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Harmonization of environmental exposure assessment for veterinary pharmaceuticals and biocides:
Influence of different experimental set-ups on observed mineralization

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Harmonization of environmental exposure assessment for veterinary pharmaceuticals and biocides: Influence of different experimental set-ups on observed mineralization.

by

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Kurzbeschreibung

Die Ausbringung von Veterinärpharmaka und Bioziden mit Gülle auf landwirtschaftlich genutzte Flächen stellt einen sehr wichtigen Eintragspfad dieser Produktgruppen in die Umwelt dar. Aktuelle Bewertungsleitfäden (zum Beispiel: „Guideline on determining the fate of veterinary medicinal products in manure“ (EMA/CVMP/ERA/430327/2009) (EMA, 2011) sehen aus diesem Grund auch experimentelle Untersuchungen zur Transformation dieser Substanzen in Gülle vor. Allerdings beinhalten die Dokumente lediglich grundlegende regulatorische Vorgaben, eine experimentelle Prüfrichtlinie zur Durchführung von Studien zum Abbauverhalten von Veterinärpharmaka und Bioziden in Gülle liegt jedoch weder auf EU- noch auf OECD-Ebene vor. Um eine einheitliche Bewertung von Studien im Zulassungsverfahren zu gewährleisten wird jedoch ein harmonisiertes, international akzeptiertes und validiertes Testverfahren benötigt.

Vor diesem Hintergrund wurde im Rahmen des F+E-Vorhabens „Entwicklung einer Testvorschrift zum Abbauverhalten von Veterinärpharmaka und Bioziden in Gülle“ (FKZ 3710 67 422) (Hennecke et al., 2015) ein Entwurf für eine experimentelle Richtlinie erarbeitet. Die experimentelle Methode wurde durch die Auswertung von Intralaborvergleichen sowie eines internationalen Interlaborvergleichs (pre-validation Ringversuch) mittels geeigneter statistischer Verfahren überprüft und überarbeitet.

Aufbauend auf diesen Vorarbeiten wurde im Vorhaben ein internationaler Ringversuch mit einem Tierarzneimittel (Florfenicol) in Schweinegülle sowie einem Biozid (Imidacloprid) in Rindergülle durchgeführt und ausgewertet. Darüber hinaus wurden zwei internationale Workshops organisiert; zu Beginn des Vorhabens in Zusammenhang mit dem Vorgängervorhaben, sowie am Ende des Projektes zur Auswertung des internationalen Ringversuchs. Basierend auf den experimentellen Ergebnissen des Ringversuchs sowie den Diskussionen und Schlussfolgerungen der beiden Workshops wurde ein überarbeiteter Prüfrichtlinienentwurf erstellt.

Abstract

The spread of veterinary medicinal products (VMP) and biocides onto agriculturally used areas represents a very important path of entry into the environment for these product groups. For this reason, current guidance (e.g. „Guideline on determining the fate of veterinary medicinal products in manure“ (EMA/CVMP/ERA/430327/2009) (EMA, 2011) stipulates experimental studies on transformation of VMPs and biocides in manure. Though, the documents only contain basic regulatory requirements, whereas an experimental test guideline is still missing, both on EU and OECD level. To allow for a consistent assessment of studies within the registration process, a harmonized internationally accepted and validated test method is needed.

A draft test guideline was developed within a previous R&D-Project “Development of test guidance for transformation of veterinary pharmaceuticals and biocides in liquid manure” (FKZ 3710 67 422) (Hennecke et al., 2015). The experimental method was examined and revised by an intra-laboratory comparisons as well as an international inter-laboratory comparison (pre-validation ring test).

In the present project, an international ring test has been performed and evaluated testing a veterinary medicinal product (florfenicol) in pig manure and a biocide (imidacloprid) in cattle manure. Moreover, two international workshops were organized; one at the beginning in connection with proceeding project (FKZ 3710 67 422) and one at the end of the project to discuss and evaluate the ring test. Based on the experimental results of the ring test as well as discussions and conclusions of both workshops, a revised draft test guideline was prepared.

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List of abbrevations

ACN	Acetonitrile
aR	applied radioactivity
ASE	accelerated solvent extraction
Bq	Bequerel (radioactive decay per second)
CAFO	Concentrated Animal Feeding Operations
CAS	Chemical Abstracts Service
COV	coefficient of variation
d	day
dm	dry matter
DMS	dimethylsulfide
DMSO	dimethylsulfoxide
DT_{50}	time [d] needed for the disappearance of 50 % of the parent compound, disappearance time
$DT_{50\ MIN}$	time [d] needed for the 50 % mineralization, i.e. for formation of 50 % of $^{14}CO_2$ + $^{14}CH_4$
DT_{90}	time [d] needed for the disappearance of 90 % of the parent compound, disappearance time
EMA	European Medicines Agency
ER	extractable residues
ESI	electrospray Ionization
FKZ	Project No. (german: Forschungskennzahl)
FOCUS	Forum of the Co-ordination of pesticide fate models and their Use
FOMC	first order multi compartment kinetics
fw	fresh weight
HCOOH	Formic acid
HPLC	high performance liquid chromatography
IQR	interquartile range
ISO	International Organization for Standardization
K_{oc}	soil adsorption coefficient
K_{ow}	octanol-water partition coefficient
LC	liquid chromatography
LSC	liquid scintillation counting
Max	Maximum
Min	Minimum
MRM	Multiple reaction monitoring

MS	mass spectromety
MSE	mild solvent extract
NaOAc	Sodium acetate
NaOH	Sodium hydroxide
NER	non-extractable residues
NH_4-N	ammonia nitrogen
N_{tot}	total nitrogen
OECD	Organisation for Economic Co-operation and Development
OM	organic matter
PEC	Predicted Environmental Concentration
PET	polyethylenterephthalat (polyester)
PIS	product ion scan
PTFE	polytetrafluorethylene
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe
SAX	strong anion exchange
SD	standard deviation
SETAC	Society of Environmental Toxicology and Chemistry
SFO	single first order kinetics
SPE	solid phase extraction
SPSF	Standard Project Submission Form
TFA	trifluoroacetic acid
TLC	thin layer chromatography
TOC	total organic carbon
TP	transformation product
VDI	The Association of German Engineers (german: Verein Deutscher Ingenieure)
VFA	volatile fatty acid
VMP	veterinary medicinal products

Zusammenfassung

Hintergrund

Das Ausbringen von Gülle ist ein wichtiger Pfad, über den Veterinärpharmaka und Biozide in die Umwelt gelangen können. Aus diesem Grund berücksichtigen aktuelle Leitfäden (z. B. „Guideline on determining the fate of veterinary medicinal products in manure” (EMA, 2011)) die Transformation der genannten Produktgruppen in Gülle. Allerdings ist für diesen Prozess bisher kein standardisiertesTestprotokoll verfügbar. Die EMA-Richtlinie (EMA 2011) enthält grundlegende regulatorische Anforderungen. Um eine konsistente Durchführung und Auswertung von Transformationsstudien im regulatorischen Kontext zu ermöglichen, ist eine harmonisierte, international akzeptierte und validierte Testmethode notwendig. In einem vorangegangenen Forschungsvorhaben wurde in mehreren Stufen ein Testprotokoll entwickelt, das die Erfahrungen von Laboratorien mit ähnlichen Studientypen (Transformation von Chemikalien in Boden und in Wasser-/Sedimentsystemen) einbezog und das Testdesign an die spezifischen Anforderungen an die Matrix Gülle anpasste.

Die experimentelle Methode wurde entwickelt, indem zwei verschiedene Veterinärpharmaka und ein Biozid getestet wurden. Die eingesetzte Gülle wurde so ausgewählt, dass sie ein möglichst repräsentatives Set zur Transformations-Testung darstellte (verschiedener Ursprung, Größe und Typ der Höfe, verschiedenes Futter etc.). Bei dem ausgewählten experimentellen Desgin handelte es sich um ein Durchflusssystem. Damit konnten $^{14}CO_2$, $^{14}CH_4$, flüchtige ^{14}C-Fettsäuren, nicht extrahierbare (NER) und extrahierbare Rückstände (ER) quantifiziert warden (Massenbilanz). Die Ausgangssubstanz sowie mögliche Transformationsprodukte wurden mittels geeigneter analytischer, chromatographischer Methoden charakterisiert und quantifiziert. Darüber hinaus wurden unter Anwendung der Empfehlungen des „Forum for the Co-ordination of pesticide fate models and their Use (FOCUS)” DT_{50}- und DT_{90}-Werte ermittelt. Statistische Auswertungen zielten darauf ab, die Einflüsse von Lagerbedingungen (Temperatur, Lagerdauer im Gülletank) und Ursprung der Gülle auf die Transformation zu beschreiben.

Aus den betreffenden Ergebnissen geht hervor, dass in fast allen Fällen signifikante Unterschiede beobachtet werden. Das bedeutet, dass Gülle desselben Typs (Rinder- bzw. Schweinegülle), die an unterschiedlichen Orten gesammelt wurde, zu signifikant unterschiedlichen Ergebnissen hinsichtlich ER und NER-Bildung sowie DTx-Werten führt. Variationskoeffizienten für die DT50-Werte lagen im Bereich von 0.04 – 0.97 (Salicylsäure), 0.08 – 0.27 (Acetaminophen) und 0.06 – 0.18 (Biozid B).

Die vorgeschlagene Methode wurde in einem vorläufigen Testprotokoll beschrieben. Um die Anwendbarkeit der Methode in anderen Laboratorien sowie die Eindeutigkeit des vorgeschlagenen Testprotokolls zu überprüfen, wurde ein internationaler Inter-Labor-Vergleich durchgeführt, an dem fünf Institute (4 aus Europa, 1 aus Nordamerika) teilnahmen. Eine wesentliche Schlussfolgerung war (Hennecke et al. (2015)): “Es konnte keine abschließende Schlussfolgerung hinsichtlich der Eignung des Testdesigns (statisch / Durchfluss) gezogen werden. Es gibt dahingehend Bedenken, dass in einem Durchfluss-System H_2 und CO_2 zu schnell ausgetrieben. Ein statisches System würde die realen Bedingungen in einem Gülletank besser widerspiegeln, und es ist darüber hinaus auch leichter handhabbar. Von daher wird ein statisches System empfohlen, bei dem der Luftzutritt während der Probenahme aus zu schließen ist. Das kann entweder durch Ventile oder durch ein spezielles Design (Replikate für einen Probenahmezeitpunkt werden in Serie und nicht parallel angelegt) erzielt werden. In weiteren Tests sollten das Durchfluss-System und das statische System miteinander verglichen werden.”

Zielsetzung

Es war Ziel des Projektes, das vorgeschlagene Testprotokoll zur Durchführung von Transformationsstudien in Rinder- und Schweinegülle weiter zu entwickeln. Insbesondere wurde der Einfluss des Testdesigns (schwerpunktmäßig: semistatisch und Durchfluss) auf die Mineralisierungsrate untersucht.

Einfluss verschiedener Testdesigns auf die Mineralisierung

Der Einfluss des Testdesigns auf die Mineralisierungsrate – ausgedrückt als Bildung von CO2 und CH4 – wurde untersucht, um die folgenden Detailfragen abzuklären:

- Beeinflusst die Nutzung eines Durchfluss-Systems (d.h. kontinuierliche Überleitung von Stickstoff über die Gülle-Proben) bzw. eines semi-statischen Systems (d.h. periodisches Überleiten von Stickstoff) die Bildungsrate von CO_2 und CH_4 sowie das CH_4/CO_2-Verhältnis?
- Beeinflusst die Fluss-Rate des Stickstoffs die die Bildungsrate von CO_2 und CH_4 sowie das CH_4/CO_2-Verhältnis?
- Beeinflusst die Menge der inkubierten Gülle die Mineralisierungsrate?
- Beeinflusst der Trockengewichtsanteil der Gülle die Mineralisierungsrate?

Aus den Ergebnissen der Tests, die mit ^{14}C-Salicylsäure als abbaubarer Substanz durchgeführt wurden, konnten die Schlussfolgerungen gezogen werden:

- Unterschiede in der Fluss-Rate des Stickstoffs im Durchfluss-System haben keinen Einfluss auf die Mineralisierungsraten von ^{14}C-Salicylsäure in Rinder- und Schweinegülle.
- Die Menge an inkubierter Gülle (50 g bzw. 300 g) beeinflusst die Mineralisierungsrate signifikant. Diese Aussage gilt für Rinder- und Schweinegülle.
- Der Trockengewichtsanteil hat in Schweinegülle signifikanten Einfluss auf die Mineralisierungsrate, nicht jedoch in Rindergülle.
- Das experimentelle Set-up (Durchfluss-System bzw. semi-statisch) hat in Rindergülle signifikanten Einfluss auf die Mineralisierungsrate, nicht jedoch in Schweinegülle.
- Grundsätzlich ist die Mineralisierungsrate in Schweinegülle etwas höher im Vergleich zur Rindergülle.

Summary

Background

Spreading of manure constitutes an important pathway by which veterinary medicinal products (VMP) and biocides enter the environment. For this reason, current guidance (e.g. „Guideline on determining the fate of veterinary medicinal products in manure" (EMA, 2011) takes transformation of VMPs and biocides in manure into account. However, currently, there is no standardized experimental test protocol available to examine the transformation of veterinary medicinal products (VMP) and biocides in liquid manure. The EMA guideline on transformation in manure (EMA, 2011) contains basic regulatory requirements. To allow for a consistent assessment of studies within regulatory frameworks, a harmonized internationally accepted and validated test method is needed In a previous research project a test protocol was developed in multiple steps taking into account experiences from labs performing simulation type studies like transformation of chemicals in soil and in water-/sediment systems and adapting their test design to the specific requirements of the matrix manure.

The experimental method was developed by testing three different VMP and biocide active ingredients. Manure to be used was chosen to represent a most diverse set for transformation testing purposes (different origins, different size/type of farms, different feed of the animals, etc.). The selected set-up was a flow-through apparatus which is a gas tight system of incubation vessels and traps set in sequence. The set-up enabled quantification of $^{14}CO_2$, $^{14}CH_4$, ^{14}C volatile fatty acids (VFA), and of extractable and non-extractable residues. Thus, complete mass balances were established which can be seen as a quality criterion for this type of study. The parent and possible transformation products were characterized and quantified by appropriate analytical, chromatographic methods. Compounds were further analyzed based on the recommendation of the Forum for the Co-ordination of pesticide fate models and their Use (FOCUS) degradation kinetics to give DT_{50}- and DT_{90}-values. 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. Differences of the endpoints (e. g. DT50-values) 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.

From the results obtained by comprehensive statistical analyses 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. COV observed for DT50 values ranged from 0.04 – 0.97 for salicylic acid, 0.08 – 0.27 for acetaminophen, and 0.06 – 0.18 for Biocide B.

The suggested test method was laid down in a draft test protocol.

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. Five institutes (4 from Europe, 1 from Northern America) registered for the ring-test. The same test substance (salicylic acid) was used. One of the summarizing remarks was (Hennecke et al. (2015)): "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."

Objective

The aim of the project was to further develop a draft test protocol for an experimental method to study the transformation of veterinary pharmaceuticals and biocides in cattle and pig liquid manure. Here specifically, the influence of different test-setups (semi-static and flow-through) were compared regarding influences on observed mineralization.

Influence of different test set-ups on mineralization

The influence of the test design on the mineralization rate – expressed as CO_2- and CH_4-formation was examined:

- Does the use of a flow-through system (that means, continuously passing nitrogen over the manure samples) versus the use of a semi-static system (that means passing nitrogen over the manure samples intermittently) influence the formation rate of CO_2 and CH_4 as well as the CH_4/CO_2-ratio?
- Does the flow rate of nitrogen influence the formation rate of CO_2 and CH_4, and of CH_4/CO_2-ratio?
- Does the amount of incubated manure have an influence on the mineralization rate?
- Does the dry matter content of the manure have an influence on the mineralization rate?

From the experiments using ^{14}C-Salicylic acid as degradable test compound it can be concluded:

- Differences in nitrogen flow rate for the flow-through system do not have an influence on the mineralization rate of ^{14}C-Salicylic acid in cattle and pig manure.
- The amount of incubated manure (50 g vs. 300 g) significantly influences the rate of mineralization. This observation is valid for both, cattle and pig manure.
- The dry matter content does not significantly influence mineralization rates in cattle manure but does in pig manure.
- Differences in the experimental setup with respect to a flow-through and semi-static design have an influence on the mineralization rate of ^{14}C-Salicylic acid in cattle manure. This was not observed in pig manure.
- In general, mineralization is slightly higher in pig manure than in cattle manure.

1 Introduction

Spreading of manure constitutes an important pathway by which veterinary medicinal products (VMP) and biocides enter the environment. For this reason, current guidance (e.g. „Guideline on determining the fate of veterinary medicinal products in manure“ (EMA, 2011) take transformation of VMPs and biocides in manure into account. However, currently, there is no standardized experimental test protocol available to examine the transformation of veterinary medicinal products (VMP) and biocides in liquid manure. The EMA guideline on transformation in manure (EMA, 2011) contains basic regulatory requirements. To allow for a consistent assessment of studies within regulatory frameworks, a harmonized internationally accepted and validated test method is needed In a previous research project a test protocol was developed in multiple steps taking into account experiences from labs performing simulation type studies like transformation of chemicals in soil and in water-/sediment systems and adapting their test design to the specific requirements of the matrix manure.

The experimental method was developed by testing three different VMP and biocide active ingredients.

2 Influence of different test set-ups on mineralization

2.1 Background

The influence of the test design on mineralization – expressed as CO_2- and CH_4-formation were studied. The following questions were addressed:

- Does the use of a flow-through system (that means, continuously passing nitrogen over the manure samples) versus the use of a semi-static system (that means passing nitrogen over the manure samples intermittently) influence the formation rate of CO_2 and CH_4 as well as the CH_4/CO_2-ratio?
- Does the flow rate of nitrogen influence the formation rate of CO_2 and CH_4, and of CH_4/CO_2-ratio?
- Does the amount of incubated manure have an influence on the mineralization rate?
- Does the dry matter content of the manure have an influence on the mineralization rate?

2.2 Material and Methods

2.2.1 General concept

Data on the formation of CO_2 and CH_4 were collected at 10 sampling points (pig manure) and 12 sampling points (cattle manure), respectively. As a radioactively labelled test compound was used, $^{14}CO_2$ and $^{14}CH_4$ were quantifiable. From the collected data on CO_2 and CH_4 formation, half-life times of mineralization rates ($DT_{50\,MIN}$) were calculated by means of KinGUI software. Beside these endpoints, the mass balance (recovery) at the end of the transformation study was determined and served as a quality check.

The following table gives an overview on the tested variations, on groups to be compared and sampling points for $^{14}CO_2$ and $^{14}CH_4$ in cattle and pig manure.

^{14}C-Salicylic acid has been used as test substance for all variations tested.

Table 1: Overview on the study design

Tested variations	Group for comparison	Sampling points [d] for $^{14}CO_2$ and $^{14}CH_4$-quantification		Test substance
		Cattle manure	Pig manure	
flow rate	slow flow (50 - 200 mL/min) versus fast flow (› 200 mL/min)	3, 7, 14, 29, 35, 42, 49, 56, 63, 70, 79, 91	3, 7, 14, 21, 29, 35, 42, 49, 56, 63	^{14}C-salicylic acid
amount of manure	50 g manure versus 300 g manure			
dry matter content (cattle)	10 % dm versus 1 % dm			
dry matter content (pig)	5 % dm versus 1 % dm			
Flow through and semi-static	slow flow (50 - 200 mL/min) versus semi-static (purged once a week).			

2.2.2 Manure collection and characterization

Manure collection

Prior to collection, the liquid manure was homogenized by mixing in the respective manure storage tank. For mixing, external devices were used. Mixing for one hour proved sufficient for homogenization of manure in the tanks independent from tank volume. Mixing was done by the farmers themselves, not by the staff of Fraunhofer IME.

Liquid pig manure and cattle manure was handled differently: Pig manure was stirred immediately before sampling as separation into liquid and solid phase easily occurs. This procedure 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 a ladle with a large beaker. 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. On-site, also the temperature, pH-value and redox-potential were measured. After filling, the 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.

Manure characterization

In the laboratory, the following parameters were determined: 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 the transformation study. pH value, redox potential and temperature were measured 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). 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, 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, 2001a).

Loss of ignition / organic dry mass 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, 2001b). The dry matter content is used to re-calculate the data to % fresh weight (fw).

Total carbon (TC) content

Total carbon content was analyzed using a CHN-analyser (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, 2001c). The dry matter content is used to re-calculate the data to % fresh weight (fw).

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, 1997). The dry matter content is used to re-calculate the data to % fresh weight (fw).

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, 1984). The dry matter content is used to re-calculate the data to % fresh weight (fw).

Redox potential

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

Microbial activity

The microbial activity of manure 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, but with the addition of ^{14}C-glucose instead of the test substance. 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.

2.2.3 Manure acclimation

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 1.3.1. 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, 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.

After the adjustment of the dry matter content, cattle manure was homogenized by gently mixing using a glass bar. Subsamples of 50 or 300 g (wet weight) each were directly filled into the incubation vessels which are used for the acclimation and transformation study. No additional measures to prevent introduction of oxygen were used during both processes of homogenization and filling of incubation vessels.

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 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. Thereafter, the dry matter content was adjusted. After a repeated homogenization under anaerobic conditions by thoroughly mixing (set up as above) subsamples of 50 or 300 g (fresh weight) were filled into the incubation vessels. Then the incubation apparatus (for details see chapter 2.2.6) 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.

2.2.4 Test substances

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.: ARC 0287

Glucose (for testing the 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: 9.4 GBq/mmol = 52 MBq/mg
Lot no.: 178517 MC144

2.2.5 Test substance application

The test substances were dissolved in an appropriate solvent (see below for details) 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 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.

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.

Glucose

An appropriate stock solution of ^{14}C-glucose in water was added to the manure subsamples in the respective incubation vessels. Approximately 940 µL solution containing 0.9 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.

2.2.6 Incubation system

A schematic presentation of the incubation system which has been used is shown in Figure 1. The incubation system was either run as a flow-through system as shown in Figure 1 or as a (semi-)static system.

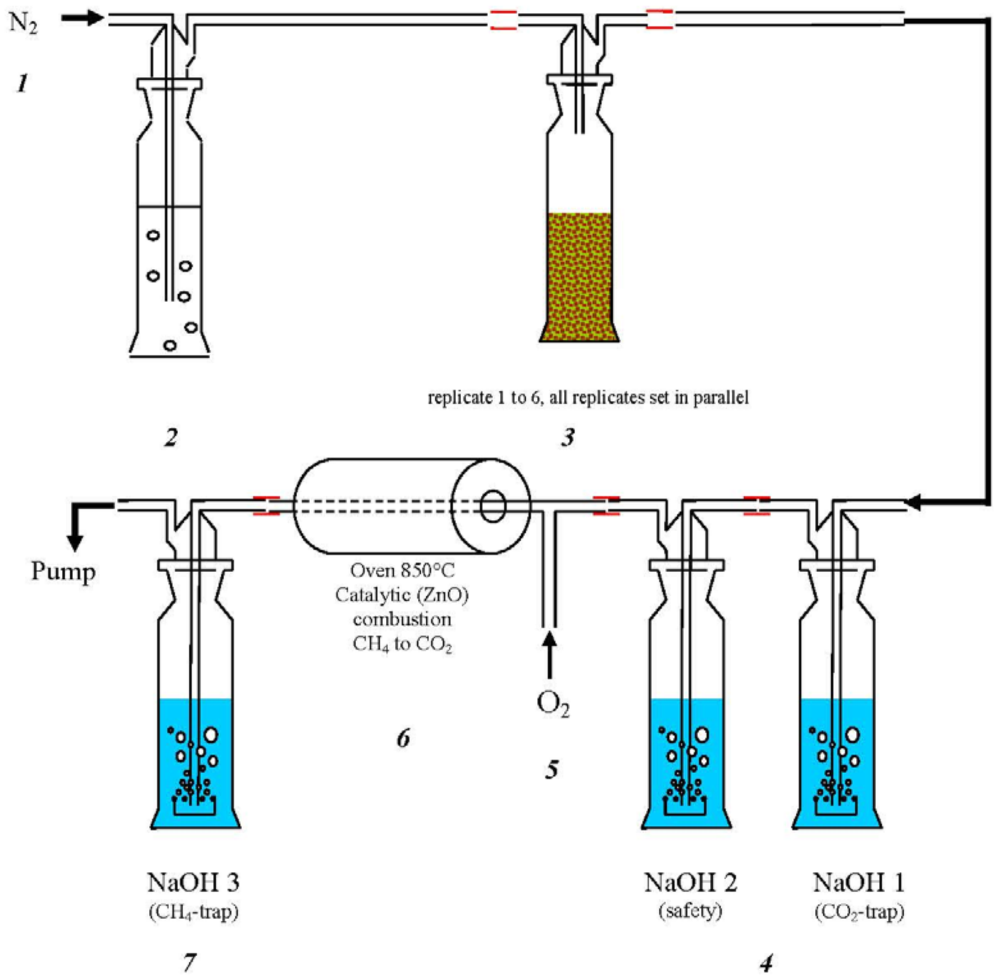
Flow-through system

In the flow-through system humidified nitrogen was gently passed over the liquid manure subsamples. The flow rate was either 50 – 200 mL/min (for the “slow flow” conditions) or > 200 mL/min (for the “fast flow” conditions). At the gas inlet nitrogen was 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.

Semi-static system

In the semi-static system no nitrogen was passed over the incubation flasks. After application of the test substance, nitrogen was passed over the sample for 0.5 hour to maintain the anaerobic conditions. Thereafter, the system was closed by valves at the gas inlet and prior to the first NaOH-filled trap. Ground glass connections were sealed airtight by applying grease. 6 replicates each were set up for all experiments. Each replicate was connected to NaOH-filled traps to trap evolved $^{14}CO_2$. 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. Mineralization was individually measured for each of the incubated replicates. Though in case of intensive $^{14}CO_2$-formation in (semi)static systems purging for 15 minutes might not be sufficient for complete $^{14}CO_2$ quantification, samples were purged for 15 minutes once a week and prior to final sampling, respectively. Based on the information on mineralization rates of ^{14}C-Salicylic acid in cattle and pig manure gained in the project Hennecke et al. (2015) such a weekly 15 minutes purging was supposed to be sufficient for a complete $^{14}CO_2$ and $^{14}CH_4$ quantification.

Figure 1: Flow-through apparatus used in the project



1: nitrogen is gently passed over the manure samples
2: gas washing bottle containing water
3: manure transformation flasks filled with 50 g or 300 g manure (fresh weight)
4: for anaerobic transformation two NaOH-filled traps in sequence are needed to trap evolving CO_2
5: addition of oxygen for subsequent catalytic combustion of CH_4
6: oven for combustion of CH_4 to form CO_2
7: NaOH-filled trap for CO_2 formed from CH_4

2.2.7 Quantification of evolved $^{14}CO_2$ and $^{14}CH_4$

Flow-through system

In the flow-through system, humidified nitrogen was passed over the manure replicates. By doing so, evolved $^{14}CO_2$ is purged from the manure samples, transported and captured in the NaOH-filled traps 1 and 2 (see Figure 1) containing 2 M NaOH. Potentially formed $^{14}CH_4$ passes the NaOH-filled traps. After the addition of oxygen $^{14}CH_4$ 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.

Quantification of trapped $^{14}CO_2$and $^{14}CH_4$ was by radio-counting (liquid scintillation counting, LSC) of aliquots of the trapping solutions. NaOH-filled traps 1, 2, and 3 (see Figure 1) were removed at the particular sampling point; the removed traps were replaced by freshly filled ones.

To verify that the radioactivity captured in the NaOH-filled 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-filled traps 1 and 2. Thereafter, 20 mL 0.25 M $BaCl_2$ (barium chloride) was added to 10 mL aliquots of NaOH trapping solution from traps 1 and 2 each. Precipitation of $Ba^{14}CO_2$ (barium carbonate) 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 $^{14}CO_2$.

Semi-static system

At each sampling point, humidified nitrogen was passed over the manure replicates for 15 minutes (for reasoning for 15 minutes purging see 2.2.6) at a flow-through of ca 150 mL/min to purge evolved CO_2 and CH_4. Further handling and quantification was identical as described for the flow-through system. Samplings were at 3 d, 7 d, and in 7 d intervals, respectively.

2.2.8 Establishment of a mass balance at end of the study

Salicylic acid

At the end of the study, the incubation vessels containing the manure samples were removed from the incubation system and cleaned-up directly after sampling. 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 quantified by LSC. 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.

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

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

Glucose

No clean-up and establishment of a mass balance was performed. The evaluation was exclusively based on the quantification of formed $^{14}CO_2$ and $^{14}CH_4$ for the determination of microbial activity.

2.2.9 Graphical presentation and statistical evaluation of data

Graphical presentation

Results on the amount of CO_2 and CH_4 as well as on the CH_4/CO_2-ratio at each single sampling point is presented graphically for each of the conditions tested. Graphs present the measured data for all six replicates as well as their mean values. Values for the measured data are given as dots; values for the means are given as open squares.

Statistical evaluation

In order to determine the significance of influences of test conditions on the formation of $^{14}CO_2$ and $^{14}CH_4$, data were subjected to a statistical evaluation. Statistical evaluation was done on $DT_{50\ MIN}$-values, i.e. on values for the time needed for formation of half of $^{14}CO_2$ plus $^{14}CH_4$. $DT_{50\ MIN}$-values were ln-transformed for calculation of means, standard deviations and the test on homogeneity of variances.

For $DT_{50\ MIN}$-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 medians, means, standard deviations and coefficients of variation are based on original data and calculated by means of Microsoft Excel 2010.

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 $DT_{50\ MIN}$ 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).

2.3 Results and discussion

2.3.1 Manure characterization

On site and after transfer to the laboratory, respectively, the manure parameters pH-value, dry matter content, organic matter content, organic carbon content, nitrogen content (total nitrogen and ammonium nitrogen), redox potential, temperature, and microbial activity were measured. Results are given in the table below.

Table 2: Manure characterisation

Off-site (laboratory)							On-site (farm)		
Dry matter content [%fw[1]]	Organic matter content [% fw[1]]	NH_4-N [mg/kg fw[1]]	Total nitrogen (Kjeldahl) [mg/kg fw[1]]	Organic carbon content [% fw[1]]	Biological activity [% mineralization of ^{14}C-glucose]		pH	Redox [mV]	T [°C]
					Start of transformation study	End of transformation study			
cattle manure; sampled December 5, 2012; site: southern part of North-Rhine-Westphalia									
9.9	7.15	2747	5062	4.37	74.8	61.3	7.2	-391	4.3
cattle manure; sampled September 15, 2014; site: southern part of North-Rhine-Westphalia (for comparison flow-through – semi-static)									
12.8	8.45	3005	5167	5.02	91.5	75.4	6.9	-323	7.8
pig manure; sampled March 12, 2013; site: southern part of North-Rhine-Westphalia									
5.4	3.73	3913	5231	2.76	83.5	68.9	7.5	-449	9.5
pig manure; sampled September 15, 2014; site: southern part of North-Rhine-Westphalia (for comparison flow-through – semi-static)									
4.7	3.16	3558	4991	2.23	88.6	67.8	7.1	-401	6.7

[1] fw = fresh weight

2.3.2 Mineralization rates of ^{14}C-Salicylic acid in cattle and pig manure using various experimental setups

Any evaluation of measured data mainly was based on the mineralization half-lives of ^{14}C-Salicylic acid under the various experimental conditions (cf. Annex 1 for raw data). Mineralization half-lives were expressed as $DT_{50\ MIN}$-values, i.e. on values for the time needed for the formation of half of maximum achievable $^{14}CO_2$ plus $^{14}CH_4$. The following table shows $DT_{50\ MIN}$ -values for all replicates and test conditions.

Table 3: $DT_{50\ MIN}$ -values for ^{14}C-Salicylic acid degraded in cattle and pig manure under various experimental setups. Results for six replicates are presented

Tested variations	$DT_{50\ MIN}$ values (d) for ^{14}C-Salicylic acid			
	Cattle manure		Pig manure	
flow rate	**slow flow I)**	**fast flow I)**	**slow flow II)**	**fast flow II)**
	45.0	51.3	14.3	13.4
	50.0	43.8	11.4	14.6
	56.0	49.7	21.3	13.7
	44.0	54.4	18.6	15.9
	44.0	50.6	11.5	13.6
	55.1	38.3	14.8	19.3
amount of manure	**50 g manure I)**	**300 g manure I)**	**50 g manure II)**	**300 g manure II)**
	45.0	60.7	14.3	31.2
	50.0	62.5	11.4	21.4
	56.0	66.9	21.3	22.6
	44.0	62.3	18.6	24.9
	44.0	62.0	11.5	33.3
	55.1	66.8	14.8	28.0
dry matter content	**10 % dm I)**	**1 % dm I)**	**5 % dm II)**	**1 % dm II)**
	45.0	47.7	14.3	18.8
	50.0	52.3	11.4	20.2
	56.0	46.9	21.3	22.6
	44.0	57.3	18.6	19.1
	44.0	37.5	11.5	21.4
	55.1	37.5	14.8	22.2
Test design	**Flow-through III)**	**Semi-static III)**	**Flow-through IV)**	**Semi-static IV)**
	28.4	40.0	15.3	20.8
	25.2	43.3	13.3	21.2
	31.0	51.3	23.8	25.1
	38.9	37.2	24.9	27.1
	33.1	39.1	16.1	22.8
	36.3	37.8	20.5	24.4

Table 3: $DT_{50\ MIN}$ -values for ^{14}C-Salicylic acid degraded in cattle and pig manure under various experimental setups. Results for six replicates are presented (continued)

Key to superscripts

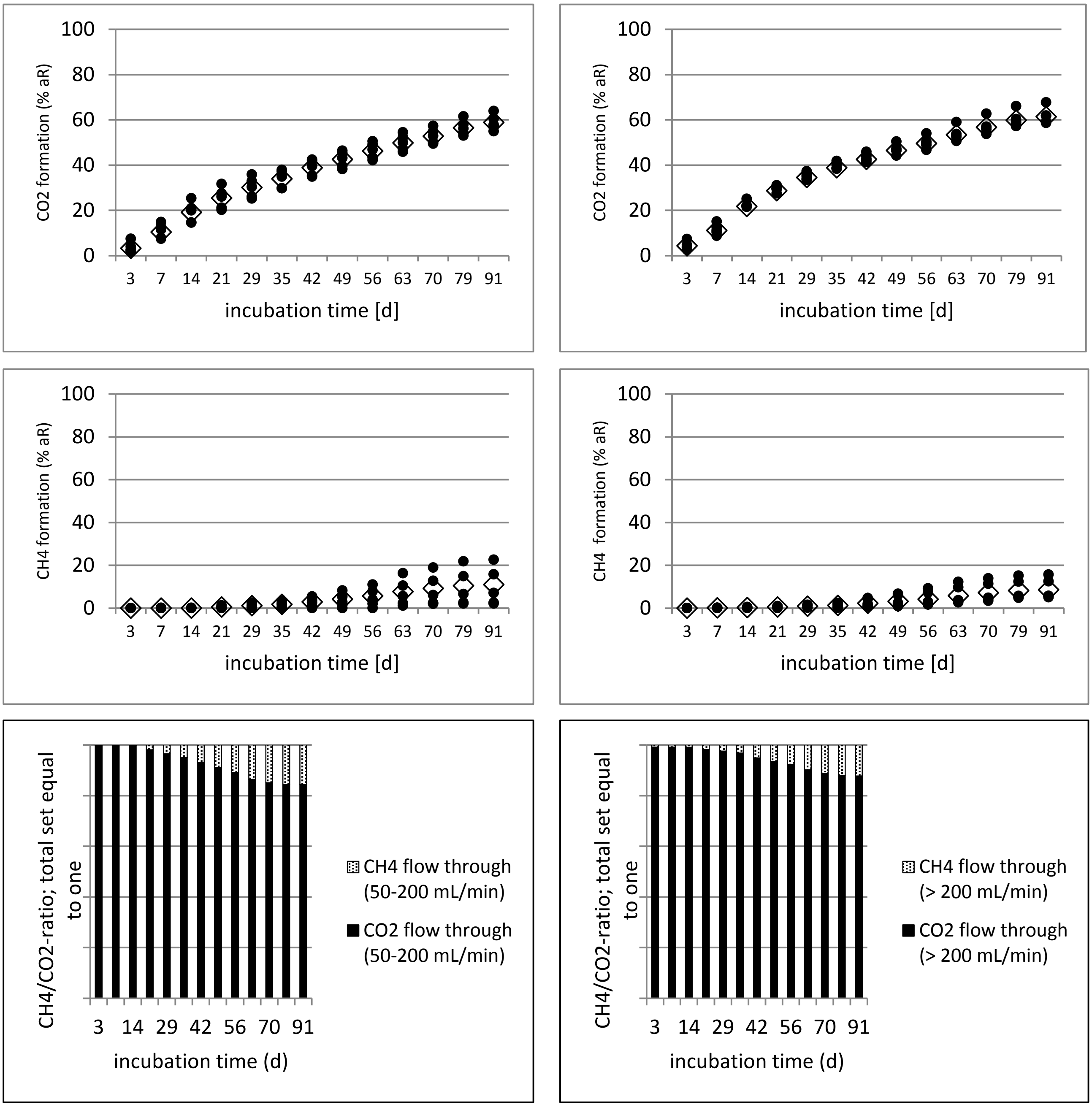
I) cattle manure sampled December 5, 2012; site: southern part of North-Rhine-Westphalia
II) pig manure sampled March 12, 2013; site: southern part of North-Rhine-Westphalia
III) cattle manure; sampled September 15, 2014; site: southern part of North-Rhine-Westphalia (for comparison flow-through – semi-static only)
IV) pig manure sampled September 15, 2014; site: southern part of North-Rhine-Westphalia (for comparison flow-through – semi-static only)

As can be seen from the superscripts, values for the comparison of flow-through and semi-static test conditions are obtained from other test series and batches of manure, respectively. Manure used for these comparisons were of the same origin (same manure tank) but sampled at another sampling time (cf Table 2). Repetition of sampling was supposed to be necessary in order to allow for a valid comparison of these experiments, which were started 21 months (cattle manure) and 18 months (pig manure) later.

All individual data on formed $^{14}CO_2$- and $^{14}CH_4$ as well as on the CH_4/CO_2-ratio and results of the statistical evaluation are also presented in the following order:

1. Graphical presentation of individual data for 6 replicates and mean values
2. Statistical evaluation comprising descriptive statistics, Levene´s test, U-test for $DT_{50\ MIN}$ values.
3. Summarizing overview on the significance of differences in medians for the pairwise comparisons.

Figure 2: Formation of $^{14}CO_2$ [% aR] (upper lane) and $^{14}CH_4$ [% aR] (middle lane) by transformation of ^{14}C-Salicylic acid in cattle manure (10 % dm, 50 g manure per replicate). Figures in lower lane show the ratio of $^{14}CO_2$ and $^{14}CH_4$.
Results for <u>slow flow</u> (constant stream of nitrogen of 50-200 mL/min) are shown on the <u>left</u> and for the <u>fast flow (> 200 mL/min)</u> on the <u>right</u>. Results for 6 replicates per sampling point (dots) and mean values (open squares) are given.

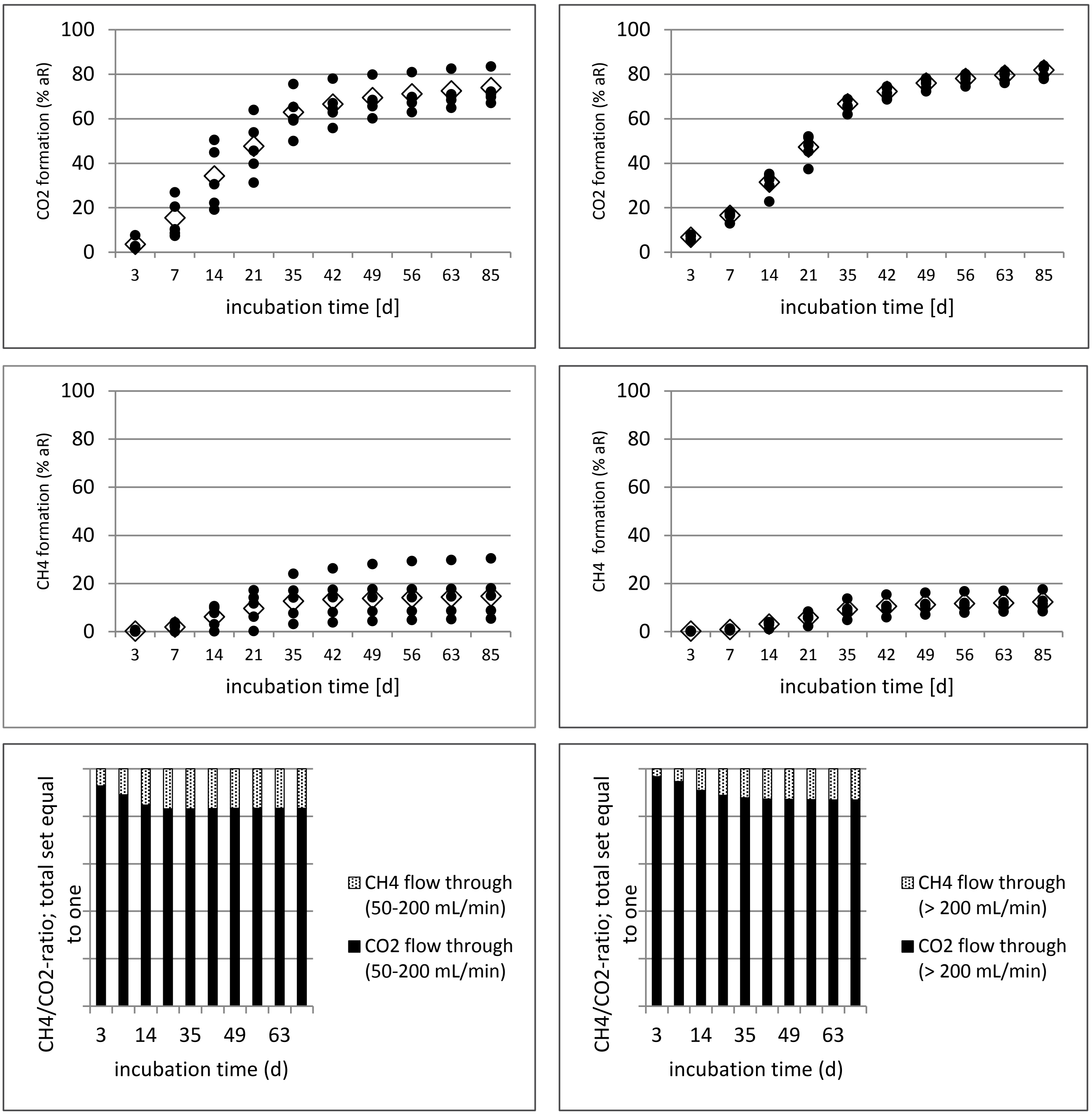


<u>Cattle manure</u>

slow flow
Mass balance at end of study: 91.6 [% aR]

fast flow
Mass balance at end of study: 89.0 [% aR

Figure 3: Formation of $^{14}CO_2$ [% aR] (upper lane) and $^{14}CH_4$ [% aR] (middle lane) by transformation of ^{14}C-Salicylic acid in pig manure (5 % dm, 50 g manure per replicate). Figures in lower lane show the ratio of $^{14}CO_2$ and $^{14}CH_4$.
Results for <u>slow flow</u> (constant stream of nitrogen of 50-200 mL/min) are shown on the <u>left</u> and for the <u>fast flow (> 200 mL/min)</u> on the <u>right</u>. Results for 6 replicates per sampling point (dots) and mean values (open squares) are given.



<u>pig manure</u>

slow flow

mass balance at end of study: 98.2 [% aR]

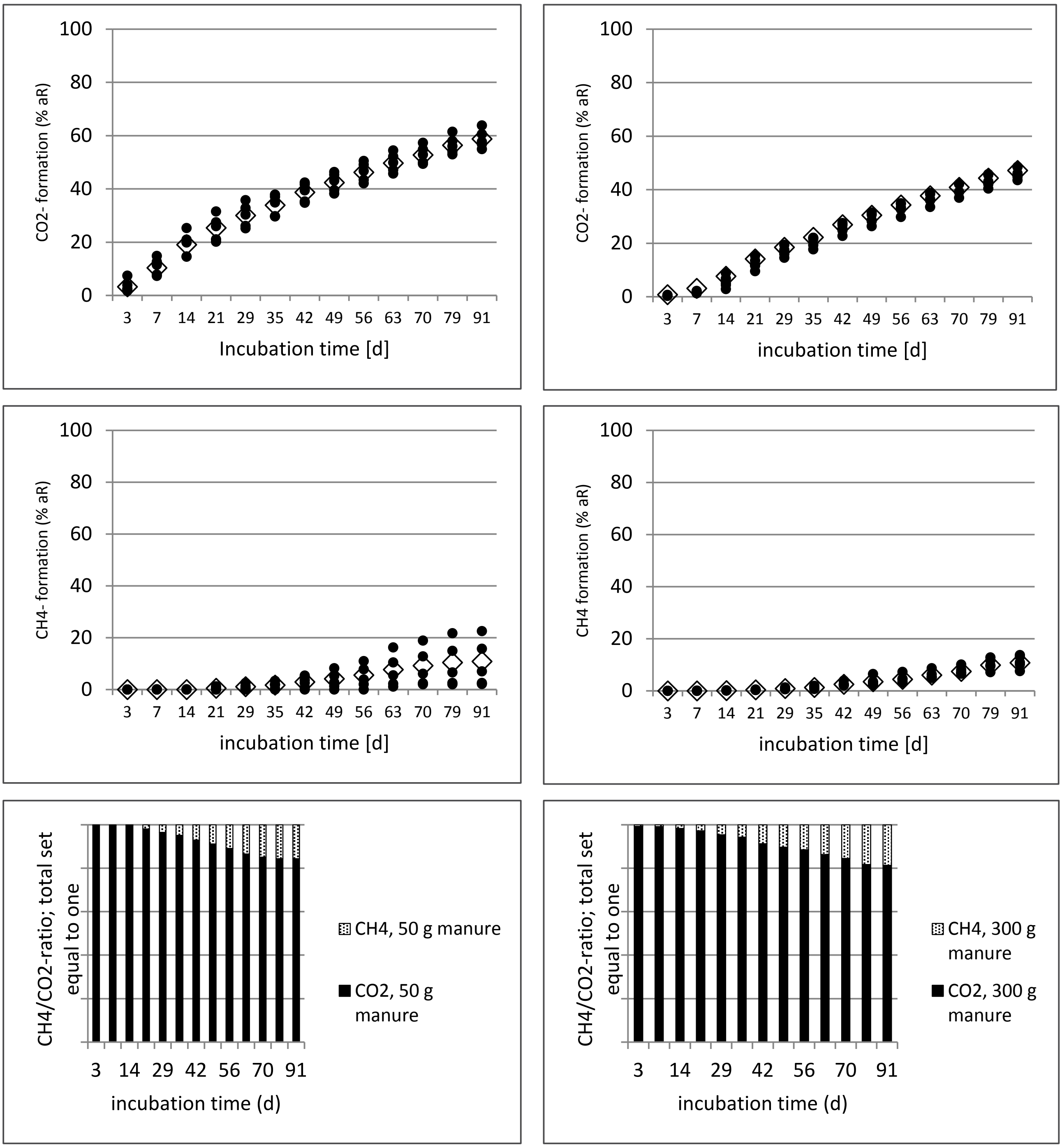
fast flow

mass balance at end of study: 105.0 [% aR]

Table 4: Statistical evaluation for $DT_{50\ MIN}$ (d) for the conditions slow flow (50 – 200 mL/min) and fast flow (› 200 mL/min)

	Cattle		Pig	
	Slow flow	**Fast flow**	**Slow flow**	**Fast flow**
Descriptive statistics				
n	6	6	6	6
Standard deviation	0.112	0.129	0.252	0.140
Mean (ln-transformed)	3.9	3.9	2.7	2.7
Mean (re-transformed)	48.8	47.7	14.9	15.0
Median	47.5	50.2	14.6	14.2
COV (%)	0.11	0.13	0.26	0.14
Levene´s test on homogeneity of variances				
p-value	0.784		0.291	
Variances are	equal		equal	
Mann- Whitney U-test on differences in means				
p-value	0.630		1.000	
Medians are	equal		equal	

Figure 4: Formation of $^{14}CO_2$ [% aR] (upper lane) and $^{14}CH_4$ [% aR] (middle lane) by transformation of ^{14}C-Salicylic acid in cattle manure (10 % dm). Figures in lower lane show the ratio of $^{14}CO_2$ and $^{14}CH_4$.
Results for <u>50 g manure</u> (constant stream of nitrogen of 50-200 mL/min) are shown on the <u>left</u> site and for <u>300 g manure</u> (constant stream of nitrogen of 50-200 mL/min) on the <u>right</u> site. Results for 6 replicates per sampling point (dots) and mean values (open squares) are given.



<u>cattle manure</u>

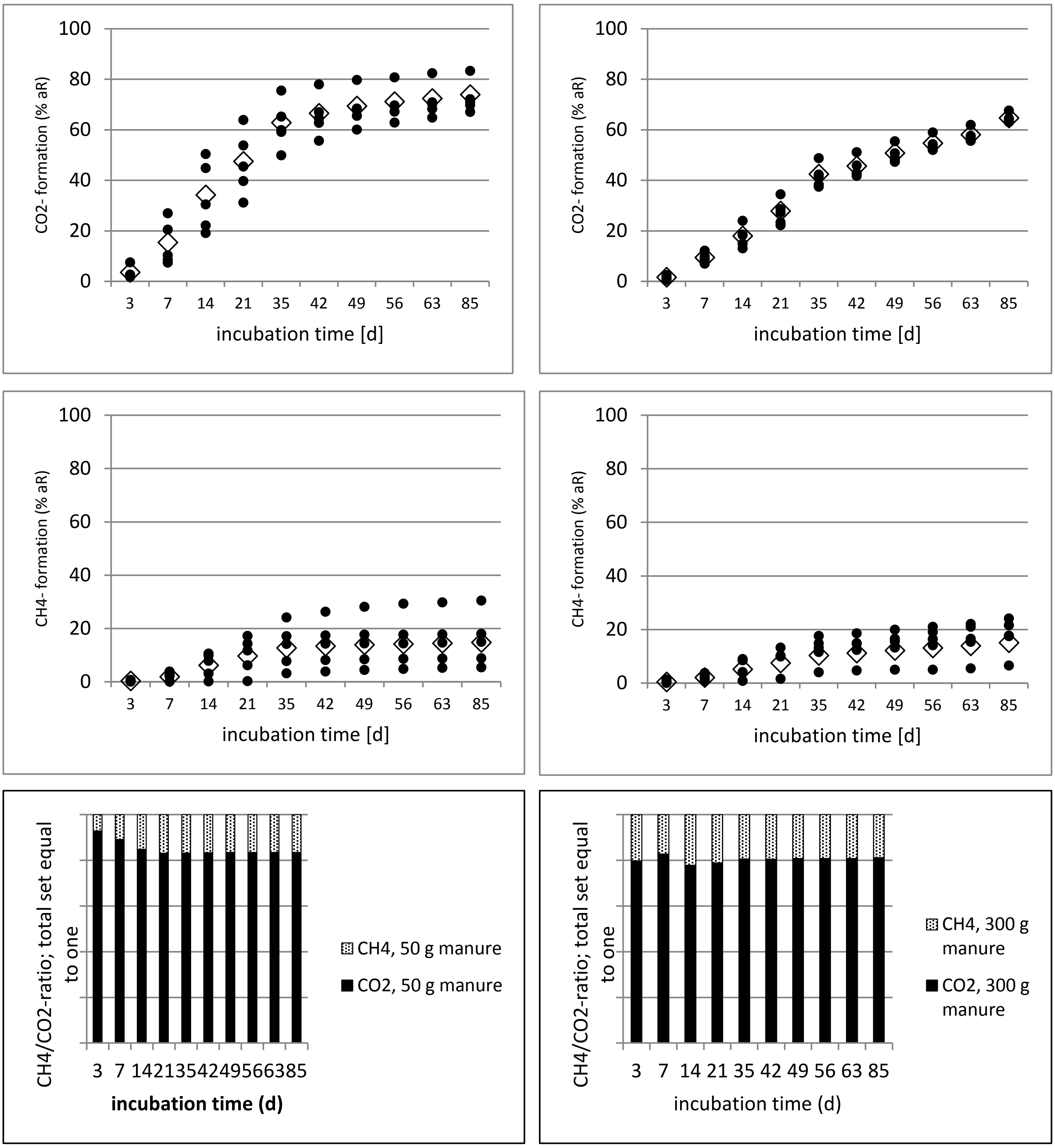
50 g manure

mass balance at end of study: 91.6 [% aR]

300 g manure

mass balance at end of study: 82.2 [% aR]

Figure 5: Formation of $^{14}CO_2$ [% aR] (upper lane) and $^{14}CH_4$ [% aR] (middle lane) by transformation of ^{14}C-Salicylic acid in pig manure (5 % dm). Figures in lower lane show the ratio of $^{14}CO_2$ and $^{14}CH_4$.
Results for 50 g manure (constant stream of nitrogen of 50-200 mL/min) are shown on the left site and for 300 g manure (constant stream of nitrogen of 50-200 mL/min) on the right site. Results for 6 replicates per sampling point (dots) and mean values (open squares) are given.



pig manure

50 g manure

mass balance at end of study: 98.2 [% aR]

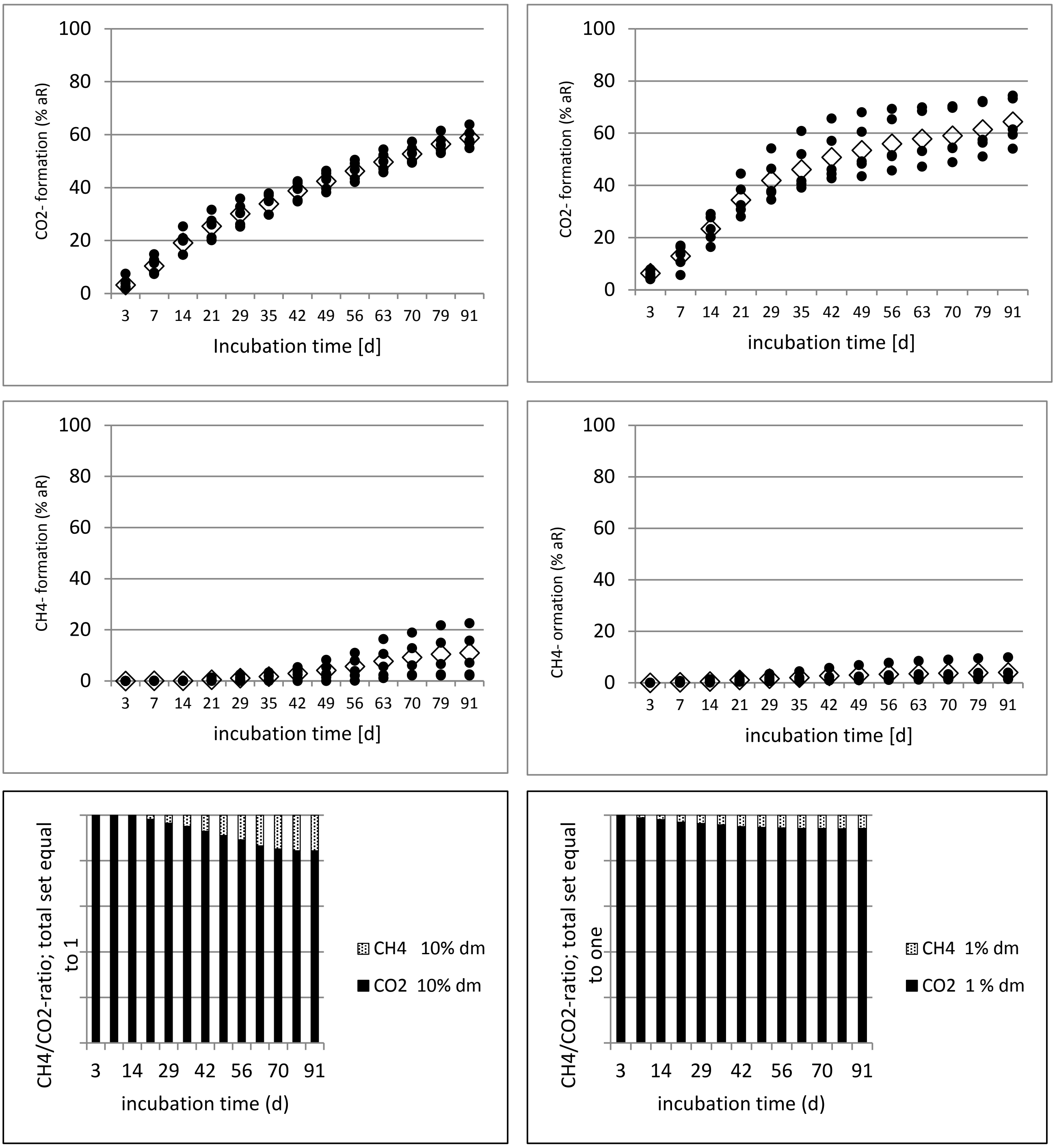
300 g manure

mass balance at end of study: 94.5[% aR]

Table 5: Statistical evaluation of $DT_{50\ MIN}$ (d) for the conditions 50 g and 300 g manure

	Cattle		Pig	
Descriptive statistics	**50 g**	**300 g**	**50 g**	**300 g**
n	6	6	6	6
Standard deviation	0.112	0.041	0.252	0.177
Mean (ln-transformed)	3.9	4.2	2.7	3.3
Mean (re-transformed)	48.8	63.5	14.9	26.6
Median	47.5	62.4	14.6	26.5
COV (%)	0.11	0.04	0.26	0.18
Levene´s test on homogeneity of variances				
p-value	0.069		0.458	
variances are	equal		equal	
Mann- Whitney U-test on differences in means				
p-value	0.004		0.002	
Medians are	not equal		not equal	

Figure 6: Formation of $^{14}CO_2$ [% aR] (upper lane) and $^{14}CH_4$ [% aR] (middle lane) by transformation of ^{14}C-Salicylic acid in cattle manure (10 % dm, 50 g manure per replicate). Figures in lower lane show the ratio of $^{14}CO_2$ and $^{14}CH_4$.
Results for <u>10 % dm</u> (constant stream of nitrogen of 50-200 mL/min) are shown on the <u>left</u> and for <u>1 % dm</u> (constant stream of nitrogen of 50-200 mL/min) on the <u>right</u>. Results for 6 replicates per sampling point (dots) and mean values (open squares) are given.



<u>cattle manure</u>

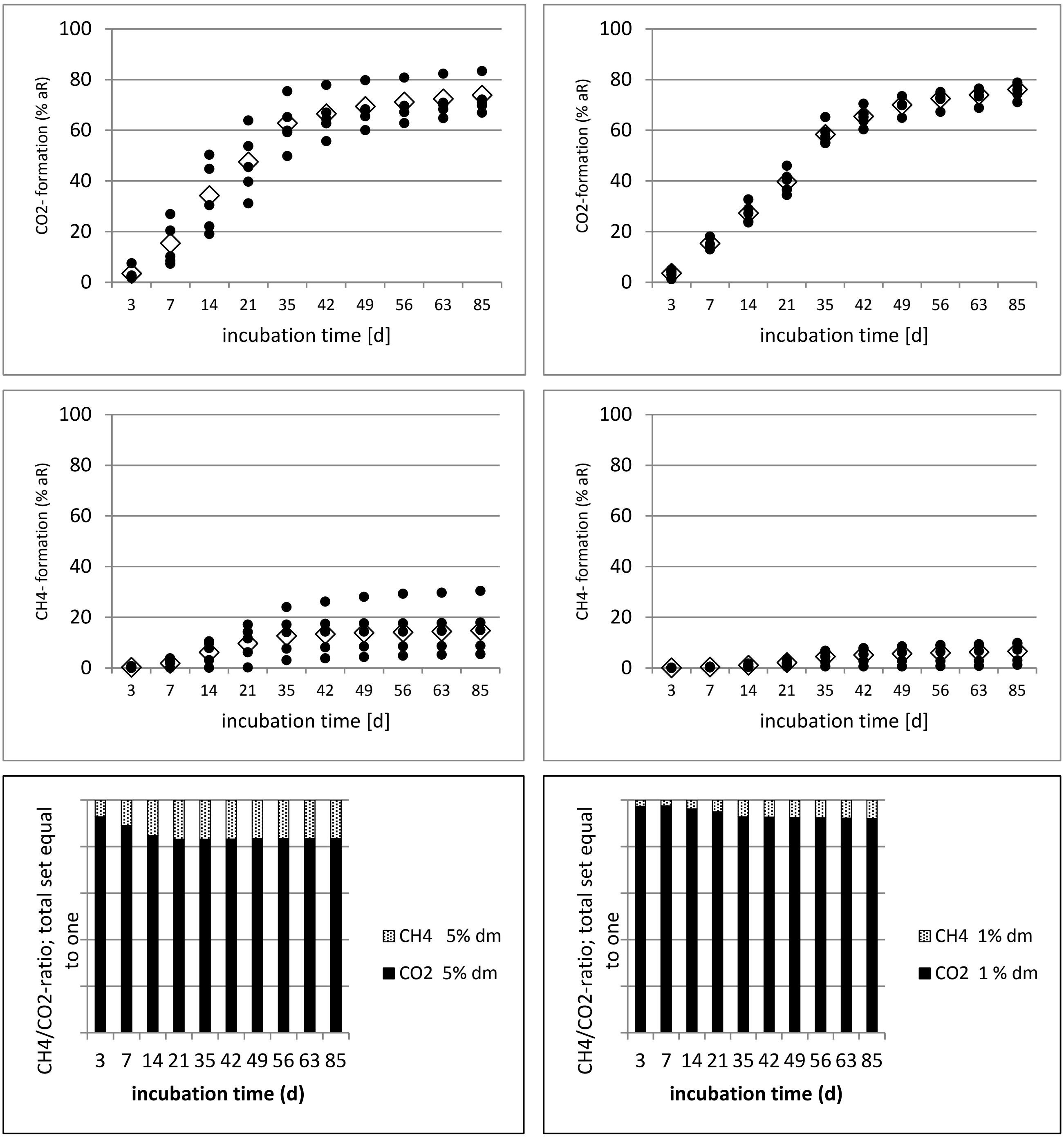
10 % dry matter content

mass balance at end of study: 91.6 [% aR]

1 % dry matter content

mass balance at end of study: 89.6 [% aR]

Figure 7: Formation of $^{14}CO_2$ [% aR] (upper lane) and $^{14}CH_4$ [% aR] (middle lane) by transformation of ^{14}C-Salicylic acid in pig manure (5 % dm, 50 g manure per replicate). Figures in lower lane show the ratio of $^{14}CO_2$ and $^{14}CH_4$.
Results for <u>5 % dm</u> (constant stream of nitrogen of 50-200 mL/min) are shown on the <u>left</u> and for <u>1 % dm</u> (constant stream of nitrogen of 50-200 mL/min) on the <u>right</u>. Results for 6 replicates per sampling point (dots) and mean values (open squares) are given.



<u>pig manure</u>

5 % dry matter

mass balance at end of study: 98.2 [% aR]

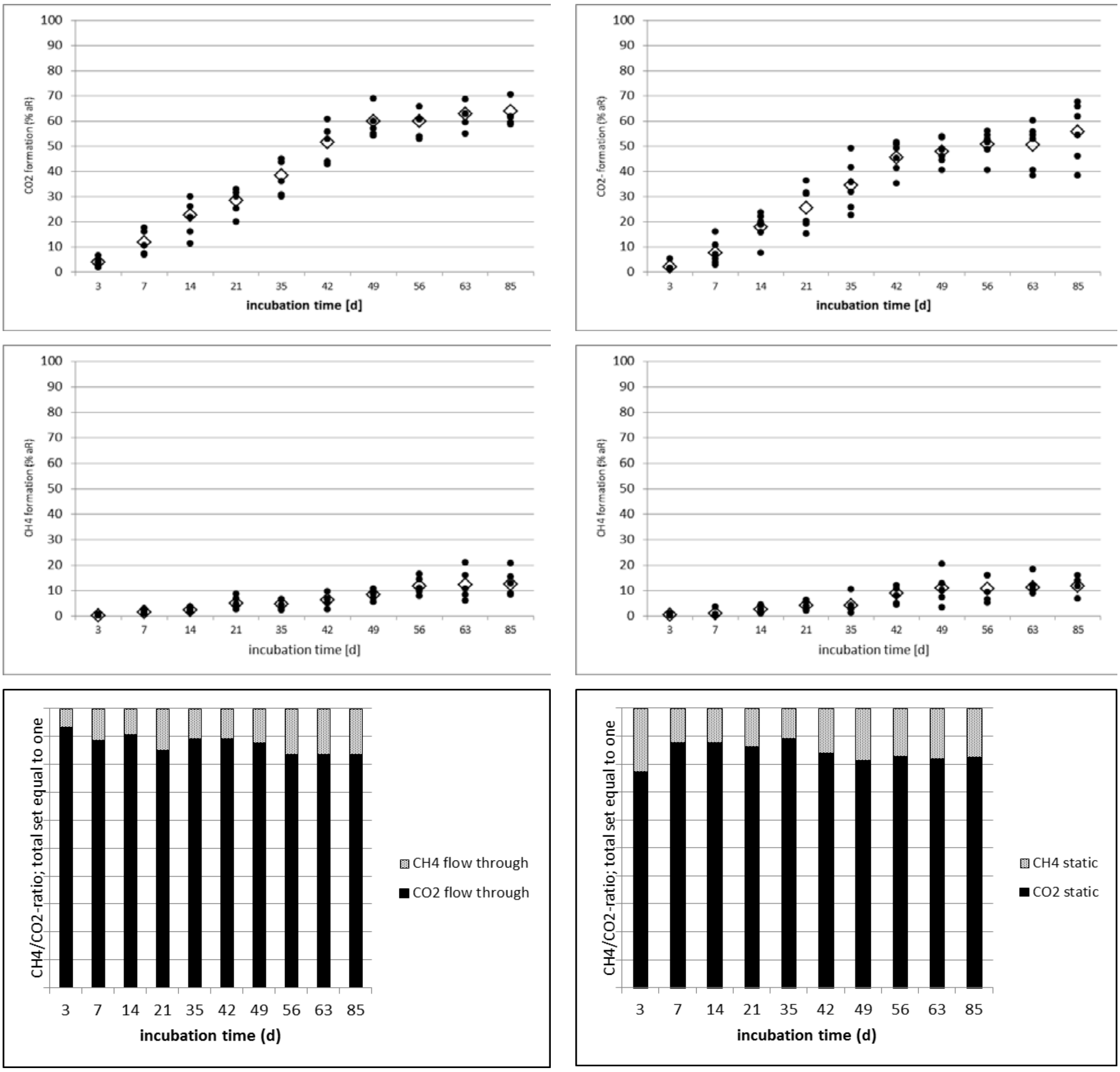
1 % dry matter

mass balance at end of study: 95.9[% aR]

Table 6: Statistical evaluation for $DT_{50\ MIN}$ (d) for the conditions 10 % (cattle)/ 5% (pig) dry matter and 1 % dry matter

	Cattle		**Pig**	
Descriptive statistics	**10 % dry matter**	**1 % dry matter**	**5 % dry matter**	**1 % dry matter**
n	6	6	6	6
Standard deviation	0.112	0.173	0.252	0.078
Mean (ln-transformed)	3.9	3.8	2.7	3.0
Mean (re-transformed)	48.8	46.0	14.9	20.7
Median	47.5	47.3	14.6	20.8
COV (%)	0.11	0.17	0.26	0.08
Levene´s test on homogeneity of variances				
p-value	0.591		0.161	
Variances are	equal		equal	
Mann- Whitney U-test on differences in means				
p-value	0.748		0.015	
Medians are	equal		not equal	

Figure 8: Formation of $^{14}CO_2$ [% aR] (upper lane) and $^{14}CH_4$ [% aR] (middle lane) by transformation of ^{14}C-Salicylic acid in cattle manure (10 % dm, 50 g manure per replicate). Figures in lower lane show the ratio of $^{14}CO_2$ and $^{14}CH_4$.
Results for the <u>flow-through system</u> (constant stream of nitrogen of 50-200 mL/min) are shown on the <u>left</u> and for <u>the semi-static system</u> (no stream of nitrogen) on the <u>right</u>. Results for 6 replicates per sampling point (dots) and mean values (open squares) are given.



<u>cattle manure</u>

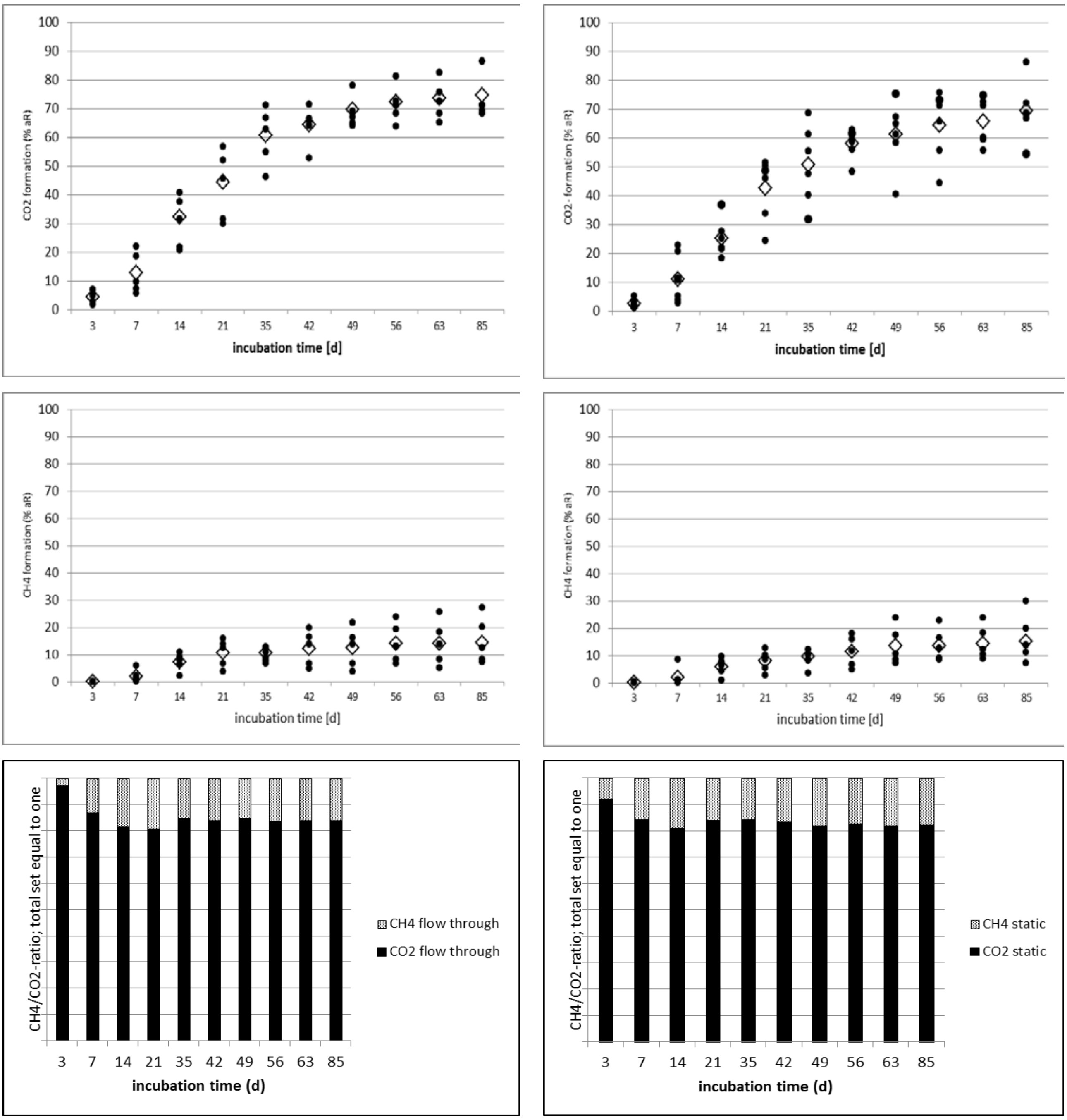
flow-through system (50 – 200 mL/min)

mass balance at end of study: 96.4 [% aR]

semi-static system

mass balance at end of study: 92.8[% aR]

Figure 9: Formation of $^{14}CO_2$ [% aR] (upper lane) and $^{14}CH_4$ [% aR] (middle lane) from transformation of ^{14}C-Salicylic acid in pig manure (5 % dm, 50 g manure per replicate). Figures in lower lane show the ratio of $^{14}CO_2$ and $^{14}CH_4$.
Results for <u>flow-through system</u> (constant stream of nitrogen of 50-200 mL/min) are shown on the <u>left</u> and for <u>the semi-static system</u> (no stream of nitrogen) on the <u>right</u>. Results for 6 replicates per sampling point (dots) and mean values (open squares) are given.



<u>Pig manure</u>

flow-through system (50 – 200 mL/min)

mass balance at end of study: 94.6 [% aR]

semi-static system

mass balance at end of study: 89.7[% aR]

Table 7: Statistical evaluation of $DT_{50\,MIN}$ (d) for the conditions flow through (50 – 200 mL/min) and semi-static

Descriptive statistics	cattle		pig	
	Flow-through	Semi-static	Flow-through	Semi-static
n	6	6	6	6
Standard deviation	0.160	0.120	0.256	0.102
Mean (ln-transformed)	3.5	3.7	2.9	3.2
Mean (re-transformed)	31.8	41.2	18.5	23.5
Median	32.1	39.6	18.3	23.6
COV (%)	0.16	0.12	0.26	0.10
Levene´s test on homogeneity of variances				
p-value	0.799		0.038	
variances are	equal		not equal	
Mann- Whitney U-test on differences				
p-value	0.009		0.093	
Medians are	not equal		equal	

2.3.3 Influence of the experimental setup on mineralization

For a better overview the results for the Man-Whitney U-test on differences in medians of mineralization half-lives for the various experimental conditions are summarized in the following table.

Table 8: Differences in medians of mineralization half-lives

Tested variations	Group for comparison	medians of mineralization half-lives in … are…	
		Cattle manure	Pig manure
flow rate	slow flow (50 - 200 mL/min) versus fast flow (› 200 mL/min)	equal	equal
amount of manure	50 g manure versus 300 g manure	not equal	not equal
dry matter content	10 % (5 %) dm versus 1 % dm	equal	not equal
flow through and semi-static	slow flow versus semi-static	not equal	equal

2.4 Conclusions

It was examined, if different experimental setups or conditions have an influence on mineralization to CO_2 and CH_4. The different setups/conditions studied were different flow-rates in the flow-through system,

- different amounts of manure,
- manure of different dry matter content,
- flow-through and semi-static system.

From the experiments using ^{14}C-Salicylic acid as test compound it can be concluded:

- Differences in nitrogen flow rate for the flow-through system do not have an influence on the mineralization rate of ^{14}C-Salicylic acid in cattle and pig manure.
- The amount of incubated manure (50 g vs. 300 g) significantly influences the rate of mineralization. This observation is valid for both, cattle and pig manure. Therefore the amount of manure used in an experiment has to be standardized to obtain comparable results.
  The dry matter content does not significantly influence mineralization rates in cattle manure but does in pig manure. This parameter also influences the outcome of transformation experiments and thus has to be standardized, as already recommended (EMA, 2011), to obtain comparable results.
- Differences in the experimental setup with respect to a flow-through and semi-static design have an influence on the mineralization rate of ^{14}C-Salicylic acid in cattle manure. This was not observed in pig manure. Therefore, for extensively mineralizing test substances, a semi-static test system should be used.
- In the experiments conducted, mineralization proceeded faster in pig manure than in cattle manure.
- The latter also was observed in the course of the project Hennecke et al. (2015). For cattle manure, $DT_{50\,MIN}$ was 44.3 d, whereas it was 30.5 d for pig manure when spiked with Salicylic acid (see Tables A1_37 and A1_38 for detailed results). Manure was collected from the same origin (i.e., manure tank) as it was in the current project.

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Annex 1 Raw data

Table A1_1: Formation of CO_2 (% applied radioactivity) in cattle manure spiked with Salicylic acid for the conditions: flow through, slow flow (50-200 mL/min), 50 g manure, 10% dm

	incubation time (d)												
rep	3	7	14	21	29	35	42	49	56	63	70	79	91
1	1.68	8.02	19.55	26.06	30.32	34.04	38.94	42.06	45.63	48.94	51.99	56.23	58.99
2	2.29	11.50	20.94	27.46	32.80	37.11	42.42	46.34	50.47	54.42	57.30	61.43	63.86
3	3.92	14.85	25.28	31.58	35.81	37.82	41.75	45.38	49.08	52.19	54.81	57.96	60.56
4	1.70	7.38	14.59	21.11	26.08	29.72	34.80	38.21	42.10	45.79	49.43	54.06	56.85
5	2.49	7.81	14.61	20.15	25.21	29.70	35.29	39.52	43.35	47.14	49.99	52.99	54.90
6	7.44	12.63	19.89	25.97	30.31	34.89	39.47	43.08	46.74	49.86	52.91	56.06	57.72

Table A1_2: Formation of CH_4 (% applied radioactivity) in cattle manure spiked with Salicylic acid for the conditions: flow through, slow flow (50-200 mL/min), 50 g manure, 10% dm

	incubation time (d)												
rep	3	7	14	21	29	35	42	49	56	63	70	79	91
1	0.01	0.06	0.06	0.90	1.96	3.04	5.25	7.13	9.01	10.77	13.08	14.87	15.56
2	0.00	0.00	0.00	0.05	0.13	0.33	0.84	1.37	2.08	2.27	2.42	2.52	2.57
3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	1.11	2.07	2.10	2.11
4	0.01	0.09	0.09	1.26	2.55	3.33	5.41	8.21	10.97	16.31	18.92	21.79	22.57
5	0.01	0.04	0.04	0.52	1.30	1.91	3.67	5.34	7.87	10.52	12.80	14.91	15.74
6	0.01	0.03	0.03	0.21	0.67	1.89	2.52	2.81	3.86	5.56	6.08	6.61	7.05

Table A1_3: Formation of CO_2 plus CH_4 (% applied radioactivity; calculated as: 100-[CO_2+CH_4]) in cattle manure for the conditions: flow through, slow flow (50-200 mL/min), 50 g manure, 10% dm

	incubation time (d)												
rep	3	7	14	21	29	35	42	49	56	63	70	79	91
1	98.31	91.92	80.39	73.04	67.72	62.92	55.81	50.81	45.36	40.29	34.93	28.90	18.42
2	97.71	88.50	79.06	72.49	67.07	62.56	56.74	52.29	47.45	43.31	40.28	36.05	17.29
3	96.08	85.15	74.72	68.42	64.19	62.18	58.25	54.62	50.87	46.70	43.12	39.94	16.34
4	98.29	92.53	85.32	77.63	71.37	66.95	59.79	53.58	46.93	37.90	31.65	24.15	18.63
5	97.50	92.15	85.35	79.33	73.49	68.39	61.04	55.14	48.78	42.34	37.21	32.10	24.24
6	92.55	87.34	80.08	73.82	69.02	63.22	58.01	54.11	49.40	44.58	41.01	37.33	22.37

Table A1_4: Formation of CO_2 (% applied radioactivity) in cattle manure spiked with Salicylic acid for the conditions: flow through, slow flow (50-200 mL/min), 50 g manure, 10% dm, sampled for the comparison flow through – semi static

	incubation time (d)												
rep	3	7	14	21	29	35	42	49	56	63	70	79	91
1	1.68	8.02	19.55	26.06	30.32	34.04	38.94	42.06	45.63	48.94	51.99	56.23	58.99
2	2.29	11.50	20.94	27.46	32.80	37.11	42.42	46.34	50.47	54.42	57.30	61.43	63.86
3	3.92	14.85	25.28	31.58	35.81	37.82	41.75	45.38	49.08	52.19	54.81	57.96	60.56
4	1.70	7.38	14.59	21.11	26.08	29.72	34.80	38.21	42.10	45.79	49.43	54.06	56.85
5	2.49	7.81	14.61	20.15	25.21	29.70	35.29	39.52	43.35	47.14	49.99	52.99	54.90
6	7.44	12.63	19.89	25.97	30.31	34.89	39.47	43.08	46.74	49.86	52.91	56.06	57.72

Table A1_5: Formation of CH_4 (% applied radioactivity) in cattle manure spiked with Salicylic acid for the conditions: flow through, slow flow (50-200 mL/min), 50 g manure, 10% dm, sampled for the comparison flow through – semi static

	incubation time (d)												
rep	3	7	14	21	29	35	42	49	56	63	70	79	91
1	0.01	0.06	0.06	0.90	1.96	3.04	5.25	7.13	9.01	10.77	13.08	14.87	15.56
2	0.00	0.00	0.00	0.05	0.13	0.33	0.84	1.37	2.08	2.27	2.42	2.52	2.57
3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	1.11	2.07	2.10	2.11
4	0.01	0.09	0.09	1.26	2.55	3.33	5.41	8.21	10.97	16.31	18.92	21.79	22.57
5	0.01	0.04	0.04	0.52	1.30	1.91	3.67	5.34	7.87	10.52	12.80	14.91	15.74
6	0.01	0.03	0.03	0.21	0.67	1.89	2.52	2.81	3.86	5.56	6.08	6.61	7.05

Table A1_6: Formation of CO_2 plus CH_4 (% applied radioactivity; calculated as: 100-[CO_2+CH_4]) in cattle manure for the conditions: flow through, slow flow (50-200 mL/min), 50 g manure, 10% dm sampled for the comparison flow through – semi static

	incubation time (d)												
rep	3	7	14	21	29	35	42	49	56	63	70	79	91
1	98.31	91.92	80.39	73.04	67.72	62.92	55.81	50.81	45.36	40.29	34.93	28.90	18.42
2	97.71	88.50	79.06	72.49	67.07	62.56	56.74	52.29	47.45	43.31	40.28	36.05	17.29
3	96.08	85.15	74.72	68.42	64.19	62.18	58.25	54.62	50.87	46.70	43.12	39.94	16.34
4	98.29	92.53	85.32	77.63	71.37	66.95	59.79	53.58	46.93	37.90	31.65	24.15	18.63
5	97.50	92.15	85.35	79.33	73.49	68.39	61.04	55.14	48.78	42.34	37.21	32.10	24.24
6	92.55	87.34	80.08	73.82	69.02	63.22	58.01	54.11	49.40	44.58	41.01	37.33	22.37

Table A1_7: Formation of CO_2 (% applied radioactivity) in cattle manure spiked with Salicylic acid for the conditions: flow through, fast flow (>200 mL/min), 50 g manure, 10% dm

	incubation time (d)												
rep	3	7	14	21	29	35	42	49	56	63	70	79	91
1	4.20	8.82	17.72	24.49	30.92	35.04	39.38	43.80	47.38	51.01	54.53	57.68	59.52
2	4.66	12.54	21.80	27.49	33.47	38.33	42.65	46.43	49.94	53.70	56.91	60.23	61.85
3	3.85	8.71	21.48	29.66	36.06	40.29	43.91	47.14	50.19	53.04	55.72	58.58	58.58
4	3.36	11.08	22.14	29.32	35.09	38.72	41.28	44.14	46.64	50.64	53.68	57.19	58.99
5	2.61	10.33	21.93	29.68	34.06	38.56	42.24	46.37	48.84	52.55	56.40	59.24	61.76
6	7.34	14.99	25.03	31.09	37.23	41.74	45.82	50.34	53.98	58.93	62.66	65.95	67.62

Table A1_8: Formation of CH_4 (% applied radioactivity) in cattle manure spiked with Salicylic acid for the conditions: flow through, fast flow (>200 mL/min), 50 g manure, 10% dm

	incubation time (d)												
rep	3	7	14	21	29	35	42	49	56	63	70	79	91
1	0.08	0.09	0.12	0.32	0.44	0.86	1.05	1.18	1.92	3.02	5.47	6.25	6.71
2	0.02	0.03	0.19	0.66	1.36	2.45	4.62	6.00	7.14	9.78	11.35	12.37	12.52
3	0.00	0.03	0.06	0.06	0.11	0.15	0.70	0.94	1.62	3.43	4.80	5.66	5.66
4	0.00	0.09	0.12	0.26	0.55	0.70	1.00	1.94	1.97	2.65	3.41	4.81	5.04
5	0.07	0.11	0.28	0.95	1.21	1.48	1.91	2.46	2.94	3.66	4.33	5.19	5.63
6	0.04	0.17	0.50	0.90	1.58	2.09	4.32	6.69	9.16	12.26	13.94	15.18	15.67

Table A1_9: Formation of CO_2 plus CH_4 (% applied radioactivity; calculated as: 100-[CO_2+CH_4]) in cattle manure spiked with Salicylic acid for the conditions: flow through, fast flow (>200 mL/min), 50 g manure, 10% dm

	incubation time (d)												
rep	3	7	14	21	29	35	42	49	56	63	70	79	91
1	95.72	91.09	82.16	75.19	68.64	64.10	59.57	55.02	50.70	45.97	40.00	36.07	16.41
2	95.32	87.43	78.01	71.85	65.17	59.22	52.73	47.57	42.92	36.52	31.74	27.40	18.91
3	96.15	91.26	78.46	70.28	63.83	59.56	55.39	51.92	48.19	43.53	39.48	35.76	18.80
4	96.64	88.83	77.74	70.42	64.36	60.58	57.72	53.92	51.39	46.71	42.91	38.00	20.09
5	97.32	89.56	77.79	69.37	64.73	59.96	55.85	51.17	48.22	43.79	39.27	35.57	21.63
6	92.62	84.84	74.47	68.01	61.19	56.17	49.86	42.97	36.86	28.81	23.40	18.87	18.68

Table A1_10: Formation of CO_2 (% applied radioactivity) in cattle manure spiked with Salicylic acid for the conditions: flow through, slow flow, 300 g manure, 10% dm

	incubation time (d)												
rep	3	7	14	21	29	35	42	49	56	63	70	79	91
1	2.67	10.34	17.87	23.03	26.53	30.65	34.26	37.46	40.60	43.43	46.08	49.21	51.75
2	0.45	1.26	8.86	15.33	19.34	21.55	25.33	28.26	32.61	35.93	39.08	42.03	44.86
3	0.49	2.12	4.97	9.56	14.59	17.69	22.72	26.33	29.82	33.51	36.98	40.45	43.59
4	0.36	1.37	4.34	11.94	16.73	22.00	27.51	31.50	34.91	38.72	42.17	45.82	48.51
5	0.31	1.76	6.80	13.45	17.47	21.99	26.88	30.68	34.74	38.34	42.03	45.48	48.59
6	0.67	1.89	2.89	11.59	15.93	19.30	24.80	28.65	33.28	36.51	39.20	43.08	46.06

Table A1_11: Formation of CH_4 (% applied radioactivity) in cattle manure spiked with Salicylic acid for the conditions: flow through, slow flow, 300 g manure, 10% dm

	incubation time (d)												
rep	3	7	14	21	29	35	42	49	56	63	70	79	91
1	0.01	0.05	0.21	0.51	1.11	1.36	2.13	2.8	3.79	5.39	6.27	8.37	9.42
2	0.001	0.03	0.17	0.54	1.31	1.891	3.951	6.41	7.22	8.63	10.11	12.80	13.69
3	0.02	0.05	0.17	0.36	0.63	1.27	2.47	3.52	4.66	6.37	8.06	11.75	13.09
4	0.001	0.02	0.101	0.39	0.94	1.74	2.03	2.17	3.11	5.21	7.13	7.14	7.61
5	0.001	0.002	0.072	0.32	0.60	0.68	2.34	3.17	4.45	5.46	6.81	9.81	10.88
6	0.001	0.01	0.071	0.28	0.70	1.11	2.43	2.73	3.66	4.96	6.601	9.52	10.17

Table A1_12: Formation of CO_2 plus CH_4 (% applied radioactivity; calculated as: 100-[CO_2+CH_4]) in cattle manure spiked with Salicylic acid for the conditions: flow through, slow flow, 300 g manure, 10% dm

	incubation time (d)												
rep	3	7	14	21	29	35	42	49	56	63	70	79	91
1	97.32	89.61	81.92	76.46	72.36	67.99	63.61	59.74	55.61	51.18	47.65	42.42	21.50
2	99.55	98.71	90.97	84.13	79.35	76.56	70.72	65.25	60.17	55.44	50.81	45.17	24.83
3	99.49	97.83	94.86	90.08	84.78	81.04	74.81	70.15	65.52	60.12	54.96	47.80	22.62
4	99.64	98.61	95.56	87.67	82.33	76.26	70.46	66.33	61.98	56.07	50.70	47.04	23.96
5	99.69	98.24	93.13	86.23	81.93	77.33	70.78	66.15	60.81	56.20	51.16	44.71	23.29
6	99.33	98.10	97.04	88.13	83.37	79.59	72.77	68.62	63.06	58.53	54.20	47.40	28.45

Table A1_13: Formation of CO_2 (% applied radioactivity)]) in cattle manure spiked with Salicylic acid for the conditions: flow through, slow flow, 50 g manure, 1% dm

	incubation time (d)											
rep	3	7	14	21	29	35	42	49	56	63	70	79	91
1	7.37	14.22	23.83	32.71	40.96	42.58	49.19	51.44	52.86	55.02	56.58	59.79	63.61
2	5.03	10.65	20.16	28.01	34.52	39.12	44.35	48.25	51.14	53.13	54.47	57.40	61.47
3	6.06	13.73	23.25	30.72	37.33	41.74	46.06	49.13	51.57	53.18	54.23	56.34	59.39
4	4.05	5.63	16.36	32.41	37.86	40.50	42.67	43.54	45.71	47.20	48.83	51.11	54.02
5	7.85	16.42	27.76	38.45	46.43	51.92	57.02	60.53	65.28	68.54	69.68	72.27	74.39
6	7.43	16.94	29.06	44.45	54.20	60.84	65.56	67.96	69.29	69.89	70.30	71.87	73.32

Table A1_14: Formation of CH_4 (% applied radioactivity) in cattle manure spiked with Salicylic acid for the conditions: flow through, slow flow, 50 g manure, 1% dm

	incubation time (d)												
rep	3	7	14	21	29	35	42	49	56	63	70	79	91
1	0.01	0.01	0.49	0.55	1.32	2.48	4.20	4.82	4.86	4.91	4.91	4.93	5.00
2	0.00	0.00	0.35	1.14	2.06	2.15	2.16	2.16	2.18	2.18	2.26	2.31	2.31
3	0.01	0.71	1.30	2.44	3.38	4.41	5.79	6.90	7.81	8.47	8.97	9.49	9.86
4	0.03	0.08	0.52	1.43	1.53	1.84	2.16	2.39	2.88	3.28	3.37	3.78	3.92
5	0.01	0.04	0.08	0.58	0.68	0.72	0.85	1.07	1.16	1.31	1.34	1.49	1.61
6	0.02	0.03	0.10	0.44	0.57	0.61	0.86	0.99	1.11	1.18	1.29	1.44	1.44

Table A1_15: Formation of CO_2 plus CH_4 (% applied radioactivity; calculated as: 100-[CO_2+CH_4]) in cattle manure spiked with Salicylic acid for the conditions: flow through, slow flow, 50 g manure, 1% dm

	incubation time (d)												
rep	3	7	14	21	29	35	42	49	56	63	70	79	91
1	92.62	85.77	75.68	66.74	57.72	54.94	46.61	43.74	42.28	40.07	38.51	35.28	21.12
2	94.97	89.35	79.49	70.85	63.42	58.73	53.49	49.59	46.68	44.69	43.27	40.29	20.43
3	93.93	85.56	75.45	66.84	59.29	53.85	48.15	43.97	40.62	38.35	36.80	34.17	23.90
4	95.92	94.29	83.12	66.16	60.61	57.66	55.17	54.07	51.41	49.52	47.80	45.11	23.39
5	92.14	83.54	72.16	60.97	52.89	47.36	42.13	38.40	33.56	30.15	28.98	26.24	15.75
6	92.55	83.03	70.84	55.11	45.23	38.55	33.58	31.05	29.60	28.93	28.41	26.69	18.00

Table A1_16: Formation of CO_2 (% applied radioactivity)]) in cattle manure spiked with Salicylic acid for the conditions: semi-static, 50 g manure, 10% dm

	incubation time (d)											
rep	3	7	14	21	29	35	42	49	56	63	70	79	91
1	0.9	7	23.5	31.5	30.8	51.6	53.3	54.3	54.3	54.3	0.9	7	23.5
2	0.9	10.8	19.8	36.2	41.6	45.3	48.5	48.5	38.2	38.2	0.9	10.8	19.8
3	1.1	2.8	20.9	20.2	49.2	49.2	40.5	40.5	40.5	45.9	1.1	2.8	20.9
4	1.5	5.6	18.7	19.2	35.6	41.1	45.9	55.9	60.3	65.7	1.5	5.6	18.7
5	1.5	2.1	15.7	30.9	25.6	35.2	44.3	51.6	55.8	61.8	1.5	2.1	15.7
6	3.9	15.9	7.6	15.2	22.5	50.7	53.8	52.9	52.9	67.5	3.9	15.9	7.6

Table A1_17: Formation of CH_4 (% applied radioactivity) in cattle manure spiked with Salicylic acid for the conditions: semi-static, 50 g manure, 10% dm

	incubation time (d)												
rep	3	7	14	21	29	35	42	49	56	63	70	79	91
1	0.8	1.1	3.4	2.8	3.7	12.8	11.7	10.8	4.9	9.8	0.8	1.1	3.4
2	0.5	3.5	1.2	3.8	12.4	10.7	12.8	15.9	11.8	10.4	0.5	3.5	1.2
3	0.4	0.4	2.4	1.9	1.1	4.4	7.2	5.1	12.3	6.8	0.4	0.4	2.4
4	0.4	0.6	4.4	5.7	2.1	11.9	10.1	9.4	11	11.7	0.4	0.6	4.4
5	1.0	0.6	1.1	3.9	2.7	8.1	3.3	15.9	18.3	13.8	1.0	0.6	1.1
6	0.1	0.7	2.8	6.1	3.1	6.8	20.4	6.6	9.1	15.9	0.1	0.7	2.8

Table A1_18: Formation of CO_2 plus CH_4 (% applied radioactivity; calculated as: 100-[CO_2+CH_4]) in cattle manure spiked with Salicylic acid for the conditions: semi-static, 50 g manure, 10% dm

	incubation time (d)												
rep	3	7	14	21	29	35	42	49	56	63	70	79	91
1	98.3	91.9	73.1	65.7	65.5	35.6	35.0	34.9	40.8	32.75	98.3	91.9	73.1
2	98.6	85.7	79.0	60.0	46.0	44.0	38.7	35.6	50.0	33.3	98.6	85.7	79.0
3	98.5	96.8	76.7	77.9	49.7	46.4	52.3	54.4	47.2	36.62	98.5	96.8	76.7
4	98.1	93.8	76.9	75.1	62.3	47.0	44.0	34.7	28.7	20.25	98.1	93.8	76.9
5	97.5	97.3	83.2	65.2	71.7	56.7	52.4	32.5	25.9	18.65	97.5	97.3	83.2
6	96.0	83.4	89.6	78.7	74.4	42.5	25.8	40.5	38.0	13.6	96.0	83.4	89.6

Table A1_19: Formation of CO_2 (% applied radioactivity) in pig manure spiked with Salicylic acid for the conditions: flow through (slow flow 5-200 mL/min), 5 % dm, 50 g manure

	incubation time (d)									
replicate	3	7	14	21	29	35	42	49	56	63
1	3.1	13.8	41.5	51.2	61.8	65.8	74.2	75.6	78.2	81.5
2	1.8	18.5	31.6	56.7	71.3	71.5	78.1	81.3	82.5	86.5
3	4.8	7.4	20.8	31.5	54.8	64.2	66.9	71.5	72.6	71.2
4	6.7	9.7	21.8	29.8	46.2	52.8	64.2	63.9	68.3	68.2
5	2.8	5.8	37.6	45.8	62.8	65.8	64.9	68.4	65.1	69.5
6	7.0	21.9	40.6	51.9	66.7	66.4	69.1	72.6	75.6	71.3

Table A1_20: Formation of CH_4 (% applied radioactivity) in pig manure spiked with Salicylic acid for the conditions: flow through (slow flow 5-200 mL/min), 5 % dm, 50 g manure

	incubation time (d)									
replicate	3	7	14	21	29	35	42	49	56	63
1	0.07	1	6.8	10.8	17.3	11.9	12.8	13.1	13.8	10.5
2	0.24	5.9	2.3	6.7	10.9	13.8	16.1	19.5	18.3	8.3
3	0.15	2.3	10.9	3.8	12.9	4.9	6.8	8.3	8.2	7.2
4	0.01	1.4	9.2	15.9	8.9	16.5	13.9	13.1	13.9	12.6
5	0.09	0.9	6.4	12.6	6.8	19.8	21.8	23.9	25.8	20.1
6	0.2	0.3	8.1	13.8	7.9	6.8	3.9	6.8	5.2	27.3

Table A1_21: Formation of CO_2 plus CH_4 (% applied radioactivity; calculated as: 100-[CO_2+CH_4]) in pig manure for the conditions: flow through (slow flow 5-200 mL/min), 5 % dm, 50 g manure

	incubation time (d)									
replicate	3	7	14	21	29	35	42	49	56	63
1	96.83	85.20	51.70	38.00	20.90	22.30	13.00	11.30	8.00	9.95
2	97.96	75.60	66.10	36.60	17.80	14.70	5.80	0.001	0.001	5.58
3	95.05	90.32	68.30	64.70	32.30	30.90	26.30	20.20	19.20	9.04
4	93.29	88.90	69.00	54.30	44.90	30.70	21.90	23.00	17.80	11.90
5	97.15	93.30	56.00	41.60	30.40	14.40	13.30	7.70	9.10	21.65
6	92.80	77.80	51.30	34.30	25.40	26.80	27.00	20.60	19.20	0.00

Table A1_22: Formation of CO_2 (% applied radioactivity) in pig manure spiked with Salicylic acid for the conditions: flow through (slow flow 5-200 mL/min), 5 % dm, 50 g manure, sampled for the comparison flow through – semi static

	incubation time (d)									
replicate	3	7	14	21	29	35	42	49	56	63
1	4.60	19.29	38.53	51.21	67.36	71.67	75.09	77.22	78.02	80.31
2	2.13	20.47	50.38	63.88	75.47	77.92	79.76	80.78	82.38	83.36
3	2.59	7.38	19.10	39.74	59.83	64.49	67.72	69.64	70.88	72.09
4	2.71	10.26	22.07	31.16	49.87	55.69	60.06	62.86	64.81	67.01
5	1.76	8.51	30.44	45.48	59.18	62.71	65.51	67.12	68.29	69.73
6	7.54	26.90	44.80	53.77	65.24	66.93	68.35	69.40	70.10	70.97

Table A1_23: Formation of CH_4 (% applied radioactivity) in pig manure spiked with Salicylic acid for the conditions: flow through (slow flow 5-200 mL/min), 5 % dm, 50 g manure, sampled for the comparison flow through – semi static

	incubation time (d)									
replicate	3	7	14	21	29	35	42	49	56	63
1	0.28	0.57	5.50	8.60	10.14	10.40	10.45	10.47	10.52	10.74
2	0.18	2.46	3.06	6.15	7.68	8.16	8.44	8.56	8.65	8.81
3	0.03	0.06	0.13	0.23	3.13	3.86	4.38	4.83	5.23	5.43
4	0.73	3.87	9.85	14.22	17.10	17.39	17.63	17.65	17.77	17.93
5	0.31	2.02	10.57	17.18	24.08	26.24	28.09	29.27	29.71	30.42
6	0.12	2.40	7.83	11.67	14.14	14.30	14.31	14.32	14.66	14.95

Table A1_24: Formation of CO_2 plus CH_4 (% applied radioactivity; calculated as: $100-[CO_2+CH_4]$) in pig manure for the conditions: flow through (slow flow 5-200 mL/min), 5 % dm, 50 g manure, sampled for the comparison flow through – semi static

	incubation time (d)									
replicate	3	7	14	21	29	35	42	49	56	63
1	95.12	80.14	55.97	40.19	22.50	17.93	14.46	12.31	11.46	10.90
2	97.69	77.07	46.56	29.97	16.85	13.92	11.80	10.66	8.97	8.21
3	97.38	92.56	80.77	60.03	37.04	31.65	27.90	25.53	23.89	9.92
4	96.56	85.87	68.08	54.62	33.03	26.92	22.31	19.49	17.42	7.76
5	97.93	89.47	58.99	37.34	16.74	11.05	6.40	3.61	2.00	11.10
6	92.34	70.70	47.37	34.56	20.62	18.77	17.34	16.28	15.24	9.80

Table A1_25: Formation of CO_2 (% applied radioactivity) in pig manure spiked with Salicylic acid for the conditions: flow through (fast flow ›200 mL/min), 5 % dm, 50 g manure

	incubation time (d)									
replicate	3	7	14	21	29	35	42	49	56	63
1	6.9	17.8	33.7	49.1	69.3	75.4	78.8	80.8	82.3	84.4
2	5.2	16.9	34.7	51.5	68.7	73.6	77.2	79.0	80.3	83.1
3	7.8	16.3	29.9	45.2	65.4	71.4	75.8	78.0	79.8	82.6
4	7.1	17.8	32.6	48.1	65.7	69.6	73.9	75.9	77.2	79.2
5	5.8	17.3	35.1	52.0	68.6	74.5	78.0	80.0	81.3	83.7
6	7.3	12.9	22.7	37.3	61.9	68.6	72.2	74.4	76.0	77.9

Table A1_26: Formation of CH_4 (% applied radioactivity) in pig manure spiked with Salicylic acid for the conditions: flow through (fast flow ›200 mL/min), 5 % dm, 50 g manure

	incubation time (d)									
replicate	3	7	14	21	29	35	42	49	56	63
1	0.28	0.90	3.63	6.99	10.42	11.86	12.50	12.79	13.10	13.58
2	0.38	1.29	3.52	5.67	7.97	9.10	9.49	9.75	9.90	10.29
3	0.22	0.97	3.69	8.37	13.71	15.39	16.20	16.71	17.01	17.54
4	0.04	0.88	2.99	5.80	9.26	10.32	10.80	11.07	11.23	11.48
5	0.28	1.19	4.01	6.37	9.41	10.70	11.45	11.88	12.18	12.80
6	0.16	0.46	1.01	2.29	4.81	6.02	7.09	7.86	8.33	8.43

Table A1_27: Formation of CO_2 plus CH_4 (% applied radioactivity; calculated as: 100-[CO_2+CH_4]) in pig manure for the conditions: flow through (fast flow ›200 mL/min), 5 % dm, 50 g manure

	incubation time (d)									
replicate	3	7	14	21	29	35	42	49	56	63
1	92.82	81.29	62.66	43.89	20.24	12.76	8.74	6.43	4.61	11.65
2	94.45	81.77	61.82	42.79	23.32	17.27	13.36	11.22	9.76	10.72
3	91.96	82.69	66.40	46.40	20.88	13.22	7.97	5.31	3.24	11.37
4	92.83	81.28	64.38	46.10	25.03	20.13	15.35	13.07	11.59	10.71
5	93.94	81.47	60.92	41.64	21.97	14.85	10.51	8.12	6.54	9.17
6	92.50	86.65	76.25	60.41	33.30	25.38	20.67	17.74	15.71	11.42

Table A1_28: Formation of CO_2 (% applied radioactivity) in pig manure spiked with Salicylic acid for the conditions: flow through, 5 % dm, 300 g manure

	incubation time (d)									
replicate	3	7	14	21	29	35	42	49	56	63
1	1.95	10.14	19.20	31.25	46.38	49.68	54.44	57.84	60.75	66.04
2	2.09	12.15	23.99	34.44	48.71	51.07	55.45	58.91	61.95	67.61
3	1.19	9.88	18.48	28.57	41.65	45.89	50.71	54.32	57.05	64.11
4	2.50	8.67	13.07	23.39	38.31	42.57	48.75	53.29	57.62	64.49
5	0.56	6.94	14.89	22.22	37.42	41.77	47.91	52.08	55.68	63.06
6	1.44	8.69	18.28	27.29	42.26	42.87	47.36	52.01	55.69	63.04

Table A1_29: Formation of CH_4 (% applied radioactivity) in pig manure spiked with Salicylic acid for the conditions: flow through, 5 % dm, 300 g manure

	incubation time (d)									
replicate	3	7	14	21	29	35	42	49	56	63
1	0.00	0.00	0.04	0.32	0.90	1.75	2.56	2.95	2.97	3.07
2	0.00	3.40	4.10	10.17	14.70	14.82	15.54	16.39	16.49	17.68
3	0.35	3.64	8.56	13.15	17.55	18.51	19.91	20.91	20.98	21.54
4	0.49	1.65	8.86	9.87	13.04	14.66	16.52	18.99	21.97	24.09
5	0.39	0.59	0.71	1.51	3.95	4.68	4.92	4.94	5.37	6.49
6	1.24	2.55	8.47	9.87	11.54	12.29	13.21	14.02	15.33	17.41

Table A1_30: Formation of CO_2 plus CH_4 (% applied radioactivity; calculated as: 100-[CO_2+CH_4]) in pig manure for the conditions: flow through, 5 % dm, 300 g manure

	incubation time (d)									
replicate	3	7	14	21	29	35	42	49	56	63
1	98.05	89.86	80.76	68.43	52.72	48.57	43.00	39.21	36.28	15.86
2	97.91	84.45	71.91	55.39	36.59	34.11	29.01	24.70	21.56	12.87
3	98.46	86.48	72.96	58.28	40.80	35.60	29.38	24.77	21.97	15.60
4	97.01	89.68	78.07	66.74	48.65	42.77	34.73	27.72	20.41	12.88
5	99.05	92.47	84.40	76.27	58.63	53.55	47.17	42.98	38.95	14.23
6	97.32	88.76	73.25	62.84	46.20	44.84	39.43	33.97	28.98	16.81

Table A1_31: Formation of CO_2 (% applied radioactivity) in pig manure spiked with Salicylic acid for the conditions: flow through, 1 % dm, 50 g manure

	incubation time (d)									
replicate	3	7	14	21	29	35	42	49	56	63
1	3.85	14.97	27.58	39.66	58.22	66.84	71.98	74.77	76.49	78.68
2	3.01	13.55	24.09	34.45	55.19	63.70	69.82	73.14	75.29	77.75
3	4.15	18.08	27.25	40.41	54.86	60.31	64.91	67.26	68.84	71.09
4	1.22	15.11	28.96	41.56	57.49	65.24	69.99	72.62	73.39	74.29
5	5.04	17.74	32.69	46.04	65.19	70.44	73.43	75.15	76.41	78.80
6	4.22	12.97	23.61	36.51	59.46	66.40	70.18	72.28	73.70	75.94

Table A1_32: Formation of CH_4 (% applied radioactivity) in pig manure spiked with Salicylic acid for the conditions: flow through, 1 % dm, 50 g manure

	incubation time (d)									
replicate	3	7	14	21	29	35	42	49	56	63
1	0.13	0.50	1.94	3.21	6.79	7.41	7.90	8.15	8.44	8.93
2	0.10	0.47	0.59	2.03	4.44	5.33	6.05	6.45	6.70	7.21
3	0.12	0.39	1.76	3.49	6.80	7.81	8.57	8.89	9.22	9.61
4	0.09	0.56	0.61	1.71	6.05	7.43	8.47	9.06	9.44	9.88
5	0.15	0.18	0.38	0.48	0.58	0.60	0.61	0.73	0.88	1.26
6	0.02	0.23	1.32	1.80	2.40	2.67	2.76	2.79	2.80	2.97

Table A1_33: Formation of CO_2 plus CH_4 (% applied radioactivity; calculated as: 100-[CO_2+CH_4]) in pig manure for the conditions: flow through, 1 % dm, 50 g manure

	incubation time (d)									
replicate	3	7	14	21	29	35	42	49	56	63
1	96.02	84.53	70.48	57.13	34.99	25.75	20.12	17.08	15.07	13.26
2	96.89	85.98	75.32	63.52	40.37	30.97	24.13	20.41	18.01	3.73
3	95.73	81.53	70.99	56.10	38.34	31.88	26.52	23.85	21.94	19.56
4	98.69	84.33	70.43	56.73	36.46	27.33	21.54	18.32	17.17	12.49
5	94.81	82.08	66.93	53.48	34.23	28.96	25.96	24.12	22.71	15.65
6	95.76	86.80	75.07	61.69	38.14	30.93	27.06	24.93	23.50	14.57

[1] negative value set equal to 0

Table A1_34: Formation of CO_2 (% applied radioactivity) in pig manure spiked with Salicylic acid for the conditions: semi static, 5 % dm, 50 g manure

	incubation time (d)									
replicate	3	7	14	21	29	35	42	49	56	63
1	1.38	11	36.8	48.5	31.8	61.5	75.1	73.1	74.6	54.3
2	2.8	20.8	25.3	51.6	55.4	58.6	61.3	65.8	71.2	86.1
3	1.25	3.8	21.9	33.8	68.5	60.7	67.3	55.8	55.8	72
4	3.15	5.2	21.6	24.5	61.3	55.9	58.2	44.3	59.4	68.5
5	1.26	2.8	27.6	45.9	40.3	48.3	40.5	75.8	60.1	68.1
6	5.3	22.8	18.3	50.4	47.6	62.9	64.8	71.1	72.5	66.8

Table A1_35: Formation of CH_4 (% applied radioactivity) in pig manure spiked with Salicylic acid for the conditions: semi static, 5 % dm, 50 g manure

	incubation time (d)									
replicate	3	7	14	21	29	35	42	49	56	63
1	0.07	1.0	5.8	9.2	13.7	12.2	12.7	12.1	12.8	8.9
2	0.24	8.5	1.0	5.4	10.4	11.7	17.5	16.5	18.3	13.8
3	0.22	1.1	9.6	2.9	12.4	6.7	7.4	9.0	10.4	7.2
4	0.5	0.5	6.5	12.8	8.7	15.9	10.7	12.7	12.4	11.2
5	0.09	0.9	7.9	8.5	3.7	18.1	23.8	22.7	23.8	19.8
6	0.2	0.3	4.4	10.1	8.4	4.8	8.7	8.7	8.9	29.8

Table A1_36: Formation of CO_2 plus CH_4 (% applied radioactivity; calculated as: 100-[CO_2+CH_4]) in pig manure for the conditions: semi static, 5 % dm, 50 g manure

	incubation time (d)									
replicate	3	7	14	21	29	35	42	49	56	63
1	98.55	88	57.4	42.3	54.5	26.3	12.2	14.8	12.6	10.32
2	96.96	70.7	73.7	43.0	34.2	29.7	21.2	17.7	10.5	1.91
3	98.53	95.1	68.5	63.3	19.1	32.6	25.3	35.2	33.8	8.6
4	96.35	94.3	71.9	62.7	30.0	28.2	31.1	43.0	28.2	5.3
5	98.65	96.3	64.5	45.6	56.0	33.6	35.7	1.5	16.1	3.3
6	94.5	76.9	77.3	39.5	44.0	32.3	26.5	20.2	18.6	2.33

Table A1_37: Formation of CO_2, CH_4, and CO_2 plus CH_4 (% applied radioactivity) in cattle manure spiked with Salicylic acid and sampled in winter 2010/2011 (conditions: flow through, 10 % dm, 50 g manure). Values taken from Hennecke et al. (2015)

	incubation time (d)						
	0	3	7	14	21	28	36
CO_2 formation (% radioactivity)	0.0	5.4	12.1	10.5	27.7	41.7	38.1
CH_4 formation (% radioactivity)	0.0	0.0	0.1	0.0	0.5	0.8	1.9
CO_2 + CH_4 formation (% radioactivity)[1]	100.0	94.6	87.8	89.5	71.8	57.5	60.0

[1] Calculated by 100 – (CO_2+CH_4)

Table A1_38: Formation of CO_2, CH_4, and CO_2 plus CH_4 (% applied radioactivity) in pig manure spiked with Salicylic acid and sampled in winter 2010/2011 (conditions: flow through, 10 % dm, 50 g manure). Values taken from Hennecke et al. (2015)

	incubation time (d)						
	0	1	3	7	14	21	35
CO_2 formation (% radioactivity)	0.0	0.1	2.8	11.5	24.6	35.8	50.7
CH_4 formation (% radioactivity)	0.0	0.0	0.0	0.5	1.1	0.5	4.1
CO_2 + CH_4 formation (% radioactivity)[1]	100.0	99.9	97.2	88	74.3	63.7	45.2

[1] Calculated by 100 – (CO_2+CH_4)