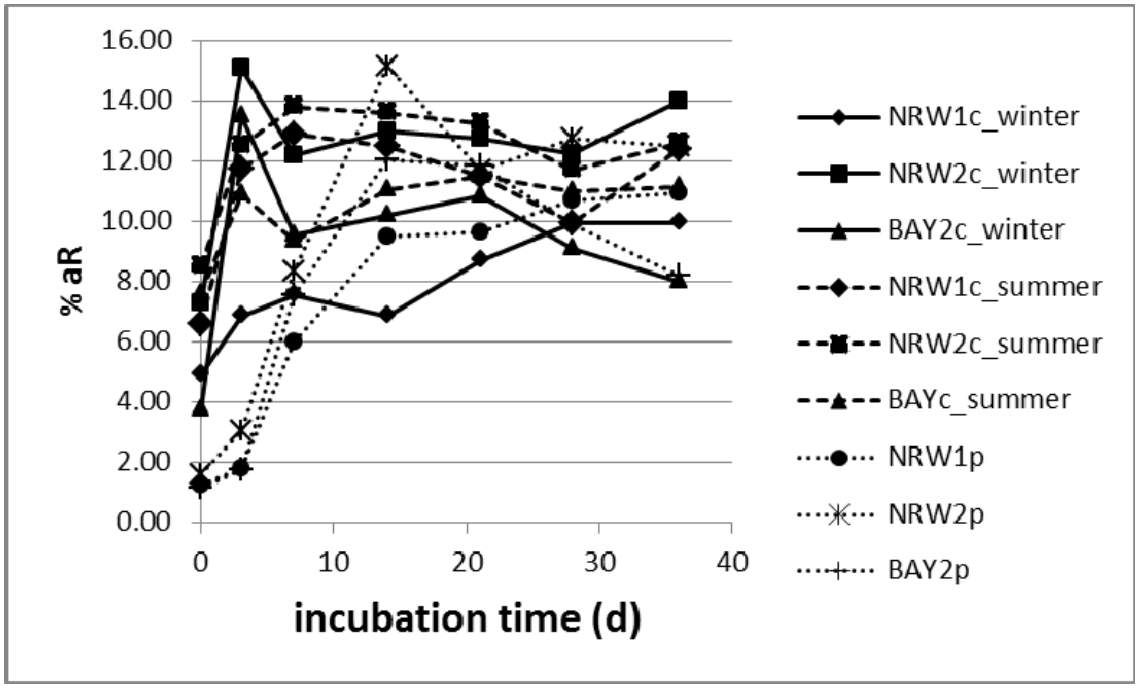

Figure 20: Non extractable residues: Salicylic acid in winter and summer manure from cattle and from pig manure

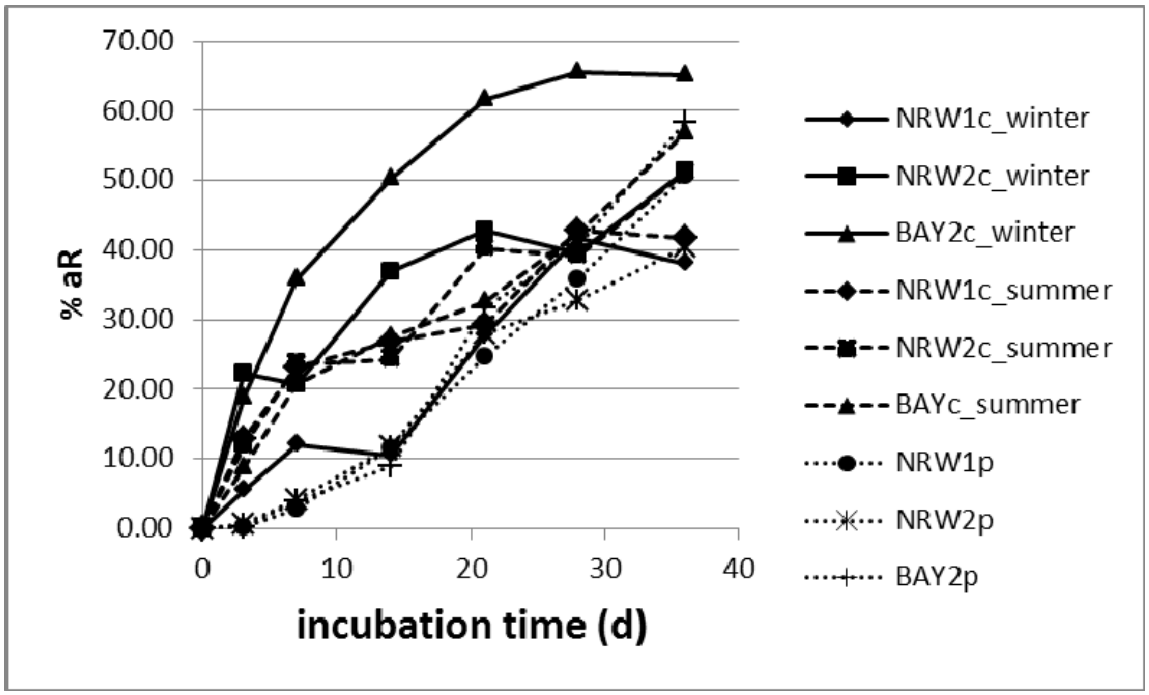

Figure 21: Mineralization: Salicylic acid in winter and summer manure from cattle and from pig manure

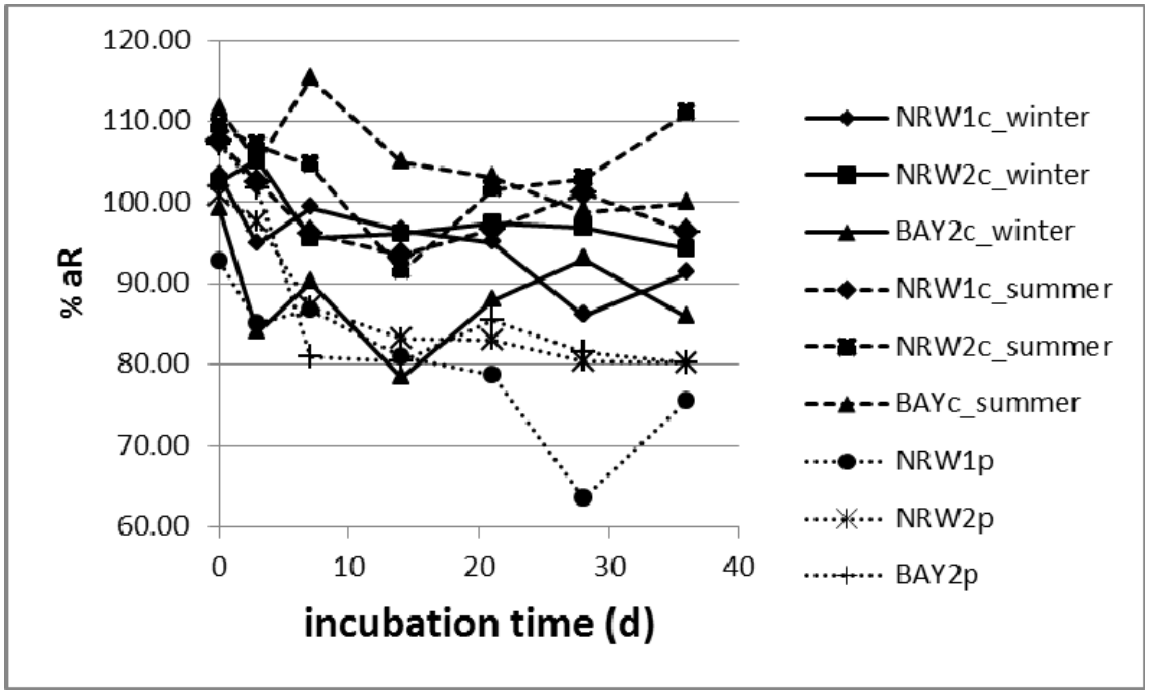

Figure 22: Mass balance: Salicylic acid in winter and summer manure from cattle and pig

Table 17: Mean-values [% aR], standard deviation (SD) and coefficient of variation (COV, [%]) for the parameter extractables for transformation of ^{14}C Salicylic acid in cattle and pig manure measured in 6 replicates, mean-values presented in the above figures.

Time [d]		0	3	7	14	21	28	36
Site NRW1c winter	Mean	97.55	93.05	71.83	78.90	58.58	43.33	44.35
	SD	2.19	2.46	8.35	5.83	7.08	5.13	3.47
	COV	2.25	2.65	11.63	7.39	12.08	11.84	7.82
Site NRW1c summer	Mean	101.00	77.82	57.27	54.02	51.48	46.00	41.32
	SD	0.53	21.36	11.52	11.72	6.93	9.16	5.75
	COV	0.52	27.45	20.12	21.69	13.46	19.92	13.91
Site NRW_2c winter	Mean	96.08	57.20	63.67	43.93	35.67	30.52	24.77
	SD	1.16	6.61	11.58	8.58	4.85	8.53	4.97
	COV	1.21	11.55	18.19	19.53	13.60	27.95	20.06
Site NRW_2c summer	Mean	100.73	82.68	64.68	53.62	45.77	51.20	46.76
	SD	3.18	9.99	7.89	7.32	5.92	9.59	3.89
	COV	3.16	12.09	12.19	13.65	12.93	18.73	8.33
Site BAY_2c winter	Mean	95.30	51.63	31.18	8.38	4.22	3.38	3.35
	SD	2.20	4.01	3.84	0.67	0.26	0.29	0.26
	COV	2.31	7.78	12.32	8.03	6.08	8.65	7.73
Site BAY_2c summer	Mean	103.87	85.30	83.60	66.30	56.77	41.05	23.48
	SD	2.06	5.73	8.49	3.45	8.41	9.57	16.15
	COV	1.99	6.72	10.16	5.20	14.82	23.31	68.76
Site NRW_1p	Mean	91.45	100.60	77.83	45.78	31.93	13.53	6.00
	SD	1.45	85.70	3.35	9.23	7.98	5.93	0.54
	COV	1.58	85.19	4.30	20.17	25.00	43.84	9.07
Site NRW_2p	Mean	99.33	93.95	74.57	33.47	24.80	9.22	10.00
	SD	3.16	2.25	5.10	5.19	5.43	3.25	3.27
	COV	3.18	2.39	6.85	15.50	21.90	35.31	32.69
Site Bay_2p	Mean	105.95	99.82	69.48	28.62	13.30	4.45	3.78
	SD	1.96	1.43	5.36	6.85	3.32	1.46	0.08
	COV	1.85	1.44	7.71	23.93	24.94	32.87	1.99

Table 18: Mean-values [% aR], standard deviation (SD) and coefficient of variation (COV, [%]) for the parameter non-extractable residues (NER) for transformation of ^{14}C Salicylic acid in cattle and pig manure measured in 6 replicates, mean-values presented in the above figures.

Time [d]		0	3	7	14	21	28	36
Site NRW1c winter	Mean	4.90	6.82	7.52	6.85	8.68	9.92	9.95
	SD	1.30	0.64	1.11	0.93	0.83	0.83	0.26
	COV	26.48	9.44	14.78	13.59	9.51	8.35	2.60
Site NRW1c summer	Mean	6.57	11.75	12.88	12.47	11.47	9.92	12.37
	SD	0.26	5.30	1.64	2.58	1.53	2.92	2.14
	COV	3.93	45.10	12.72	20.66	13.35	29.44	17.30
Site NRW_2c winter	Mean	7.27	15.07	12.17	13.00	12.72	12.25	13.97
	SD	0.30	1.86	2.33	1.01	0.32	0.84	1.31
	COV	4.14	12.36	19.14	7.74	2.51	6.82	9.39
Site NRW_2c summer	Mean	8.47	12.47	13.80	13.58	13.25	11.70	12.58
	SD	0.62	2.10	2.36	1.95	2.00	3.17	2.62
	COV	7.27	16.82	17.11	14.37	15.12	27.13	20.85
Site BAY_2c winter	Mean	3.75	13.47	9.52	10.20	10.85	9.10	8.00
	SD	0.36	3.00	1.02	1.65	0.94	0.92	1.18
	COV	9.50	22.29	10.68	16.17	8.66	10.10	14.79
Site BAY_2c summer	Mean	7.58	10.88	9.32	11.03	11.50	10.97	11.15
	SD	0.43	1.20	1.94	1.23	0.69	1.84	1.56
	COV	5.62	11.02	20.82	11.18	5.97	16.76	13.96
Site NRW_1p	Mean	1.25	1.80	6.00	9.47	9.63	10.67	10.95
	SD	0.34	1.80	0.81	3.62	2.09	1.84	0.67
	COV	27.13	100.00	13.50	38.21	21.70	17.29	6.13
Site NRW_2p	Mean	1.60	3.05	8.33	15.12	11.70	12.72	12.50
	SD	0.24	0.63	1.45	0.89	1.04	2.37	2.69
	COV	14.79	20.61	17.37	5.88	8.92	18.64	21.53
Site Bay_2p	Mean	1.13	1.75	7.53	12.05	11.83	9.90	8.17
	SD	0.21	0.22	1.03	1.31	1.79	1.12	1.22
	COV	18.23	12.39	13.61	10.87	15.09	11.30	14.97

Table 19: Measured values for the parameter mineralization for transformation of ^{14}C Salicylic acid in cattle and pig manure, -values presented in the above figures. Single values measured and thus no SD and COV were calculated

Time [d]	0	3	7	14	21	28	36
Mean Site NRW1c winter	0.00	5.40	12.10	10.50	27.70	41.70	38.10
Mean Site NRW1c summer	0.00	12.90	23.10	26.90	29.20	42.80	41.80
Mean Site NRW_2c winter	0.00	22.30	20.70	36.90	42.90	39.50	51.40
Mean Site NRW_2c summer	0.00	11.80	23.60	24.30	40.20	39.00	51.20
Mean Site BAY_2c winter	0.00	18.70	35.90	50.20	61.60	65.60	65.20
Mean Site BAY_2c summer	0.00	8.80	20.70	27.50	32.50	42.40	56.80
Mean Site NRW_1p	0.00	0.10	2.80	11.50	24.60	35.80	50.70
Mean Site NRW_2p	0.00	0.60	4.20	11.70	28.00	32.80	40.40
Mean Site Bay_ 2p	0.00	0.20	3.90	9.00	31.60	40.70	58.30

Table 20: Mean-values [% aR], standard deviation (SD) and coefficient of variation (COV, [%]) for the parameter mass balance for transformation of ^{14}C Salicylic acid in cattle and pig manure measured in 6 replicates, mean-values presented in the above figures.

Time [d]		0	3	7	14	21	28	36
Site NRW1c winter	Mean	103.33	94.77	99.32	96.47	94.95	85.95	91.23
	SD	1.53	2.26	7.51	4.95	6,75	4.52	3.28
	COV	1.49	2.15	7.86	5.15	6.92	4.67	3.48
Site NRW1c summer	Mean	107.57	102.43	96.22	93.60	96.75	101.12	96.35
	SD	0.41	16.12	10.49	10.40	7.30	10.40	6.25
	COV	0.38	15.73	10.90	11.11	7.54	10.29	6.48
Site NRW_2c winter	Mean	102.60	105.27	95.60	96.22	97.45	96.80	94.32
	SD	1.36	5.41	9.60	8.21	4.85	8.23	4.30
	COV	1.32	5.71	9.67	8.51	5.11	9.58	4.71
Site NRW_2c summer	Mean	109.20	106.92	104.50	91.55	101.52	102.80	110.96
	SD	3.21	8.29	6.15	5.79	6.77	8.18	4.27
	COV	2.94	7.76	5.88	6.32	6.67	7.96	3.85
Site BAY_2c winter	Mean	99.05	83.90	90.23	78.42	87.96	93.05	85.90
	SD	2.48	3.77	3.86	2.02	2.75	1.20	1.25
	COV	2.50	4.49	4.27	2.58	3.13	1.29	1.45
Site BAY_2c summer	Mean	111.48	104.98	115.17	104.90	103.10	98.82	99.82
	SD	1.76	4.52	6.62	4.10	8.36	8.11	15.41
	COV	1.58	4.31	5.74	3.91	8.11	8.20	15.44
Site NRW_1p	Mean	92.70	85.15	86.70	80.92	78.78	63.63	75.55
	SD	1.55	16.99	3.16	9.98	9.43	6.59	1.03
	COV	1.67	19.95	3.65	12.34	11.96	10.35	1.36

Table 20: Mean-values [% aR], standard deviation (SD) and coefficient of variation (COV, [%]) for the parameter mass balance for transformation of 14C Salicylic acid in cattle and pig manure, mean-values presented in the above figures (ctn.)

Time [d]		0	3	7	14	21	28	36
Site NRW_2p	Mean	100.92	97.60	87.10	83.17	82.95	80.45	80.28
	SD	3.18	1.70	3.84	4.74	6.43	5.46	5.75
	COV	3.15	1.74	4.41	5.70	7.75	6.79	7.16
Site Bay_2p	Mean	107.12	101.77	80.97	80.45	85.53	81.55	80.22
	SD	1.28	4.36	7.24	4.39	4.39	2.45	1.16
	COV	1.92	1.26	5.38	8.99	5.14	3.00	1.45

Summary of transformation of ^{14}C-Salicylic acid in manure:

Cattle manure

The mass balance is in the range of 85.9 – 111.5 % aR (COV: 0.4 – 15.7 %) over the testing period. Poorer mass balances are mostly observed at the end of the incubation period.

The amount of extractable radioactivity steadily decreases from 96.3 – 103.9 % aR (COV: 0.52 – 3.16 %) at the beginning of the transformation study to 3.4 – 46.8 % aR (COV: 7.7 – 68.8 %) after 28 days of incubation.

In parallel, the formation of $^{14}CO_2$ + $^{14}CH_4$ (mineralization; cumulative presentation at each sampling interval) increases from 0 % aR at the beginning to 38.1 -65.2 % aR at the end of the incubation period.

The formation of non-extractable residues (NER) also increases in the course of incubation from 3.8 – 8.5 % aR (COV: 4.0 – 26.5 %) to 8.0 – 14.0 % aR (COV: 2.6 – 20.9 %).

Pig manure

The mass balance is in the range of 75.6 – 107.1 % aR (COV: 1.4 – 20.0 %) over the testing period.

The amount of extractable radioactivity steadily decreases from 91.5 – 106.0 % aR (COV: 1.6 – 3.2 %) at the beginning of the transformation study to 3.8 – 10.0 % aR (COV: 2.0 – 32.7 %) after 28 days of incubation.

In parallel, the formation of $^{14}CO_2$ + $^{14}CH_4$ (mineralization; cumulative presentation at each sampling interval) increases from 0 % aR at the beginning to 40.4 – 58.3 % aR at the end of the incubation period.

The formation of non-extractable residues (NER) also increases in the course of incubation from 1.1 – 1.6 % aR (COV: 14.8 – 27.1 %) to 8.2 – 12.5 % aR (COV: 6.3 – 21.5 %)

In summary it can be stated that the transformation studies of ^{14}C-Salicylic acid are comparable for cattle and pig manure. Trends are comparable and also coefficients of variation are in the same range for the same parameter and stage of the incubation period. In particular, for NERs with values around or even < 10% aR, COVs are quite high (often > 10%).

^{14}C Paracetamol

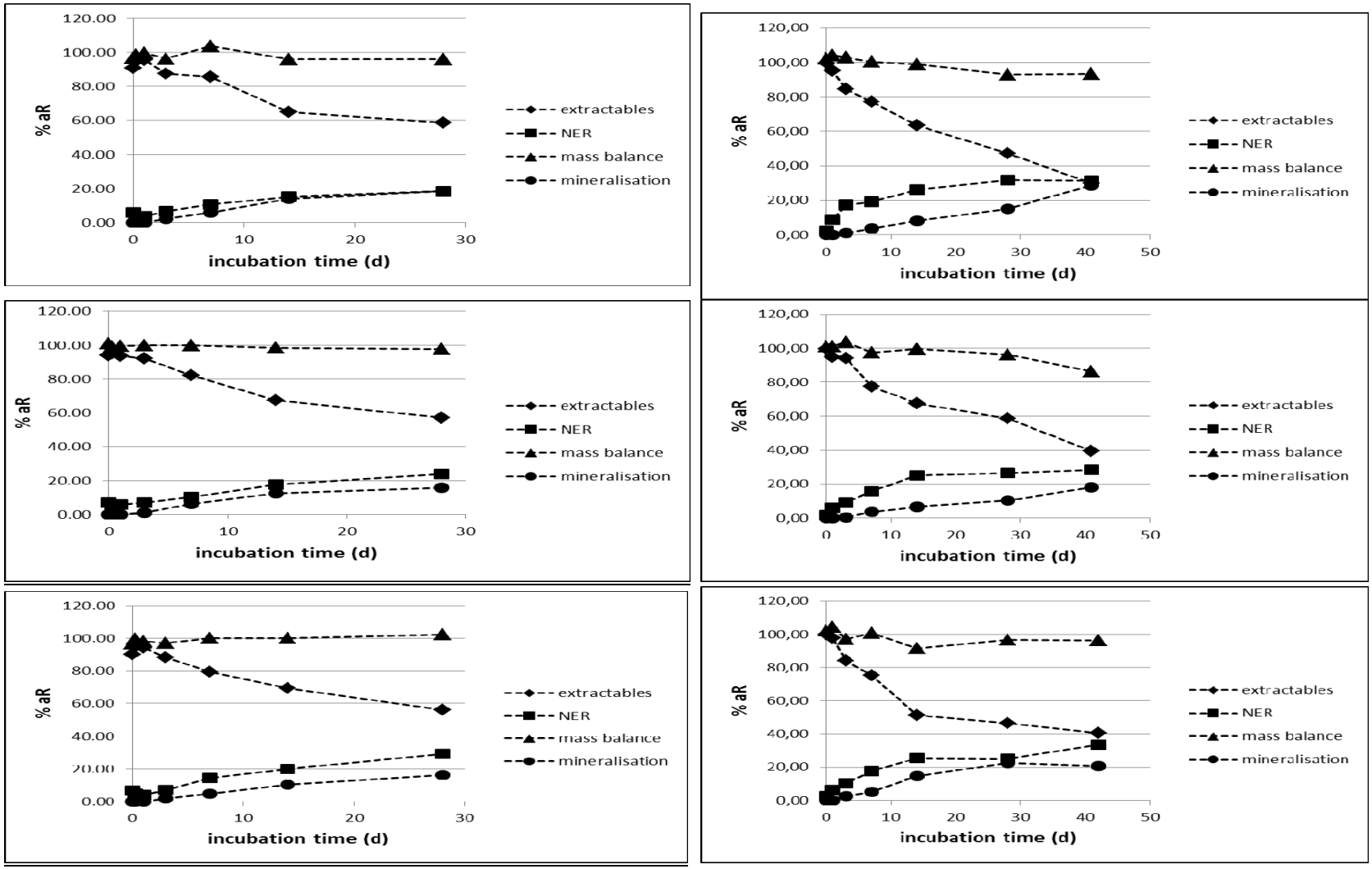

Figure 23: Radioactivity distribution for the transformation of ^{14}C-Paracetamol in cattle and pig manure. Sites and sampling times are: left side, top to bottom = NRW_1c; NRW_2c; BAY_2c. Right side, top t bottom = NRW_1p; NRW_2p; BAY_2p

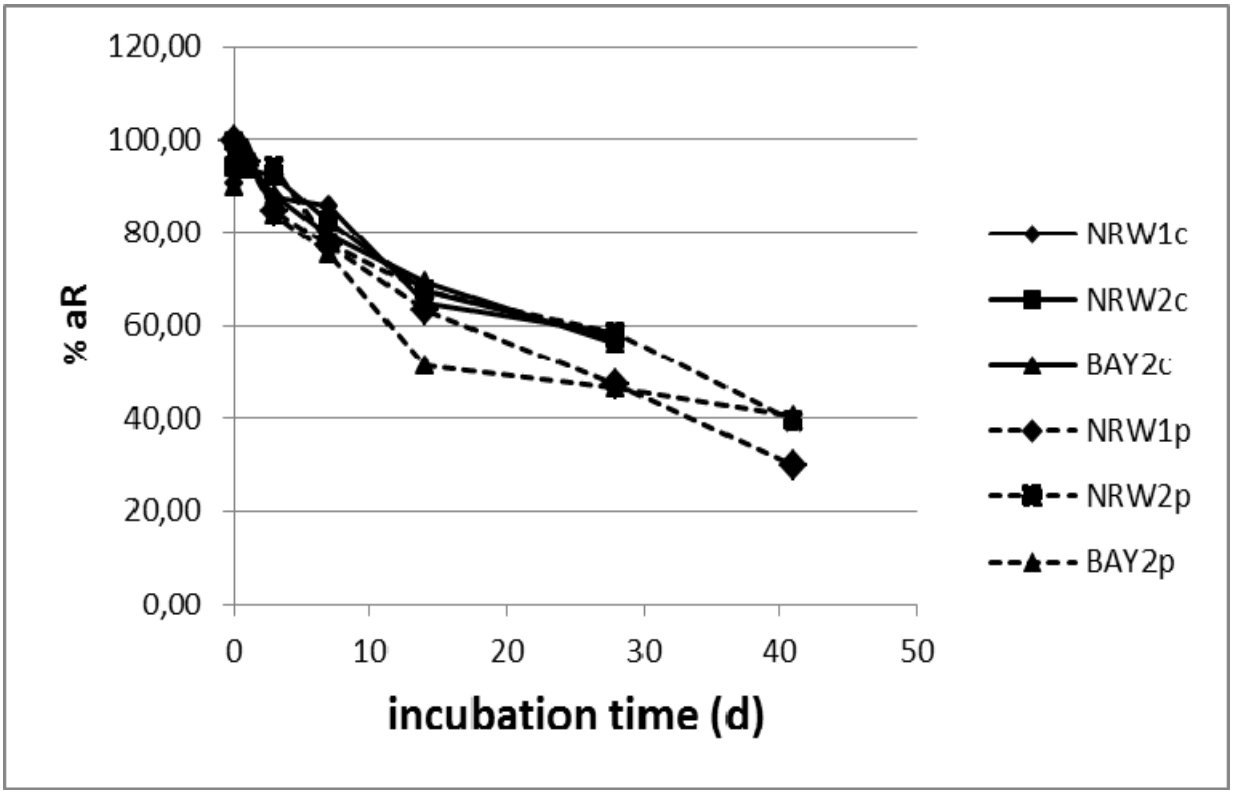

Figure 24: Extractable residues: Paracetamol in cattle and pig manure

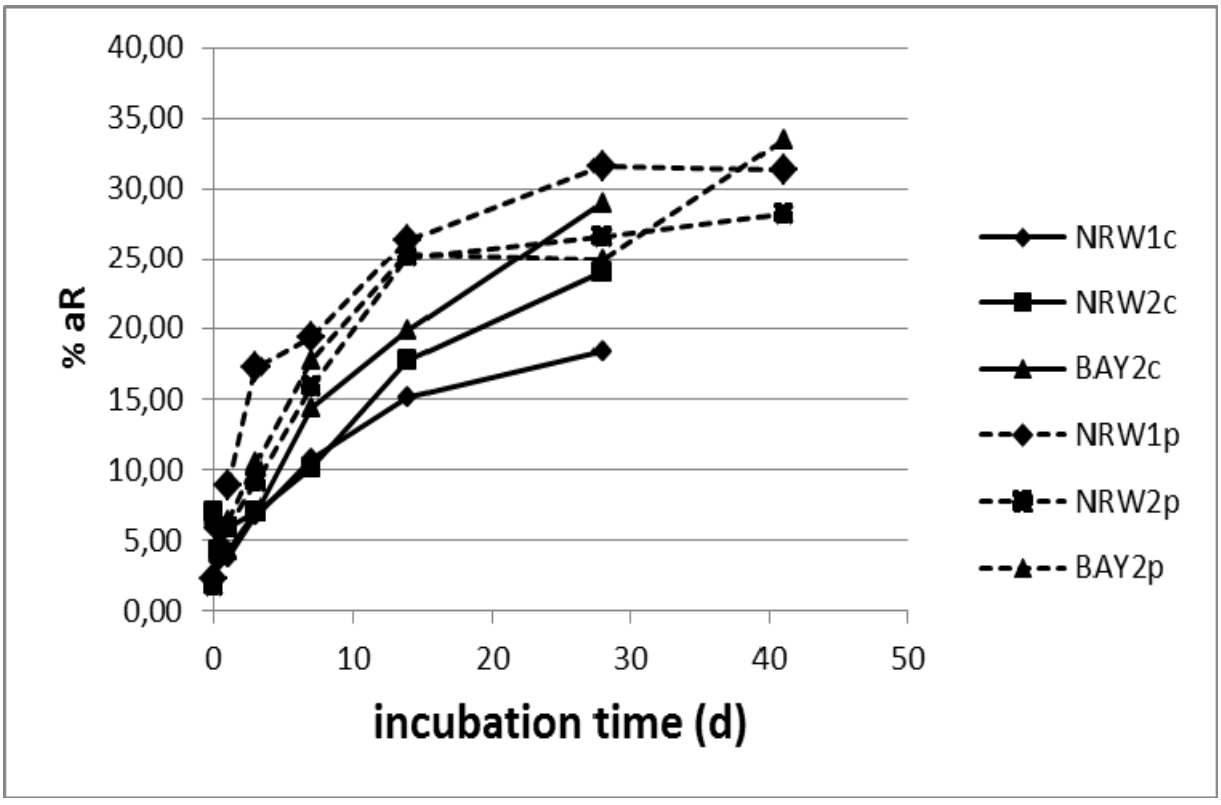

Figure 25: Non extractable residues: Paracetamol in cattle and pig manure

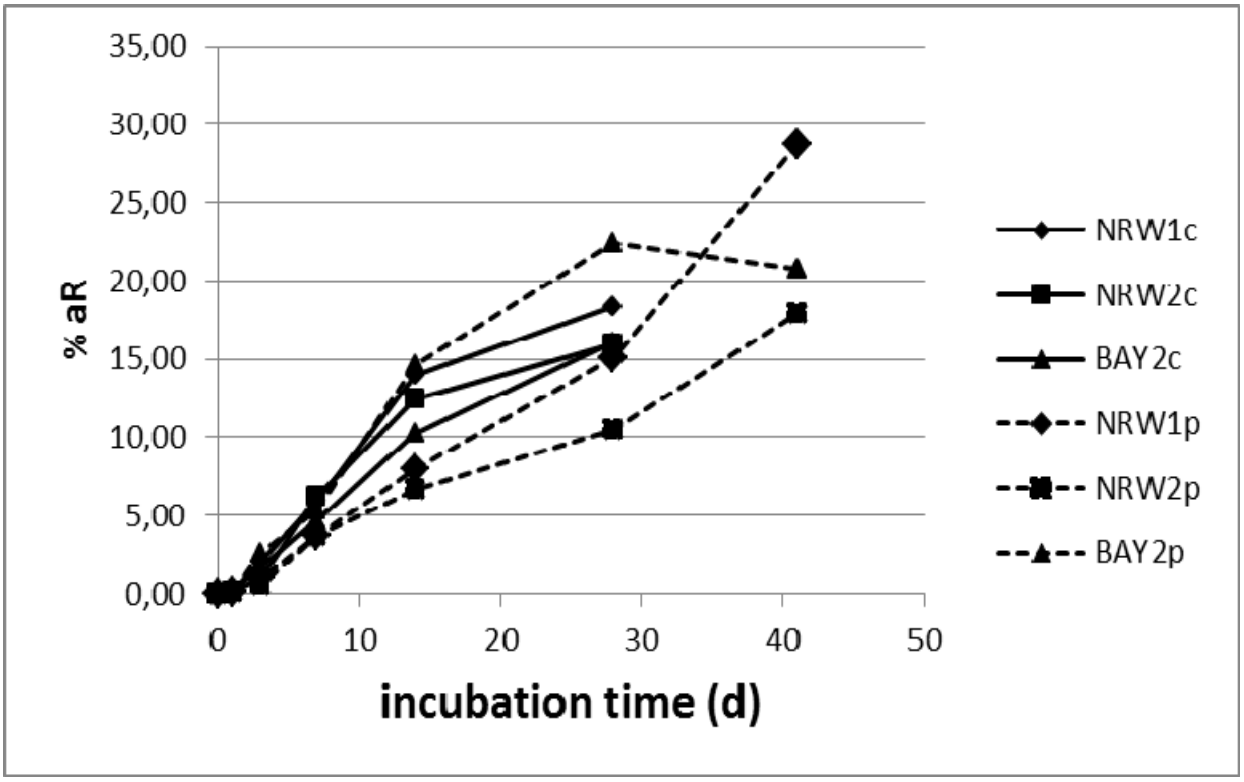

Figure 26: Mineralization: Paracetamol in cattle and pig manure

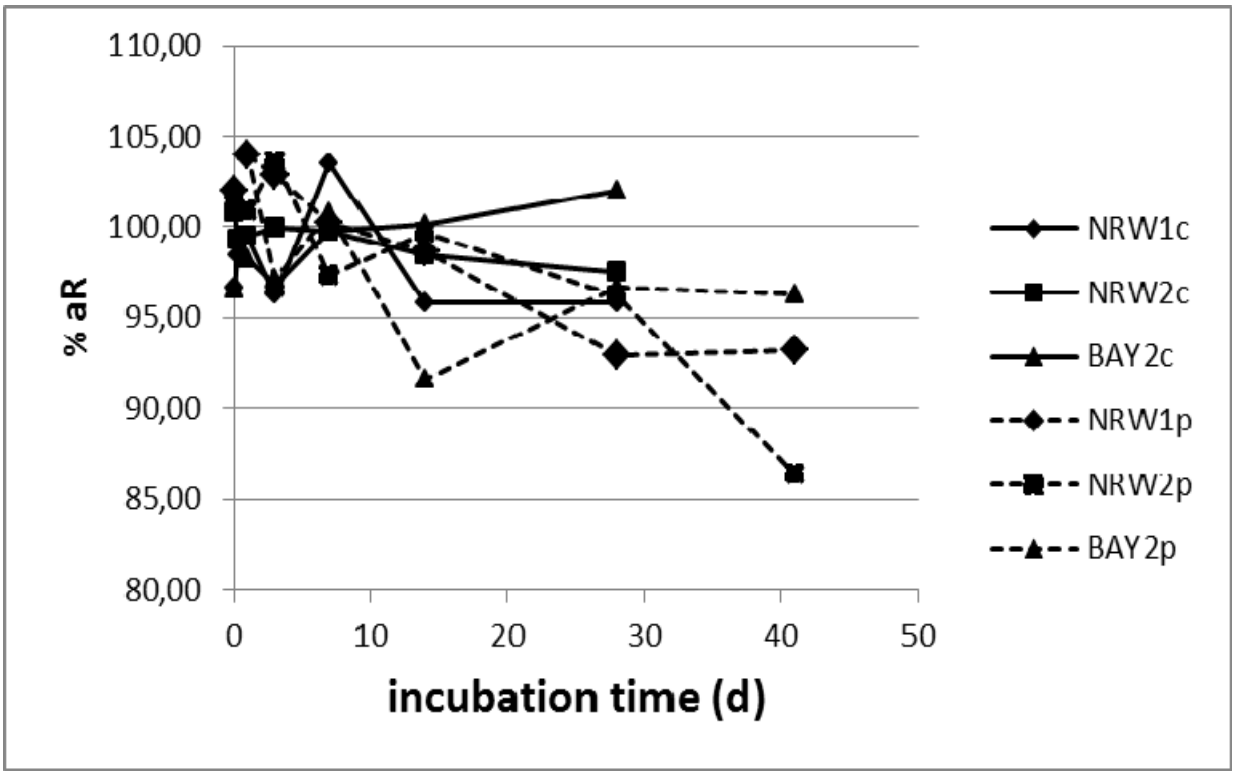

Figure 27: Mass balance: Paracetamol in cattle and pig manure

Table 21: Mean-values [% aR], standard deviation (SD) and coefficient of variation (COV, [%]) for the parameter extractables for transformation of ^{14}C Paracetamol in cattle and pig manure measured in 6 replicates, mean-values presented in the above figures.

Time [d]		0	0.3	1	3	7	14	28
Site NRW_1c	Mean	90.77	94.93	95.48	87.47	85.60	64.95	58.48
	SD	1.75	1.15	95.48	4.83	8.31	5.56	5.09
	COV	1.93	1.21	100.00	5.52	9.70	8.56	8.71
Site NRW_2c	Mean	93.85	95.10	93.48	91.97	82.30	67.27	57.17
	SD	0.97	1.22	1.60	4.43	1.48	1.44	4.19
	COV	1.03	1.29	1.71	4.82	1.79	2.15	7.32
Site BAY_2c	Mean	89.82	94.55	93.93	88.07	79.40	69.33	56.23
	SD	3.26	3.94	93.93	3.34	3.91	5.78	6.69
	COV	3.63	4.17	100.00	3.79	4.93	8.34	11.89
Time [d]		0	1	3	7	14	28	41
Site NRW_1p	Mean	99.72	95.02	84.60	77.08	63.30	47.12	29.87
	SD	2.74	1.63	1.77	1.42	2.54	6.57	4.57
	COV	2.75	1.71	2.09	1.84	4.02	13.93	15.30
Site NRW_2p	Mean	99.40	94.90	94.03	77.50	67.45	58.47	39.42
	SD	0.99	1.67	1.89	3.64	3.84	5.62	6.50
	COV	1.00	1.76	2.01	4.70	5.70	9.61	16.48
Site Bay_2p	Mean	99.83	97.83	84.00	75.37	51.13	46.48	40.58
	SD	2.20	2.49	2.00	4.55	3.99	8.21	7.29
	COV	2.21	2.55	2.38	6.04	7.80	17.65	17.97

Table 22: Mean-values [% aR], standard deviation (SD) and coefficient of variation (COV, [%]) for the parameter non-extractable residues (NER) for transformation of ^{14}C Paracetamol in cattle and pig manure measured in 6 replicates, mean-values presented in the above figures.

Time [d]		0	0.3	1	3	7	14	28
Site NRW_1c	Mean	5.90	3.52	3.72	6.73	10.72	15.08	18.38
	SD	0.24	0.28	0.39	0.94	1.71	1.39	1.75
	COV	4.01	7.92	10.55	13.89	15.97	9.21	9.52
Site NRW_2c	Mean	6.95	4.22	5.83	7.00	10.12	17.67	23.97
	SD	0.34	1.28	0.92	0.79	0.97	1.05	1.79
	COV	4.96	30.39	15.81	11.36	9.63	5.95	7.47
Site BAY_2c	Mean	6.77	4.92	4.18	7.03	14.33	19.82	28.98
	SD	0.36	0.67	0.21	0.48	2.67	1.43	3.34
	COV	5.34	13.58	5.11	6.89	18.59	7.22	11.52
Time [d]		0	1	3	7	14	28	41
Site NRW_1p	Mean	2.28	8.87	17.22	19.36	26.35	31.63	31.40
	SD	0.34	1.00	1.77	3.28	2.08	2.61	2.98
	COV	14.77	11.25	10.30	16.96	7.91	8.24	9.48
Site NRW_2p	Mean	1.77	5.87	9.07	15.85	25.15	26.65	28.25
	SD	0.14	0.57	0.98	1.41	3.40	2.57	1.52
	COV	7.73	9.68	10.78	8.89	13.51	9.66	5.37
Site Bay_2p	Mean	2.42	6.33	10.52	17.75	25.28	24.97	33.52
	SD	0.16	0.29	0.78	1.54	4.98	5.58	6.41
	COV	6.63	4.54	7.38	8.70	19.70	22.35	19.11

Table 23: Measured values for the parameter mineralization for transformation of ^{14}C Paracetamol in cattle and pig manure, -values presented in the above figures. Single values measured and thus no SD and COV were calculated.

Time [d]	0	0.3	1	3	7	14	28
Site NRW_1c	0.00	0.01	0.08	2.10	5.90	14.00	18.40
Site NRW_2c	0.00	0.01	0.10	1.00	6.20	12.40	15.90
Site BAY_2c	0.00	0.01	0.10	1.70	4.70	10.30	16.10
Time [d]	0	1	3	7	14	28	41
Site NRW_1p	0.00	0.10	1.00	3.70	8.00	15.10	28.70
Site NRW_2p	0.00	0.10	0.50	3.70	6.70	10.50	17.90
Site BAY_2p	0.00	0.10	2.60	5.30	14.60	22.40	20.80

Table 24: Mean-values [% aR], standard deviation (SD) and coefficient of variation (COV, [%]) for the parameter mass balance for transformation of ^{14}C Paracetamol in cattle and pig manure measured in 6 replicates, mean-values presented in the above figures.

Time [d] ++)		0	0.3	1	3	7	14	28
Site NRW_1c	Mean	96.67	98.45	99.50	96.30	103.52	95.78	95.83
	SD	1.67	1.11	1.95	4.88	8.43	4.42	3.68
	COV	1.73	1.13	1.96	5.06	8.15	4.61	3.84
Site NRW_2c	Mean	100.80	99.28	99.45	99.95	99.72	98.47	97.47
	SD	1.16	2.22	1.54	4.26	0.73	1.55	3.10
	COV	1.15	2.24	1.55	4.26	0.73	1.57	3.18
Site BAY_2c	Mean	96.58	99.48	98.23	96.83	99.80	100.12	101.98
	SD	3.24	4.20	1.95	3.21	3.19	4.44	9.60
	COV	3.35	4.22	1.98	3.31	3.20	4.43	9.42
Time [d]		0	1	3	7	14	28	41
Site NRW_1p	Mean	102.00	103.98	102.83	100.22	98.67	92.88	93.24
	SD	2.71	1.17	0.84	2.82	2.42	9.71	6.64
	COV	2.65	1.13	0.82	2.81	2.45	10.46	7.13
Site NRW_2p	Mean	101.17	100.88	103.60	97.28	99.62	96.20	86.40
	SD	0.92	1.81	2.23	3.04	5.07	6.80	8.61
	COV	0.94	1.82	2.31	2.96	5.05	6.54	7.44
Site Bay_2p	Mean	102.23	104.20	97.13	100.80	91.63	96.63	96.32
	SD	2.30	2.69	1.69	3.70	7.64	12.13	12.77
	COV	2.25	2.58	1.74	3.67	8.34	12.55	13.26

Summary of transformation of ^{14}C Paracetamol in cattle and pig manure:

Cattle manure

The mass balance is in the range of 96.6 – 103.5 % aR (COV: 0.7 – 5.1 %) over the testing period. No trend of decreasing mass balances towards the end of the incubation period is observed.

The amount of extractable radioactivity steadily decreases from 89.8 – 93.6 % aR (COV: 1.0 – 3.6 %) at the beginning of the transformation study to 56.2 – 58.5 % aR (COV: 7.3 – 11.9 %) after 28 days of incubation.

In parallel, the formation of $^{14}CO_2$ + $^{14}CH_4$ (mineralization; cumulative presentation at each sampling interval) increases from 0 % aR at the beginning to 15.9 – 18.4 % aR at the end of the incubation period.

The formation of non-extractable residues (NER) increases in the course of incubation from 5.9 – 7.0 % aR (COV: 4.0 – 5.2 %) to 18.4 – 29.0 % aR (COV: 7.5 – 11.5 %).

Pig manure

The mass balance is in the range of 86.4 – 104.2 % aR (COV: 0.8 – 13.3 %) over the testing period without any trend. An exception might be site NRW_2p with a mass balance of 86.4 % aR at the end of the test.

The amount of extractable radioactivity steadily decreases from 99.4 – 99.8 % aR (COV: 1.0 – 2.8 %) at the beginning of the transformation study to 29.9 – 40.6 % aR (COV: 15.3 – 18.0 %) after 41 days of incubation.

In parallel, the formation of $^{14}CO_2 + {}^{14}CH_4$ (mineralization; cumulative presentation at each sampling interval) increases from 0 % aR at the beginning to17.9 – 28.7 % aR at the end of the incubation period.

The formation of non-extractable residues (NER) also increases in the course of incubation from 1.2 – 2.4 % aR (COV: 14.8 – 27.1 %) to 28.3 – 33.5 % aR (COV: 5.4 – 19.1 %).

The transformation studies of ^{14}C-Paracetamol are comparable for cattle and pig manure. Trends are comparable and also coefficients of variation are in the same range for the same parameter and stage of the incubation period. An exception is the high COV of 100.0 % which is observed for the degradation in cattle manure (BAY_2c, 3 days of incubation).

^{14}C-Biocide B

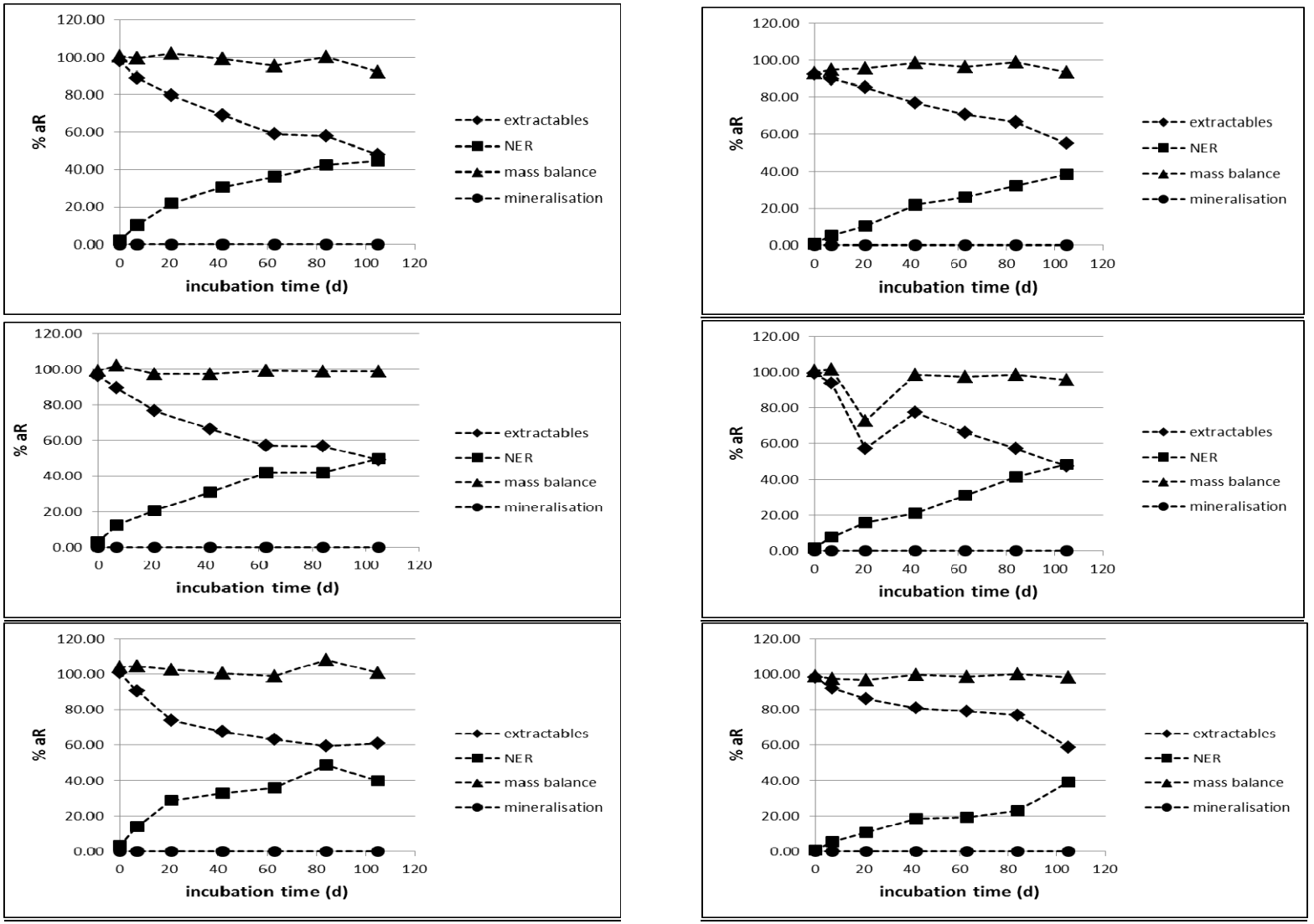

Figure 28: Radioactivity distribution for the transformation of ^{14}C-Biocide B in cattle and pig manure. Sites and sampling times are: left side, top to bottom = NRW_1c; NRW_2c; BAY_2c. Right side, top t bottom = NRW_1p; NRW_2p; BAY_2p

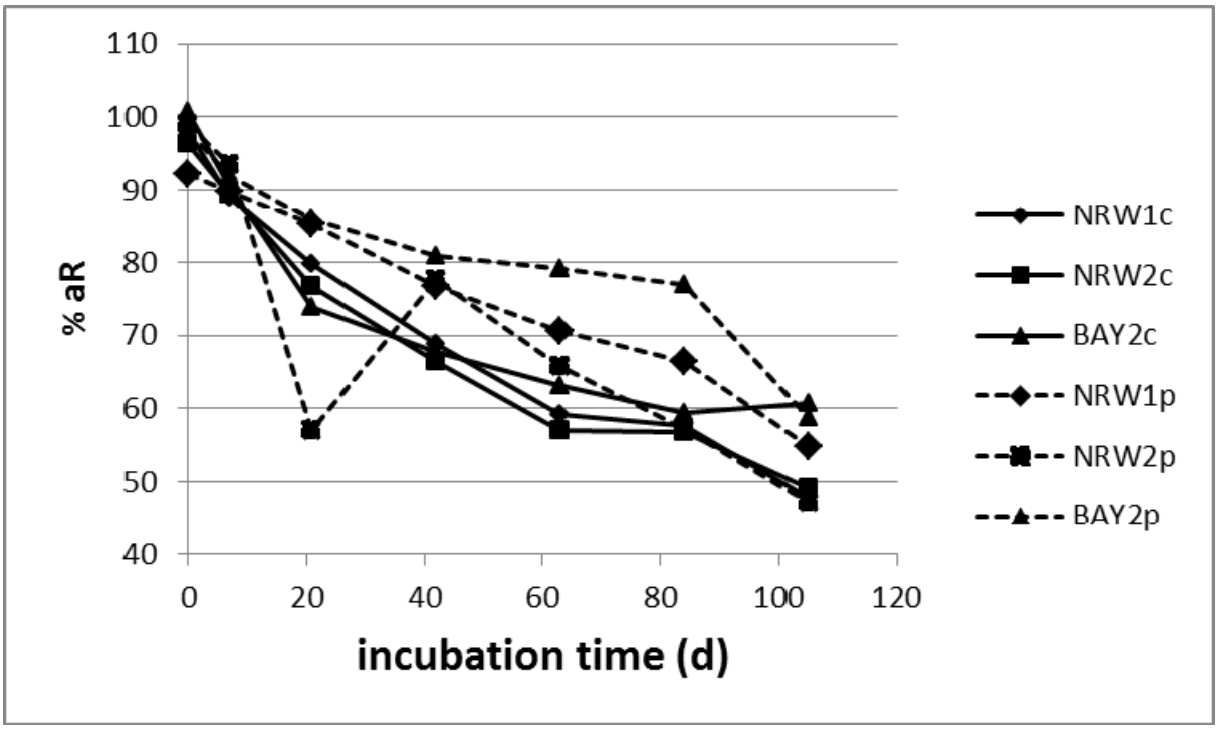

Figure 29: Extractable residues: Biocide B in cattle and pig manure

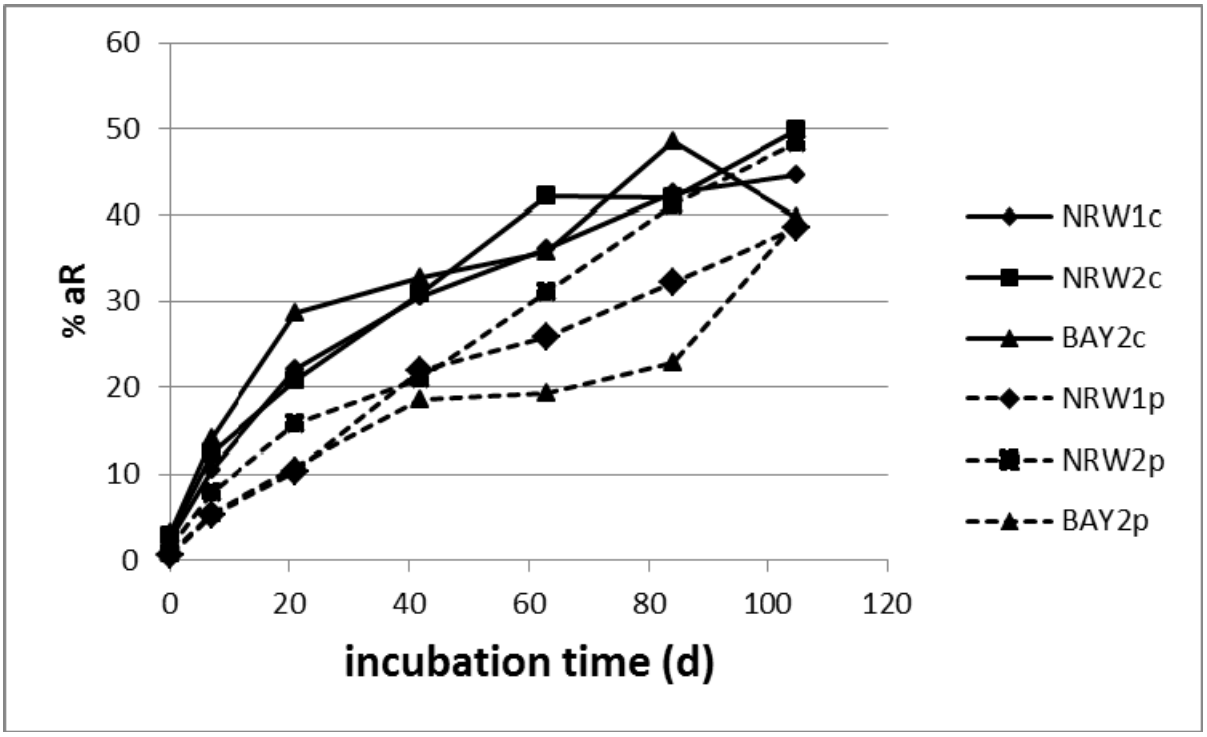

Figure 30: Non extractable residues: Biocide B in cattle and pig manure

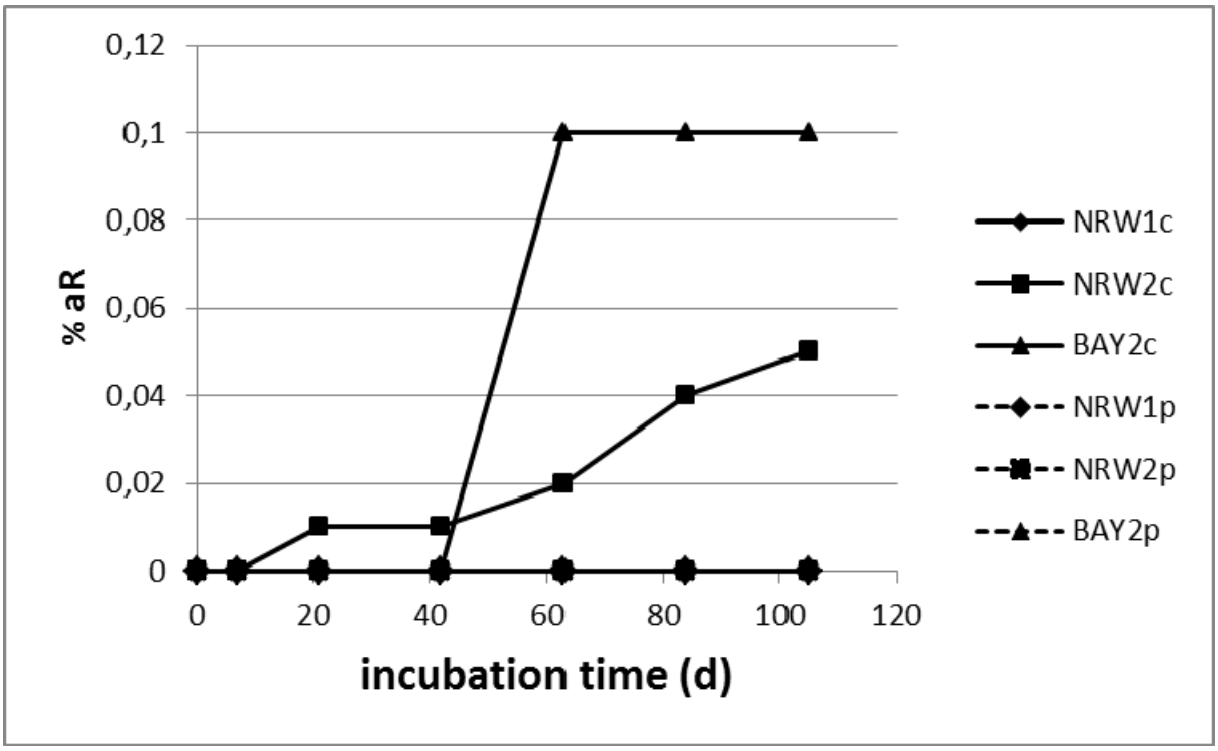

Figure 31: Mineralization: Biocide B in cattle and pig manure

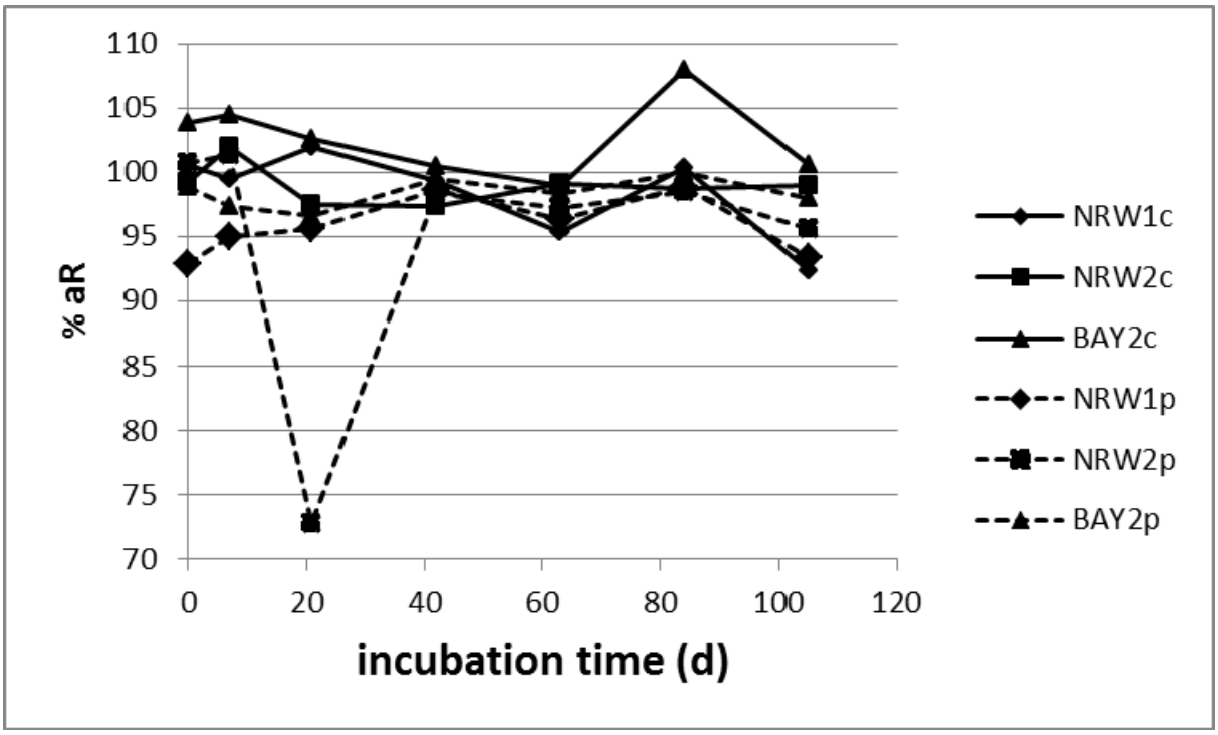

Figure 32: mass balance: Biocide B in cattle and pig manure

Table 25: Mean-values [% aR], standard deviation (SD) and coefficient of variation (COV, [%]) for the parameter extractables for transformation of ^{14}C Biocide B in cattle and pig manure measured in 6 replicates, mean-values presented in the above figures.

Time [d] ++)		0	7	21	42	63	84	105
Site NRW_1c	Mean	98.18	89.02	79.81	68.81	59.14	57.79	47.86
	SD	0.90	1.35	1.48	4.14	3.08	1.57	0.79
	COV	0.92	1.52	1.86	6.01	5.20	2.71	1.66
Site NRW_2c	Mean	96.34	89.48	76.69	66.48	56.98	56.73	49.07
	SD	0.85	3.78	2.20	6.57	1.75	2.50	0.94
	COV	0.88	4.22	2.87	9.88	3.08	4.41	1.91
Site BAY_2c	Mean	100.69	90.52	73.87	67.68	63.07	59.36	60.77
	SD	1.04	0.94	1.29	0.96	1.58	1.91	2.25
	COV	1.04	1.03	1.75	1.42	2.51	3.22	3.71
Site NRW_1p	Mean	92.22	89.72	85.20	76.68	70.52	66.52	54.83
	SD	2.54	2.62	0.56	2.29	1.51	3.30	3.26
	COV	2.75	2.92	0.66	2.98	2.15	4.96	5.94
Site NRW_2p	Mean	99.10	93.57	72.57	72.41	65.87	57.38	47.41
	SD	1.88	2.48	8.25	3.20	2.13	1.96	1.94
	COV	1.89	2.65	11.37	4.41	3.23	3.41	4.10
Site Bay_2p	Mean	98.07	91.92	85.78	80.93	79.05	76.83	61.12
	SD	2.89	0.80	1.43	2.92	1.70	7.84	4.42
	COV	2.95	0.87	1.67	3.61	2.15	10.20	7.23

Table 26: Mean-values [% aR], standard deviation (SD) and coefficient of variation (COV, [%]) for the parameter non-extractable residues (NER) for transformation of ^{14}C Biocide B in cattle and pig manure measured in 6 replicates, mean-values presented in the above figures.

Time [d]		0	7	21	42	63	84	105
Site NRW_1c	Mean	2.34	10.41	22.16	30.47	36.21	42.48	44.49
	SD	0.35	0.95	1.26	4.28	0.87	1.61	0.90
	COV	15.01	9.13	5.70	14.06	2.42	3.78	2.02
Site NRW_2c	Mean	12.43	20.73	30.82	42.05	41.90	49.85	12.43
	SD	0.36	1.53	0.61	4.67	1.66	2.13	1.65
	COV	12.69	12.34	2.95	15.16	3.96	5.09	3.31
Site BAY_2c	Mean	14.02	28.66	32.74	35.83	48.52	39.72	14.02
	SD	0.66	1.60	4.28	5.32	0.83	5.58	1.55
	COV	20.50	11.41	14.93	16.26	2.33	11.50	3.89
Site NRW_1p	Mean	5.20	10.33	21.87	25.87	32.18	38.53	5.20
	SD	0.04	0.68	0.48	1.03	2.56	3.23	4.83
	COV	7.00	13.10	4.69	4.70	9.88	10.03	12.55
Site NRW_2p	Mean	7.80	15.70	20.88	31.02	41.17	48.38	7.80
	SD	2.51	1.38	1.90	4.02	3.64	3.23	2.03
	COV	158.55	17.69	12.11	19.25	11.73	7.83	4.19
Site Bay_2p	Mean	5.40	10.77	18.53	19.33	22.90	39.28	5.40
	SD	0.05	0.18	0.47	2.33	1.29	6.91	2.12
	COV	6.20	3.31	4.34	12.60	6.69	30.17	5.40

Table 27: Measured values for the parameter mineralization for transformation of ^{14}C Biocide B in cattle and pig manure, -values presented in the above figures. Single values measured and thus no SD and COV were calculated

Time [d]	0	7	21	42	63	84	105
Site NRW_1c	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Site NRW_2c	0.00	0.00	0.01	0.01	0.02	0.04	0.05
Site BAY_2c	0.00	0.00	0.00	0.00	0.10	0.10	0.10
Site NRW_1p	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Site NRW_2p	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Site BAY_2p	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 28: Mean-values [% aR], standard deviation (SD) and coefficient of variation (COV, [%]) for the parameter mass balance for transformation of ^{14}C Biocide B in cattle and pig manure measured in 6 replicates, mean-values presented in the above figures.

Time [d]		0	7	21	42	63	84	105
Site NRW_1c	Mean	100.53	99.43	102.00	99.28	95.37	100.28	92.37
	SD	0.96	1.64	1.02	3.68	2.45	1.41	1.14
	COV	0.95	1.65	1.00	3.71	2.57	1.41	1.24
Site NRW_2c	Mean	101.93	97.43	97.32	99.07	98.70	98.92	101.93
	SD	1.11	2.81	2.60	2.71	1.11	1.38	1.76
	COV	1.11	2.76	2.66	2.78	1.12	1.40	1.78
Site BAY_2c	Mean	104.52	102.53	100.47	98.97	107.97	100.65	104.52
	SD	1.24	0.85	3.11	5.95	1.85	6.13	1.64
	COV	1.19	0.81	3.03	5.92	1.87	5.67	1.62
Site NRW_1p	Mean	94.95	95.57	98.58	96.38	98.82	93.37	94.95
	SD	2.50	2.65	0.57	2.33	3.24	2.72	3.12
	COV	2.69	2.79	0.59	2.36	3.36	2.75	3.34
Site NRW_2p	Mean	101.38	72.70	98.40	97.25	98.42	95.65	101.38
	SD	4.23	2.02	12.59	4.11	2.28	2.81	1.27
	COV	4.20	1.99	17.32	4.17	2.34	2.86	1.33
Site Bay_2p	Mean	97.30	96.60	99.52	98.38	99.97	97.97	97.30
	SD	1.89	0.98	1.19	0.83	2.10	1.16	1.60
	COV	2.92	1.01	1.23	0.83	2.13	1.16	1.63

Summary of transformation of ^{14}C Biocide B in cattle and pig manure:

Cattle manure

The mass balance is in the range of 92.4 – 108.0 % aR (COV: 0.8 – 5.9 %) over the testing period without a trend to poorer mass balances at the end of the incubation period.

The amount of extractable radioactivity decreases from 96.4 – 100.7 % aR (COV: 0.9 – 1.0 %) at the beginning of the transformation study to 47.9 – 60.8 % aR (COV: 1.7 – 3.7 %) after 105 days of incubation.

Mineralization, i.e. formation of $^{14}CO_2$ plus $^{14}CH_4$, is negligible. 0.0 – 0.1 % aR have been formed at termination of the study. Since no replicates have been measured no COVs can be given.

The formation of non-extractable residues (NER) increases in the course of incubation from 2.3 – 3.2 % aR (COV: 12.7 – 20.5 %) to 39.7 – 49.9 % aR (COV: 2.0 – 3.9 %).

Pig manure

The mass balance is in the range of 72.7 – 101.4 % aR (COV: 0.6– 17.3 %) over the testing period. The high COV of 17.3 % is allocated to the mean value of 72.7 % aR (site NRW_2p) and might be attributed to a very low value of 51.1 % aR in one of the six replicates.

The amount of extractable radioactivity decreases from 92.2 – 99.1 % aR (COV: 1.9 – 3.0 %) at the beginning of the transformation study to 47.3 – 58.7 % aR (COV: 2.5 – 5.9 %) after 28 days of incubation.

No mineralization, i.e. formation of $^{14}CO_2$ plus $^{14}CH_4$, is observed over the entire testing period.

The formation of non-extractable residues (NER) also increases in the course of incubation from 0.6 – 1.6 % aR (COV: 6.2 – 158.6 %) to 38.5 – 48.4 % aR (COV: 4.2 – 12.6 %). Noteworthy is the very high COV of 158.6 % (site NRW_2p) which can be attributed to the low absolute values of NER.

It can be summarized that the result for the transformation studies of ^{14}C-Biocide B in cattle and pig manure are comparable. That means that temporal trends are comparable, and also coefficients of variation are in the same range for the same parameter and stage of the incubation period.

4.2 Kinetic evaluation of test substance dissipation (DT50 values)

By use of the KinGUI-software tool (Mikolasch et al., 2006), DT_{50}-values and DT_{90}-values were calculated for the parent compound and for transformation products (TP) both in cattle and pig manure and using all kinetic models. Chi^2-values were compared, and furthermore, a visual check of the graphs of all models was performed. From both comparisons it was obvious, that none of the kinetic models was better compared to the SFO-model for the entire set of all transformation studies. Though from a mechanistic point of view the DFOP-model might be more appropriate, the SFO-model was used in the course of project in order to have a uniform basis for further comparisons and conclusions.

The following figures and tables show arithmetic means, standard deviation, coefficients of variation (COV, [%]) and medians for all DT_{50}-values obtained for six replicates each as well as chi^2-values.

Further information - including original data of the six replicates, ln-transformed data, and also the results of the variance analyses are presented in tables A3_12 to A3_18 in annex 3.

Salicylic acid

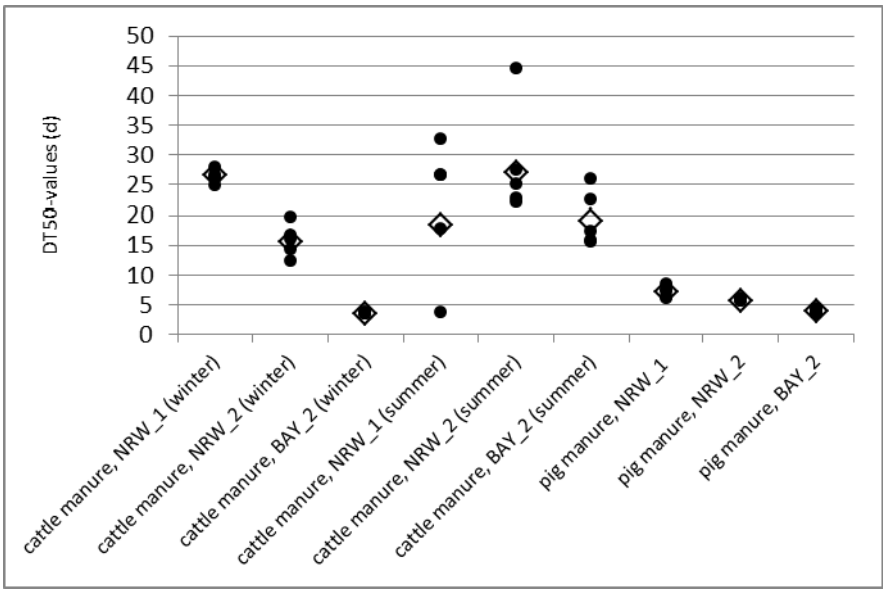

Figure 33: Means (open squares) and individual data (black dots) of DT_{50}-values [d] for transformation of ^{14}C-Salicylic acid in cattle and pig manure.

Table 29: Mean-values, standard deviation (SD), coefficient of variation (COV, [%]), and median of DT50-values [d] for transformation of ^{14}C-Salicylic acid in cattle and pig manure.

Data set	Cattle NRW_1c (winter)	Cattle NRW_2c (winter)	Cattle BAY_2c (winter)	Cattle NRW_1c (summer)	Cattle NRW_2c (summer)	Cattle BAY_2c (summer)	Pig NRW_1p	Pig NRW_2p	Pig BAY_2p
N	6	6	6	6	6	6	6	6	6
Mean	26.5	15.8	3.6	22.3	27.9	19.3	7.2	5.6	3.9
SD	1.07	2.47	0.12	10.28	8.40	4.13	0.83	0.51	0.28
COV [%]	4.03	15.65	3.45	46.17	30.10	21.36	11.49	9.11	7.2
Median	26.5	15.9	3.5	26.4	25.4	18.0	7.4	5.8	3.9

Transformation of ^{14}C-Salicylic acid was studied in cattle manure sampled in winter and in summer as well as in pig manure sampled in March to June (for pig manure no differentiation between "winter manure" and "summer manure" was made, see chapter 2.1). For both types of manure, sampling was at three different sites named as NRW_1c, NRW_2c, and BAY_2c for cattle manure and NRW_1p, NRW_2p, and BAY_2p for pig manure. Six replicates each were analyzed, and data discussed in the following paragraph are means (re-transformed) and standard deviation (given ln-transformed) obtained for these replicates.

DT_{50}-values for the transformation in cattle manure are in the range of 3.6 d to 27.9 d. Standard deviations range between 0.12 – 10.28. Coefficients of variation are in the range of 4.0 – 46.17 %.

Dissipation in pig manure is faster compared to that in cattle manure. The range of DT_{50}-values is 3.9 – 7.2 d. Standard deviations range between 0.28 – 0.83; coefficients of variation are between 7.2 – 11.49 %.

The range of standard deviation and coefficients of variation are much narrow for pig manure compared to cattle manure. The highest COV for pig manure is much lower than that for cattle manure.

Paracetamol

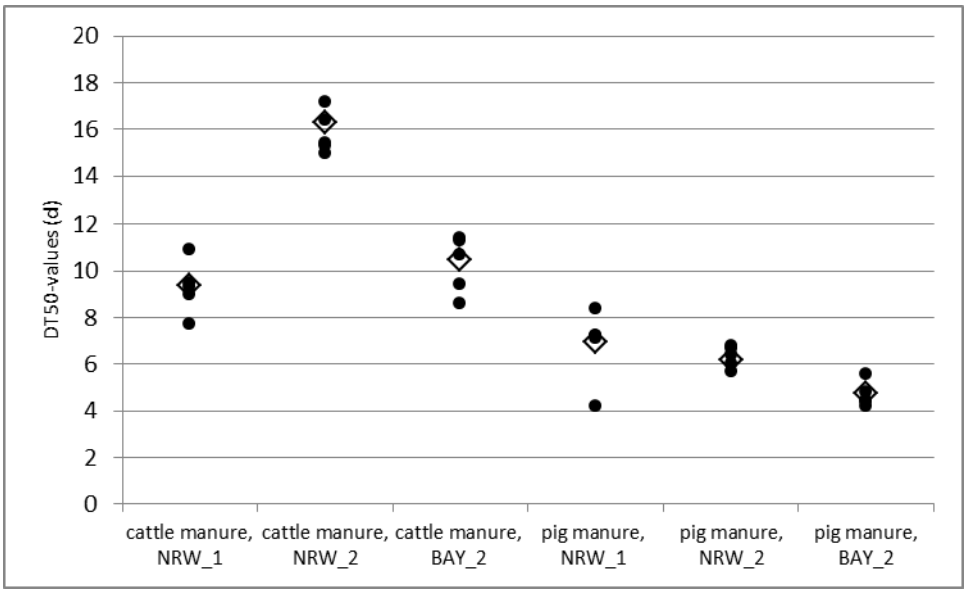

Figure 34: Means (open squares) and individual data (black dots) of DT_{50}-values [d] for transformation of ^{14}C-Paracetamol in cattle and pig manure.

Table 30: Mean-values, standard deviation (SD), coefficient of variation (COV, [%]), and median of DT50-values [d] for transformation of ^{14}C- Paracetamol in cattle and pig manure.

Data set	Cattle NRW_1c	Cattle NRW_2c	Cattle BAY_2 c	Pig NRW_1p	Pig NRW_2p	Pig BAY_2p
N	6	6	6	6	6	6
Mean	9.4	16.3	10.6	7.2	6.2	4.8
SD	1.09	1.42	1.31	1.61	0.51	0.54
COV [%]	11.57	8.69	12.36	22.55	8.22	11.39
Median	9.4	15.9	11.0	7.2	6.2	4.6

Transformation of ^{14}C-Paracetamol was studied in cattle manure (sampled in July and August) and in pig manure (sampled in November, February and March). For both types of manure, sampling was at three different sites, namely NRW_1c, NRW_2c, and BAY_2c (for cattle manure) and NRW_1p, NRW_2p, and BAY_2p (for pig manure). Six replicates each were analyzed.

DT_{50}-values for the transformation in cattle manure are in the range of 9.4 – 16.3 d. Standard deviations are between 1.09 and 1.42, coefficients of variation between 8.69 – 12.36 %.

Means of DT_{50}-values obtained in pig manure were somewhat lower. They were in the range of 4.8 – 7.2 d. Standard deviations ranged between 0.51 – 1.61, coefficients of variation between 8.22 -22.55 %.

Biocide B

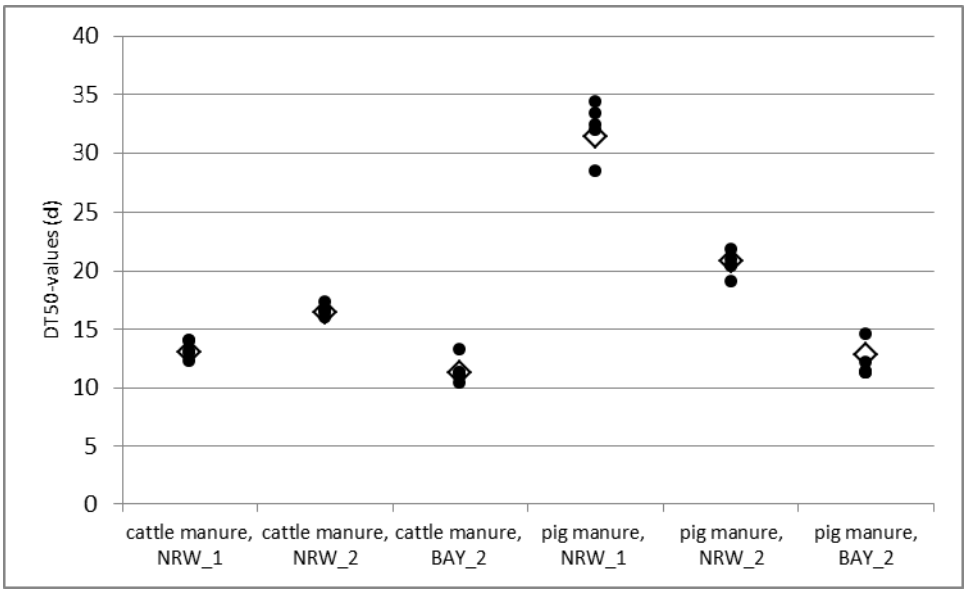

Figure 35: Means (open squares) and individual data (black dots) of DT_{50}-values [d] for transformation of ^{14}C-Biocide B in cattle and pig manure.

Table 31: Mean-values, standard deviation (SD), coefficient of variation (COV, [%]), and median of DT50-values [d] for transformation of ^{14}C- Biocide B in cattle and pig manure.

Data set	Cattle NRW_1c	Cattle NRW_2c	Cattle BAY_2c	Pig NRW_1p	Pig NRW_2p	Pig BAY_2p
N	6	6	6	6	6	6
Mean	13.0	16.5	11.3	31.5	20.9	13.0
SD	0.89	0.49	1.04	2.54	1.18	2.53
COV [%]	6.83	2.96	9.22	8.06	5.68	19.40
Median	13.0	16.45	11.1	32.15	20.7	11.75

Transformation of the biocide ^{14}C-Biocide B also was studied in cattle manure (sampled in July, August and November) and in pig manure (sampled in June and July). As for the other tested chemicals, sampling was at three different sites, NRW_1c, NRW_2c, and BAY_2c (for cattle manure) and NRW_1p, NRW_2p, and BAY_2p (for pig manure). Six replicates each were analyzed.

DT_{50}-values for the transformation in cattle manure range between 11.3 d and 16.5 d. Standard deviations are rather low and in the range of 0.49 – 1.04, whereas coefficients of variation are in range of 2.96 – 9.22 %

For the sites NRW_1p and NRW_2p means of DT_{50}-values obtained in pig manure are higher compared to cattle manure, and are 20.9 d and 31.5 d, respectively. The DT_{50}-value for the dissipation of Biocide B in pig manure sampled at the site BAY_2p is 13.0 d. Again, standard deviations are rather low and between 1.18 and 2.54; coefficients of variation are in the range of 5.68 and 19.4 %.

Chi^2-values are a measure of the goodness of fit of the kinetic model selected. FOCUS, 2006 states that chi^2-values < 15% indicate that the respective model is well suited to explain the observed data. The following Table gives a summary of all chi^2-values.

Table 32: Overview on chi²-values of all samples and measurements (SFO-kinetics).

	Cattle NRW_1c winter	Cattle NRW_2c winter	Cattle BAY_2c winter	Cattle NRW_1c summer	Cattle NRW_2c summer	Cattle BAY_2c summer	Pig NRW_1p	Pig NRW_2p	Pig BAY_2p
Salicylic acid									
Replicate 1	10.42	12.64	12.31	3.5	4.92	7.49	24.49	6.36	7.9
Replicate 2	7.92	12.86	11.97	16.44	17.3	8.75	7.84	3.54	5.62
Replicate 3	8.49	16.72	5.32	12.11	12.77	13.48	5.04	6.43	11.19
Replicate 4	9.38	19.64	3.34	11.91	13.49	11.31	9.73	10.19	13.42
Replicate 5	10.78	21.13	9	8.68	10.43	6.82	17.71	9.66	8.34
Replicate 6	8.87	17.92	3.5	18.98	16.76	10.61	11.75	12.46	6.29

	Cattle NRW_1c	Cattle NRW_2c	Cattle BAY_2c	Pig NRW_1p	Pig NRW_2p	Pig BAY_2p
Paracetamol						
Replicate 1	8.50	1.85	2.75	17.50	3.30	8.10
Replicate 2	7.41	2.10	6.30	21.10	5.80	6.30
Replicate 3	10.99	6.59	3.60	21.70	2.90	7.30
Replicate 4	14.14	3.25	4.30	19.60	8.00	5.20
Replicate 5	9.21	2.77	7.60	23.80	11.90	5.10
Replicate 6	10.07	2.9	6.90	20.90	5.70	4.00

Table 32 (ctn.): Overview on chi²-values of all samples and measurements (SFO-kinetics).

	Cattle NRW_1c	Cattle NRW_2c	Cattle BAY_2c	Pig NRW_1p	Pig NRW_2p	Pig BAY_2p
Biocide B						
Replicate 1	12.89	12.77	17.91	3.37	16.38	11.34
Replicate 2	15.3	14.48	17.07	7.33	14.57	6.29
Replicate 3	15.7	10.15	19.07	6.60	15.07	5.54
Replicate 4	11.21	12.56	16.2	3.96	11.92	5.43
Replicate 5	11.00	10.60	19.47	6.66	12.92	6.49
Replicate 6	13.1	11.85	18.9	7.91	12.50	10.03

4.3 Statistical analyses

Comprehensive statistical analyses were performed as described in 2.7, i.e. tests whether manure of different type and origin yields significantly different results for the transformation studies. Several parameters were selected for these statistical analyses as indicated in table 33.

From the results it is obvious, that in nearly all cases significant differences in either median or dispersion were observed. That means, that manure of the same type (cattle or pig manure, respectively) sampled from different sites resulted in significant differences in median values of NER, extractables, sum of TP and DTx-values.

Results can be detailed as follows:

Table 33: Overview of statistical analysis of significant differences ($p < 0.05$) in dispersion tested by Levene´s Test and median values tested by U-Test, for indicated comparisons of datasets and parameters. n.a. not applicable; (D) significant difference in dispersion and U-Test is not applicable; (M) significant different medians were observed in at least one comparison; (-) no significant difference;

Comparison	Parameter tested	Results: significant differences in dispersion (D) an median (M)			Refer to table in Annex 3		
		Salicylic acid	*Para-cetamol*	*Biocide B*	*Salicylic acid*	*Para-cetamol*	*Biocide B*
3 sampling sites, winter manure, cattle	NER at end of the study	D	n.a.	n.a.	A3_19		
	Extractables at half of the study	D	n.a.	n.a.	A3_19		
	Sum of all TP at end of study duration	D	n.a.	n.a.	A3_19		
	DT_{50}-values	M	n.a.	n.a.	A3_12		
3 sampling sites, manure, cattle, (for Salicylic acid sampled in summer)	NER at end of the study	-	M	M	A3_20	A3_22	A3_24
	Extractables at half of the study	-	-	-	A3_20	A3_22	A3_24
	Sum of all TP at end of study duration	D	M	M	A3_20	A3_22	A3_24
	DT_{50}-values	-	M	M	A3_13	A3_15	A3_17
3 sampling sites, pig	NER at end of the study	M	D	M	A3_21	A3_23	A3_25
	Extractables at half of the study	M	M	M	A3_21	A3_23	A3_25
	Sum of all TP at end of study duration	D	M	M	A3_21	A3_23	A3_25
	DT_{50}-values	M	M	M	A3_14	A3_16	A3_18

4.4 Conclusions on transformation of ^{14}C-Salicylic acid, ^{14}C-Paracetamol and ^{14}C-Biocide B in manures of different origin

- The test protocol developed proved to be applicable for different test substances.
- Comprehensive statistical analyses were applied to test whether manures of different type and origin yield significantly different results for the transformation studies. Several parameters were selected for these analyses. From the results it is obvious, that in nearly all cases significant differences were observed. That means, that manure of the same type (cattle or pig manure, respectively) sampled from different sites resulted in significant differences in NER, extractables, sum of transformation products and DTx-values.
- A further analysis of original data of NER, ER, and mass balances over the incubation period showed that trends are comparable and also coefficients of variation are in the same range for the same parameter and stage of the incubation period. For cattle manure, COV were in the range of 0.4 – 100 % (all test compounds considered), and for pig manure the respective range was 0.6 – 158%. Transformation of Salicylic acid showed COV in the range of 0.4 – 100% (cattle and pig manure considered), for Paracetamol the values were between 0.8 – 100 %, and for Biocide B between 0.6 – 158.6 %.
- High coefficients of variation mostly were observed for NER occurring in small quantities. COV of 100 % and 158.6 % were obtained for NER with arithmetic means between 1.8 – 7.8 [% aR]. This might be due to methodological difficulties when analyzing NER of small quantities. From this observation the conclusion is drawn, that NER in quantities below 10 [% aR] can be determined with a fairly high uncertainty only.

5 Results of the inter-laboratory comparison (ring test)

5.1 Presentation of results

The pre-validation ring test experiments were performed at five international laboratories (see chapter 2.8 for further experimental details) between October 2012 and April 2013. Flow-through test systems were used by IME, ECT and IES, whereas IBACON and AAFC used static test systems. Table 34 gives an overview of the collected data.

Table 34: Parameters and endpoints determined by the participants during the pre-validation ring test

Institute	cattle manure						pig manure					
	$^{14}CO_2$	$^{14}CH_4$	extracts	NER	chemical analyses	microbial activity	$^{14}CO_2$	$^{14}CH_4$	extracts	NER	chemical analyses	microbial activity
IME	Yes	yes	yes	Yes	yes	yes	yes	yes	yes	yes	yes	yes
ECT	Yes	yes	yes	Yes	yes	yes	yes	yes	yes	yes	yes	yes
IES	Yes	no	yes	Yes	yes	yes	yes	no	yes	yes	yes	yes
IBACON	Yes	yes	yes	Yes	yes [1)]	yes	no	no	no	no	no	no
AAFC	Yes	no	yes	Yes	no	yes [2)]	yes	no	yes	no [3)]	no	yes [2)]

1) Chemical analysis was performed using HPLC.
2) Glucose test to determine microbial activity of the manure was run for 35 days instead of 7 days and therefore cannot be compared to the other results for glucose.
3) Combustion of pig manure samples was not possible due to the very low dry matter content of 0.8%.

Remark: The experiments with cattle manure were performed using the same batch of manure (sampled in September 2012) at all European institutes. However, experimental work at IES and IBACON was started in February 2013 and March 2013, respectively. Thus, the recommended maximum storage period of one month was exceeded at the beginning of these experiments.

In detail, storage duration was for:

Table 35: Storage in the laboratory prior to testing

Institute	Storage duration [d]	
	Cattle manure	Pig manure
IME	20	7
ECT	20	12
IES	152	61
IBACON	183	Not performed
AAFC	Not reported	Not reported

All results and evaluations are based on the provided evaluation sheets filled by each participating institute. Endpoints and results are reported herein under in the following order.

Chapter 5.1.1 Redox-potential and pH-values
Chapter 5.1.2 Mineralization
Chapter 5.1.3 Mass balances and distribution of ^{14}C-radioactivity
Annex 4, figures A4_28 to A4_45 individual values on mass balances for each participant, mean values for mineralization, extractables and non-extractables for each participant.

5.1.1 Redox-potential and pH-values

The redox-potential and pH-value are two important parameters which reflect the test conditions. Especially the redox potential is important as it is suggested as a validity criterion to be in the redox range typically observed in manure tanks. The results from one participant do not meet the specifications (always below -100 mV).

Table 36: pH-values of manure at the given sampling points

Time [d]	0	3	7	14	21	28	35
N	3	3	3	3	3	3	3
Participant: IME							
Cattle	8.03	n.a.	n.a.	7.9	n.a.	n.a.	7.7
Pig	9.06	n.a.	n.a.	8.9	n.a.	n.a.	9.08
Participant: ECT							
Cattle	8.18	8.6	8.28	8.3	n.a.	n.a.	8.02
Pig	8.51	8.45	8.06	7.14	n.a.	n.a.	8.44
Participant: IES							
Cattle	8.19	9.1	8.92	8.77	8.87	8.57	8.59
Pig	7.83	6.95	7.34	7.37	7.53	7.95	8.09
Participant: IBACON							
Cattle	7.4	n.a.	n.a.	7.8	n.a.	n.a.	7.09
Pig	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Participant: AAFC							
Cattle	7.0	7.0	7.0	7.0	7.0	7.0	7.0
Pig	7.0	7.0	7.0	7.0	7.0	7.0	7.0

n.a. = not analyzed

Table 37: Redox-potential [mV] of manure at the given sampling points

Time [d]	0	3	7	14	21	28	35
N	3	3	3	3	3	3	3
Participant: IME							
Cattle	-470	n.a.	n.a.	-406	n.a.	n.a.	-398
Pig	-408	n.a.	n.a.	-425	n.a.	n.a.	-450
Participant: ECT							
Cattle	< -400	< -300	< -280	< -320	n.a.	n.a.	< -260
Pig	< -300	< -285	< -320	< -340	n.a.	n.a.	< -310
Participant: IES							
Cattle	-161	-158	-180	-166	-175	-124	-159
Pig	-94	-114	-120	-115	-91	-144	-170
Participant: IBACON							
Cattle	-243 to -297	n.a.	n.a.	-167	n.a.	n.a.	-176 to -274
Pig	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Participant: AAFC							
Cattle[1)]	20	-10	-8	-10	-9		50
Pig [1)]	70	66	70	20	40		10

n.a. = not analyzed
[1)] Redox potential rather high compared to values obtained by the other participants.

5.1.2. Mineralization

Extend of mineralization is expressed as $^{14}CO_2$-formation, $^{14}CH_4$-formation as well as the sum thereof ($^{14}CO_2$ plus $^{14}CH_4$-formation). In that order results are presented as tables and graphically followed by a summary at the end of the chapter.

As the participants IME and IES did not measure replicates but single values per sampling point, the data shown in the table and figures are the measured data, but no means. For the other participants, arithmetic mean values, standard deviations (SD) and coefficients of variation (COV) of three replicates are given.

$^{14}CO_2$-Formation:

Table 38: Summarizing presentation of arithmetic mean-values, standard deviation (SD) and coefficients of variation (COV, (%)) of $^{14}CO_2$-formation measured for the transformation of ^{14}C- Salicylic acid in cattle and pig manure, participant: all.

Time [d]	Type of manure	0	3	7	14	21	28	35
N		3	3	3	3	3	3	3
Participant: IME (single values; no replicates measured)								
single value	Cattle	n.a.	1.8	14.0	17.9	23.4	31.0	40.3
single value	Pig	n.a.	6.88	22.81	47.23	55.73	57.35	67.93
Participant: ECT								
Mean	Cattle	n.a.	3.73	5.12	5.45	6.17	6.87	7.86
SD		n.a.	3.97	5.76	4.79	4.44	4.24	4.05
COV		n.a.	106.35	112.48	87.86	72.03	61.77	51.51
Mean	Pig	n.a.	0.87	1.24	2.45	4.34	6.03	8.24
SD		n.a.	0.29	0.15	0.51	0.82	1.16	1.51
COV		n.a.	33.40	11.73	20.91	18.96	19.21	18.33
Participant: IES (single values; no replicates measured)								
single value	Cattle	n.a.	26.6	29.3	18.5	32.8	41.0	45.2
single value	Pig	n.a.	0.4	1.8	2.7	3.8	11.1	22.3
Participant: IBACON								
Mean	Cattle	4.41	6.97	7.72	29.42	19.61	16.73	25.60
SD		6.65	4.35	2.00	13.78	18.17	11.99	10.39
COV		150.93	62.31	25.97	46.85	92.63	71.67	40.59
Participant: AAFC								
Mean	Cattle	n.a.	2.37	3.64	16.22	3.44	n.a.	7.27
SD		n.a.	0.98	0.72	2.60	1.06	n.a.	0.32
COV		n.a.	41.30	19.71	16.00	30.75	n.a.	4.34
Mean	Pig	n.a.	0.96	2.67	13.64	6.63	n.a.	17.53
SD		n.a.	0.27	1.22	6.80	1.21	n.a.	1.50
COV		n.a.	28.48	45.90	49.84	18.24	n.a.	8.55
Participant: all								
Mean	Cattle	n.a.[1)]	6.1	8.4	17.2	13.1	17.9	18.9
SD		n.a. [1)]	7.6	8.0	11.4	13.2	14.1	15.2
COV		n.a. [1)]	122.9	95.3	66.3	101.1	79.2	80.4
Mean	Pig	n.a.	1.6	4.5	11.0	11.6	17.3	20.9
SD		n.a.	2.2	7.4	12.5	17.9	22.5	19.8
COV		n.a.	135.2	163.8	113.2	155.0	130.1	94.4

[1)] Results for IBACON not considered

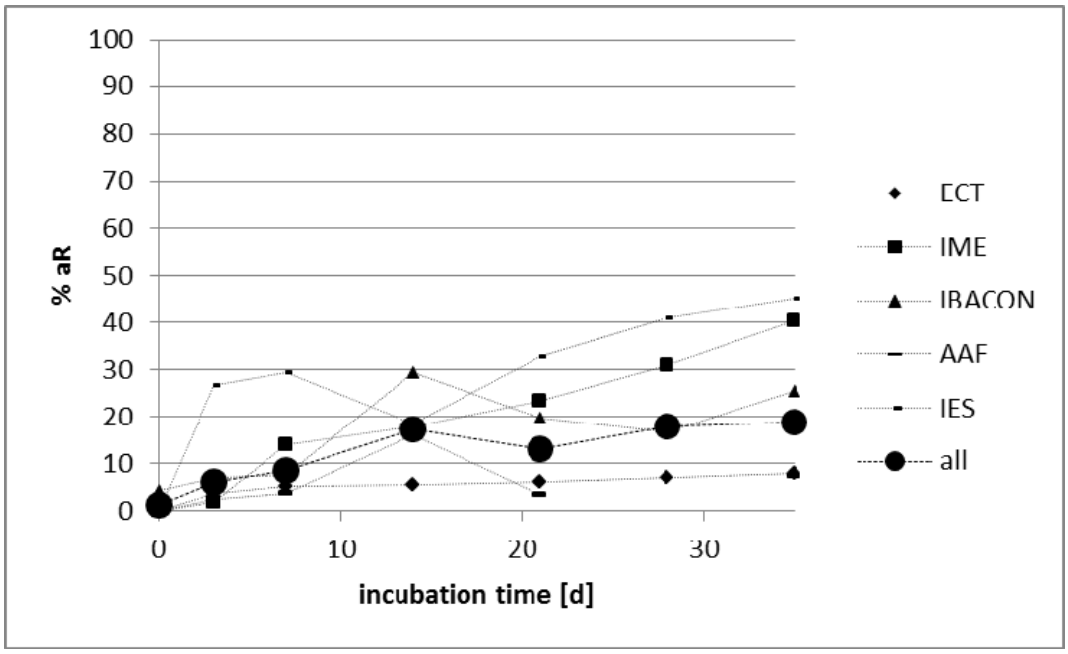

Figure 36: $^{14}CO_2$-formation obtained from transformation of ^{14}C-Salicylic acid in cattle manure for the participants (means of each and of all) of the ringtest. The amount of radioactivity [% applied radioactivity, % aR] per sampling interval is given as arithmetic mean or as single value in case NaOH-filled traps were used for all replicates.

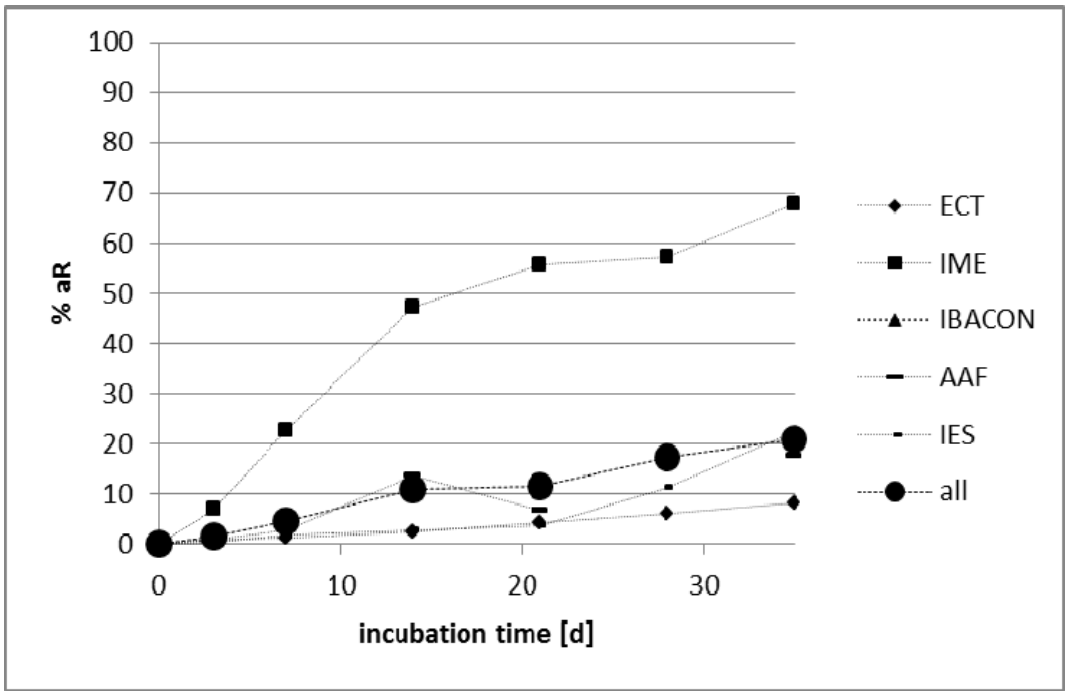

Figure 37: $^{14}CO_2$-formation obtained from transformation of ^{14}C-Salicylic acid in pig manure for the participants of the ringtest. The amount of radioactivity [% applied radioactivity, % aR] per sampling interval is given as arithmetic mean or as single value in case NaOH-filled traps were used for all replicates.

$^{14}CH_4$-Formation:

Table 39: Summarizing presentation of arithmetic mean-values, standard deviation and coefficients of variation (COV, (%) of $^{14}CH_4$-formation measured for the transformation of ^{14}C- Salicylic acid in cattle and pig manure, participant: all.

Time [d]	Type of manure	0	3	7	14	21	28	35
N		3	3	3	3	3	3	3
Participant: IME (single values; no replicates measured)								
single value	Cattle	n.a.	0.01	0.15	0.56	1.11	2.34	3.54
single value	Pig	n.a.	0.1	0.2	2.0	2.7	3.3	4.1
Participant: ECT								
Mean	Cattle	n.a.	0.05	0.33	1.18	2.26	3.31	4.76
SD		n.a.	0.01	0.16	0.23	0.17	0.43	0.99
COV		n.a.	11.56	49.06	19.72	7.58	13.14	20.86
Mean	Pig	n.a.	0.03	0.12	0.56	1.50	3.00	3.00
SD		n.a.	0.00	0.03	0.18	0.49	0.52	0.52
COV		n.a.	15.56	23.66	32.06	32.63	17.25	17.25
Participant: IES (single values; no replicates measured)								
single value	Cattle	n.a.	0.0	0.0	0.0	0.0	0.0	0.0
single value	Pig	n.a.	0.0	0.0	0.0	0.0	0.0	0.0
Participant: IBACON								
Mean	Cattle	n.a.	0.65	0.48	0.32	0.28	0.22	0.31
SD		n.a.	0.06	0.16	0.14	0.11	0.12	0.08
COV		n.a.	8.93	33.59	45.14	38.63	54.09	25.70
Participant: AAFC								
Mean	Cattle	n.a.	0.01	0.02	0.13	0.08	n.a.	0.0
SD		n.a.	0.01	0.02	0.03	0.01	n.a.	0.0
COV		n.a.	100.00	86.60	22.22	10.19	n.a.	-
Mean	Pig	n.a.	0.0	0.0	0.12	0.06	n.a.	0.0
SD		n.a.	0.0	0.0	0.13	0.01	n.a.	0.0
COV		n.a.	-	-	111.50	25.00	n.a.	-
Participant: all								
Mean	Cattle	n.a.	0.2	0.2	0.5	0.8	1.6	1.7
SD		n.a.	0.3	0.2	0.5	1.0	1.6	2.3
COV		n.a.	151.2	94.5	97.1	120.0	99.1	132.1
Mean	Pig	n.a.	0.0	0.1	0.3	0.6	1.6	1.6
SD		n.a.	0.0	0.1	0.7	0.9	1.2	1.8
COV		n.a.	152.0	116.2	200.1	159.1	78.1	110.4

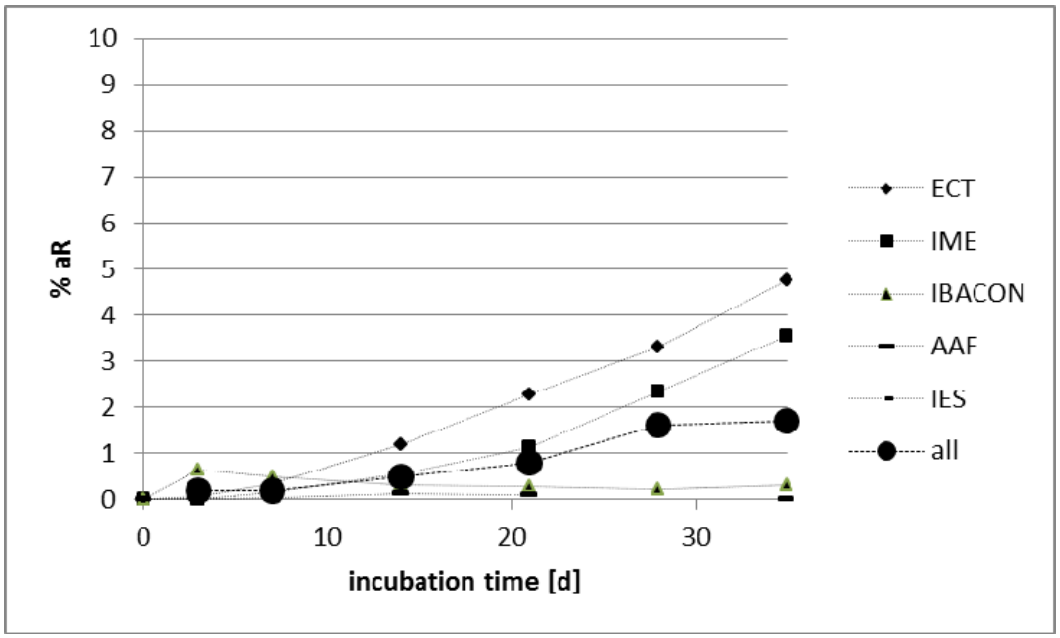

Figure 38: $^{14}CH_4$-formation obtained from transformation of ^{14}C-Salicylic acid in cattle manure for the participants (means of each and of all) of the ringtest. The amount of radioactivity [% applied radioactivity, % aR] per sampling interval is given as arithmetic mean or as single value in case NaOH-filled traps were used for all replicates.

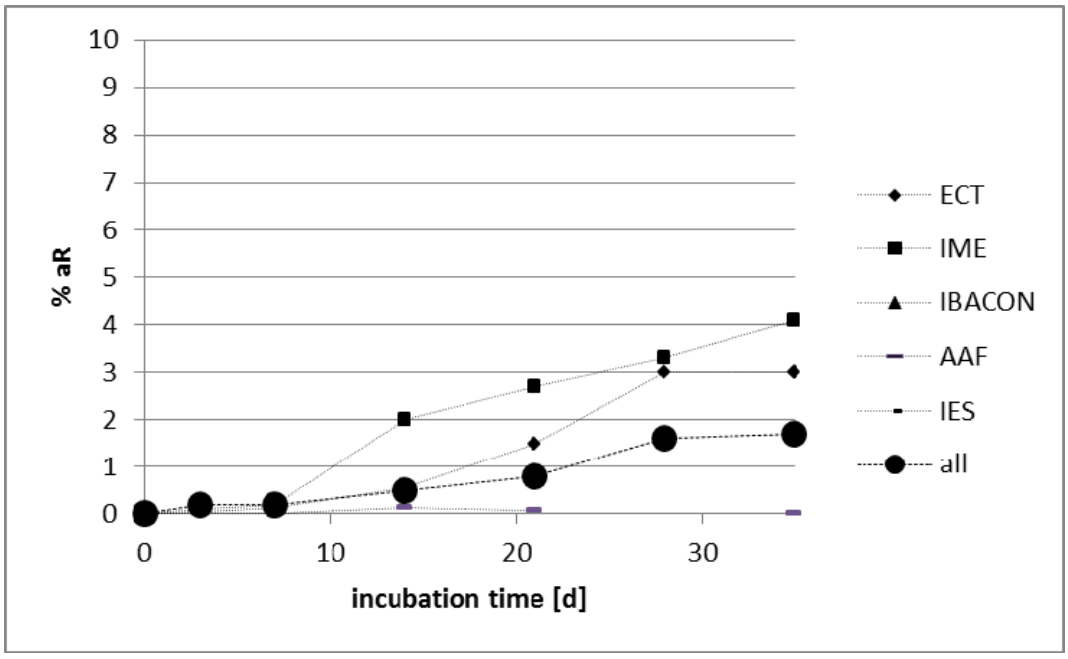

Figure 39: $^{14}CH_4$-formation obtained from transformation of ^{14}C-Salicylic acid in pig manure for the participants of the ringtest. The amount of radioactivity [% applied radioactivity, % aR] per sampling interval is given as arithmetic mean or as single value in case NaOH-filled traps were used for all replicates.

Sum of $^{14}CO_2$ plus $^{14}CH_4$:

Table 40: Summarizing presentation of arithmetic mean-values, standard deviation (SD) and coefficient of variation (COV, (%) of $^{14}CO_2$ + $^{14}CH_4$-formation measured for the transformation of ^{14}C- Salicylic acid in cattle and pig manure, participant: all.

Time [d]	Type of manure	0	3	7	14	21	28	35
N		3	3	3	3	3	3	3
Participant: IME (single values; no replicates measured)								
single value	Cattle	n.a.	1.9	14.2	18.5	24.5	33.3	43.8
single value	Pig	n.a.	6.98	23.01	49.23	58.43	60.65	72.03
Participant: ECT								
Mean	Cattle	n.a.	3.78	5.45	6.63	8.42	10.18	12.62
SD		n.a.	3.97	5.91	4.61	4.31	3.81	3.49
COV		n.a.	105.08	108.30	69.54	51.15	37.46	27.65
Mean	Pig	n.a.	0.89	1.36	3.00	5.84	9.03	14.20
SD		n.a.	0.29	0.17	0.52	0.65	1.38	2.39
COV		n.a.	32.62	12.57	17.26	11.12	15.25	16.85
Participant: IES (single values; no replicates measured)								
single value	Cattle	n.a.	26.6	29.3	18.5	32.8	41.0	45.2
single value	Pig	n.a.	0.4	1.8	2.7	3.8	11.1	22.3
Participant: IBACON								
Mean	Cattle	4.41	7.62	8.20	29.74	19.89	16.95	25.91
SD		6.65	4.40	1.96	13.69	18.24	12.11	10.32
COV		150.93	57.68	23.89	46.02	91.70	71.43	39.84
Participant: AAFC								
Mean	Cattle	n.a.	2.39	3.66	16.35	3.52	n.a.	7.27
SD		n.a.	0.99	0.73	2.62	1.06	n.a.	0.32
COV		n.a.	41.56	20.05	16.00	30.02	n.a.	4.34
Mean	Pig	n.a.	0.96	2.67	13.76	6.69	n.a.	17.53
SD		n.a.	0.27	1.22	6.93	1.22	n.a.	1.50
COV		n.a.	28.48	45.90	50.37	18.25	n.a.	8.55
Participant: all								
Mean	Cattle	n.a.[1)]	6.4	8.7	17.7	13.9	19.5	20.6
SD		n.a.[1)]	7.6	8.0	11.1	13.0	13.4	14.8
COV		n.a.[1)]	119.3	92.3	62.8	93.7	68.8	71.9
Mean	Pig	n.a.	1.6	4.6	12.8	12.5	19.8	23.7
SD		n.a.	2.2	7.5	16.1	18.6	22.9	19.8
COV		n.a.	135.2	162.4	126.1	149.1	115.8	83.5

[1)] Results for IBACON not considered

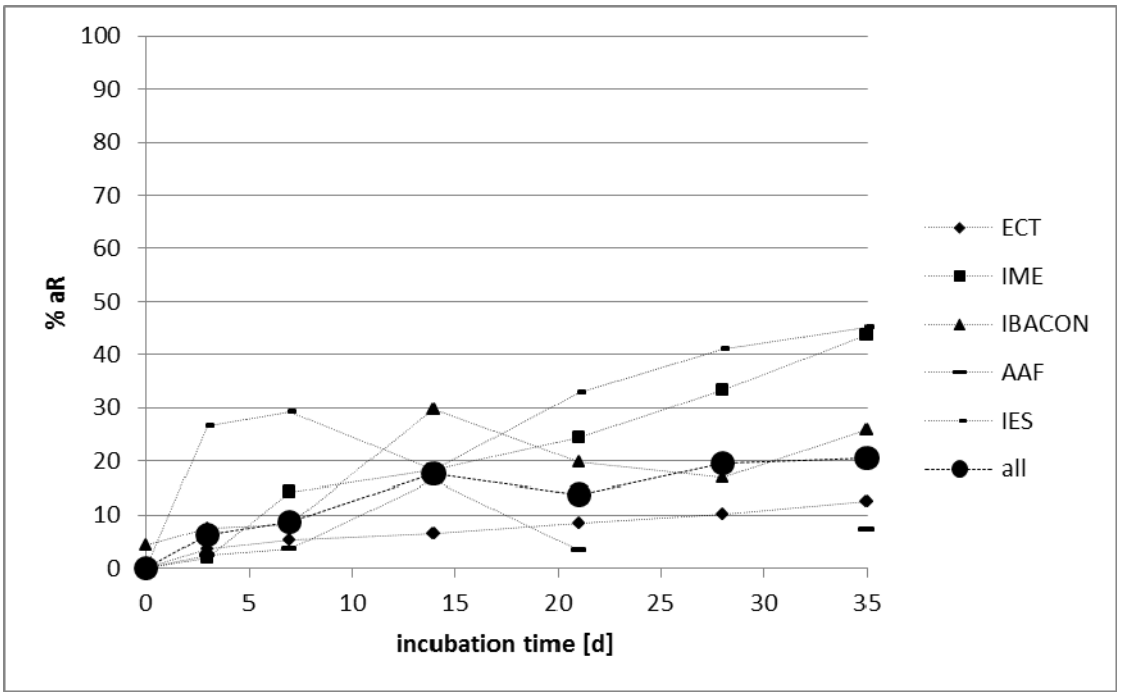

Figure 40: $^{14}CO_2$+ $^{14}CH_4$-formation obtained from transformation of ^{14}C-Salicylic acid in cattle manure for the participants (means of each and of all) of the ringtest. The amount of radioactivity [% applied radioactivity, % aR] per sampling interval is given as arithmetic mean or as single value in case NaOH-filled traps were used for all replicates.

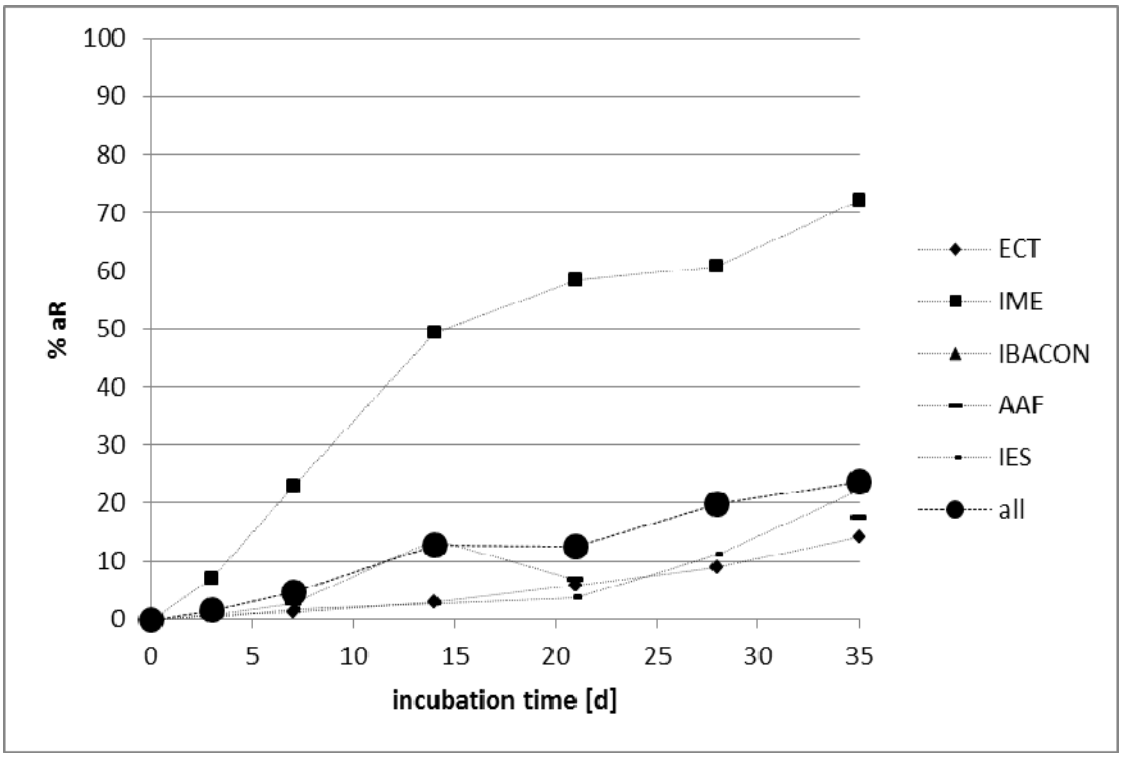

Figure 41: $^{14}CO_2$+ $^{14}CH_4$-formation obtained from transformation of ^{14}C-Salicylic acid in pig manure for the participants of the ringtest. The amount of radioactivity [% applied radioactivity, % aR] per sampling interval is given as arithmetic mean or as single value in case NaOH-filled traps were used for all replicates.

Summary: Mineralization

$^{14}CO_2$-formation obtained in ring test

For cattle manure $^{14}CO_2$-formation rates at the end of the test are in the range of 7.3 % aR to 45.2 % aR. Few of the measured values are scattering in the course of the test – e.g. for IBACON and AAFC – whereas most of the participants measured increasing amount of formed $^{14}CO_2$ over the incubation period. The COVs range between 4.3 – 150.9 %.

For pig manure, the amount of $^{14}CO_2$ which has been formed until the end of the test also differs for the participants and is in the range of 8.2 % aR (ECT) and 67.9 % aR (IME). COVs are in the range of 8.6 – 45.9 %.

Arithmetic means (all participants) of $^{14}CO_2$ which has been formed at the end of the studies are 18.9 % aR (cattle manure) and 20.9 % aR (pig manure), respectively. COVs are in a range of 66.3 – 122.9 % (cattle manure) and 94.4 – 163.8 % (pig manure).

$^{14}CH_4$-formation obtained in ring test

CH_4-formation rates in both types of manure are much lower compared to CO_2-formation.

In cattle manure, the formed amount at the end of the studies is between 0.0 – 4.8 % aR. COVs are between 7.6 – 86.6 %.

For pig manure, values have been reported by IME and ECT. The amount of $^{14}CH_4$, which has been formed until the end of the test, was 4.1 % aR (IME) and 5.8 % aR (ECT).

Arithmetic means (all participants) of $^{14}CH_4$ which has been formed at the end of the studies are 1.7 % aR (cattle manure) and 1.6 % aR (pig manure), respectively. COVs are in a range of 94.5 – 151.2 % (cattle manure) and 78.1 – 200.1 % (pig manure).

Mineralization ($^{14}CO_2$ + $^{14}CH_4$) obtained in ring test

The mineralization rates for salicylic acid at the end of the test are in the range between 7.2 % aR and 45.2 % aR for cattle manure and in the range between 17.5 % aR and 60 % aR for pig manure. Particularly the differences for cattle manure between IME and ECT are surprising, because identical manure, a similar test setup (flow-through test system) and similar storage conditions have been used. The differences cannot be explained by killing of methanogenic bacteria because the low mineralization rates at ECT are mainly caused by very low amounts of evolved $^{14}CO_2$, whereas higher amounts of evolved $^{14}CH_4$ were measured. No obvious reasons for low mineralization rates could be found.

For pig manure, it seems that mineralization increases at the end of the test period for ECT and IES, which might be explained by a long lag-phase. Thus, the observed variability with regard to mineralization at the end of the test might be reduced by prolonged test duration.

Arithmetic means (all participants) of total mineralization at the end of the studies are 20.6 % aR (cattle manure) and 23.7 % aR (pig manure), respectively. COVs are in a range of 62.8 – 119.3 % (cattle manure) and 83.5 – 162.4 % (pig manure).

5.1.3 Mass balances and distribution of ^{14}C-radioactivity

First, graphical presentations comparing the results each for extractable and non-extractable residues are shown. The amount of radioactivity is given as mean of three replicates. Results for mass balances are detailed more than those for extractable and non-extractable residues as they could be suitable as a quality criterion of the study. Thus, for the parameter “mass balance” results are presented as tables and graphically followed by a summary for all parameter at the end of this chapter.

Extractable residues:

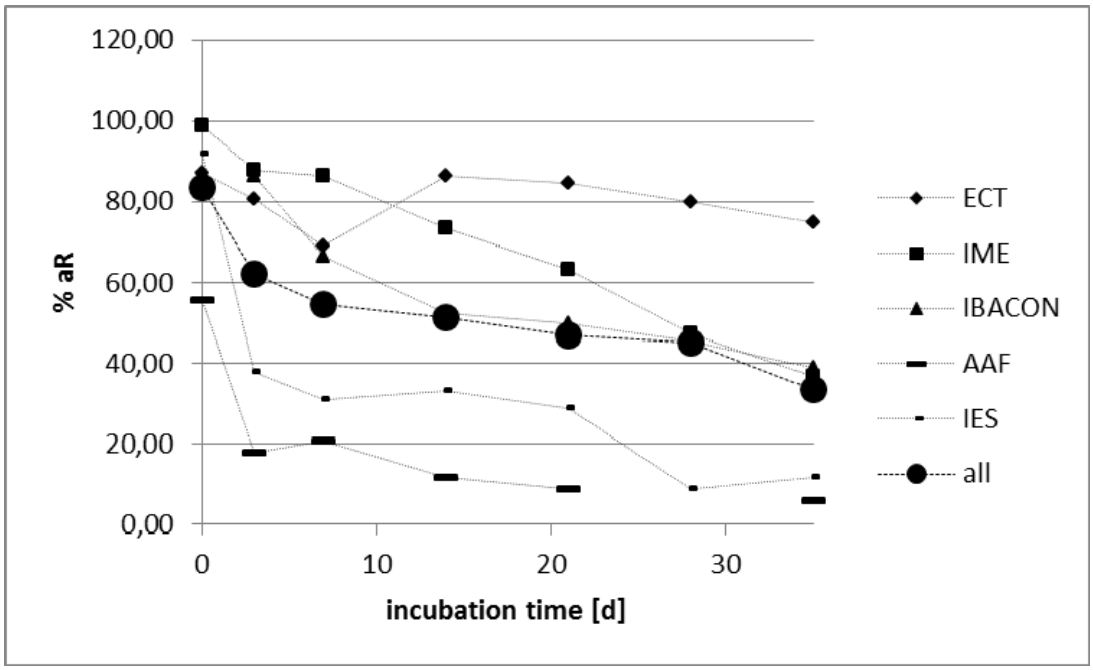

Figure 42: Comparison of extractable residues obtained from transformation of ^{14}C-Salicylic acid in cattle manure for the participants (means of each and of all) of the ringtest. The amount of radioactivity [% applied radioactivity, % aR] per sampling interval is given as arithmetic mean.

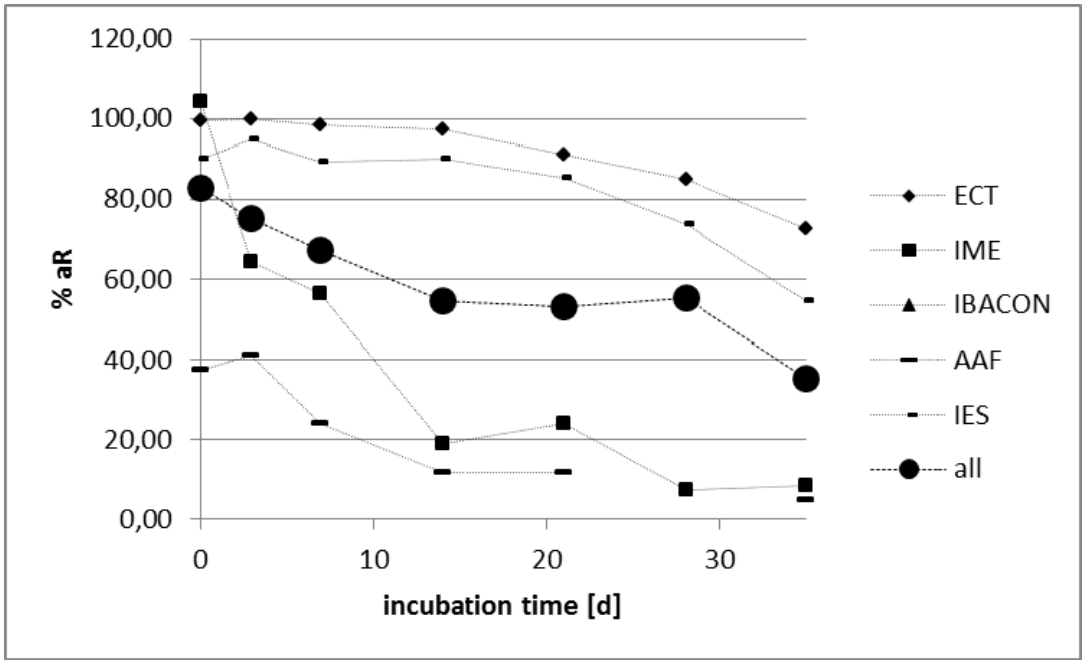

Figure 43: Comparison of extractable residues obtained from transformation of ^{14}C-Salicylic acid in pig manure for the participants of the ringtest. The amount of radioactivity [% applied radioactivity, % aR] per sampling interval is given as arithmetic mean.

Non-extractable residues:

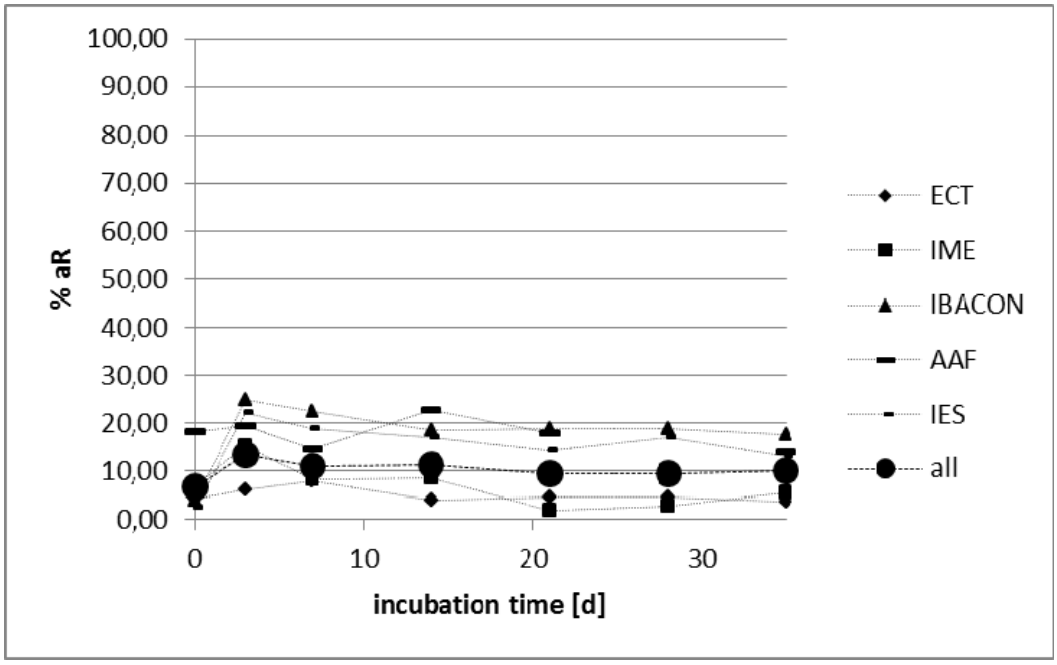

Figure 44: Comparison of non-extractable residues obtained from transformation of ^{14}C-Salicylic acid in cattle manure for the participants (means of each and of all) of the ringtest. The amount of radioactivity [% applied radioactivity, % aR] per sampling interval is given as arithmetic mean.

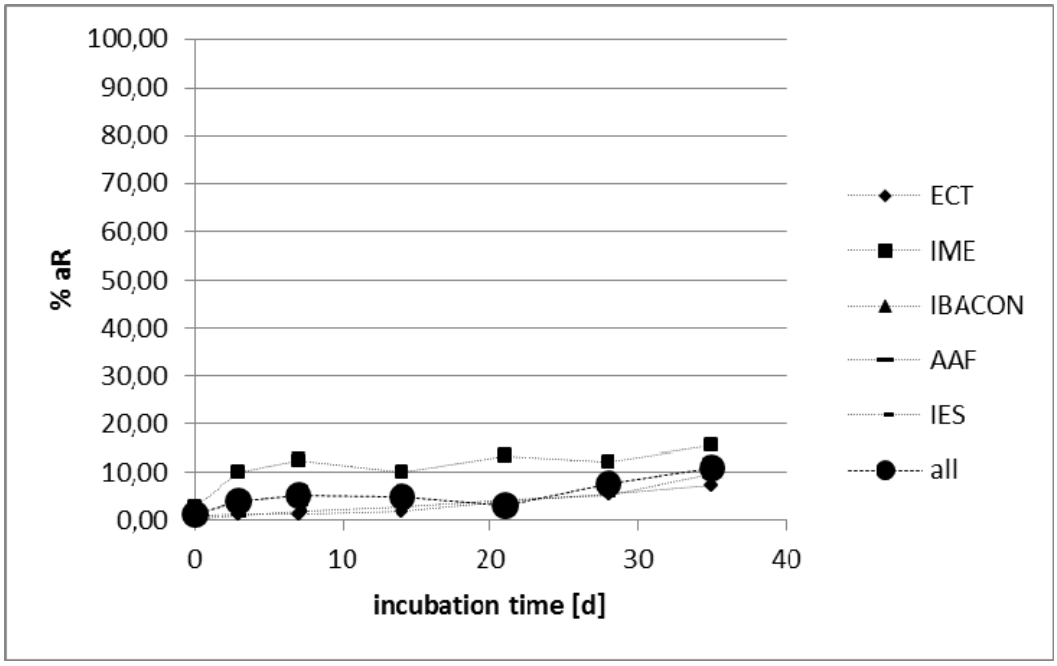

Figure 45: Comparison of non-extractable residues obtained from transformation of ^{14}C-Salicylic acid in pig manure for the participants of the ringtest. The amount of radioactivity [% applied radioactivity, % aR] per sampling interval is given as arithmetic mean.

Mass balances:

Table 41: Summarizing presentation of all mean-values, standard deviation (SD) and coefficients of variation (COV, (%) of mass balances for the transformation of ^{14}C- Salicylic acid in cattle and pig manure, participant: all. Furthermore, exemplary results of the transformation of Salicylic acid which are obtained in the study development part of the project (see chapter 4) are presented for comparative purposes.

Time [d]	Type of manure	0	3	7	14	21	28	35
N		3	3	3	3	3	3	3
Participant: IME								
Mean	Cattle	105.94	105.05	108.81	100.48	89.54	83.44	86.06
SD		0.62	1.14	4.46	0.69	3.81	3.16	0.25
COV		0.59	1.08	4.10	0.69	4.26	3.79	0.29
Mean	Pig	106.73	81.44	91.77	78.32	82.98	80.00	95.89
SD		1.76	13.44	4.73	5.58	12.31	6.06	2.79
COV		1.65	16.51	5.16	7.12	14.83	7.58	2.90
Participant: ECT								
Mean	Cattle	91.61	90.70	82.38	97.11	97.53	94.46	91.17
SD		0.85	7.26	18.47	5.80	6.84	8.99	7.42
COV		0.93	8.01	22.42	5.97	7.01	9.52	8.14
Mean	Pig	100.53	102.11	101.28	102.39	101.21	99.54	94.19
SD		1.43	0.76	0.65	0.48	1.05	3.19	3.12
COV		1.42	0.75	0.64	0.47	1.04	3.21	3.31
Participant: IES								
Mean	Cattle	94.51	86.46	79.10	68.42	75.68	66.62	70.16
SD		1.59	11.50	3.69	8.53	12.95	3.55	7.24
COV		1.68	13.30	4.66	12.46	17.11	5.33	10.32
Mean	Pig	90.41	96.06	93.27	95.30	93.72	90.07	86.82
SD		2.04	2.01	4.45	10.18	7.85	19.17	7.85
COV		2.26	2.09	4.77	10.68	8.37	21.28	9.05
Participant: IBACON								
Mean	Cattle	967.67	118.95	97.08	100.36	88.51	81.16	82.39
SD		22.04	7.56	5.20	23.27	16.47	20.27	10.43
COV		2.28	6.36	5.36	23.19	18.61	24.97	12.66
	Pig	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Participant: AAFC								
Mean	Cattle	73.80	39.72	38.87	50.77	30.23	n.a.	27.06
SD		7.39	7.92	4.97	4.60	7.54	n.a.	11.26
COV		10.01	19.94	12.80	9.07	24.93	n.a.	41.61
Mean	Pig	37.44	42.21	26.80	25.54	18.25	n.a.	22.20
SD		12.54	6.46	3.44	4.12	1.27	n.a.	1.33
COV		33.48	15.31	12.84	16.14	6.96	n.a.	6.01

Table 40: Summarizing presentation of all mean-values, standard deviation (SD) and coefficients of variation (COV, (%) of mass balances for the transformation of ^{14}C- Salicylic acid in cattle and pig manure, participant: all. Furthermore, exemplary results of the transformation of Salicylic acid which are obtained in the study development part of the project (see chapter 4) are presented for comparative purposes (ctn.).

Time [d]	Type of manure	0	3	7	14	21	28	35
N		3	3	3	3	3	3	3
Participant: all								
Mean	Cattle	266.7	88.2	81.2	83.4	76.3	81.4	71.4
SD		363.0	28.5	25.8	23.2	26.5	14.2	25.0
COV		136.1	32.3	31.7	27.8	34.7	17.4	35.1
Mean	Pig	83.8	80.5	78.3	75.4	74.0	89.9	74.8
SD		29.1	25.2	31.4	31.9	34.9	13.2	32.1
COV		34.8	31.3	40.1	42.3	47.1	14.7	43.0
Transformation of Salicylic acid performed by IME. first part of the project								
N		6	6	6	6	6	6	6
Mean	Cattle. Winter manure. site: NRW_1c	103.33	94.77	99.32	96.47	94.95	85.95	91.23
SD		1.53	2.26	7.51	4.95	6.75	4.52	3.28
COV		1.49	2.15	7.86	5.15	6.92	4.67	3.48
Mean	Cattle. Summer manure. site: NRW_1c	107.57	102.43	96.22	93.60	96.75	101.12	96.35
SD		0.41	16.12	10.49	10.40	7.30	10.40	6.25
COV		0.38	15.73	10.90	11.11	7.54	10.29	6.48
Mean	Pig. site: NRW_1p	92.70	85.15	86.70	80.92	78.78	63.63	75.55
SD		1.55	16.99	3.16	9.98	9.43	6.59	1.03
COV		1.67	19.95	3.65	12.34	11.96	10.35	1.36

n.a. = not analyzed

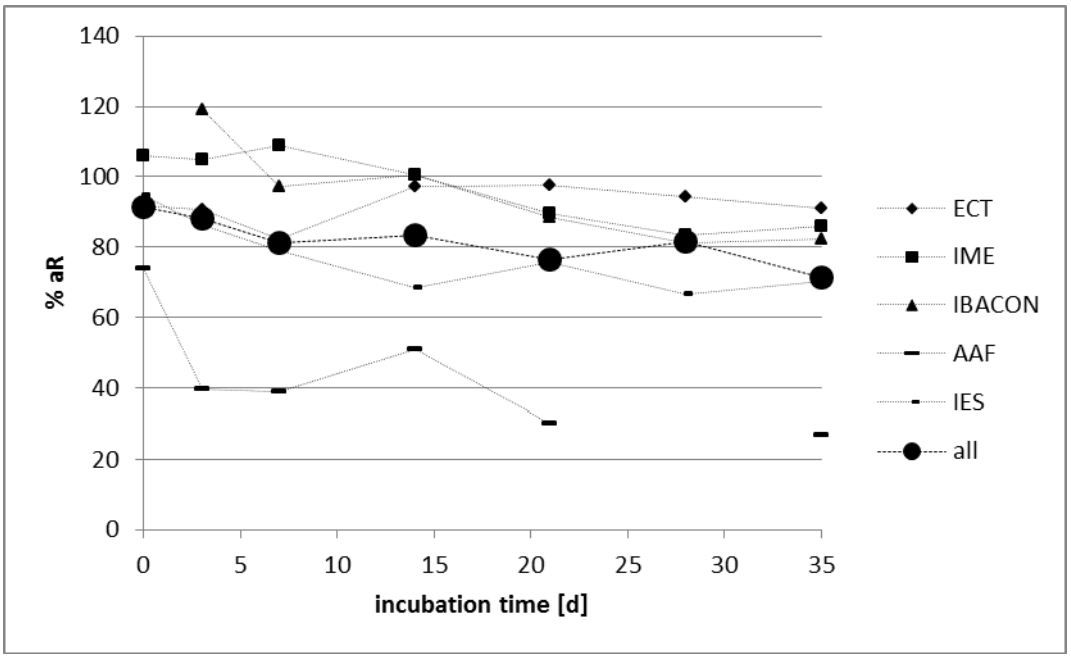

Figure 46: Mass balances for transformation of ^{14}C-Salicylic acid in cattle manure for all participants (means of each and of all) of the ringtest. The amount of radioactivity [% applied radioactivity, % aR] per sampling interval is given as re-transformed mean.

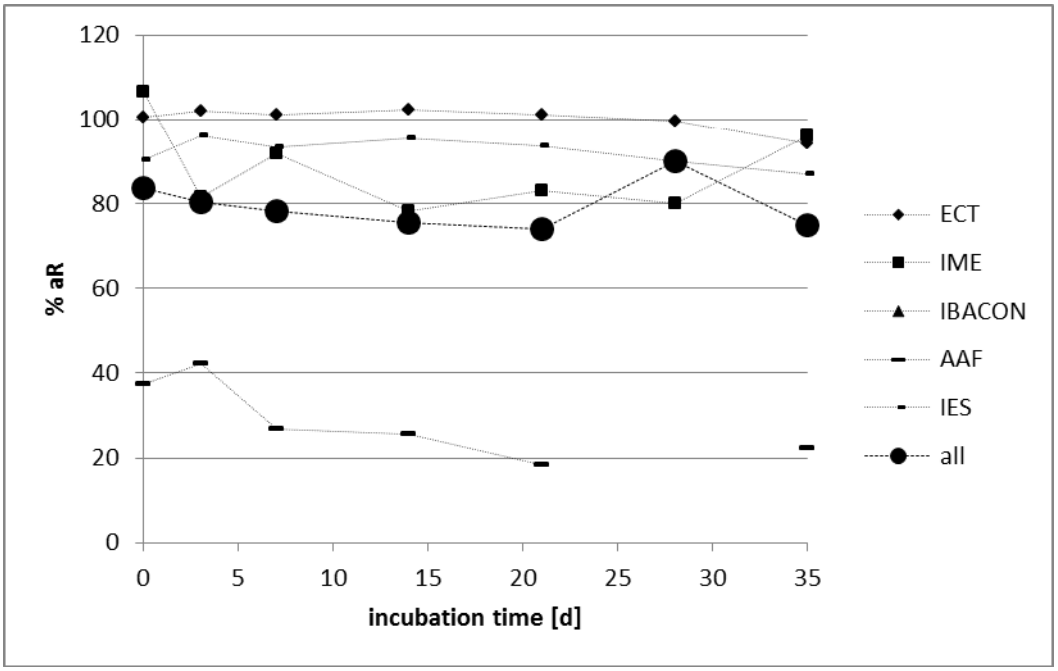

Figure 47: Mass balances for transformation of ^{14}C-Salicylic acid in pig manure for all participants of the ringtest. The amount of radioactivity [% applied radioactivity, % aR] per sampling interval is given as re-transformed mean.

Summary: extractable and non-extractable residues, mass balances

Extractable residues

A typical time dependent behaviour of extractable residues (ER) can be observed in both, cattle and pig manure showing a decrease over time.

In cattle manure, the participants AAFC and IES observed a fast decrease of ER within 4 days after application of the test substance followed by a modest decrease. The amount of extractable residues descended from 55.7 % aR to 5.8 % aR (AAFC) and from 92.0 % aR to 11.8 % aR (IES). The amount of 55.7 % aR (AAFC) at the beginning of the transformation study is remarkably low, and much lower compared to the results obtained by the other participants (87.3 % aR - 99.0 % aR).
The decrease of ER during the transformation study observed by the participants IME, ECT, and IBACON was slower compared to those just described. The decrease was from 99.0 % aR to 36.8 % aR (IME), from 87.3 % aR to 74.8 % aR (ECT), and from >> 100 % aR to 39.0 % aR (IBACON). The extreme value of >> 100 % aR (IBACON) was not explainable and thus not considered in further evaluations.

In pig manure, the participant IME measured a pronounced reduction of ER from 104.1 % aR at the beginning of the transformation study to 8.4 % aR at the end. The participant AAFC also measured minor amounts of ER at the end of the study, namely 4.7 % aR. Reduction of ER measured by the participant ECT and IES was less pronounced. Reduction was from 99.5 % aR to 72.7 %aR (ECT), and 90.0 % aR to 54.7 % aR (IES).

Non-extractable residues

A typical time dependent behaviour of non extractable residues (NER) can be observed in cattle manure showing an increase of NERs at the beginning of the test, followed by a decrease until the end of the experiment. This could be explained by degradation of solid organic material in the course of the test, whereby the bound test compound is released. The amount of NER at the end of the study was around 5 % aR (ECT and IME) and around 15 % aR (AAFC, IBACON, IES), respectively. Apart from AAFC, all participants used the identical cattle manure, though storage duration at IES and IBACON was above the recommended duration of 2 months. In the first part of the project, IME obtained results for NER at the end of the study in the range of 8.0 – 14.0 % aR.

For pig manure an increase of NER within the test period was observed. Both, the trend and the absolute numbers measured by the participants ECT and IES were comparable. In the course of the incubation period NER increased from 1.1 % aR at the beginning to 7.3 % aR after 35 days of incubation (ECT), and from 0.4 % aR to 9.8 % aR (IES). Higher NER were observed by the participant IME. NER increased from 2.6 % aR to 15.4 % aR. In the first part of the project means obtained by IME were in the range of 8.2 – 12.5 % aR at the end of the study.

Results for NER at the end of the study including respective COVs are summarized in table 41.

Figure 48 compares all results obtained in the course of the ringtest and during the first part of the study.

Table 42: NER at the end of the incubation period

	Fh-IME		ECT		IES		IBACON	AAFC	All participants	
	Cattle	Pig	Cattle	Pig	Cattle	Pig	Cattle	Cattle	Cattle	Pig
Original data [% aR]										
1	2.8	14.6	3.2	9.2	15.3	7.7	17.6	4.3	NER all partici-pants	NER all partici-pants
2	2.7	15.0	3.8	6.6	11.1	13.6	17.8	25.0		
3	11.0	16.6	4.3	6.3	13.1	8.1	17.0	12.6		
N	3	3	3	3	3	3	3	3	15	9
Mean	6.85	15.80	4.05	6.45	12.10	10.85	17.40	18.80	10.77	10.86
Standard deviation	5.87	1.13	0.35	0.21	1.41	3.89	0.57	8.77	7.00	4.04
COV (%)	85.68	7.16	8.73	3.29	11.69	35.84	3.25	46.64	64.93	37.26

Mass balance

Mass balances can be seen as a quality criterion of a transformation study performed with radiolabelled test substance. In the guidelines OECD 307 (aerobic and anaerobic transformation in soil, OECD (2002b)) and OECD 308 (aerobic and anaerobic transformation in aquatic systems, OECD (2002a)) it is stated that the mass balance should be between 90 % aR and 110 % aR.

However, none of the participants met this quality criterion for all samples and sampling points. Overall mass balances were above 80% aR except for IES and AAFC, where lower recoveries can be observed at several time points. However, $^{14}CH_4$ was not determined at these laboratories, which might explain these differences.

The comparatively low recovery at ECT for cattle manure at day 7 (82.4 % aR) can be explained as follows: the test replicates for the different sampling time points were arranged in series and not in parallel. At day 7, a high amount of $^{14}CO_2$ was detected in one of the three test replicates for that sampling time point. However, the measured radioactivity was evenly allocated to all remaining test replicates at that time point (day 7, 14, 21, 28 and 35), resulting in a lower recovery at day 7 and comparatively higher recoveries at the following sampling time points.

Mean values of mass balances for all participants were between 71.4 to 266.7 % aR for cattle manure and between 74.0 to 89.9 % aR for pig manure. COV were in the range of 17.4 – 136.1 % (cattle manure) and 14.7 – 47.1 % (pig manure).

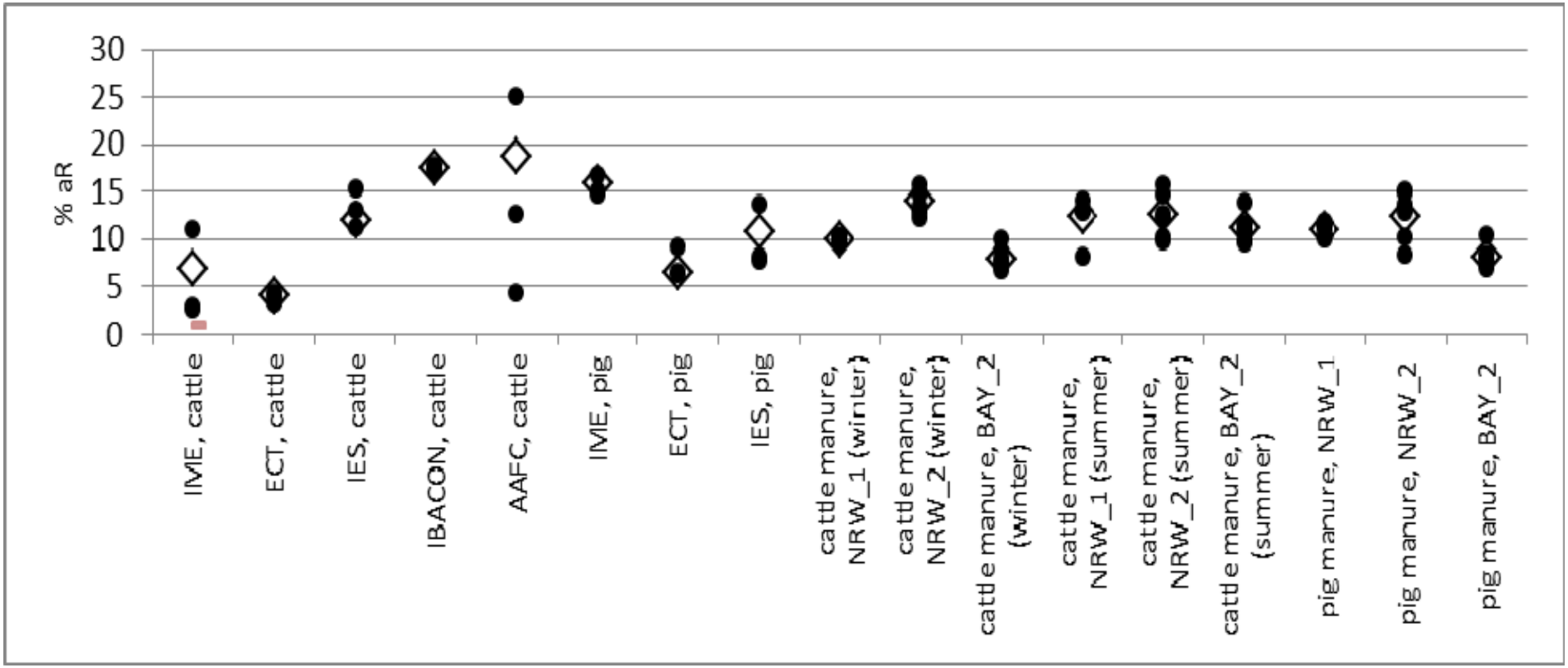

Figure 48 Means (open squares) and individual data (black dots) of non-extractable residues (NER) at the end of the study for the transformation of ^{14}C-Salicylic acid in cattle and pig manure as obtained by the participants IME, ECT, IES, IBACON and AAF and – for comparative purposes – data obtained by IME in the first part of the project (named as cattle manure, NRW_1 (winter) etc. on the right hand site of the figure.

Summary: Coefficients of variation

A measure for the variability of results obtained from all participants of the ringtest is the coefficient of variation (COV). The following table shows COV (%) for the mass balance, mineralization, extractable residues, and non-extractable residues for all participants.

Table 43: Presentation of COV (%) for mass balance, mineralization ($^{14}CO_2$ + $^{14}CH_4$-formation), extractables and non-extractable residues obtained for all participants.

Time [d]	0	3	7	14	21	28	35
Mass balance							
Cattle	136.1	32.3	31.7	27.8	34.7	17.4	35.1
pig	34.8	31.3	40.1	42.3	47.1	14.7	43.0
$^{14}CO_2$-formation							
Cattle	n.a.	122.9	95.3	66.3	101.1	79.2	80.4
pig	n.a.	135.2	163.8	113.2	155.0	130.1	94.4
$^{14}CH_4$-formation							
Cattle	n.a.	151.2	94.5	97.1	120.0	99.1	132.1
pig	n.a.	152.0	116.2	200.1	159.1	78.1	110.4
Mineralization ($^{14}CO_2$ + $^{14}CH_4$)							
Cattle	n.a.	119.3	92.3	62.8	93.7	68.8	71.9
pig	n.a.	135.2	162.4	126.1	149.1	115.8	83.5
Extractable residues							
Cattle	21.3	50.5	52.2	55.7	59.6	58.9	76.1
pig	34.3	34.5	46.2	76.0	71.4	69.4	88.5
Non-extractable residues							
Cattle	92.6	58.7	64.7	65.5	80.7	78.8	69.1
pig	72.9	114.9	108.6	82.9	77.3	60.9	37.3

5.1.3 Degradation kinetics and statistical evaluation

The substance-specific chemical analysis was performed with regard to the test substance salicylic acid and potential transformation products. The test results from AAFC were not considered for evaluation because the data cannot be compared to the results from the other participating institutes due to differing dry matter contents. Dry matter contents were not adjusted, resulting in lower dry matter contents compared to recommended values: 7.4% for cattle manure and 0.8% for pig manure instead of 10% and 5%, respectively.

DT_{50}-values

The DT_{50} values were determined by means of the SFO-kinetic (see chapter 2.6) using the software KinGUI. The data were ln-transformed and means, standard deviation (SD), re-transformed means, coefficient of variation and the medians were calculated. Analyses at IBACON were performed using HPLC. However, only for day 0, a value above the detection limit is available and therefore, no DT_x-values were determined.

Table 44: Statistical evaluation of DT_{50}- values determined in the pre-validation ring test for the test substance salicylic acid

	Participant							
	IME		ECT		IES		All participants	
	Cattle	Pig	Cattle	Pig	Cattle	Pig	Cattle	Pig
Original DT_{50}-values [d]								
Replicate 1	27.6	6.1	116.4	24.3	1.1	39.7	DT_{50}-values from all participants	DT_{50}-values from all participants
Replicate 2	27.0	6.6	1000	35.0	8.0	53.5		
Replicate 3	24.1	6.6	203.7	50.0	10.2	128.2		
N	3	3	3	3	3	3	9	9
Mean (d)	26.23	6.43	440.03	36.43	6.43	73.80	157.6	38.9
Standard deviation	1.87	0.29	486.91	12.91	4.75	47.61	322.8	38.2
Coefficient of variation (%)	7.13	4.49	110.65	35.43	73.80	64.52	204.9	98.3

The three participants Fh-IME, ECT and IES determined DT_{50}-values for both the transformation of Salicylic acid in cattle and pig manure. In cattle manure a broad range of DT_{50}-values can be observed; it is in the range of 4.5 [d] to 287.3 [d]. For pig manure, the range observed is between 6.4 [d] and 64.8 [d]. The participants used the same cattle manure, whereas pig manure was of different origin. At the workshop held with the study participants, it was considered likely that different handling at different steps of the experimental procedure caused these differences. As a consequence further details for test set-up and handling of samples was included into the protocol and demonstrations of some of the steps, e.g. addition of the test substance were given for all participants.

Standard deviations and coefficients of variation were calculated both for the participants each ("intra-laboratory comparison") and for all participants ("inter-laboratory comparison"):
Coefficients of variation are rather low for the DT_{50}-values determined at Fh-IME; they are in the range of 4.5 – 7.1 %. Coefficients of variation for DT_{50}-values determined by the other participants are between 35.4 – 110.7%. Coefficients of variation are by far higher when using all data of all participants as basis for calculation. Both right hand columns in table 42 show the respective results. COVs are 98.3% for the transformation in pig manure, and 204.9% in cattle manure.

The results (DT_{50}-values only) for IME, ECT, and IES are presented in the following figure. For comparative purposes DT_{50}-values for the transformation of Salicylic acid in cattle and pig manure as obtained by IME in the first part of the project (see chapter 4.2) are also shown in the same figure.

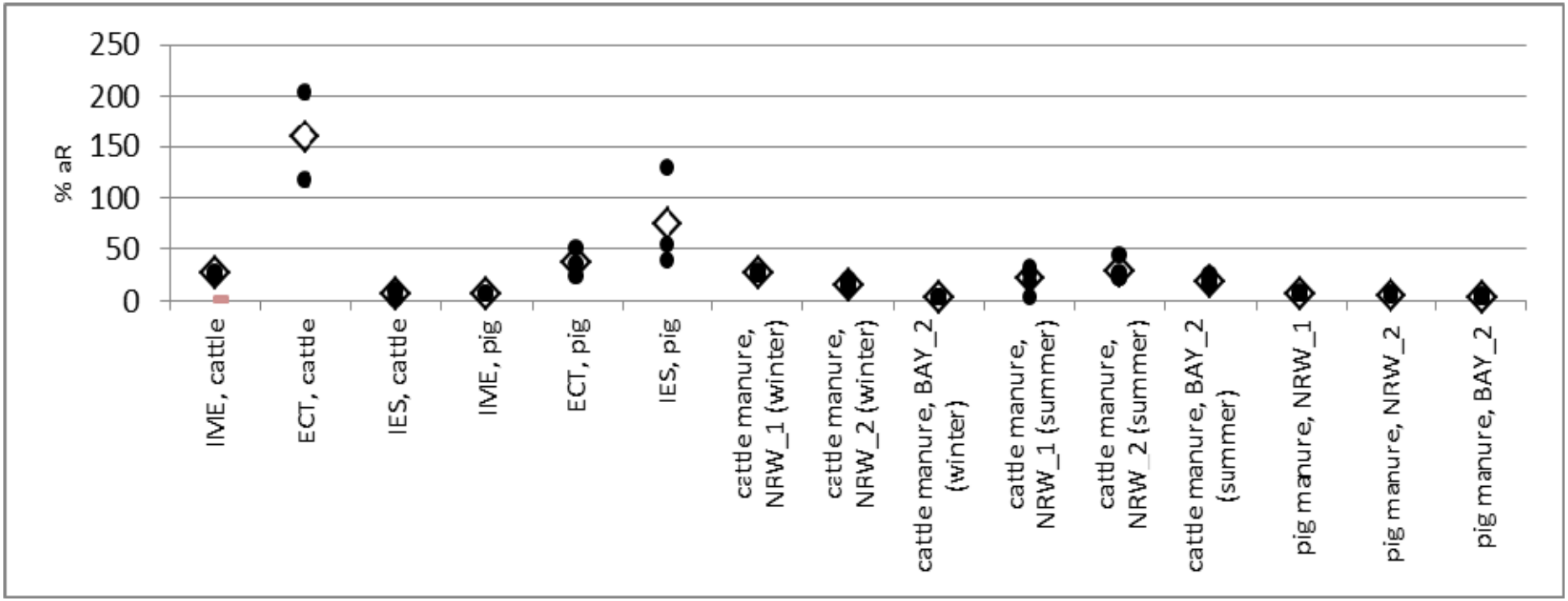

Figure 49 Means (open squares) and individual data (black dots) of DT_{50}-values of the transformation of ^{14}C-Salicylic acid in cattle and pig manure as obtained by the participants IME, ECT, and IES (above) and - for comparative purposes - DT_{50}-values as obtained by IME in the first part of the project.

5.1.3 Microbial activity

The results of the mineralization of ^{14}C-glucose in cattle and pig manure at the start and at the end of the pre-validation ring test experiments – as a measure of the microbial activity of the manure – are summarised in the Table 45. The glucose test to determine microbial activity of the manure at AAFC was erroneously run for 35 days instead of 7 days and therefore cannot be compared with the other results for ^{14}C-glucose. Furthermore, these mineralization rates as obtained from the test on biological activity using ^{14}C-glucose are compared with the mineralization rates for the test substance ^{14}C-Salicylic acid. The comparison is shown in the following figures for cattle and pig manure.

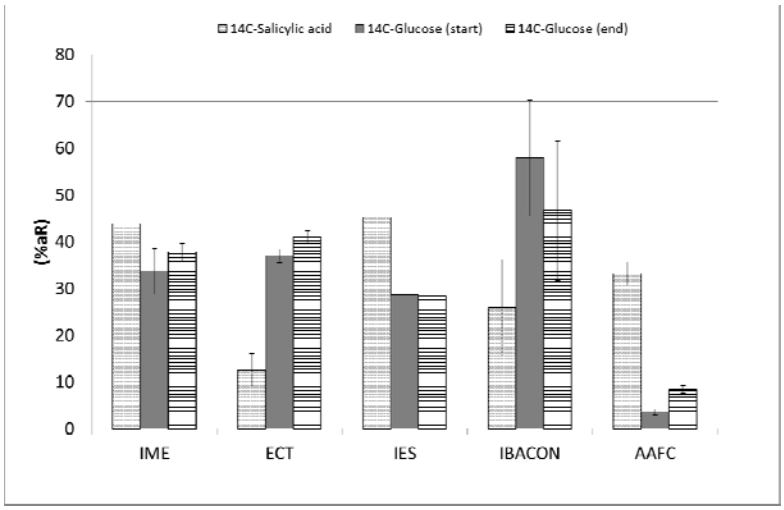

Figure 50: Mineralization rates as obtained from the test on biological activity using ^{14}C-glucose at the start of the transformation study and at the end of the transformation study for ^{14}C-Salicylic acid. Furthermore, the comparison with the mineralization rate for ^{14}C-Salicylic acid at the end of the transformation study is shown.

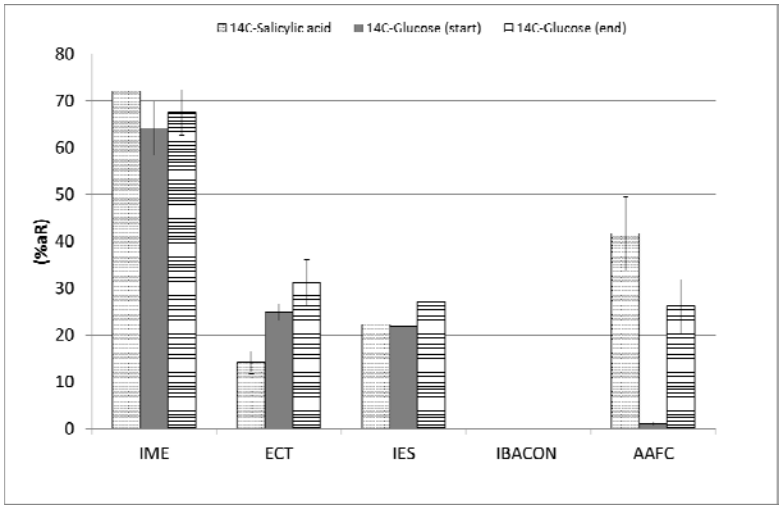

Figure 51: Mineralization rates as obtained from the test on biological activity using ^{14}C-glucose at the start of the transformation study and at the end of the transformation study for ^{14}C-Salicylic acid. Furthermore, the comparison with the mineralization rate for ^{14}C-Salicylic acid at the end of the transformation study is shown.

Table 45: Results of the mineralization of ^{14}C-glucose in cattle manure (mean values ± standard deviation)

	Cattle manure				pig manure		
	Mineralization [%AR]				Mineralization [%AR]		
	IME	ECT	IES	IBACON	IME	ECT	IES
Start	33.7 ± 4.8	37.0 ± 1.4	28.7	57.9 ± 12.4	64.1 ± 5.7	24.9 ± 1.8	21.8
End	37.8 ± 1.9	41.0 ± 1.3	28.4	46.6 ± 14.8	67.5 ± 4.8	31.2 ± 4.9	27.1

In a series of previous transformation studies using cattle and pig manure at IME, at least approximately 50% mineralization of ^{14}C-glucose were measured after 7 days, independent from the stage of the transformation study with a test substance (i.e. at the start or at the end of the transformation study).

However, 50% mineralization of ^{14}C-glucose was not reached by all ring test participants and the results do not correlate with the results for the mineralization of the test substance ^{14}C-salicylic acid.

Thus, the outcome of ^{14}C-glucose mineralization seems not to be predictive for the test results with the active ingredient and thus seems not to be suitable for measurements of biological activity of the manure. As an example, mineralization rates in cattle manure for ^{14}C-salicylic acid at IME (43.8 [% aR]) and at ECT (12.6 [% aR]) are quite different, whereas mineralization of ^{14}C-glucose is in the same range (IME: 33.7 – 37.8 [% aR]; ECT: 37.0 – 41.0 [% aR]). Further testing is required to check the suitability of the glucose test for measuring microbial activity of the manure. However, no other methods are available so far that are better suited. A simple measurement of gas production over time might be used alternatively as an indicator for microbial activity.

5.2 Outcome of the workshop

In addition to the discussion of the results of the pre-validation ring test, the present version of the draft test method as well as necessary changes or problems were discussed at the workshop. The main results are summarised in the following.

Incubation conditions

Flow-through as well as static test systems have to maintain anaerobic/methanogenic conditions. It was questioned whether the flow-through conditions are realistic, because tanks or lagoons are more or less static systems. Furthermore, problems may occur, if H_2 is removed from the system with the gas flow since hydrogen is then missing for other processes, e.g. formation of CH_4.

The option for using a static test system will be added to the description of the test method.

Dry matter content

Although the dry matter content might vary in the tank or lagoon due to different layers, the adjustment of the dry matter content to a uniform, representative value for testing purposes seems to be very important.

Extensive increase of the dry matter content by centrifugation was criticised because microorganisms are removed together with supernatant. Therefore, the minimum initial dry matter content will be added to the description of the test method (e.g. 8% for cattle manure and 3% for pig manure). Another option to increase the dry matter content could be to add the lowermost layer of settled manure.

Test item concentration /application

The test item concentration should always be mentioned when presenting the results.

Different test results might be obtained due to different application techniques (locally high test concentrations might be toxic to the microorganisms). Therefore, the application of the test item into the manure was simulated in a practical demonstration. Conclusion: comparable application techniques have been used by all participants of the pre-validation ringtest. Differences in the results cannot be explained by different application techniques.

Measurements and Analysis

Problems may occur with regard to chemical analysis if transformation products are detected at very low but continuously increasing concentrations during the study (e.g. 1% of applied activity (AR) continuously increasing up to 1.5%AR at the end of the study).

Beyond that, the required test item purity of ≥95% means that up to 5% impurities might be present. Therefore, it would not make sense to identify transformation products below 5%AR. Suggestion for modified wording: “Transformation products once detected at ≥5%AR for which concentrations are continuously increasing during the study afterwards should also be identified, even if their concentrations do not exceed the limit given above, as this may indicate persistence.”

Quality Criteria

The redox conditions throughout the study have to guarantee methanogenic conditions as is observed in a manure tank. Care has to be taken to fulfil these conditions. A high mass balance is important for the acceptability of the study. Nevertheless the criterion of 90-110% mass balance as specified in [OECD (2002a), OECD (2002b)] was not met by all participants and also seemed to be difficult to achieve in the other experiments.

5.3 Conclusions on the inter-laboratory comparison

Based on the results of the pre-validation ring test and the discussions at the workshop, the following conclusions can be drawn:

The ring test was a first attempt to do an inter-laboratory comparison for a simulation type study. Many of the laboratories did not have previous experience with manure. Therefore the primary intention was to get information on problematic steps and feed-back on the developed methodology. The results have to be interpreted with care and are listed for informative purposes, as some important quality criteria, as redox conditions or acceptable mass balances, were not met by all participants. Nevertheless, this exercise was very

fruitful for adapting the method further and describing important handling steps for the manure and the samples in more detail and informing further research activities, which is described below.

- Variability of the test results seems to be mainly caused by differences in the test design and test procedures at the different laboratories. For that reason, a more precise description of manure handling has been added to the protocol.
- A prolongation of the test duration (e.g. up to 90 days) is required and already considered for further studies.
- Adjusting the dry matter content seems to be a crucial point: Centrifugation does not only mean a removal of water but also of DOC and micro-organisms. Hence, a limit of minimum dry matter content was included in the draft test protocol which states: “If the dry matter content is below the recommended value, it can be concentrated by careful centrifugation (e.g. for 10 minutes at 740 x g). However, the initial dry matter content should not be below 8% (cattle) or 3% (pig). If dry matter content is too high, water (de-ionized water, bubbled with nitrogen for 30 min) should be added as needed“.
- In further studies the influence of different dry matter contents on the parameters mineralization, DT50, and NER will be examined.
- The outcome of ^{14}C-glucose mineralization as a measure of microbial activity of the manure seems not to be predictive for the test results with the active ingredient.
- No final conclusion could be drawn on the suitability of the test design (static/flow-through systems). There are concerns about a too fast stripping of H_2 and CO_2 in a flow-through system. A static system does rather represent the real conditions in a manure storage tank and can be handled more easily by laboratories. It is recommended to prevent air entering the system during removal of single replicates. This might be done by using valves or a special set-up design (replicates for one sampling time point in series instead of in parallel). In further tests static systems will be compared to the flow-through system.

6 Conclusions

- A homogeneity check of manure parameters determined for 10 replicates showed excellent homogeneity. Thus it can be concluded that it is feasible to use manures sampled directly from a tank for testing purposes and that the sampling methodology developed and applied in the project is suitable for this purpose.
- A comparison of ^{14}C-glucose mineralization for different storage temperatures of -20°C, +4 °C and +20 °C showed that differences in mineralization cannot be neglected. Therefore storage in the laboratory prior to use for a transformation study should be at test temperature.
- A comparison of ^{14}C-glucose mineralization for different storage periods after sampling of 28, 63 and 105 d showed that the storage period after sampling influences the mineralization. Therefore it is necessary to establish a maximum storage period to ensure comparable testing conditions. If sampling is not possible prior to the start of the study, a maximum storage period of 2 months in the laboratory prior to use for a transformation study is recommended.
- A comparison of ^{14}C-glucose mineralization for the tested acclimation periods of 3 days and 21 days showed that the length of the acclimation (or pre-incubation) period influences the mineralization. Therefore it is recommended to use an acclimation period of 21 days in order to ensure comparable testing conditions.
- The suggested and tested study design is applicable and can be handled for routine measurements of the transformation of veterinary medicines and biocides in manure.
- Standard deviations for six replicates are low. Thus, the obtained results per sampling point are reliable. It is necessary to test manure species specific. A transfer of results from pig to cattle manure or vice versa is not possible.
- Variability of the test results in the ring test seems to be mainly caused by differences in the test design and test procedures at the different laboratories. For that reason, a precise description of manure handling, especially for critical steps, is needed and the test protocol has been adapted with regard to further ring testing exercises.
- A prolongation of the test duration (e.g. up to 90 days) is required and already considered for further ring test in the framework of the follow-up project.
- Adjusting the dry matter content seems to be a crucial point: Centrifugation does not only mean a removal of water but also of DOM and microorganisms. If the dry matter content is below the recommended value of 10 ± 1% (cattle) and 5 ± 1% (pig), it can be concentrated by careful centrifugation (e.g. for 10 minutes at 740 x g). However, the initial dry matter content should not be below 8% (cattle) or 3% (pig). If this threshold is not reached, fresh manure should be collected. If dry matter content is too high, water (de-ionized water, bubbled with nitrogen for 30 min) should be added as needed. In further studies the influence of different dry matter contents on the parameters mineralization DT_{50} and NER will be examined.
- The outcome of ^{14}C-glucose mineralization as a measure of microbial activity of the manure seems not to be predictive for the test results with the active ingredient.
- No final conclusion could be drawn on the suitability of the test design (static/flow-through systems). There are concerns about a too fast stripping of H_2 and CO_2 in a flow-through system. A static system does rather represent the real conditions in a manure storage tank and can be handled more easily by laboratories. Maintaining anaerobic/methanogenic conditions is a prerequisite for an acceptable study. Therefore great care has to be taken to prevent air entering the system.

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