"Bound" residues from biomass and CO_2 in soils – formation, fate and stability during biotic incubation

Der Fakultät für Mathematik, Informatik und Naturwissenschaften der Rheinisch-Westfälischen Technischen Hochschule Aachen vorgelegte Dissertation zur Erlangung des akademischen Grades einer Doktorin der Naturwissenschaften

von
Diplom-Ingenieurin im Umweltschutz

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Parts of this thesis are published or submitted for publication in scientific journals:

NOWAK, **K.M.**; MILTNER, A.; GEHRE, M.; SCHÄFFER, A.; KÄSTNER, M. Formation and fate of bound residues from microbial biomass during 2,4-D degradation in soil, *Environ. Sci. Technol.*, in press;

NOWAK, **K.M.**; GIRARDI, C.; MILTNER, A.; GEHRE, M.; SCHÄFFER, A.; KÄSTNER, M. Formation and fate of non-extractable residues during microbial degradation of $^{13}C_6$-ibuprofen in soil (in preparation);

GIRARDI, C.; **NOWAK**, **K.M.**; LEWKOW, B.; MILTNER, A.; GEHRE, M.; KÄSTNER, M. Comparison of microbial degradation of the C-isotope-labelled pharmaceutical ibuprofen and the herbicide 2,4-D in water and soil, submitted to *Environ. Pollut.*

This thesis is dedicated to the memory of my father,
Prof. Dr. habil. Grzegorz Nowak (1946 – 2002),
who inspired my fascination in environmental science

LIST OF ABBREVIATIONS

^{13}C	Labelled (stable isotope)
^{14}C	Labelled (radioactive isotope)
2,4-D	2,4-Dichlorophenoxyacetic acid
2,4-DCP	2,4-Dichlorophenol
2-OH-ibu	Hydroxyibuprofen
a (for FA)	Anteiso
AA	Amino Acids
AC	Acetylchloride
ACN	Acetone
Ala	Alanine
ASE	Accelerated Solvent Extraction
Asp/Asg	Aspartate/Asparagine
BAME	Bacterial Acid Methyl Esters
bioAA	Biomass Amino Acids
br (for FA)	Branched
C_{org}	Organic Carbon
cy (for FA)	cyclopropyl
DCM	Dichloromethane
Dept	Department
DT_{50}	Half -life in soil
DW	Dry Weight
EA-C-irMS	Elemental Analyser-Combustion-isotope ratio Mass Spectrometry
EDTA	Ethylenediaminetetraacetic acid
FA	Fatty Acids
FAME	Fatty Acid Methyl Esther
FAME 21:0	Heneicosanoate methyl ester
GC-C-irMS	Gas Chromatography Combustion-isotope ratio Mass Spectrometry
GC-MS	Gas Chromatography Mass Spectrometry
Glu/Gln	Glutamate/Glutamine
Gly	Glycine
i (for FA)	Iso
Ibu	Ibuprofen

Ile	Isoleucine
IP	Isopropanol
IUPAC	The International Union of Pure and Applied Chemistry
Koc, $logK_{oc}$	Soil-sorption coefficient
Kow, $logK_{ow}$	Octanol/water partition coefficient
Leu	Leucine
Lys	Lysine
M	Molar concentration
Me (for FA)	Mid-chain branched
MeOH	Methanol
MM	Minimum Medium
Na_2CO_3	Sodium bicarbonate
n.d.	not detectable
NER	Non-Extractable Residues
NH_4OH	Ammonium Hydroxide
nm	Nanometre
N_{Total}	Total Nitrogen
OD	Optical Density
OECD	Organization for Economic Co-operation and Development
OM	Organic Matter
PAH	Polycyclic Aromatic Hydrocarbons
PB	Phosphate Buffer
PEG 600	Polyethylenglycol 600
Phe	Phenylalanine
PLFA	Phospholipid Fatty Acids
Pro	Proline
Ser	Serine
SOM	Soil Organic Matter
SPE	Solid Phase Extraction
tAA	Total Amino Acids
TCC	Tricarboxylic acid Cycle
tFA	Total Fatty Acids
TFAA	Trifluroacetic Acid Anhydride
Threo	Threonine

TM	Tryptone Medium
TMCS	Trimethylchlorosilane
TNT	Trinitrotoluene
TOC	Total Organic Carbon
UFZ	Centre for Environmental Research
USA	United States of America
UV/VIS	Ultraviolet-Visible Spectrophotometry
Val	Valine
WHC	Water Holding Capacity
WWTP	Wastewater treatment plant

SUMMARY

Biodegradation of organic pollutants in soil generally results in the formation of metabolites, microbial biomass, mineralisation products and bound or non-extractable residues (NER). It is speculated that NER can pose a risk for humans due to the remobilisation and further distribution of active parent compounds or their metabolites over the food web (BARRACLOUGH ET AL., 2005). Although there are many studies on NER formation available, their chemical structures are still unknown. However, without this knowledge, a proper risk assessment for the pollutant and the related NER during its transformation in soil is impossible. Part of the NER may be biogenic, since the pollutant-derived C and CO_2 released from its mineralisation are assimilated by microorganisms into their cellular components [e.g. fatty acids (FA) and amino acids (AA)], which are subsequently incorporated into soil organic matter (SOM) after cell death

In order to study the microbial biomass contribution to NER formation, soil was incubated with either ^{13}C-labelled 2,4-Dichlorophenoxy acetic acid (2,4-D) or ibuprofen (Ibu) for 64 and 90 days, respectively. At different sampling dates, the soil was analysed for the presence of the ^{13}C label in FA and AA. The ^{13}C label assimilated in biomolecules was determined in both the living and the non-living SOM fractions. Moreover, for investigating the relevance of heterotrophic CO_2 fixation for the incorporation of ^{13}C into biogenic NER in soil and to distinguish between formations of NER directly from 2,4-D and via CO_2, an experiment with unlabelled 2,4-D and labelled CO_2 was conducted. In addition, to understand both the processes of the biogenic NER formation and the incorporation of ^{13}C label into FA and AA from either $^{13}C_6$-2,4-D or $^{13}CO_2$ in the complex soil environment, a simple biological model system with the known 2,4-D degrader (*Cupriavidus necator* JMP 134), was studied.

After 64 days of $^{13}C_6$-2,4-D incubation in soil, the total contents of ^{13}C detected in AA in SOM indicated that 44% of the initially applied $^{13}C_6$-2,4-D equivalents had been converted to microbial biomass and finally to biogenic residues. The intermediate maximum of ^{13}C-FA in SOM indicated a 20% conversion of $^{13}C_6$-2,4-D to biomass. However, ^{13}C-FA in the non-living SOM fraction decreased to 50% indicating their metabolisation and their further distribution within the food web. Contrary to the ^{13}C-FA, ^{13}C-AA in non-living SOM pool were surprisingly stable.

The soil experiment with $^{13}CO_2$ showed that after 16 days of incubation, the heterotrophic CO_2 fixation was relevant in the assimilation of ^{13}C label into biomass components, in particular FA. In addition, the liquid culture experiment with *C. necator* JMP 134 demonstrated the high importance of CO_2 fixation in the incorporation of ^{13}C label into

the biomass components in the later phase of incubation. In this study about 4% of ^{13}C derived from $^{13}C_6$-2,4-D was assimilated in the biomass of this bacterial strain via CO_2 fixation.

A total content of biogenic NER of 54% of $^{13}C_6$-ibu equivalents at the end of the $^{13}C_6$-ibu soil experiment was indicated by the amount of ^{13}C found in AA in SOM. From the maximum content of ^{13}C-FA detected in this experiment, at least 24% (of $^{13}C_6$-ibu equivalents) biomass must have been formed. Contrary to the $^{13}C_6$-2,4-D soil incubation experiment, the ^{13}C-FA remained stable until the end. However, the formation of NER in the soil incubated with $^{13}C_6$-ibu started much later than that in the $^{13}C_6$-2,4-D study, due to the longer lag phase and the interruption of the incubation before the destabilisation of biogenic NER. The ^{13}C-AA in SOM remained also stable as found in $^{13}C_6$-2,4-D experiment.

The results from these soil biodegradation experiments provide the first evidence that nearly all NER both from 2,4-D and Ibu are biogenic, containing only natural microbial biomass components stabilised in SOM. However, biogenic residues are formed if the respective organic contaminant is readily degraded by microorganisms under significant formation of CO_2. Depending on the yield coefficients of C conversion into biomass, we expect ratios of biomass plus biogenic residues to CO_2 of about 0.2 to 1. In the $^{13}C_6$-2,4-D study, the ratio was ~ 0.8 and in the $^{13}C_6$-ibu was 1.2.

Biogenic residues are clearly excluded from the definition of NER according to IUPAC. Due to biogenic residue formation, the potential risk of NER from the readily metabolised organic contaminants in soils is thus highly overestimated in many cases. Therefore, the formation of biogenic residues must be taken into consideration, when determining the mass balances of contaminants during their microbial degradation in soil.

ZUSAMMENFASSUNG

Biologischer Abbau von organischen Schadstoffen im Boden führt generell zur Mineralisierung und zur Bildung von Metaboliten und gebundenen oder nicht-extrahierbaren Rückständen (NER). Es wird spekuliert, dass NER ein Risiko für den Menschen darstellen können, da sie möglicherweise remobilisiert werden können und die aktiven Ausgangsverbindungen oder deren Metabolite weiter in der Nahrungskette verbreitet werden können (BARRACLOUGH AT AL., 2005). Trotz vieler Studien über die NER-Bildung sind ihre genauen chemischen Strukturen noch nicht bekannt. Allerdings ist ohne dieses Wissen eine korrekte Risikobewertung für den Schadstoff und die damit verbundenen NER während der Transformation im Boden unmöglich. Ein Teil der NER könnten biogen sein, weil der schadstoffbürtige Kohlenstoff und das durch Mineralisierung freigesetzte CO_2 von Mikroorganismen in ihre zellulären Bestandteile eingebaut werden, z.B. Fettsäuren (FS) und Aminosäuren (AS), die nach dem Zelltod in der organischen Bodensubstanz (OBS) festgelegt werden können.

Um den Beitrag der mikrobiellen Biomasse zur NER-Bildung zu untersuchen, wurde ein Boden mit ^{13}C-markierter 2,4-Dichlorphenoxyessigsäure (2,4-D) bzw. Ibuprofen (Ibu) für 64 bzw. 90 Tagen inkubiert. Die Bodenproben wurden zu verschiedenen Probenahmezeitpunkten auf die ^{13}C-Anreicherung in FS und AS analysiert. Die in den Biomolekülen gebundene ^{13}C-Markierung wurde sowohl in der lebenden als auch in der toten OBS Fraktionen bestimmt. Außerdem wurden Experimente mit unmarkiertem 2,4-D und markiertem CO_2 durchgeführt, um den Beitrag der heterotrophen CO_2-Fixierung zum Einbau von ^{13}C in biogene NER in Böden zu untersuchen. Damit kann zwischen direkter NER-Bildung aus 2,4-D und indirekter über CO_2 unterschieden werden. Zudem wurde ein einfaches biologisches Modellsystem mit dem bekannten 2,4-D Abbauer *Cupriavidus necator* JMP 134 untersucht, um sowohl die an der Bildung von biogenen NER beteiligten Prozesse als auch den Einbau von ^{13}C aus $^{13}C_6$-2,4-D bzw. $^{13}CO_2$ in FS und AS in der komplexen Bodenumwelt besser zu verstehen.

Die Menge der gesamten ^{13}C-AS in SOM nach 64-tägiger Inkubation mit $^{13}C_6$-2,4-D im Boden deutet darauf hin, dass 44% der anfänglichen $^{13}C_6$-2,4-D-Äquivalente in mikrobielle Biomasse und schließlich in biogene Rückstände umgesetzt wurden. Das zwischenzeitliche Maximum der ^{13}C-FS in der organischen Bodensubstanz (SOM) zeigte, dass 20% des $^{13}C_6$-2,4-D in mikrobielle Biomasse umgesetzt wurden. Allerdings sanken die ^{13}C-FS in der toten SOM Fraktion wegen ihrer Metabolisierung und ihrer weiteren Verbreitung im Nahrungsnetz

auf 50% ab. Im Gegensatz zu den ^{13}C-FS waren ^{13}C-AS im toten SOM Pool überraschend stabil.

Das Bodenexperiment mit $^{13}CO_2$ zeigte nach 16-tägiger Inkubation die Relevanz der heterotrophen CO_2-Fixierung bei der Assimilation von ^{13}C in die Bestandteile der mikrobiellen Biomasse, insbesondere in die FA. Zudem wies das Flüssigkultur Experiment mit *C. necator* JMP 134 auf die hohe Bedeutung der CO_2-Fixierung für den Einbau der ^{13}C-Markierung in die Biomasse-Bestandteile während der späteren Phase der Inkubation hin. In dieser Studie wurde etwa 4% des 2,4-D-bürtigen Kohlenstoffs über CO_2-Fixierung in die Biomasse dieses Bakterienstamms assimiliert.

Am Ende des Bodenexperiments mit $^{13}C_6$-Ibu wies die Menge der ^{13}C-AS in der SOM auf einen gesamten biogenen NER-Gehalt von 54% der $^{13}C_6$-Ibu-Äquivalente hin. Auf der Basis des maximalen ^{13}C-FA Gehalts, der in diesem Experiment gefunden wurde, müssen mindestens 24% (der $^{13}C_6$-Ibu-Äquivalente) Biomasse gebildet worden sein. Im Gegensatz zur Inkubation des Bodens mit $^{13}C_6$-2,4-D blieben die ^{13}C-FA im Falle von $^{13}C_6$-Ibu bis zum Ende des Experiments stabil. Allerdings begann die NER-Bildung in dem Boden, der mit $^{13}C_6$-Ibu inkubiert wurde, viel später als in der $^{13}C_6$-2,4-D-Studie, was auf der längeren Lag-Phase und dem Abbruch der Inkubation vor der Destabilisierung der biogenen NER beruht. Die ^{13}C-AA in der SOM blieben ebenfalls stabil, wie bereits im $^{13}C_6$-2,4-D Experiment.

Die Ergebnisse dieser Abbauexperimente im Boden liefern den ersten Beweis dafür, dass sowohl im Falle des 2,4-D als auch des Ibu fast die gesamten NER biogen sind. Sie enthalten deshalb nur natürliche Bestandteile der mikrobiellen Biomasse, die in der SOM stabilisiert sind. Die Bildung biogener Rückstände ist vor allem dann von Bedeutung, wenn der jeweilige organische Schadstoff durch Mikroorganismen leicht abbaubar ist und dabei erhebliche Mengen CO_2 gebildet werden. Abhängig vom Ertragskoeffizienten der C Umwandlung in Biomasse, erwarten wir Verhältnisse von Biomasse plus biogene Rückstände zu CO_2 von etwa 0,2 bis 1. In der $^{13}C_6$-2,4-D-Studie war dieses Verhältnis ~ 0,8 und in der $^{13}C_6$-ibu betrug es 1,2.

Biogene Rückstände sind eindeutig von der NER-Definition nach IUPAC ausgeschlossen. Da sie aber analytisch in der Regel nicht von direkt schadstoffbürtigen NER abgetrennt werden, wird das potenzielle Risiko von NER aufgrund der biogenen Rückstandsbildung aus leicht abbaubaren organischen Schadstoffen in Böden oft weit überschätzt Deswegen sollte die biogene Rückstandsbildung bei der Erstellung von Massenbilanzen für Schadstoffe während ihres mikrobiellen Abbaus im Boden berücksichtigt werden.

1 INTRODUCTION

Soil as a very complex medium with a large number of interaction sites is a major sink for xenobiotics (KÄSTNER, 2000), which are released in huge amounts due to human activities. Organic contaminants, which enter the soil environment harbouring an enormous number and high diversity of bacteria, are generally subject to microbial degradation. Microbial degradation of these contaminants is often the main pathway of their disappearance from soils (WALDMAN AND SHEVAH, 1993; EDGEHILL AND FIN, 1983).

Microbial degradation of organic pollutants in soil generally results in the formation of metabolites, microbial biomass, mineralisation products (CO_2 and H_2O) and bound or Non-Extractable Residues (NER; KÄSTNER, 2000). The process of the NER formation from organic contaminants as well as their stability in soils over time has gained strong interest as a subject of research in the recent years. It is generally believed that NER are formed as a result of the various physicochemical interactions between parent compound or its metabolites and soil organic matter [(SOM); BOLLAG ET AL., 1992; SENESI, 1992; VERSTRAETE AND DEVLIEGHER, 1996; FÜHR, 1998; ALEXANDER, 2000; GEVAO ET AL., 2000; LOISEAU AND BARRIUSO, 2002; MORDAUNT ET AL., 2005]. The formation of these residues in soil decreases the bioavailability of contaminants and thereby reduces their toxicity (FÜHR, 1998; NORTHCOTT AND JONES 2000). Enhanced organic contaminants transformation into NER is thus actually suggested as highly desirable process for the natural elimination of the contaminants from soils (BOLLAG, 1992; BERRY AND BOYD, 1985; VERSTRAETE AND DEVLIEGHER, 1996). On the other hand, recent studies on the stability of these residues have revealed that they are not always irreversibly bound to SOM and can be remobilised (BURAUEL AND FÜHR, 2000; BOIVIN ET AL., 2005; GEVAO ET AL., 2005; LERCH ET AL., 2009B). Thus toxic acive compounds and their derivatives of unknown structure can be spread to other compartments of the environment, such as surface- and ground waters, which may finally result in their distribution over the food web (BARRACLOUGH ET AL., 2005). In terms of the public health safety these potential risks related to NER formation in soils make this natural detoxification process one of the hot topics of the world-wide scientific debate.

Most of the available studies on the formation and the fate of NER are performed with radioactive tracer compounds. However, this labelling technique only allows the quantitative distribution analyses and does not provide any information of the chemical nature of tested compounds (RICHNOW ET AL., 1999). Therefore it is not clear, how NER formed during biodegradation of contaminants in soils are composed and if they really pose a risk for the environment and humans. Stable isotopes allow tracing the flux of pollutant-derived C into

different chemical structures using more sophisticated analytical techniques such as gas-chromatography-mass spectrometry (RICHNOW ET AL., 1999). Therefore, detailed elucidation of the formation and chemical nature of NER during the biodegradation of organic pollutants in soil is possible using these isotopes as a tracer.

Some part of NER formed during the microbial degradation of organic contaminants may be biogenic. It is generally known that a wide variety of microorganisms utilise the pollutant-derived C during its biodegradation in soils for their growth and biomass formation (KÄSTNER AND RICHNOW, 2001). The biomass components are incorporated into the non-living SOM fraction after their death and cell lysis (KINDLER ET AL., 2006, 2009), and thus may contribute to the formation of "biogenic" form of NER.

Contrary to the xenobiotic-derived NER, biogenic residues are composed only of non-toxic microbial components stabilised in the SOM pool, which do not pose any risks for the environment. However, soil is a very complex system, which is not fully understood, thus it is difficult to analyse the NER in SOM and the mechanisms of their formation are not elucidated yet. In addition, all available studies on NER formation during biodegradation of organic contaminants are limited to quantitative analyses. Therefore, it is essential to study the NER structure in detail in order to assess properly the risks related with the NER formation during biodegradation of contaminants in soil.

The overall objective of the present study was to trace the NER formation during biodegradation of organic contaminants in soil, and 2,4-Dichlorophenoxyacetic acid (2,4-D) and Ibuprofen (Ibu) were selected as the model compounds. In order to investigate in detail the fate of these contaminants in the complex soil system, stable isotope tracers (^{13}C) were used for the proper quantitative analyses and for tracing the flux of the pollutant-derived C. The transformation of ^{13}C label from $^{13}C_6$-2,4-D or $^{13}C_6$-ibu into CO_2, biomass components, metabolites, biogenic NER and non-biogenic NER was determined. For assessing the risks related to the NER formation from $^{13}C_6$-2,4-D or $^{13}C_6$-ibu, the estimated biogenic NER contents were compared with the non-biogenic NER amounts.

2 STATE OF THE ART

2.1 Complexity of the soil system

Organic contaminants, which enter the soil system, are subject to various interactions within the complex soil matrix (KÄSTNER, 2000). Basically, these contaminants can be degraded by microorganisms, immobilised in the form of non-extractable residues (NER) via binding processes to soil components, volatilised, leached to the groundwater or taken up by living organisms (**Figure 1**). Besides the physico-chemical properties of the contaminant itself, also the soil components, which create the soil environment, affect the fate of contaminants in this system. These respective components present in soil define the overall structure and the physico-chemical properties of this whole complex system.

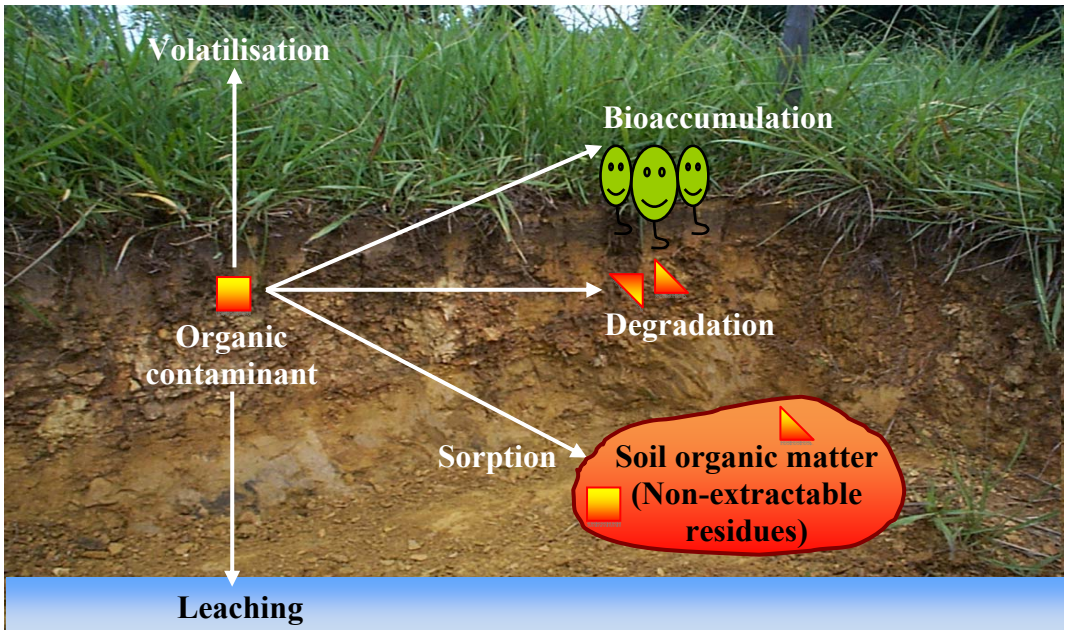

Figure 1. Possible fate of organic contaminant in the soil system (adapted from SEMPLE ET AL., 2003; STOKES ET AL., 2006)

Soil is a heterogeneous system consisting of four phases; the volumetric proportions of which varies over a wide range depending on the soil type (SIMS ET AL., 1990):

1. ***inorganic solids*** (38–45%): which are represented by larger-sized quartz, sand, silt and very fine clay. The larger mineral particles ensure the drainage in soils via formation of macropores, which enable both the transport of water and the distribution of gases within components of soil aggregates;
2. ***organic solids*** (1–12%): so-called soil organic matter (SOM). Although this component makes up the smallest part of the soil system, it is very important, because it affects all physical, chemical and biological soil properties (STEVENSON,

1994). Furthermore, it plays a crucial role in the protection of soil from degradation and erosion (PICCOLO, 1996);

3. ***soil water*** (15–35%): is present in pore and capillary spaces. Water in pore spaces is a solvent for nutrients and salts, which support microbial activity in soil (HAIDER AND SCHÄFFER, 2009). Therefore, microbial biomass tends to concentrate along water flow paths in the soil (VINTHER ET AL., 1999; BUNDT ET AL., 2003). Moreover, it affects the soil aeration status, the soil water osmotic pressure and the pH of soil solution (PAUL AND CLARK; 1989);
4. ***soil gases*** (15–35%): are distributed within pore spaces and are necessary for microbial and plant root respiration.

The distribution and type of solid particles in the soil system defines its overall structure and thus the active surface area affecting the fate of organic compounds (GAVRILESCU, 2005). The interaction of SOM with clay in presence of polyvalent cations (e.g. Fe^{3+}, Ca^{2+}, Al^{3+}) results in the formation of stable clay-organic complexes and thus microaggregates (BRONICK AND LAL, 2005; HAIDER AND SCHÄFFER, 2009). Microaggregates are joined with larger soil particles (e.g. quartz and sand) forming macroaggregates (TISDALL, 1996). This aggregation process plays an important role in shaping the physical, chemical and biological properties of the soil system. Adhesive exopolymers excreted by living microorganisms on soil particles and fungal hyphae on roots stabilise macroaggregates in the form of "sticky string bags" (KÄSTNER, 2000, see **Figure 2**).

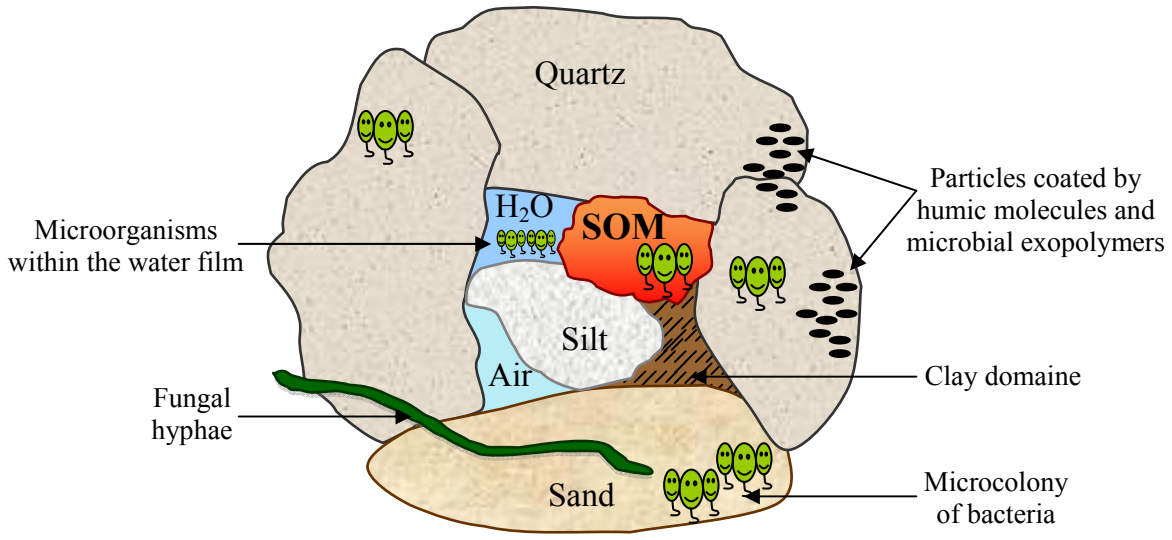

Figure 2. Aggregation of soil constituents (KÄSTNER 2000, ATLAS AND BARTHA 1997)

The water content, the presence of soil biota and the composition of solid components (in particular clays and SOM) affect the aggregate size (BRONICK AND LAL, 2005). Pore sizes and

relative proportions of water and air in the soil system have an impact on the mobility of contaminants (SIMS ET AL., 1990).

The main actors in the interactions with organic contaminants in soils are small-sized soil particles clays and soil organic matter (CALDERBANK, 1989; SIMS ET AL., 1990; STEVENSON, 1994). Clay particles have a large reactive charged surface, thus they are believed to be involved in physico-chemical interactions with organic contaminants (SIMS ET AL., 1990). Interactions with pesticides and other organic chemicals have also been reported (WHITE, 1976; THENG, 1982; MORTLAND, 1986; ZIELKE ET AL., 1989).

SOM possessing many highly reactive functional groups (SIMS ET AL., 1990) was proposed as the dominant factor affecting the organic contaminants interactions within soil systems (BRUSSEAU ET AL., 1991; CORNELISSEN ET AL., 1998). This soil fraction, called "non-living" SOM is a complex mixture of organic materials derived from fragments and decomposed products of plant residues (e.g. proteins, starch, polysaccharides, lignins, lipids), microbial cell components (e.g. lipids, carbohydrates, proteins) and humic substances (KÖGEL-KNABNER, 2002; STEVENSON, 1994; SOLLINS ET AL., 1996; ZECH ET AL., 1997). Because of their small size, microorganisms cannot be separated from SOM and thus form a living fraction of it. The living fraction (1–5% of total SOM) containing a wide variety of microorganisms, higher animals and plant roots (KLEBER ET AL., 1998; OADES, 1995), constitutes a primary source for SOM formation (KELLEHER ET AL., 2006).

Humic substances are considered to be dominant components of SOM (50–60%, PAUL AND CLARK, 1996, SCHNITZER, 1978; STEVENSON, 1994) and show molecular weights ranging from a few hundred to several hundred thousand Daltons (HUANG ET AL., 2003). They are believed to form from decaying plant biomass in a process called "humification" (HAIDER, 1998). The main components of the dead plant biomass, high-molecular-weight polysaccharides and lignin are supposed to affect the size of humic substances (HAIDER, 1998). Humic substances are commonly classified depending on their solubility in alkaline or acidic solutions into humins (insoluble in both solvents), fulvic acids (soluble in acid and alkali) and humic acids [(soluble in alkali and insoluble in acid); NORTHCOTT AND JONES, 2000; STEVENSON, 1994]. This classification into these three fractions is only operational and does not indicate any chemical behaviour or structure of humic acids (HAYES ET AL., 1989; STEVENSON, 1994). Due to the fact that humic substances are very complex heterogenous organic compounds, their overall chemical structure has not been clarified yet (ZIECHMANN, 1994). PAULI (1967) suggested a model, in which humic substances are present as complex soil colloids with micellar structure. In this model, hydrophobic aromatic and aliphatic

building blocks, which are linked by covalent bonding, carry reactive functional groups with a hydrophilic character. Both hydrophilic and hydrophobic sites of humic substances are commonly considered to be involved in various interactions with organic contaminants (KÄSTNER, 2000). Other authors have recently described humic substances as supramolecular associations of low-molecular-mass organic biomolecules (SUTTON AND SPOSITO, 2005), which have been excluded from traditional definitions of humic substances (STEVENSON, 1994; HAIDER, 1998). These low-molecular-mass organic molecules include for example branched and linear alkanes, alkenes, fatty acids, dicarboxylic acids, and long chain alcohols/ethers, ketones/aldehydes, amino acids and esters (FABBRI ET AL., 1996; SCHULTEN, 1999; SCHNITZER, 2000; KRAMER ET AL., 2001; CHEFETZ ET AL., 2002; GRASSET ET AL., 2002; STENSON ET AL., 2002; MUGO AND BOTTARO, 2004). In addition, the hydrophobic properties of humic substances, which have been suggested in the proposed models of humic substances, have not yet been identified (SUTTON AND SPOSITO, 2005). Therefore, SUTTON AND SPOSITO (2005) proposed to reevaluate the biogeochemical pathways of their formation and to redefine the concept of "humification".

KÄSTNER (2000) proposed an other definition of "humification", which is in reality a prolonged stabilisation process of organic components from non-living components of SOM leading to the formation of refractory SOM. This refractory SOM is formed by complex processes, in which metabolites and fragments of microbial and plant biomass metabolised by microorganisms rearrange to form macromolecular aggregates. The continuous process of biomolecules incorporation results in increasing molecular weight and thus in the formation of macromolecules, which are characterised commonly as humins, humic acids and fulvic acids. These macromolecules rearrange with each other, fragments of biomass and clay minerals and finally form larger aggregates. In cultivated soils, 50–75% of SOM is associated with clay-sized organo-mineral particles (CHRISTENSEN, 2001). Due to the fact that SOM is tightly bound to clays in the form of clay-organic complexes (HAIDER AND SCHÄFFER, 2009), it is difficult to distinguish between the contributions of clay and SOM to the interactions with organic contaminants (KÄSTNER, 2000).

2.2 Microbial activity in the soil

Microorganisms are known to play a crucial role in biogeochemical cycles and in sustainable development of the biosphere (VAN HAMME, 2004; ADRIANO AND BOLLAG, 1999). Soil microbes need to cover their energy expenditure by uptake of the products of enzymatic breakdown of organic substrate in order to survive in an active stage (EKSCHMITT ET AL., 2005). The microbial transformation of organic compounds into various metabolites, which

become integral parts of the soil after stabilisation processes, can ultimately define the overall structure and thus the physico-chemical properties of this system (YOUNG AND CRAWFORD, 2004).

Microorganisms, which might be detected in the soil system, are eubacteria, actinomycetes, archaea, fungi, algae, protozoa and viruses (KÄSTNER, 2000). Scientists have estimated that in one gram of fertile soil between 5000–7000 different bacterial species can exist and that their populations can often exceed one hundred million individuals (GAVRILESCU, 2005). The soil systems are heterogeneous habitats, therefore it is difficult to characterise their microbial community in detail and only 1–10% of the microscopically visible soil bacteria were estimated to be actually culturable by artificial media (PICKUP, 1991; LEADBETTER, 1997).

Due to the high abundance and diversity of microorganisms present in soils, most of the natural and anthropogenic organic compounds are subject to microbial attack (KÄSTNER, 2000). Owing to the presence of small-sized microorganisms (few μm) at hardly accessible places within the soil system, organic contaminant, even if occluded within pore spaces, can be degraded. Therefore, soil microbes are the major agents in the disappearance of organic contaminants from soil in the so-called biodegradation process. This decontamination mechanism is considered to be environmentally friendly (GOLOVLEVA ET AL., 1990; SINGH AND WALKER, 2006; MÜLLER ET AL., 2007), because they are capable of degrading of a vast diversity of compounds without hazardous by products. Among the microbial communities, bacteria, fungi, archaea and actinomycetes are the main organic compound degraders (HÄGGBLOM, 1992; MÜLLER ET AL., 2007).

The microbial degradation (biodegradation) of organic compound in soil is dependent on many factors (BOLLAG AND LIU, 1990; SIMS AT AL., 1990; GAVRILESCU, 2005):

- ***Soil conditions:*** temperature, aeration, pH, moisture and SOM content, that influence the activity of soil microorganisms, their size and diversity. In addition, the organic compound availability for microorganisms is closely linked to the soil conditions (e.g. decrease by sorption to SOM or lower pH);
- ***Organic compound characteristics***, which include solubility in water, tendency to adsorb to the soil matrix and persistence in the soil (half-life);
- ***Frequency of organic compound application:*** repeated application of a compound may result in the development of a microbial community capable of the organic compound degradation;

- ***Concentration of organic compound:*** very low concentrations may not provide maintenance energy levels sufficient for microorganisms. Very high concentrations may either be toxic and thus inhibit the microbial activity or, as an easily available substrate, stimulate their growth.

The organic compound characteristics are important in determining its transformation, fate and persistence in the soil system. Among others these characteristics include (AGA AND THURMAN, 2001; ANDREU AND PICÓ, 2004; GAVRILESCU, 2005):

- ***water solubility***, which strongly depends on temperature and pH of the solution. It controls the mobility of organic compound in soil;
- ***soil-adsorption coefficient (K_{oc}, log K_{oc})***, which describes the tendency of a compound to be adsorbed to soil particles. The higher the K_{oc} value, the more strongly the contaminant is sorbed, which leads to reduced availability and mobility;
- ***octanol/water partition coefficient (Kow, log K_{ow})***, which is related to water solubility and soil/sediment sorption coefficients (K_{oc}), for instance: log $K_{ow} < 2.5$ indicates low sorption potential, values log K_{ow} between 2.5 and 4.0 indicate intermediate sorption potential, whereas log$K_{ow} > 4.0$ indicates high sorption potential (JONES-LEPP AND STEVENS, 2007). In addition, K_{ow}, log K_{ow} is widely accepted to describe bioconcentration in aquatic organisms, which is indicative of the tendency for bioaccumulation of a contaminant in organisms;
- ***half-life in soil (DT_{50})***, is the amount of time necessary for disappearance of 50 % of parent compound from soil.

Depending on the chemical nature of a compound, the degradation rate can be different. Easily degradable compounds are usually degraded immediately accompanied by the formation of biomass and mineralisation products (H_2O and CO_2), whereas readily and hardly degradable xenobiotics are degraded at slow rates from the beginning or at higher rates, but after only prolonged phases of adaptation (KÄSTNER, 2000). During degradation, the C derived from the pollutant is used as energy and C source by microorganisms to form their biomass components (MÜLLER ET AL., 2007). However, many organic contaminants are also degraded cometabolically (MÜLLER ET AL., 2007). In this cometabolic process, the organic contaminants do not serve as C or energy source and are metabolised together with another substrate used for growth (SIMS AT AL., 1990; HÄGGBLOM, 1992). This type of transformation is frequently based on metabolic reactions catalysed by extracellular enzymes present in soils (MÜLLER ET AL., 2007; SIMS ET AL., 1990).

2.2.1 Natural organic compounds biodegradation in soil

The high-molecular-weight organic compounds derived from plant and microbial biomass can be mineralised to CO_2 and H_2O with the formation of microbial biomass (see **Figure 3**). After the death of microorganisms, low-molecular-weight microbial compounds are incorporated into SOM and form so-called biogenic residues. Thereafter, these residues are stabilised in SOM, which leads to the formation of refractory SOM (humic substances in "humification"). Biogenic residues, their metabolites and biomass components are also probably parent materials for renewed degradation and conversion reactions (KÄSTNER AND RICHNOW, 2001).

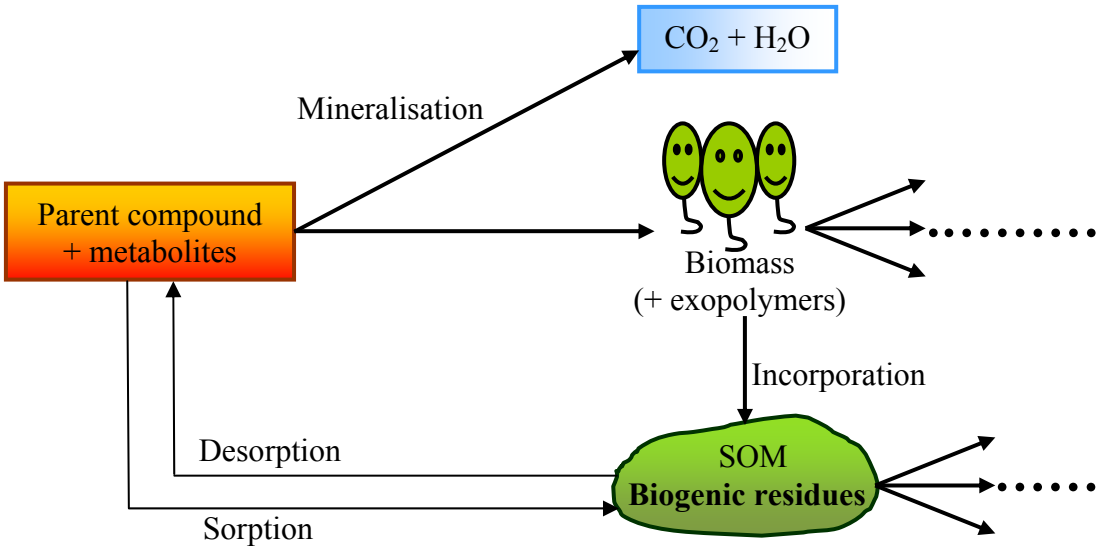

Figure 3. Scheme of C flow during microbial degradation of natural organic compounds in soil (adapted from KÄSTNER AND RICHNOW, 2001)

Formation of biogenic residues (up to 35%) in soil was observed during the biodegradation of plant biomass residues (STOTT ET AL., 1983). In addition, even after a one-year incubation of soil with ^{14}C-glucose, residues derived from this easily degradable compound were still detected in SOM (10%; BALDOCK ET AL. 1989).

Plant residues are suggested to be the primary source for refractory SOM formation (STEVENSON, 1994; SCHOLES ET AL., 1997; HAIDER, 1998; KÖGEL-KNABNER, 2002). This is caused by the relative high inputs to soil and the fact that plant components, in particular high-molecular-weight compounds (e.g. lignin) are believed to be highly resistant to microbial degradation (STEVENSON, 1994; HAIDER, 1998; KÖGEL-KNABNER, 2002). However, recent findings show that lignin is less persistent than the average of bulk SOM (VON LÜTZOW ET AL., 2008; MARSCHNER ET AL., 2008). The importance of microbial biomass C in the formation of SOM is considered to be minor (SCHOLES AND SCHOLES, 1995), because of both its small pool size and fast turnover (JENKINSON AND LADD, 1981; COLEMAN ET AL., 1983).

However, studies on the molecular composition of SOM proved that plant-derived organic matter (OM) was not stored in arable soils, but was transformed to microbial residues, i.e. carbohydrates and proteins, which were kept in soil by organo-mineral interactions (BOL ET AL., 2009). Several recent studies on SOM showed that the contribution of microbial biomass to SOM formation in the generally accepted range of 1-5% is highly underestimated (SIMPSON ET AL., 2007). For instance, soil incubation with ^{13}C-labelled *Escherichia coli* indicated that microbial biomass-derived C significantly contributed to the formation of refractory SOM (LÜDERS ET AL., 2006; KINDLER ET AL., 2006, 2009; MILTNER ET AL., 2009). In this experiment about 56% of the bulk C of *E. coli* was mineralised, the residual 44% was stabilised in the soil after 224 days (KINDLER ET AL., 2006). Further research reported that SOM was predominantly of microbial origin (> 50% of extractable humic acids; SIMPSON ET AL., 2007). The high content of *E. coli* biomass-derived C stabilised in SOM after 224 days thus indicates that microorganisms may form significant amounts of biogenic residues in soil and finally the refractory SOM during biodegradation of organic compounds.

2.2.2 Anthropogenic organic compounds biodegradation in soil

The microbial degradation of organic contaminants in soil is generally understood as their transformation into mineralisation products, metabolites, microbial biomass and non-extractable residues (NER; see mass balance in **Figure 4**).

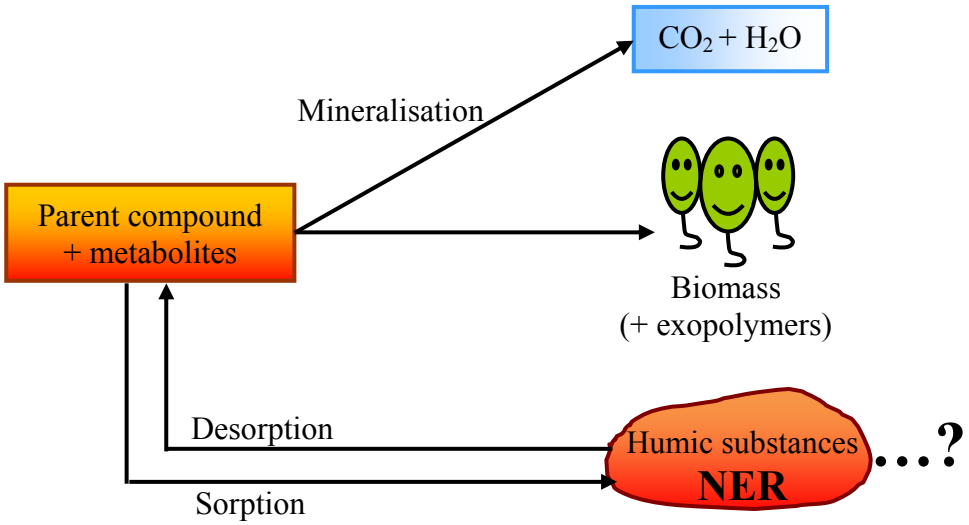

Figure 4. Conventional model of C flow during microbial degradation of organic contaminants in soil (mass balance)

The organic contaminant or products of its partial biodegradation are believed to sorb to SOM via various physico-chemical mechanisms leading to the NER formation (ALEXANDER, 2000; GEVAO ET AL., 2000; GAVRILESCU, 2005; LOISEAU AND BARRIUSO, 2002; MORDAUNT ET AL.,

2005). The sorption of contaminants to SOM is considered as the major factor preventing their complete biodegradation in soil (BÜYÜKSÖNMEZ ET AL., 1999). The mass balance of organic contaminant within soil fractions during its biodegradation is usually determined using radioactive tracers (^{14}C-labelled compounds; GERST AND KLIGER, 1990; KUBIAK ET AL., 1990; RICHNOW, 1999). The rate of microbial transformation of an organic compound is estimated only by the determination of the residual concentrations in soil (FOGARTY AND TUOVINEN, 1991). The analyses of parent compounds and their metabolites remaining in soil solution are usually accomplished using different extraction methods such as batch solvent shaking, Soxhlet extraction, supercritical fluid extraction or accelerated solvent extraction (HAWTHORNE ET AL., 2000; NORTHCOTT AND JONES, 2000) with aqueous or organic solvents. However, these conventional and enhanced solvent extraction techniques do not extract contaminants that are strongly bound or sequestered into the components of soil matrix like SOM (NORTHCOTT AND JONES, 2000).

Therefore, the total amount of unextracted contaminant residues is mostly quantified as $^{14}CO_2$ released from combustion of soil samples (WAIS, 1998; BARRIUSO ET AL., 2008). Using this destructive approach, it is not possible to check if NER are intact contaminants, their metabolites or ^{14}C accumulated in microbial biomass or in humic substances (BARRIUSO ET AL., 2008). Alternatively, the distribution of the ^{14}C in humic acids, fulvic acids and the insoluble humins of soil containing non-extractable contaminant residues is often analysed after the extraction of both fulvic and humic acids (NORTHCOTT AND JONES, 2000). A major obstacle for deeper analysis of these residues is that no proper method for the identification of the chemical nature of the transformation products bound to complex soil matrix using radiotracers is available. Therefore, due to the problems with NER analyses, the detailed biodegradation pathways of many organic contaminants in these complex soil systems have not yet been elucidated (FOGARTY AND TUOVINEN, 1991).

2.3 Bioavailability of organic contaminants in soil

Bioavailability of contaminants is the key factor, which controls their overall fate in soil, in particular their biodegradability and toxicity for biota (SEMPLE ET AL., 2007). Bioavailability is affected by many factors such as: the properties of contaminant and soil, aging time in the soil, climate and organisms of concern (KATAYAMA ET AL., 2010). On the whole, the assessment of the bioavailability of contaminants in soil is necessary both for understanding the risks, which may be posed by these contaminants and for the proper choice of the method for soil remediation (SEMPLE ET AL., 2003).

The term bioavailability has been used in many different scientific fields, thus there are many definitions (SEMPLE ET AL., 2007), which generally consider the interactions between an organism and a chemical (SEMPLE ET AL., 2004). Toxicologists term bioavailability as the fraction of chemical absorbed and able to reach systemic circulation in an organism (SEMPLE ET AL., 2004). For instance, the National Research Council reports:

> "Bioavailability may represent the fraction of a chemical accessible to an organism for absorption, the rate at which a substance is absorbed into a living system or a measure of the potential to cause a toxic effect" (NRC, 2002).

EHLERS AND LUTHY (2003) have stated that:

> "Bioavailability refers to the extent to which humans and ecological receptors are exposed to contaminants in soil or sediment".

Environmental scientists consider bioavailability as the accessibility of soil-bound chemicals for assimilation and possible toxicity (ALEXANDER, 2000). SEMPLE ET AL. (2004) identified the lack of clarity of the term "bioavailability" among environmental scientists, and thus proposed the distinction of two terms bioavailability and bioaccessibility:

> "Bioavailability represents, at a given time, the fraction of the chemical that is freely available to cross an organism's membrane from the medium which the organism inhabits." Bioaccessibility was defined as: "that which is available to cross an organism's (cellular) membrane from the environment it inhabits, if the organism had access to it; however, it may be either physically removed from the organism or only bioavailable after a period of time. Bioaccessibility encompasses what is actually bioavailable now and what is potentially bioavailable" (SEMPLE ET AL., 2004).

Not only bioavailable compounds present in the water-soluble fraction of soil are available, but also these actually desorbed from soil during the time when a target organism is in direct contact with the soil (HARMSEN, 2007). Bioaccessibility includes both the readily available contaminants present in the water-soluble fraction of soil and the contaminants which can become available after desorption from the soil matrix (**Figure 5**). This includes also the contaminants, which may be released after longer timescale (slowly reversible). The accessibility depends on the desorption conditions (e.g. shaking, temperature) and desorption time (REICHENBERG AND MAYER, 2006). Both bioavailability and bioaccessibility of contaminants decrease with increasing contact time of contaminants with soil matrix [(so-called "aging"); REID ET AL., 2000].

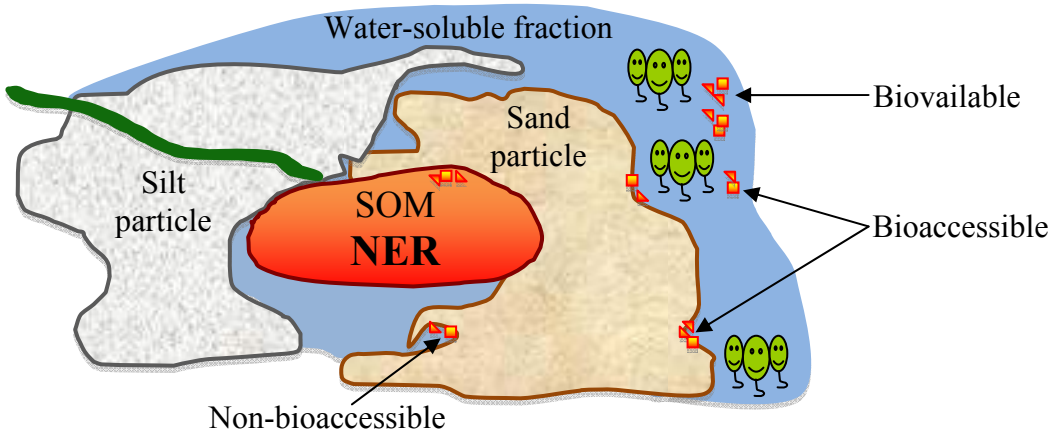

Figure 5. Bioavailability and bioaccessibility of organic contaminants in soil system (adapted from SEMPLE ET AL., 2004); (■) parent compound; (◣) metabolites

A chemical immobilised in SOM or present in the soil solution can become bioaccessible. This involves a number of consecutive steps until eventually the compound is absorbed into an organism (see bioavailability processes A-D in **Figure 6**; EHLERS AND LUTHY, 2003; SEMPLE ET AL., 2004).

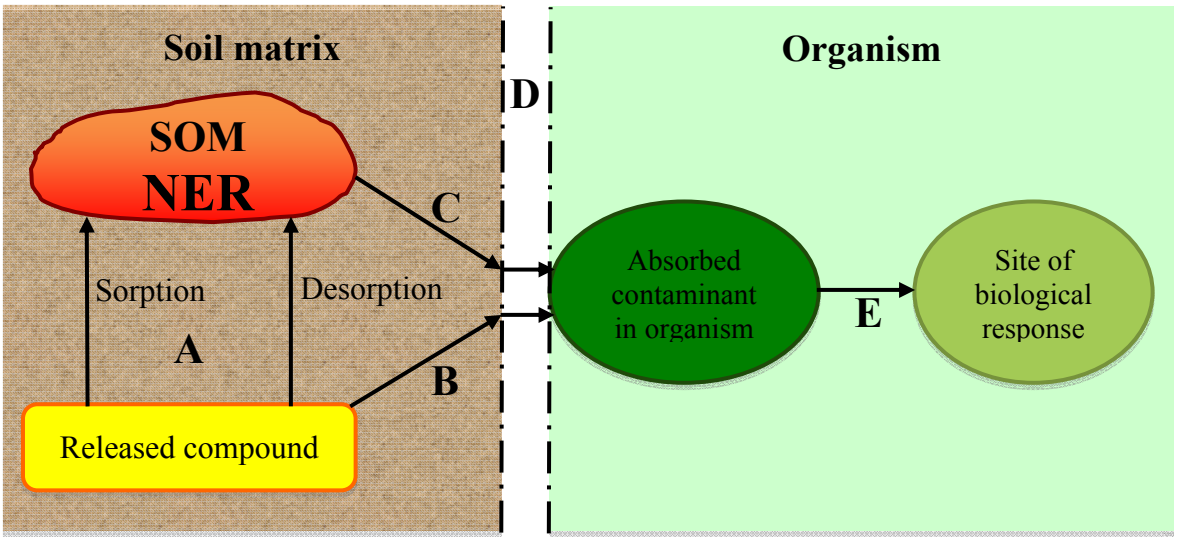

Figure 6. Bioavailability processes in soil system (EHLERS AND LUTHY, 2003; SEMPLE ET AL., 2004)

Process "A" encompasses the possible behaviour of the contaminant within the soil matrix (sorption or desorption). When environmental conditions are changed (e.g. changes in water saturation, pH or temperature) immobilised contaminant in SOM (NER), may be released or transformed into more stable associations over time (aging) and become non-accessible. The release of immobilised contaminant from SOM is the key step toward the assessment of its bioaccessibility and thus the toxicity for living organisms (HARMSEN, 2007). Therefore, the

formation of NER during microbial degradation of organic contaminants in soils is actually discussed in terms of the probability of the risks which they can pose to the environment (BARRACLOUGH ET AL., 2005). Processes B and C include the transport of the contaminant, which is present in the soil solution (B) or bound to soil components (C). D shows the barrier between external environment (soil matrix) and an organism and represents the uptake of the contaminant through the cell membrane into the target organism. E refers to paths taken by contaminant after uptake into the cells (e.g. metabolic transformation or exerting toxic effects within cells) and addresses toxicological bioavailability (EHLERS AND LUTHY, 2003).

Various chemical or biological measurements are employed to assess the bioavailability of an organic contaminant for living organisms. Chemical measurements involve various extraction methods of the contaminant from soil, whereas biological ones are based on monitoring the toxic effects of a contaminant taken up by the target living organism (HARMSEN, 2007).

2.4 Definition of the non-extractable residues (NER)

The term NER has been defined in different ways over the years (MORDAUNT ET AL., 2005). In the past, NER were considered to be pesticide residues remaining in the soil after application until the next growing season or the planting of the following crop (CRAVEN, 2000). Pesticides were and are still of particular interest in this regard, since they have selective activity and are deliberately manufactured for application in terrestrial systems (GEVAO ET AL., 2000).

The first official definition of the term NER was provided in 1975 by the American Institute of Biological Sciences – Environmental Task Group (NORTHCOTT AND JONES, 2000), which stated:

> "bound pesticide residues in the soil are unextractable and chemically unidentifiable pesticide residues remaining in the fulvic, humic acids and humin fractions after exhaustive sequential extraction with nonpolar and polar solvents".

The most accepted and widely used NER definition, proposed by the Applied Chemistry Division, Commission on Pesticide Chemistry of the International Union of Pure and Applied Chemistry (IUPAC), is the following:

> "bound residues (also referred as "non-extractable" residues or "non-extracted" residues) in plants and soils as chemical species originating from pesticides, used according to good agricultural practice, that are unextracted by methods which do not significantly change the chemical nature of these residues. **These residues are**

considered to exclude fragments recycled through metabolic pathways leading to natural products" (ROBERTS, 1984).

Biogenic residues formed during biodegradation of organic compounds are thus excluded from the IUPAC NER definition, because they are composed only of natural compounds derived from microbial biomass stabilised in SOM. However, with the use of radiotracers for estimating of mass balance of the tested contaminant in soil, these natural compounds cannot be excluded from the quantitative analysis of NER.

The IUPAC NER definition was later slightly modified but not substantially changed (NORTHCOTT AND JONES, 2000) and was extended to include that "the NER formation reduces the bioaccessibility and bioavailability significantly" (CALDERBANK, 1989; FÜHR ET AL., 1998).

2.5 Determination of NER in soil

The determination of NER is based on quantitative analyses accomplished after the extraction of residual parent compound and its metabolites from soil as mentioned in section 2.2.2. The distinction between extractable and non-extractable residues depends on extraction methods and conditions employed (KHAN 1991; NORTHCOTT AND JONES, 2000; MORDAUNT ET AL., 2005). Numerous "exhaustive" (harsh) and "non-exhaustive" (mild) extraction methods employed for the assessment of bioavailability of organic contaminants for living organisms show different quantitative recovery of a target compound (NORTHCOTT AND JONES, 2000; SEMPLE ET AL., 2007). The main aim of harsh extraction methods measuring the total concentration of contaminant in soil is to recover all or as much as possible of the contaminant from environmental samples (HATZINGER AND ALEXANDER, 1995; NOORDKAMP ET AL., 1997; ALEXANDER, 2000; REID ET AL., 2000; STOKES ET AL., 2006). These harsh methods involving mostly heated organic solvents include soxhlet extraction, microwave extraction, supercritical fluid extraction, ultrasonication and accelerated solvent extraction [(ASE); NORTHCOTT AND JONES, 2000]. It is generally assumed that the amount of compound recovered by exhaustive soil extraction is 100% available when assessing its potential risk for environment (ALEXANDER, 2000). However, the measurement of total concentration besides the accessible fraction of contaminant may also include parts or the entire recalcitrant fraction of contaminants (e.g. aged or sorbed strongly to SOM). In addition, the soil matrix by these extraction techniques is altered thus no longer represents a real soil (MORDAUNT ET AL., 2005). This occurs, because used organic solvents remove H_2O and SOM (mostly humic acids) from the soil matrix (MORDAUNT ET AL., 2003). Therefore, these harsh methods were considered to be improper for the assessment of the toxicity and thus the risk of soil

associated organic contaminants for the environment (KELSEY ET AL., 1997; GEVAO ET AL., 2003). Mild extractions methods that remove only the "labile" pool of the contaminant (water-soluble and loosely adsorbed contaminants on surface of soil particles) are more useful for the measurement of bioaccessibility of contaminants in soils and their potential risks (KELSEY ET AL., 1997; REID ET AL., 2000; SEMPLE ET AL., 2007). These mild extraction methods involving natural extractants (e.g. ionic solution of $CaCl_2$) mimic solutions likely to be present in the soil (MORDAUNT ET AL., 2003). These methods are aqueous-based extractions including solid-phase extraction (e.g. Tenax TA) and the cyclodextrin technique (REID ET AL., 2000; LISTE AND ALEXANDER, 2002; SEMPLE ET AL., 2007).

Besides the physico-chemical properties of compound and soil, the amount of NER also strongly depends on the extraction method (MORDAUNT ET AL., 2005). Therefore, the methods used for the extraction of contaminants from soil affects also the quantitative analyses of NER. For instance, harsh extraction methods result in lower estimates of NER, whereas the mild ones give higher estimates for NER in soils. Hence, the numerous presented data on the total amounts of NER formed during the microbial degradation of the same model organic contaminant in soils show high variations. Summing up the above, the NER term is defined operationally by the extraction method employed; hence it is important to clarify how the method is developed from this definition and what information this method will provide (MORDAUNT ET AL., 2005).

2.6 Formation of NER in soil

It is believed that formation of NER during biodegradation of a contaminant is based on the various physico-chemical interactions between parent compounds or its metabolites and SOM (BOLLAG ET AL., 1992; SENESI, 1992; VERSTRAETE AND DEVLIEGHER, 1996; FÜHR, 1998; ALEXANDER, 2000; GEVAO ET AL., 2000; LOISEAU AND BARRIUSO, 2002; MORDAUNT ET AL., 2005). However, biodegradation of natural organic compounds in soil results in the formation of biogenic residues (as shown in **Figure 3** in section 2.2.1). Organic contaminants similar to the natural organic compounds are also subject to microbial degradation, which can lead to the formation of organic contaminant-derived biogenic residues. These contaminant-derived biogenic residues are only quantified as NER and no detailed information about their chemical structure is available (RICHNOW ET AL., 2000; KÄSTNER AND RICHNOW, 2001; BARRIUSO, 2008). Therefore, it is necessary to consider the formation of biogenic residues during biodegradation of organic contaminants and to distinguish between formations of NER via components of microbial biomass and via parent compound or its metabolites in soils.

The amounts of NER formed during the biodegradation of organic contaminants depend on the previously mentioned physico-chemical properties of both the contaminant and the soil system. In addition, the position of the label in the molecule of the tested contaminant has an impact on the results on the fate of this contaminant in general including the formation of NER (BARRIUSO ET AL., 2008). For instance, if the label is positioned in a labile molecular fragment of contaminant (which is easily evolved as $^{14}CO_2$), the mineralisation will be overestimated, whereas the detected amounts of NER tend to be low (BARRIUSO ET AL., 2008).

2.6.1 Parent compounds and metabolites

Humic acids as major components of SOM were previously regarded as the main agents leading to the formation of NER in soil (SENESI, 1992; WAIS, 1998). This is caused by the fact that it is believed that humic acids are large aromatic polymers with many binding sites for organic contaminants (hydrophilic and hydrophobic) in their molecular structure (SENESI, 1992).

Investigations of the interactions between organic contaminants and SOM involved various extraction techniques basing mostly on the isolation and fractionation of soil humic substances. To characterise NER, several attempts to cleave bonds of the contaminant with SOM were used. For instance, alkaline hydrolysis was used to cleave organic compounds bound to SOM via ester bonds (RICHNOW ET AL., 1998), and acid hydrolysis to release compounds linked to SOM by ether bonds (RICHNOW ET AL., 1997). However, in most cases, the physico-chemical interactions of contaminants with humic substances were studied in simple systems with solutions containing only humic acids (mainly synthetic) and the contaminant (BOLLAG, 1991; BOLLAG 1992; HATCHER ET AL., 1993; PICCOLO ET AL., 2001). These approaches follow only a specific single reaction or binding mechanism of the respective contaminant to humic acids (NORTHCOTT AND JONES, 2000). The physico-chemical interactions were thus described mainly theoretically. Therefore, these data on the mechanisms of NER formation cannot necessarily be extrapolated to the realistic processes, which take place in complex soil systems, especially when considering the fact that the molecular structure of humic acids is not yet clear.

Organic contaminants are believed to bind to humic acids via various mechanisms of adsorption, covalent bonds and sequestration (BOLLAG ET AL., 1992; WAIS, 1998; GEVAO ET AL., 2000).

Adsorption

Adsorption is the binding of the organic compound to the surface of solid particles through weak and reversible bonds (BÜYÜKSÖNMEZ ET AL., 1999). Minerals, in particular clays and SOM can take part in the sorption of the organic contaminants. The clay minerals are responsible for adsorption of polar and hydrophilic compounds, whereas SOM has both hydrophilic and hydrophobic sites and therefore can interact with polar, charged as well as apolar and lipophilic contaminants (VERSTRAETE AND DEVLIEGHER, 1996). This type of association is reversible and leads to the formation of unstable NER subject to microbial degradation (VERSTRAETE AND DEVLIEGHER, 1996). This can be caused by the fact that microorganisms, which colonise surfaces of soil particles, might have direct access to adsorbed chemicals and degrade them.

The extent of adsorption depends on the properties of the soil (e.g. SOM content) and the nature of contaminant e.g. if it is acidic or alkaline (GEVAO ET AL., 2000). Adsorption occurs via several mechanisms like ionic and hydrogen bonding, charge-transfer, ligand exchange, van der Waals forces and hydrophobic bonding (KHAN, 1978; PIGNATELLO, 1989; GEVAO ET AL., 2000).

Ionic binding: this type of binding occurs with those contaminants and their metabolites that exist in the cationic form or can become cationic upon protonation (SENESI, 1992; WAIS, 1998; GEVAO ET AL., 2000). These contaminants react with ionised or easily ionisable carboxylic and phenolic hydroxyl groups of humic acids or hydroxyl groups of minerals (SENESI, 1992).

Hydrogen binding: nonionic contaminants can interact with oxygen- and nitrogen-containing functional groups at the surfaces of humic substances or oxygen- and nitrogen-containing functional groups of the contaminant can react with hydrogen containing groups of SOM (WAIS, 1998).

Charge-transfer complexes: also called donor-acceptor complexes are formed when molecules with a high electron density (e.g. π-electrons in aromatic systems) react with electron-deficient molecules (e.g. quinones; WAIS, 1998).

Ligand exchange: in this type metal ions complexed by humic acids are usually associated with water molecules, which can be replaced by functional groups (e.g. carboxylic or amino groups) of organic contaminants (WAIS, 1998). However, this bonding is rare and weak and thus plays minor role.

Van der Waals forces: are relatively weak short-range dipolar or induced-dipolar attractions that exist in presence of stronger binding forces, in all adsorbent-adsorbate interactions (GEVAO ET AL., 2000).

Hydrophobic bonding: is the interaction mechanism between hydrophobic groups of humic substances and non-polar contaminants (WAIS, 1998). Active partners are aliphatic side chains, fat constituents or lignin components of humic substances (SENESI, 1993).

Covalent bonding

Covalent bonding is a chemical interaction between a contaminant or its metabolites and humic substances driven by strong bonding forces (> 300kJ/mol; BLASCHETTE, 1974). This type of bonding is generally accepted to be an irreversible and very stable association (SENESI, 1992; WAIS, 1998), which results in the formation of persistent contaminant NER in soil. These interactions can be mediated by enzymatic, chemical or photochemical catalysts (SENESI, 1992; GEVAO ET AL, 2000) and result in ester, ether or carbon-carbon linkages (KÄSTNER AND RICHNOW, 2001). The organic contaminants, which are bound covalently to soil humic acids, are integral components of humic substances (BOLLAG ET AL., 1992; SENESI, 1992). Thereafter, they are subject to all further transformation processes involved in humification (WAIS, 1998). The residues bound chemically to soil humic acids are considered to be toxicologically inactive (CALDERBANK, 1989), because they are not bioavailable. However, the exact chemical nature and structure of this type of NER in SOM have not yet been elucidated for all xenobiotics (KATAYAMA ET AL., 2010). The studies on NER formation by covalent bonding are limited to simple humic acids-contaminant systems (BOLLAG, 1991, 1992; HATCHER ET AL., 1993).

Sequestration (Aging)

Sequestration (aging) contrary to the adsorption mechanisms does not include reactions that alter the structure of an organic contaminant (HATZINGER AND ALEXANDER, 1995). Sequestration is also referred to a slow sorption/diffusion (PIGNATELLO AND XING, 1996; DEC AND BOLLAG, 1997) of non-polar and hydrophobic compounds, which in comparison to adsorption is a very long-term process (GEVAO ET AL., 2000). During aging, the molecule becomes progressively more tightly bound or entrapped in SOM and correspondingly less bioavailable (BARRACLOUGH ET AL., 2005). The size distribution of micropores is a factor in aging: the smaller the micropore, the slower aging proceeds, but the effect is stronger (ALEXANDER, 2000; KATAYAMA ET AL., 2010). Aging may also result from covalent bonding of contaminant with soil humic acids after sorption inside or within micropores in soil aggregates (ALEXANDER, 2000; KATAYAMA ET AL., 2010).

2.6.2 Components of microbial biomass

Several studies showed that the formation of NER from degradation of most pesticides is often related to soil biological activity and to the amount of SOM present in the soil (KAUFMANN AND BLAKE, 1973; ABDELHAFID ET AL., 2000A, B). For instance, soil microbial activity, which is generally higher in topsoil layers, induces the formation of NER in higher amounts than in deeper soil horizons (SCHIAVON, 1988; BALUCH ET AL., 1993; STOLPE AND SHEA, 1995; RICE ET AL., 2002). In addition, soil amendments with organic materials such as manure and straw enhanced the formation of NER and the dissipation of pesticides (DOYLE ET AL., 1978; PRINTZ ET AL., 1995). For example, during the incubation of composted non-sterile straws with 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4-dichlorophenol (2,4-DCP) or 4-chlorophenol, the contents of NER were high, whereas in sterile ones these values were negligible (BENOIT AND BARRIUSO, 1997). The addition of glucose, which usually induces the microbial activity, increased the rate of NER formation in soil amended with atrazine in comparison to a soil without glucose application (ABDELHAFID ET AL., 2000A, B).

Also for Polycyclic Aromatic Hydrocarbons (PAH) adsorption and covalent bonding were proposed as possible mechanisms of NER formation in soils. However, the molecular analyses of SOM by pyrolysis gas chromatography-mass spectrometry after alkaline hydrolysis of ester bonds revealed that the contents of both parent compounds and known metabolites of PAH were very low (0.5% of the initial amount of PAH; RICHNOW, ET AL., 1994). Other study on the ^{15}N-labelled simazine-derived NER in ^{15}N-depleted plant compost indicated that these NER contained no parent compound and were composed of degradation products resulting from N-dealkylation and triazine ring destruction (BERNS ET AL., 2005). KÄSTNER ET AL. (1999) suggested for the first time that NER formed during biodegradation of PAH (9-[^{14}C]-anthracene) could be of "biogenic" origin. In their experiments, addition of compost led to higher mineralisation rate of anthracene and lower amounts of NER in comparison with the native soil (see **Table 1**). The amount of NER in the experiment with 9-[^{14}C]-anthracene was relatively high, in spite of the fact that the C at the labelled position is subject to release as CO_2. In addition, an experiment with $^{14}CO_2$ (in the amount corresponding to the final mineralisation of 9-[^{14}C]-anthracene) showed that the label was found mostly in NER. This indicates that they were not directly formed from anthracene, but via CO_2 fixation and microbial biomass. In addition, the partitioning of ^{14}C in humines, humic and fulvic acids in the $^{14}CO_2$ experiment was similar to the one in the 9-[^{14}C]-anthracene experiment. The amounts of ^{14}C originating from $^{14}CO_2$ in NER of soil inhibited with $CHCl_3$ were significantly lower than in soil without $CHCl_3$ treatment.

Table 1. Distribution of ^{14}C after microbial degradation of 9-[^{14}C]-anthracene and in native soil and soil-compost-mixture (after 176 days) and after incubation under $^{14}CO_2$-atmosphere [(after 90 days); from KÄSTNER ET AL. 1999]

Mass balance	Applied radioactivity [%] 9-[^{14}C]-anthracene		$^{14}CO_2$	
	Native soil	Soil-compost [a]	Soil-compost [a,b]	+$CHCl_3$ [a,b,c]
Total recovery	98.7	91.9	99.8	98.7
CO_2	43.8	67.2	4.6 [e]	11.3 [e]
Total soil	54.9	24.6	95.2 [f]	87.4
Extraction+alk. hydrolysis	9.5	3.9	9.4	44.2
"bound" residues	45.4	20.7 [d]	85.8	43.2

[a] soil-compost-mixture (80% : 20% dry weight); [b] 90d; [c] microflora inhibited by fumigation with $CHCl_3$
[d] amount represents 25.5 Bq/g of soil; [e] recovered $^{14}CO_2$ (initially applied amount: 24.8 Bq/g of soil)
[f] 31% bound in acid labile carbonates, 67% bound to SOM, < 2% bound to clays and silicates

The above-mentioned studies clearly indicate that in many cases microorganisms mediate NER formation in soils. As already mentioned, the C derived from organic contaminants can be used for the formation of microbial biomass during their biodegradation in soil (MÜLLER ET AL., 2007). The CO_2 evolved during mineralisation of organic contaminants can be utilised as an additional C source by soil microorganisms, since even heterotrophic microorganisms need CO_2 for their growth (KREBS, 1941). PEREZ AND MATIN (1982) showed that during heterotrophic growth about 10% of the cell C originated from CO_2. Another experiment with labelled CO_2 in the atmosphere proved that soil microorganisms assimilated C and incorporated the label into SOM (MILTNER ET AL. 2004, 2005). Therefore, NER formed during biodegradation of organic contaminants in soil, can in reality be biogenic residues (KÄSTNER AND RICHNOW, 2001; **Figure 7**), the formation of which is also observed during biodegradation of natural organic compounds (**Figure 3** in section 2.2.1; KÄSTNER, 2000).

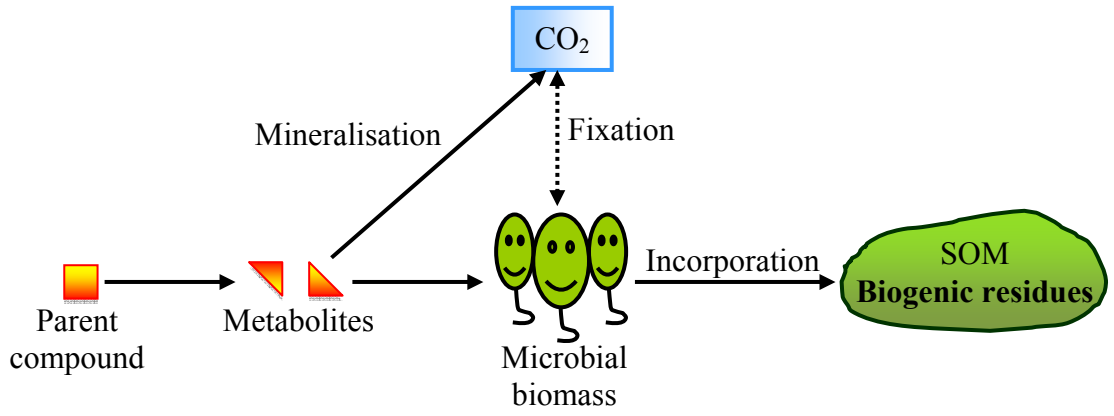

Figure 7. Biogenic residues formation during microbial degradation of organic contaminants in soil

In addition, when labelled contaminants are used for soil biodegradation studies, a certain amount of this label incorporated into microbial biomass components, which thereafter are

stabilised in SOM, will be detected as NER. Biogenic residues can be formed via direct incorporation of C from pollutant or indirectly via fixation of CO_2 released during mineralisation of the contaminant (see **Figure 7**).

Fatty acids and amino acids as representatives for biogenic residues

Fatty acids (FA) and amino acids (AA) that are known microbial biomarkers (BOSCHKER AND MIDDELBURG, 2002) can be incorporated into SOM and thus form biogenic residues. Free and bound lipids, amino acids and carbohydrates form the major fraction of analytically recognisable compounds in SOM of various origin (ALLARD, 2006). In addition, the extraction of known microbial biomarkers (e.g. FA and AA) from soil after addition of C isotope tracer allows an estimation of microbial activity in the biogeochemical cycling of C (PELZ ET AL., 1998). Biomarker analysis is thus also useful in studying the microbial transformation of labelled organic contaminants in soil (BOSCHKER AND MIDDELBURG, 2002). Therefore, the analysis of C isotope label distributions in FA and AA within the living and non-living SOM fraction enables understanding the formation and the fate of biogenic residues during biodegradation of a labelled organic contaminant in soil.

FA are major constituents of the soil lipids (PAUL AND CLARK, 1996) and may originate from both plant residues and soil organisms (STEVENSON, 1994). This biomarker is used for identification of microbial populations involved in specific geochemical transformations, e.g. organic contaminant degradation in soils after deliberately added tracers (BOSCHKER AND MIDDELBURG, 2002). Short-chain FA ($C:_4$-$C:_{20}$) are typical for microorganisms (SCHNITZER ET AL., 1986). On average, FA represent about of 5% of the dry weight (DW) of the microbial biomass (BAS ET AL., 2003). Phospholipid fatty acids (PLFA) are characteristic for the lipids of living cells (ZELLES, 1999). PLFA are known to be stable only in intact cells and are hydrolysed within weeks after their death (VESTAL AND WHITE ET AL., 1989). They therefore can be used as biomarkers for representative groups of the living microorganisms in the environment (GREEN AND SCOW, 2000; KAUR ET AL., 2005; ZELLES, 1999). The characteristic chain lengths of ester-linked saturated FA of PLFA range from $C_{14:0}$ to $C_{18:0}$ for bacteria and from $C_{14:0}$-$C_{24:0}$ for fungi (ZELLES, 1997). Several authors (GREEN AND SCOW, 2000; KAUR ET AL., 2005; ZELLES, 1999) proposed that the following five classes of PLFA are typical for specific groups of microorganisms: (1) saturated straight chain PLFA for all microorganisms, (2) saturated methyl branched PLFA for Gram-positive bacteria, (3) monounsaturated PLFA for Gram-negative bacteria, (4) polyunsaturated PLFA for fungi and (5) saturated cyclopropyl PLFA for starving Gram-negative bacteria.

AA, which account for 10–20% of total C and 30–50% of total N in SOM, are mainly present as polymers e.g. proteins, protein-humic complexes and peptides (STEVENSON, 1982). AA are also the most abundant components (55% of dry weight) of bacterial cells (MADIGAN AND MARTINKO, 2006). Total free AA concentrations in soil are very low representing < 0.05% of total soil N (JONES AT AL., 2005) and have very short turnover times (JONES, 1999), because they are easily available and degraded immediately by soil microorganisms.

Two biodegradation studies with labelled organic contaminants showed that the label was incorporated into AA. In the first biodegradation experiment with tar oil contaminated soil spiked with 1-[^{13}C]-phenanthrene significant amounts of the label were found in hydrolysable AA, which represented 11% of total NER (RICHNOW ET AL., 2000). The second experiment studying the fungal degradation of [^{15}N]-Trinitrotoluene (^{15}N-TNT) indicated that 1.7% of the ^{15}N label was detected as "biogenic" AA in the wheat straw containing the fungus (WEIß ET AL., 2004). In contrast to the wheat straw with fungus, no incorporation of the label from ^{15}N-TNT into AA was observed in the soil layer without fungus. A study on the fate of ^{13}C-labelled *E. coli* demonstrated high stability of ^{13}C-amino acids, whose contents remained constant even after 224 days of incubation (MILTNER ET AL., 2009). This is caused by the fact that proteins are amphiphilic molecules with a strong tendency to bind to surfaces (HLADY AND BUIJS, 1996), especially to mineral surfaces, over long periods of time (KLEBER ET AL., 2007).

2.7 Stability of NER in soil

The stability of NER formed during microbial degradation of organic contaminants in soils has been investigated since the late 1960s (HATZINGER AND ALEXANDER, 1995). Since then, it has been observed that aged organic compounds in NER are not always immobilised irreversibly in the soil. Therefore, many scientists focused on the possible release of NER from soils under certain conditions (BURAUEL AND FÜHR, 2000; BOIVIN ET AL., 2005; GEVAO ET AL., 2005; LERCH ET AL., 2009) and the long-term environmental impact of these residues (GEVAO ET AL., 2001).

The release of organic contaminant or its various metabolites immobilised in SOM is related closely to SOM decomposition as a result of either biochemical processes or physico-chemical mechanisms (GEVAO ET AL., 2000; BARRACLOUGH ET AL., 2005). However, the activity of microorganisms is considered as the primary factor responsible for the release of NER (GEVAO ET AL., 2000). To explore the possible scenarios of long-term behaviour of contaminant NER in soils during SOM turnover, several biodegradation studies were conducted. Their overall aim was to simulate enhanced SOM decay by stimulation of

microorganisms. Microbial activity was induced by modification of the physicochemical properties of soil like pH or by an increase of SOM content. The addition of fresh soil to soil containing [U-^{14}C]3,4-dichloroaniline-derived NER induced a slight mineralisation of these NER to $^{14}CO_2$ (HSU AND BARTHA, 1974). A similar effect was also observed in a soil experiment with [^{14}C]prometryn, in which the addition of the fresh soil inoculum remobilised 27% of the initial amount of [^{14}C]prometryn-derived NER, which could be extracted with methanol (KHAN AND IVARSON, 1981). No release of [^{14}C]prometryn-derived NER in the sterile control experiment highlighted the importance of microorganisms in the destabilisation of NER derived from this contaminant (KHAN AND IVARSON, 1981). Another experiments with prometryn, in which the effect of pH was studied, revealed that an increase of pH from 4 to 8 caused a release of up to 25% of the initial amount of prometryn NER from soil (YEE ET AL., 1985). Addition of either glucose or cow manure, both known to induce the microbial activity, resulted in higher release of [^{14}C]parathion NER from soil compared to controls (RACKE AND LICHTENSTEIN, 1985). In an inverse experiment, the microbial activity in soil was supressed by either the bactericide chloramphenicol or the fungicide captafol. Both treatments led to the significant decrease of the mineralisation rate of [^{14}C]parathion NER in soil (RACKE AND LICHTENSTEIN, 1985).

ESCHENBACH ET AL. (1998) and WEISS ET AL. (2004) investigated the stability of NER formed during microbial degradation of 9-[^{14}C]-anthracene or ^{14}C-TNT by simulation of extreme physical, chemical or biological situations. They used the following treatments:

1. Physical treatment = simulation of climatic effects by changing the soil texture via grinding, freezing and thawing of soil;
2. Chemical treatment = extraction of soil with the metal complexing agent EDTA, in order to estimate the effects of bivalent cations on the aggregation of macromolecular organic compounds and their potential release from soil. The extraction of soil with acidified H_2O was used to simulate the acid rain impact on the release of ^{14}C-TNT NER;
3. Biological treatment = simulation of increased turnover of SOM by addition of the compost and incubation of the soil containing NER with ligninolytic fungi. The ligninolytic fungi are capable of depolymerising lignin and humic substances (KÄSTNER AND HOFRICHTER, 2001). Additionally, the impact of the plants on the uptake or mobilisation of NER was studied.

Neither physical nor biological treatments caused a mobilisation of “bound” 9-[^{14}C]-anthracene and ^{14}C-TNT residues in soils. In contrast, a low (< 15% of initial amounts

in soil) release of NER from soil containing residues from microbial degradation of either 9-[^{14}C]-anthracene or ^{14}C-TNT was observed after addition of the complexing agent EDTA. Simulation of acid rain also mobilised ^{14}C-TNT residues in soil. Neither 9-[^{14}C]-anthracene nor ^{14}C-TNT was released from SOM after EDTA or the acid rain treatment.

However, the NER released by these mobilisation treatments were analysed only for the presence of the parent compounds and their known main transformation products, which are supposed to be responsible for their formation. A detailed characterisation of the chemical nature of other compounds, which may form NER, is still lacking. However, the absence of the relevant toxic components clearly indicates that they were further transformed into different kind of compounds during residue formation. On the whole, the NER formed during microbial degradation of ^{14}C-TNT or 9-[^{14}C]-anthracene were very stable (ESCHENBACH ET AL., 1998; WEISS ET AL., 2004). The stability of NER is strongly dependant on their "age", as it was shown in soil experiments with 15 years-old NER and 90 years-old NER derived from 2,4-D (LERCH ET AL., 2009). The addition of fresh soil to younger NER induced their mineralisation, whereas the older ones were stable and turned over at similar rates as SOM (LERCH ET AL., 2009).

2.8 Risk assessment of NER

Independent of the binding mechanism, the formation of NER leads to the limitation of bioavailability of contaminants (NORTHCOTT AND JONES 2000). In addition, the binding of contaminants to humic substances was reported to reduce the amounts which may interact with the biota and thus their complexed products should be less toxic than the free parent compounds (BOLLAG ET AL., 1992). Therefore, the enhanced transformation of organic contaminants into NER has been proposed as an alternative remediation method for polluted soils (BERRY AND BOYD, 1985; BOLLAG, 1992; VERSTRAETE AND DEVLIEGHER, 1996). On the other hand, under certain conditions the release of NER was observed; therefore, it is speculated that the compounds of unknown structure released from SOM can become bioavailable and thus toxic for the living organisms (BARRACLOUGH ET AL., 2005). Due to the difficulties in the identification of NER, the complications of multiple applications of a range of compounds and the uncertainties around their bioavailability it has been suggested to include them in the "conventional" environmental risk assessments (BARRACLOUGH ET AL., 2008).

The "conventional" risk assessment comprises the followings steps (ISO/DIS, 2006):

1. The hazard identification: recognition of the potential of the compound, which may cause harm to human health or the environment.

2. The exposure assessment: estimation whether and how much exposure will occur between a receptor and a contaminated source.
3. The dose-response assessment: characterisation of the relationship between the dose of a chemical and the anticipated incidence of health and environmental effect in an exposed population.
4. The risk characterisation: description of nature and magnitude of health or environmental risk.

In step 1, to identify the hazard, respective samples containing the contaminant are taken from the investigation site and their total concentrations are measured. For that purpose, different soil extraction methods are employed. These concentrations are compared with threshold values from dose-response relationships (step 3). Crossing the threshold is considered to be a risk. This procedure leads to overestimation of the risks (HARMSEN, 2007). In step 2, both the bioavailability of the contaminant and the exposure of a target receptor to the contaminant are analysed. In the assessment of the environmental risk, which may be posed by the contaminant, the receptor or protection goal is a leading principle (HARMSEN, 2007). These receptors can be human and higher animals or soil-living organisms. Furthermore, soils and sediments act as buffers, since they may absorb contaminants and prevent their transport to groundwater, surface water and the terrestrial food chain (HARMSEN, 2007).

Using this model for the assessment of environmental risk associated with NER is more complex than for human health risk assessment (EHLERS AND LUTHY, 2003). Therefore, EHLERS AND LUTHY (2003) proposed to redefine "bioavailability" into "bioavailability processes" in order to allow the accurate assessment of the environmental risk posed by a chemical in soil (as shown in **Figure 6**). Up to date, however, there are still many factors, which may impede the proper risk assessment of a target compound in soils, for example:

1. The complex and heterogeneous soil matrix, makes the direct analysis of the chemical nature of NER without application of any specific techniques and separation methods impossible;
2. Measurement of bioavailability of organic contaminants in soils is often inappropriate. The prediction of the potential risk of chemicals in soils is conducted by using various solvent extraction methods (GEVAO ET AL., 2003) for the assessment of "bioavailability". However, these chemical methods used for extraction of the target compound from soil show still large uncertainties in the estimated NER content (EHLERS AND LUTHY, 2003; total concentration or bioaccessibility are measured). Furthermore, apart from parent compounds and their metabolites

extracted from soils, other unidentified small molecular fragments may be present in extracts, in forms that do not pose ecological risks (KELSEY ET AL., 1997; CHUNG AND ALEXANDER, 1998; ROBERTSON AND ALEXANDER, 1998);

3. **Proper knowledge about possible mechanisms of NER formation during microbial degradation of organic contaminants in the soil system is missing.**

Available studies on the NER formation during biodegradation of organic contaminants in soil are mostly limited to quantitative analyses using radioactive tracers (GERST AND KLIGER, 1990; KUBIAK ET AL., 1990; RICHNOW, 1999). It is suggested that NER are formed by sorption or sequestration of parent compound or metabolites within SOM components and their possible release is considered as a potential risk for both environment and humans (ALEXANDER, 2000; BARRACLOUGH ET AL, 2005), since their chemical nature is still not clear (see conventional model in **Figure 8**).

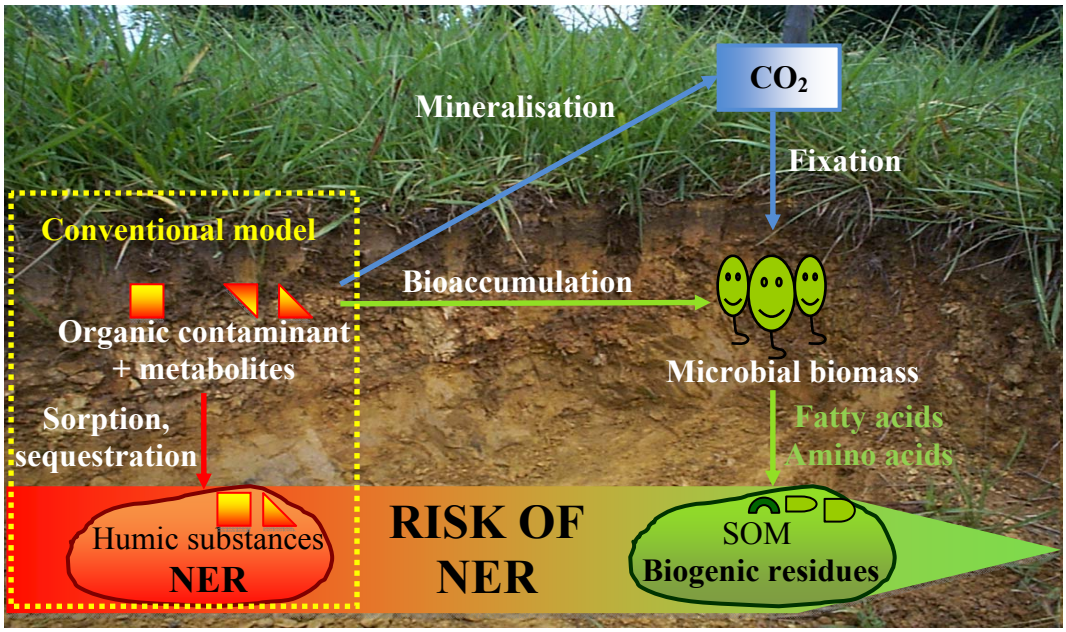

Figure 8. Proposed concept and the conventional model of the risk assessment related to NER formation during the microbial degradation of organic contaminants in soil

In case of readily biodegradable contaminants, mainly the misunderstanding of the processes of NER formation in soil is thus the key problem affecting the proper assessment of the risk which they can pose. It should be kept in mind that NER formed during microbial degradation of organic contaminants can be also biogenic residues (**Figure 8**). Biogenic residues are composed only of natural compounds derived from microbial biomass, such as FA and AA, which are incorporated into the SOM pool. Over time these residues are more and more irreversibly bound to SOM by the stabilisation process. Residues from biomass, however, do not present any toxicological hazard and thus would lead to an overestimation of the risk

related to NER in soil. Although biogenic residues are clearly excluded by the IUPAC definition (ROBERTS ET AL. 1984), they are generally not analysed and considered in studies on the fate and in the risk assessment concepts.

Therefore, the detailed study on the formation of biogenic residues during the microbial degradation of organic contaminants in soil is needed to establish both their realistic degradation rates and a proper assessment of their potential risk.

3 AIMS OF THE STUDY

Studies on the formation of NER during microbial degradation of various organic contaminants in soils presented in the literature are mostly limited to quantitative analyses. Therefore, the chemical nature of NER is not clear yet. However, the soil environment is a very complex system, thus the detailed analysis of NER structure needs a thorough understanding of the mechanisms of their formation and specific extraction methods. In the present study, it has been assumed that some part of NER formed during biodegradation of organic contaminants in soils is composed of microbial components. This is based on the fact that microorganisms utilise C derived from organic compounds or from CO_2 for biomass synthesis and after death their cell constituents are incorporated into SOM and form biogenic residues. This mechanism occurs for both natural organic compounds and readily degradable contaminants in soil.

The overall aim of this study was to elucidate the pathways of biogenic residue formation originating from microbial degradation of organic contaminants in soil and to study their stability in time course. The formation and fate of two representatives for biogenic residues, FA and AA during biodegradation of either $^{13}C_6$-2,4-Dichlorophenoxyacetic acid (2,4-D) or $^{13}C_6$-Ibuprofen (Ibu) in soil were investigated. In addition, an incubation experiment with $^{13}CO_2$ and unlabelled 2,4-D was carried out in order to study the incorporation of the labelled C into FA and AA via heterotrophic CO_2 fixation. A simple model experiment with a well-known 2,4-D degrader in the pure culture was performed to obtain a better view on the pathways of biogenic residue formation in complex soil systems. FA and AA were extracted from soil and analysed for their concentration and isotopic composition.

The specific objectives of this study were:

1. To quantify the extent and the kinetics of biogenic residue formation during microbial degradation of two model organic contaminants. Up to date, there is no report on the contribution of microorganisms to biogenic residue formation. This is caused by the fact that it is generally accepted that the population of microorganisms colonising soil particles is negligible in comparison with the large surfaces of both clays and SOM with many binding sites. The main questions adressing this point are:
   - Are biogenic residues formed during microbial degradation of organic contaminants in soil?

- If yes, how much biogenic residues are formed and what is their contribution to the total NER content in soil? Are these values really important for the environment?

2. To evaluate if specific biomass components are preferentially incorporated into NER. Are FA or AA more relevant for biogenic residues formation in soil?
3. To assess if heterotrophic CO_2 fixation plays any role for the incorporation of C into biogenic residues in soil.
4. To check the stability of biogenic residues in soil and their possible remobilisation in time course. Several studies have revealed that some part of the NER was released as CO_2 even after the target compound was completely depleted. Therefore in this study, besides the quantification of the total content of biogenic residues at respective sampling dates, we also focused on the monitoring of the labelled C distribution within the living and the non-living SOM fraction. The non-living fraction provides information about the stabilisation process of biogenic residues in the SOM pool.

4 MODEL COMPOUNDS

2,4-Dichlorophenoxyacetic acid (2,4-D) and Ibuprofen (Ibu) were selected as model compounds for the biodegradation studies. These compounds are aromatic and acidic molecules, and are used extensively world-wide. In addition, both 2,4-D and Ibu are reported to degrade readily in soil and to form NER, which potentially may pose a risk for the environment. Detailed description and current state of the art of these compounds are presented in two next sections.

4.1 2,4-Dichlorophenoxyacetic acid (2,4-D)

2,4-Dichlorophenoxyacetic acid (2,4-D) as a member of the phenoxy group compounds is among the most widely used herbicids worldwide to control broad leaf weeds in cereal and grass seed crops (MCGHEE AND BURNS, 1995; VOOS AND GROFFMAN, 1997; BOIVIN ET AL., 2005) with an annual production of more than 100,000 tons (MERINI ET AL., 2008). This herbicide is a weak by acidic molecule (**Figure 9**) with a relatively low molecular mass of 221 g/mol and a high solubility of 0.6 mg/L in H_2O (VILLAVERDE AT AL., 2008). The octanol/water partition coefficient (log K_{ow}) 2.83 indicates a moderate sorption potential of 2,4-D (BOIVIN AT AL., 2005). The persistence of 2,4-D is considered low to moderate (DT_{50} 5-59 days; VILLAVERDE AT AL., 2008).

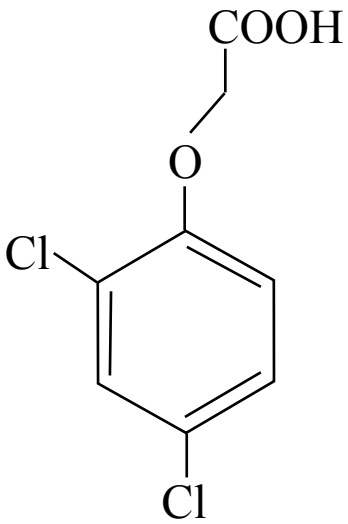

Figure 9. The molecular structure of 2,4-Dichlorophenoxyacetic acid

The microbial degradation of 2,4-D is well described (FULTHORPE ET AL., 1996; KAMAGATA ET AL., 1997; VOOS AND GROFFMAN, 1997B; CRESPÍN ET AL., 2001) and this compound is classified as readily biodegradable. 2,4-D is used as C and energy source (SANDMANN ET AL., 1988; SMITH AND LAFOND, 1990 LERCH ET AL., 2009A) by a wide range of microorganisms even in pristine soils (FULTHORPE ET AL., 1996; KAMAGATA ET AL., 1997). Bacteria genera, which have been reported to degrade 2,4-D include: *Pseudomonas* (KILPI ET AL., 1980),

Alcaligenes (PIEPER ET AL., 1988), *Arthrobacter* (BEADLE AND SMITH, 1982) and *Flavobacterium* (CHAUDHRY AND HUANG, 1988). Microbial breakdown of 2,4-D in soils is initiated by the removal of the carboxyl side chain or the cleavage of the ether bond (FORSTER AND MCKERCHER, 1973; ROBERTS ET AL., 1998) leading to the formation of 2,4-dichlorophenol (2,4-DCP) and other phenolic metabolites that are further degraded by cleavage of the phenyl ring (SMITH AND AUBIN, 1991; ROBERTS ET AL., 1998). The final products of 2,4-D degradation are succinyl-CoA and Acetyl-CoA, which enter the tricarboxylic acid cycle [(TCC); MANZANO ET AL., 2007; see **Figure 10**). Besides 2,4-DCP, the removal of the two-C side chain from 2,4-D also results in the formation of glyoxylate (AMY ET AL., 1985), which joins the TCC.

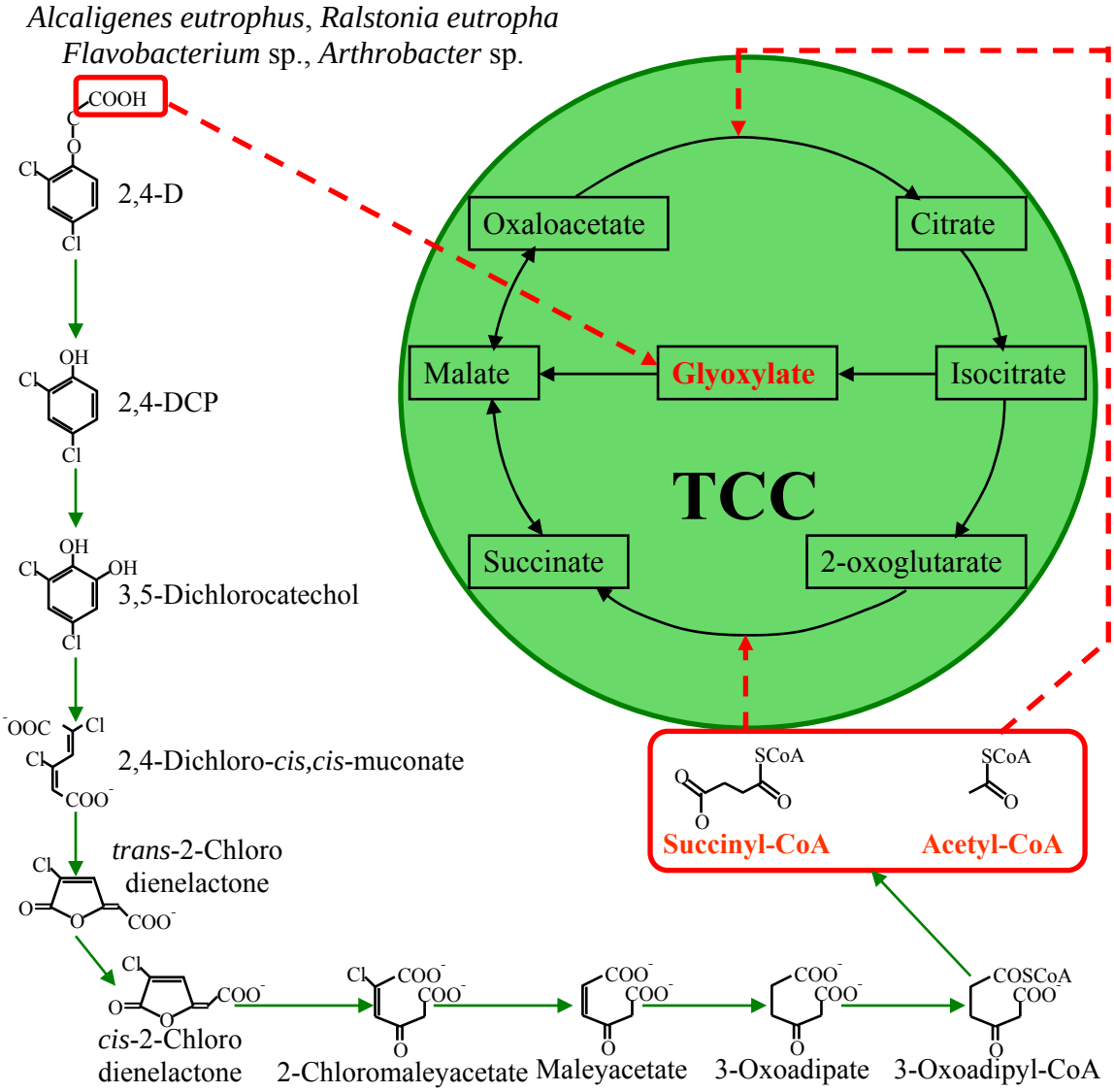

Figure 10. Scheme of 2,4-D degradation pathway (adapted from E. Young; source: map http://umbbd.msi.umn.edu/2,4-d/2,4-d_map.html)

Most laboratory studies on the fate of 2,4-D in soils are limited to radiotracer experiment with [ring-U-^{14}C] 2,4-D (BENOIT AND BARRIUSO, 1997; BOIVIN ET AL., 2005; GAULTIER ET AL., 2008). Under non-sterile conditions the mineralisation of 2,4-D in soil reached about 50–60% of the initially applied compound (BENOIT AND BARRIUSO, 1997; BOIVIN ET AL., 2005). The degradation rate of 2,4-D in soils with higher total organic C (TOC) content was significantly higher than in soils with lower amount of TOC (GETENGA ET AL., 2004). It is generally known that the TOC content of a soil affects the microbial activity, which is higher in soils containing more TOC (GETENGA ET AL., 2004). However, it is speculated that 2,4-D is only partially degraded in soil, since a portion of 2,4-D and its metabolites are "bound" to SOM forming NER, which become stable over time (BARRIUSO ET AL., 1997; BENOIT AND BARRIUSO, 1997; BOIVIN ET AL., 2005). The amounts of NER formed during microbial degradation of 2,4-D in soil were reported to be relatively high, in a range of 20–56% of the initially added pollutant (BARRIUSO ET AL, 1997; BENOIT AND BARRIUSO, 1997; XIE ET AL., 1997).

A very reactive metabolite of 2,4-D degradation 2,4-DCP, is considered to be a major precursor for NER formation by binding to SOM (BARRIUSO ET AL., 1997; BENOIT AND BARRIUSO, 1997). Binding of 2,4-DCP to SOM occurs mainly by covalent bonding, which is catalysed by oxidoreductive enzymes, such as laccases and peroxidases (BOLLAG, 1991; BOLLAG 1992; HATCHER ET AL., 1993; DEC AND BOLLAG, 2000; XU AND BHANDARI, 2003A, B). The complexes resulting from covalent binding of 2,4-DCP to humic material were reported to be highly persistent (SAXENA AND BARTHA, 1983; BOLLAG, 1991, 1992). KLIBANOV ET AL. (1983) thus proposed using horseradish peroxidase for an in-situ stabilisation of phenolic compounds at contaminated sites. Horseradish peroxidase affected the sorption potential of 2,4-DCP in soil slurries; more 2,4-DCP was bound to SOM in presence of horseradish peroxidase than in soil slurries without this enzyme (PALOMO AND BHANDARI, 2005, 2006). However, horseradish peroxidase is a very reactive enzyme, which may react differently than peroxidases of microbial origin. In addition, soils used in these experiments were autoclaved several times; both the soil structure and physicochemical properties are changed during autoclaving. SHAW ET AL. (1999) reported that autoclaving soil increased dissolved organic C concentration 20-fold and reduced soil pH. This resulted in higher 2,4-DCP adsorption to autoclaved soil than to non-autoclaved one in their experiments. They also suggested using both re-inoculated sterile and sterile soils for a proper investigation of sorption-biodegradation interactions. Other study also demonstrated that autoclaving

changes also the aggregation state of the soil, which may result in a higher surface area of soils and thus may lead to an increased sorption potential (BERNS ET AL., 2008).

The humification of straw was reported to increase the sorption potential of pollutants by increasing the proportion of constituents with sorption capacities relative to cellulosic constituents with low sorption capacities (BENOIT ET AL., 1996). However, even the presence of highly humified sterile straw did not induce a sorption of [U-ring-^{14}C] 2,4-D and [U-ring-^{14}C] 2,4-DCP to SOM (BENOIT AND BARRIUSO, 1997). XIE ET AL. (1997) fractionated [U-ring-^{14}C] 2,4-D residues bound to humin after previous removal of soluble fulvic and humic acids from soil. They showed that most of the label was associated with the aqueous bound-humic acid solution and a very low amount was bound to the mineral fractions. Most recent studies have revealed that aged NER formed during 2,4-D biodegradation in soils are still available to microorganisms (BOIVIN, 2005; LERCH ET AL., 2009B) and that the stability was dependent on their age (LERCH ET AL., 2009B).

All studies on NER formation by sorption of either 2,4-D or its main metabolite 2,4-DCP are limited to simple humic acids-contaminant models (BOLLAG, 1991; BOLLAG 1992; HATCHER ET AL., 1993; PICCOLO ET AL., 2001) or to autoclaved soil slurries (PALOMO AND BHANDARI, 2005; 2006). In addition, NER formed during 2,4-D biodegradation in the complex soil systems were only quantified, thus their chemical structure is still unknown. It is difficult to compare the processes of NER formation occurring in the simple systems with the ones which may take place in the complex soil environment. Moreover, the comparison of biotic experiment with abiotic one is perhaps not appropriate, since these soils have different soil physico-chemical properties and a contaminant may thus behave differently. To conclude, in spite of many studies on NER formation during microbial degradation of 2,4-D in soil, a satisfying explanation on the mechanism of their formation is still missing.

4.2 Ibuprofen (Ibu)

Ibuprofen (Ibu) represents a novel environmentally relevant compound; is an important anti-inflammatory and analgesic non-prescription drug (BUSER ET AL., 1999), with a global annual production of several kilotons and a high daily dose of 600–1200 mg (ZWIENER ET AL., 2002). Ibu is derived from propionic acid (**Figure 11**), has a relatively low molecular mass of 206.28 g/mol and a moderate solubility of 21 mg/L in H_2O (YALKOWSKY AND DANNENFELSER, 1992). The log K_{ow} of Ibu is 3.5 (STUER-LAURIDSEN ET AL., 2000), indicating its moderate sorption potential. However, a study on the environmental fate of ibuprofen [carboxyl-^{14}C] in water/sediment systems pointed to a low affinity for sorption onto sediment

(LÖFFLER ET AL., 2005). Ibuprofen displayed a low persistence in soils with DT_{50} between 0.91 and 6.09 days (XU ET AL., 2009).

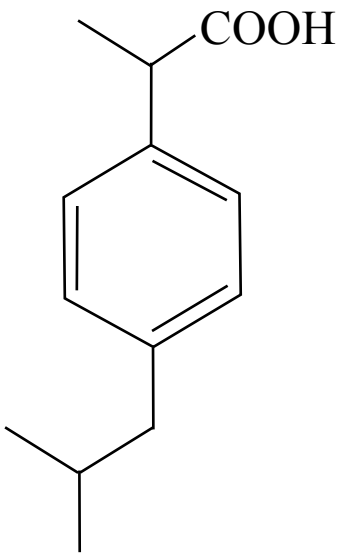

Figure 11. The molecular structure of ibuprofen

After normal therapeutic use, large amounts of Ibu are excreted as the parent compound or its various metabolites, e.g. hydroxyibuprofen, carboxyibuprofen and carboxyhydratropic acid to the wastewater treatment plant (WWTP; ZWIENER ET AL., 2002). The main mechanisms involved in the removal of pharmaceuticals in WWTP are biodegradation, sorption on sludge or particulate matter, filtration and chemical oxidation (MIÈGE ET AL., 2009). Ibu is eliminated in WWTP efficiently (between 75% to more than 98% [CLARA ET AL., 2005; JOSS ET AL., 2005; CASTIGLIONI ET AL., 2006; GÓMEZ ET AL., 2007; JONES ET AL., 2007; KOSJEK ET AL., 2007; MATAMOROS ET AL., 2008]). However, presence of this pharmaceutical was analysed only in the water phase of effluents, thus its exact removal mechanism cannot be fully elucidated. After chemical step of WWTP using $FeCl_3$ only a small decrease (25%) of Ibu concentration was reported (ZORITA ET AL., 2009). The last step of WWTP sand filtration eliminated up to 20% of the initial concentration of Ibu (ZORITA ET AL., 2009). Despite the fact that Ibu is mostly removed during biological step of WWTP (JONES ET AL., 2007; ZORITA ET AL., 2009), this pharmaceutical is still detected at high concentration in biosolids (246–750 ng/g of dry weight of dewatered municipal biosolids [EDWARDS ET AL., 2009; MCCLELLAN AND HALDEN, 2010]). Application of biosolids in agriculture as a fertiliser may introduce Ibu into soils (EDWARDS ET AL., 2009).

In comparison with 2,4-D, up to date, there are only few studies on the fate of Ibu in soils after sewage sludge application. Ibu was easily degraded in the water/sediment system (77% was evolved as CO_2 after 100 days) and its metabolite 2-hydroxyibuprofen (2-OH-ibu)

dissipated already after 28 days of incubation (LÖFFLER ET AL., 2005). The degradation of Ibu was also quick in four agricultural soils with different OM contents; its dissipation kinetics depended on OM content and was much slower in soils containing more OM (XU ET AL., 2009). The degradation of this pharmaceutical in non-sterile soils was 34.5-fold faster than in sterile ones (XU ET AL. 2009) clearly indicating the contribution of microorganisms to its transformation. Another study focussing on the leaching potential of Ibu from soil demonstrated the absence of this compound in the leachate already after 20 hours (LAPEN ET AL., 2008), suggesting its microbial degradation or sorption to soil components. The first study on the label distribution within the soil system during $^{14}C_3$-ibu biodegradation was presented by RICHTER ET AL. (2007). In their studies, after 100 days, ~ 35 % of $^{14}C_3$-ibu equivalents was mineralised, ~ 50% of label was found in $^{14}C_3$-ibu-derived NER and 11% was found as unidentified extractable $^{14}C_3$-ibu residues. The formation of $^{14}C_3$-ibu-derived NER was quick, reaching a maximum on day 4, until when the extractable $^{14}C_3$-ibu residues were detectable in soil. Thereafter, their contents decreased at a very slow rate (~ 5% of $^{14}C_3$-ibu equivalents after 100 days).

Microbial degradation of Ibu and NER formation in soil, up to date, are not studied in detail. In addition, neither data on the chemical structure of NER formed by Ibu nor information about the mechanisms of their formation in soil is available.

5 MATERIAL AND METHODS

5.1 Chemicals and materials

All chemicals and materials, except where otherwise specified (see **Table 2** and **Table 3**) were purchased from VWR/Merck (Darmstadt, Germany) at the highest quality commercially available.

Table 2. Chemicals

Chemical	Company
$^{13}C_6$-2,4-D	Alsachim, Illkirch, France; (isotopic purity: 99 at%; chemical purity: ~ 98%; ^{13}C label in 6 positions)
$Na_2{}^{13}CO_3$	Chemotrade, Leipzig, Germany; (isotopic purity: 99 at%; chemical purity: ~ 98%)
$^{13}C_6$-ibu	Alsachim, Illkirch, France; (isotopic purity: 99 at%; chemical purity: ~ 98%; ^{13}C label in 6 positions)
2-OH-ibu	LGC GmbH (Luckenwalde, Germany); chemical purity 99.8%)
Bacterial Acid Methyl Esters (BAME)	Supelco, München, Germany
Heneicosanoate methyl ester (FAME 21:0)	Sigma-Aldrich, Munich, Germany
Sodium deoxycholate	Sigma-Steinheim, Germany
Polyethylenglycol (PEG 6000)	Carl Roth, Karlsruhe, Germany

Table 3. Materials

Material	Company
CHROMABOND® EASY, 3ml 200 mg (for 2,4-D + metabolites)	Macherey-Nagel, Düren, Germany
Silicagel (for PLFA-analysis)	Unisil, Clarkson Chromatography Products, South Wiliamsport, USA
Silicagel (for tFA-analysis)	Mallinckrodt Baker, Griesheim, Germany
glass fiber membrane filters (for AA-analysis)	0.7 µm, Puradisc 25 GF/F, Whatman Inc., Cliffton, USA
Cupriavidus necator JMP 134	Environmental Microbiology Department (UFZ, Leipzig); DMSZ 4058, Braunschweig, Germany

5.2 Liquid culture experiments

In order to understand the processes of biogenic residue formation during microbial degradation of 2,4-D in the complex soil system, a simple model experiment with the bacterial strain *Cupriavidus necator* JMP 134 was performed. *C. necator* JMP 134, which is a well-described 2,4-D degrader in soil, was selected for biodegradation studies in pure culture. As mentioned in section 2.6.2, biogenic residues can be formed directly via the incorporation of C derived from an organic contaminant or indirectly from CO_2 evolved during contaminant mineralisation (CO_2 fixation). To distinquish between these two processes, different ^{13}C-labelled compounds were used. To study ^{13}C incorporation into biomass of *C. necator* directly from a labelled contaminant, $^{13}C_6$-labelled 2,4-D was used as a model compound. An incubation experiment with *C. necator* JMP 134 grown on unlabelled 2,4-D and in the presence of $^{13}CO_2$ was prepared to investigate the incorporation of ^{13}C into biomass components via CO_2 fixation.

5.2.1 Strain

The strain used for medium inoculation was *Cupriavidus necator* JMP 134. *C. necator* JMP 134 (formerly *Alcaligenes eutrophus, Ralstonia eutropha, Wautersia eutropha*) was isolated from an Australian soil by its ability to grow on 2,4-D (DON AND PEMBERTON, 1981) and up to date is the best-studied 2,4-D-degrading soil ß-proteobacterium (Manzano et al., 2007). *C. necator* JMP 134 is gram-negative bacterium with short rod-shaped cells [see microscopic cells visualised at 100x magnification using a laboratory microscope (Carl Zeiss, Jena, Germany) in **Figure 12**].

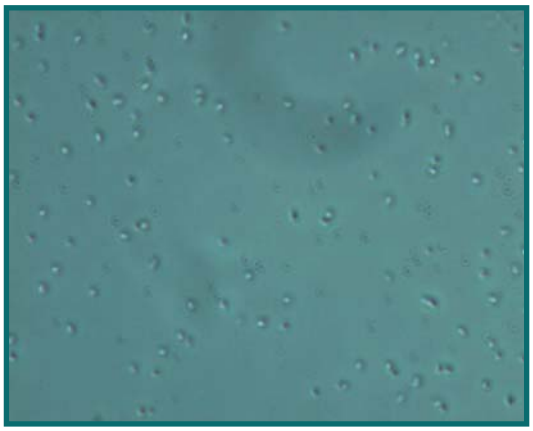

Figure 12. Microscopic view of single cells of *Cupriavidus necator* JMP 134

C. necator JMP 134 was tested in bioaugmentation procedures performed to remediate 2,4-D polluted environments (DEJONGHE ET AL., 2000; DIGIOVANNI ET AL., 1996). However, limited success of bioremediation with *C. necator* JMP 134 in soil was observed (DIGIOVANNI ET AL.,

1996). This is caused by the fact that various biotic (e.g. presence of eukaryotic microbiota) and abiotic factors in the complex soil environment may affect both the survival and catabolic performance of this strain in bioremediation processes (MANZANO ET AL., 2007).

5.2.2 Medium

The cultures were grown in a Minimal Medium (MM) prepared according to LERCH ET AL. (2007). 2,4-D was added as the sole C source at a total concentration of 73 mg/L (1978.3 µmol/L). MM components are presented in **Table 4**. MM was autoclaved (Systec Autoclave, Wettenberg, Germany) one time at 121°C for 20 min before use for the incubation experiment.

Table 4. MM components for cultivation of *C. necator* JMP 134 (LERCH ET AL., 2007)

Component	Concentration
K_2HPO_4	1.5 g/L
KH_2PO_4	0.5 g/L
$(NH_4)_2SO_4$	1 g/L
$MgSO_4 (H_2O)_7$	207 mg/L
$ZnSO_4 (H_2O)_7$	200 µg/L
$MgCl_2 (H_2O)_4$	10 µg/L
H_3BO_3	5 µg/L
$CoCl_2 (H_2O)_6$	25 µg/L
$CuSO_4$	100 µg/L
$NiCl_2 (H_2O)_6$	5 µg/L
$FeSO_4 (H_2O)_7$	250 µg/L
EDTA	125 µg/L

5.2.3 Preculture incubation

Prior to the incubation experiment, to increase the biomass content and to adapt *C. necator* JMP 134 for 2,4-D degradation, this strain was pre-cultivated on tryptone medium (TM) as described previously by LERCH ET AL. (2007), which consisted of 5 g/L tryptone, 3 g/L yeast extract and 50 mg/L 2,4-D. Unlabelled 2,4-D dissolved in acetone (ACN) was placed in 500 mL Erlenmeyer flasks. In order to prevent killing the bacteria, the solvent was evaporated under the gentle air stream in a sterile bench. Both the tryptone and the yeast extract were dissolved in de-ionised water, autoclaved and transferred into the Erlenmeyer flasks after removal of ACN. After inoculation of the TM with cells of *C. necator* JMP 134, the batch preculture was incubated on a rotary shaker (~ 70 min^{-1}; Infors HT, Bottmingen, Switzerland) for 48 hours, in the dark and at 30°C. After 48 hours of incubation, an aliquot of 10 mL of the culture was centrifuged (11,000 min^{-1} for 15 min at 4°C; Jouan KR25i, DBJ Labcare Ltd, Buckinghamshire, UK). After centrifugation, the biomass pellets were rinsed once with

Phosphate Buffer (PB) prior to the inoculation of the MM to avoid carryover of C from TM to MM.

5.2.4 Incubation experiment with $^{13}C_6$-2,4-D

Three different incubation experiments were prepared, in which MM (for details see section 5.2.2) was spiked with:

1. $^{13}C_6$-2,4-D (271 µmol/L) and $^{12}C_6$-2,4-D (1707.3 µmol/L). The ^{13}C-labelled 2,4-D was mixed with the unlabelled one in order to increase the 2,4-D concentration for formation of the minimum biomass necessary for analysis (^{13}C-labelled compounds are expensive, therefore $^{13}C_6$-2,4-D was diluted with unlabelled 2,4-D);
2. $^{12}C_6$-2,4-D (1978.3 µmol/L; control);
3. abiotic (sterile) containing $^{13}C_6$-2,4-D (271 µmol/L) and $^{12}C_6$-2,4-D (1707.3 µmol/L).

Control samples provided the information on the natural abundance of ^{13}C in the medium after addition of unlabelled 2,4-D. The abiotic system was performed to prove that the incorporation of ^{13}C-label into the biomass during $^{13}C_6$-2,4-D degradation is a result of biotic processes. ^{13}C labelled or unlabelled 2,4-D were dissolved separately in ACN and then placed in 500 mL of Duran glass bottles. The ACN was evaporated and 250 mL (final volume of culture) of MM was placed in the glass bottles and the content of the bottle was mixed for 10 min. The medium in each bottle was inoculated with cells of *C. necator* JMP 134 (~ 10 mg/bottle) from the preculture on TM (see previous section 5.2.3). The abiotic systems were then autoclaved once. Duran glass bottles were sealed with Teflon-lined caps and incubated on the rotary shaker for 14 days under controlled laboratory conditions (in the dark and constant temperature 30 ± 2°C). To ensure a sufficient amount of O_2 for bacterial respiration, the systems were flushed periodically with humidified (bottle with de-ionised H_2O) and CO_2-free air. During aeration of systems, CO_2 evolved during 2,4-D mineralisation was trapped in two vials containing 2 M NaOH (see **Figure 13**).

The ^{13}C label incorporation into biomass components such as fatty acids (FA) and amino acids (AA) and into total biomass of *C. necator* during $^{13}C_6$-2,4-D biodegradation were determined after 1, 2, 3, 7 and 14 days of incubation. In addition, to set up a detailed mass balance of ^{13}C in the system, the ^{13}C in the CO_2 produced during mineralisation of 2,4-D in the gas space of the bottle and in the medium was also monitored. At each sampling date, the optical density (OD) indicating bacterial biomass growth was determined using a Lambda 2S spectrophotometer UV/VIS (λ = 560 nm; Perkin Elmer, Massachutes, USA).

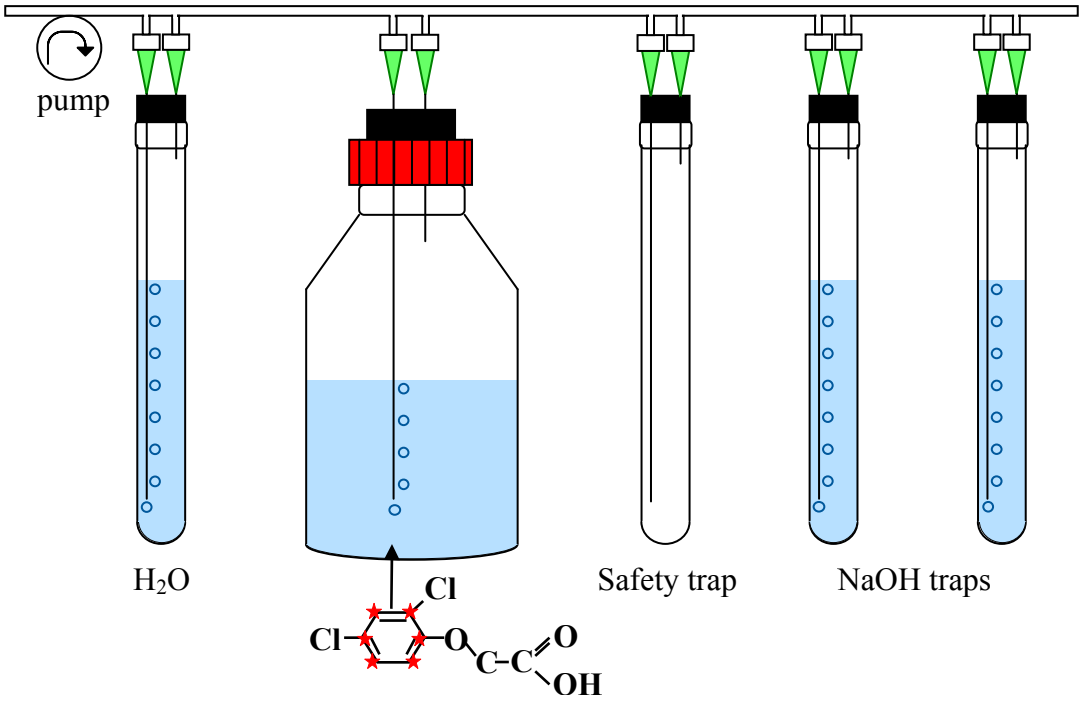

Figure 13. The liquid culture incubation experiment with $^{13}C_6$-2,4-D in intermittent aeration systems

5.2.5 Incubation experiment under $^{13}CO_2$ atmosphere

To study the incorporation of C into biogenic residues via CO_2 released during 2,4-D mineralisation (CO_2 fixation), MM in Duran glass bottles spiked previously with unlabelled 2,4-D at a concentration of 1978.3 µmol/L was incubated as three different incubation experiments under:

1. $^{13}CO_2$ (33.93 µmol/bottle);
2. $^{12}CO_2$ (33.93 µmol/bottle; control);
3. $^{13}CO_2$ (33.93 µmol/bottle) in an abiotic system (sterile).

Small tubes with either labelled $Na_2{}^{13}CO_3$ or unlabelled Na_2CO_3 dissolved in de-ionised H_2O, were placed in each Duran glass bottle (**Figure 14**). To ensure conditions of $^{13}CO_2$ fixation experiment as close as possible to those in the $^{13}C_6$-2,4-D incubation experiment, the same concentration of unlabelled 2,4-D was used. The initial amount of CO_2 in each bottle was adjusted to 33.93 µmol and corresponded to an assumed 50% mineralisation of the initial amount of 2,4-D in the $^{13}C_6$-2,4-D experiment (67.75 µmol/bottle). After closing the Duran bottle, CO_2 was released from Na_2CO_3 by adding 6M HCl drop wise into this tube using a syringe. Thereafter, the bottle was incubated in the same way as for the $^{13}C_6$-2,4-D incubation experiment. The bottles were destructively sampled after 2, 3, 7 and 14 days and the ^{13}C label was analysed in FA, AA and in the total biomass of *C. necator*. However, no samples were taken after 1 day of incubation as it was done for $^{13}C_6$-2,4-D incubation experiment. This is

caused by the fact that CO_2 fixation plays a secondary role at the initial phase of incubation. One triplicate set of liquid culture systems with $^{13}CO_2$ (33.93 µmol/bottle) and without 2,4-D for 7 days was incubated to check if CO_2 fixation takes place in the absence of the main C source. At each sampling date, the OD of the bacterial biomass suspension was measured.

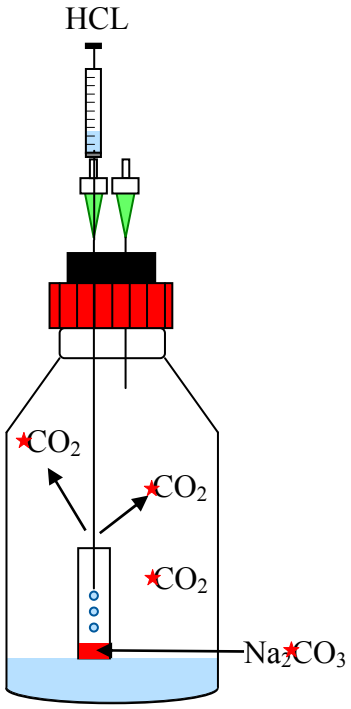

Figure 14. The preparation of $^{13}CO_2$ fixation liquid culture incubation experiment treated with unlabelled 2,4-D

5.3 Soil experiments

The formation of biogenic residues in soil systems during microbial degradation of two most widely used organic contaminants 2,4-D and Ibu was investigated. The herbicide 2,4-D was selected as a first model compound, due to numerous studies on its microbial degradation and reported high NER content in the soil. In spite of many studies on NER formation during 2,4-D biodegradation, their chemical structure is still not known and there is no report on the contribution of microorganisms to biogenic residues formation. Therefore, to trace biogenic residues formation directly from 2,4-D, $^{13}C_6$-2,4-D incubation experiments were prepared. In addition, also the indirect incorporation of C into biogenic residues via CO_2 fixation process during 2,4-D biodegradation was studied in the soils amended with unlabelled 2,4-D and incubated in presence of $^{13}CO_2$. The second model compound Ibu is a emerging contaminant, which both microbial degradation and the processes of NER formation are not studied in detail compared to 2,4-D. In order to investigate biogenic residues formation via the direct incorporation of C from Ibu, soil systems were incubated with $^{13}C_6$-ibu.

Summing up the above, the mechanisms of NER formation and thus the risks for an environment related to these two extensively used organic contaminants are not elucidated yet. Therefore, the knowledge on biogenic residues formation is necessary for a proper risk assessment and a realistic degradation rate of these contaminants in the soil.

5.3.1 Soil

The soil was collected from A horizon of the agricultural long-term experiment "Statischer Düngungversuch" located in Bad Lauchstädt, Germany (BLAIR ET AL., 2006). It is a Haplic Chernozem, which has been cultivated continuously with a crop rotation (sugar beet, summer barley, potatoes and winter wheat) and fertilised every second year with a farmyard manure (30 t/ha) since 1902. Detailed soil characteristics are presented in **Table 5**.

Table 5. Soil characteristics used for an incubation experiment (KÖRSCHENS ET AL., 2000)

Parameter	Content
Clay	21%
Silt	68%
Sand	11%
N_{total}	0.17%
C_{org}	2.1%
pH	6.6
WHC	37.5%

5.3.2 Incubation experiment

Three different biotic soil experiments with $^{13}C_6$-2,4-D, $^{13}C_6$-2,4-ibu or $^{13}CO_2$ were prepared. In order to distinguish between the abiotic and the biotic NER formations, one or two triplicate sets of abiotic systems for each experiment were also incubated. All soil incubation experiments were prepared according to the OECD guideline 307 addressing the aerobic and anaerobic transformation of chemicals in the soil (OECD, 2002). The experimental setup of the soil systems (**Figure 15)** was the same as for the liquid culture experiments (**Figure 13**; section 5.2.4).

To prevent killing all microorganisms, first only a small amount (10% of the total amount used for an experiment) of soil ($^{13}C_6$-2,4-D experiment) or soil-sludge mixture ($^{13}C_6$-ibu experiment) was spiked. Either ^{13}C-labelled or unlabelled model compound was dissolved in MeOH. This initially spiked portion was then mixed with the rest of the soil to yield a final compound concentration of 20 mg/kg. The water content of the soil was adjusted to 60% of its maximum water holding capacity (WHC). Finally 40 gram of this soil was weighed using laboratory balance (Sartorius AG, Göttingen, Germany) and placed in 1000 mL Duran glass bottles sealed with Teflon-lined caps.

Figure 15. Incubation experiment of soil treated with organic contaminant (according to OECD 307)

The soil systems were incubated in modified intermittent aeration systems under controlled laboratory conditions in the darkness and a constant temperature of 20°C (± 2°C). Similar to the liquid culture systems, soil systems were aerated periodically and the CO_2 was trapped in 2 M NaOH solutions.

5.3.3 Soil incubation experiment with $^{13}C_6$-2,4-D

Three different soil incubations were prepared:

1. soil spiked with $^{13}C_6$-2,4-D,
2. soil spiked with $^{12}C_6$-2,4-D (control),
3. non-amended (blank, without 2,4-D).

Blank and control samples allow the correction for the natural abundance of ^{13}C in soil. The final concentration of spiked 2,4-D in the soil was 20 mg/kg (542.5 µmol ^{13}C/kg) of soil. The soil was incubated for 64 days. After 2, 4, 8, 16, 32 and 64 days of incubation, the bottles were sampled destructively and the soil was analysed for the amount and the isotopic composition (^{13}C) of FA and AA as representatives for biogenic residues. In addition, two triplicate sets of abiotic soil systems with $^{13}C_6$-2,4-D were also incubated and sampled destructively after 32 and 64 days.

5.3.4 Soil incubation experiment under $^{13}CO_2$ atmosphere

In order to investigate CO_2 fixation (from mineralisation of 2,4-D residues) by soil microorganisms and its incorporation into biogenic residues, three soil incubations were prepared:

1. with unlabelled 2,4-D (20 mg/kg) and $^{13}CO_2$ (12.6 µmol/bottle);
2. with unlabelled 2,4-D (20 mg/kg) and unlabelled CO_2 (12.6 µmol/bottle);

3. with neither 2,4-D nor additional CO_2.

The preparation of an incubation experiment under CO_2 atmosphere was already presented in detail in section 5.2.5 (**Figure 14**). The initial amount of CO_2 in each bottle was adjusted to the final concentration of $^{13}CO_2$ evolved during mineralisation of $^{13}C_6$-2,4-D. To estimate the incorporation of ^{13}C-label into FA, the soil was extracted and analysed after 8, 16, 32 and 64 days of incubation. No samples were taken on days 2 and 4 due to a minor role of CO_2 fixation in the initial phase of incubation. In addition, because of a low level of the isotopic enrichment (in a range of the natural isotopic abundance) in the extracted AA and tFA from soils, they were not further quantified. One triplicate set of soil systems with $^{13}CO_2$ (12.6 µmol/bottle) without 2,4-D and abiotic systems with $^{13}CO_2$ and unlabelled 2,4-D were also incubated for 64 and 32 days, respectively. The soil systems without 2,4-D were prepared to check if the presence of this readily available organic substrate stimulates CO_2 fixation.

5.3.5 Soil incubation experiment with $^{13}C_6$-ibu

Similar to the soil incubation experiment with $^{13}C_6$-2,4-D, also three types of soil systems were prepared:

1. soil spiked with $^{13}C_6$-ibu;
2. soil spiked with $^{12}C_6$-ibu (control);
3. non-amended (blank, without Ibu).

Contrary to $^{13}C_6$-2,4-D soil incubation experiment, the soil was amended additionally with stabilised sewage sludge (Klärwerk Rosental, Leipzig, Germany), because Ibu can only be introduced into the soil by sewage sludge application (EDWARDS ET AL., 2009). Soil and sludge were mixed at a 9:1 (DW/DW) ratio. The initial Ibu concentration was 20 mg/kg (581.5 µmol/kg) of soil. The soil systems were incubated for 90 days and sampled destructively after 2, 7, 14, 28, 59 and 90 days of incubation and analysed for the amount and the isotopic composition of FA and AA. Additionally, two triplicate sets of abiotic soil systems with $^{13}C_6$-2,4-ibu were also incubated and sampled destructively after 28 and 90 days of incubation.

5.4 Chemical analyses

The main focus of all experiments was to study the ^{13}C incorporation into two representatives for biogenic residues, fatty acids (FA) and amino acids (AA). FA and AA are known microbial biomarkers (BOSCHKER AND MIDDELBURG, 2002), which represent the major fraction of the analytically recognisable compounds in SOM (ALLARD, 2006). Owing to FA

and AA extraction and their amount and isotopic composition analyses, we can outline the formation and the fate of biogenic residues in the complex soil system.

However, for a better understanding of biogenic residue formation, additional information on the ^{13}C label distribution within a system (mass balance) is necessary. In order to set up a detailed mass balance of ^{13}C in a system, the amount and the isotopic composition of the following components should be considered: CO_2, solvent-extractable parent compound and its metabolites and finally either ^{13}C incorporation into total biomass (liquid culture experiment) or total NER (soil experiments).

5.4.1 CO_2 measurement

The $^{13}CO_2$ trapped in 2 M NaOH solution was quantified by measurement of the total inorganic C using a Total Organic Carbon Analyser (Schimadzu TOC-5050, Duisburg, Germany). The isotopic composition (atom %) of CO_2 was determined using a GC-Combustion-isotope ratio Mass Spectrometry (GC-C-irMS; Finnigan MAT 252, Thermo Electron, Bremen, Germany, coupled to Hewlett Packard 6890 GC, Agilent Technologies, Germany), equipped with Porabondt Q-HT Plot FS column (50 m × 0.32 m × 5 µm; Chrompack, Middburg, Netherlands). In order to measure the isotopic composition, 2 ml of the NaOH solution was transferred to 15 ml crimp cap vials and acidified with 400 µl of phosphoric acid (85%). Headspace samples of 100-250 µl were then injected into the GC-C-irMS. CO_2 was separated isothermally at a temperature of 40°C. The temperature of the oxidation oven was 940°C and the one of the reduction oven was 640°C. Both the C content and isotopic composition of CO_2 trapped in MM after removal of *C. necator* biomass by centrifugation was determined in the same way as CO_2 trapped in the NaOH solutions.

5.4.2 Parent compound and metabolites measurement

$^{13}C_6$-2,4-D or $^{13}C_6$-ibu residues were extracted from soil using an Accelerated Solvent Extraction (ASE) 200 system (Dionex, Sunnyvale, CA) equipped with 11 mL stainless steel extraction cells according to RADJENOVIĆ ET AL. (2009) with few modifications. 5 gram of soil was placed in the extraction cell and mixed with diatomaceous earth (Hydromatrix™, Varian Associates, Inc., Hansen Way, Palo Alto, USA) and 20 µg of the internal standard 2-methyl-4-chlorophenoxyacetic acid (MCPA) used for quantitative analyses. The soil samples were extracted three times with methanol-water (1:1, v/v) at the following operating conditions: extraction temperature, 100ºC; pressure 100 bar; preheating period, 5 min; static extraction period, 10 min; solvent flush, 150% of the cell volume; and nitrogen purge, 150 s.

The biomass of *C. necator* was separated from MM by cetrifugation (11,000 min^{-1} for 15 min at 4°C, Jouan KR52i). The supernatant containing $^{13}C_6$-2,4-D and $^{13}C_6$-2,4-D-derived metabolites was then purified.

MM after centrifugation and soil extracts were acidified to pH 2 prior to purification by Solid Phase Extraction (SPE, Macherey Nagel, Düren, Germany). The SPE cartridges (CHROMABOND® EASY) were conditioned with 3 mL of MeOH and 3 mL of de-ionised H_2O. After the conditioning step, the acidified sample was passed slowly through the column applying a slight vacuum. The column then was washed with 10 mL of de-ionised H_2O and dried under vacuum using a vacuum pump (KNF Neuberger, Frankfurt, Germany) for 15 min. The elution of 2,4-D and its metabolites from column was performed 2 times with 5 mL of methanol (MeOH)-ACN mixture (1:1, v/v). A mixture of MeOH-tetrahydrofuran (1:1, v/v; 5 mL; 2 times) was used for the elution of ibu and ibu-derived metabolites. The eluates were then evaporated under a gentle stream of N_2. Dried sample was resuspended in the mixture of acetonitrile/trimethylsilyltrifluoroacetamide (1:2, v/v) for silylation. This mixture was then incubated for 10 min at 60 ºC, next cooled down at room temperature and finally transferred into a GC vial.

Parent compounds and their metabolites were quantified and identified using Gas Chromatography-Mass Spectrometry (GC-MS, Hewlett Packard 6890 GC, Agilent Technologies, Germany) equipped with a BPX-5 column (30 m × 0.32 m × 0.25 µm, SGE, Darmstadt, Germany) with the following temperature program: initial temperature 60°C (1.5 min), heat to 120°C (0 min) at 20°C/min, to 160°C (0 min) at 4°C/min and finally to 260°C (5 min) at 16°C/min (ZWIENER AT EL., 2002). The injector was set at 260°C and the He flow was 1.5 mL/min. The identification of 2,4-D, 2,4-DCP, Ibu and 2-OH-ibu in sample was done by comparison of their retention times with those of a standard.

5.4.3 Total biomass and NER measurement

At each sampling date, bacterial cells of *C. necator* were harvested from MM by centrifugation (11,000 min^{-1} for 15 min at 4°C; Jouan KR25i) and then freeze-dried (2 h; Christ RVC 2-25, Osterode am Harz, Germany). About of 2-5 mg of either air-dried soil sample (after removal of solvent-extractable parent compound and its metabolites) or freeze-dried biomass was weighed. Both the amount of ^{13}C and isotopic composition in either total biomass or total NER were determined using Elemental Analyser-Combustion-isotope ratio Mass Spectrometry (EA-C-irMS; Finnigan MAT 253, Thermo Electron, Bremen, Germany) coupled to Euro EA 3000, Eurovector, Milano, Italy). The temperature of the oxidation oven

was 1020°C and the one of the reduction oven was 650°C. In addition, bulk extracts from soil before purification by SPE were also analysed for ^{13}C content and the isotopic composition.

5.4.4 Fatty acids (FA) analyses

We analysed the ^{13}C label incorporation into FA of living biomass of *C. necator* and into FA of living soil microbial biomass. To distinguish between ^{13}C distributions in FA within the living (PLFA) and the non-living SOM fraction, total fatty acids (tFA) in the soil were also determined. The tFA contains FA both from the living and the dead biomass stabilised in SOM (non-living SOM; DRENOVSKY ET AL., 2004).

However, the PLFA extraction procedure from the soil microbial biomass is different than from the biomass of *C. necator* and includes two purification steps. Therefore, PLFA extractions from *C. necator* and the soil are presented below separately.

Biomass of *C. necator* was harvested from MM by centrifugation (3300 min^{-1} for 15 min at 4°C; Jouan KR25i) prior to PLFA extraction. The PLFA were extracted with a mixture of PB/MeOH/chloroform from the *C. necator* (0.5 mL/1 mL/2 mL) according to BLIGH AND DYER (1959). The PB/MeOH/chloroform mixture containing cells of *C. necator* was votexed for 3 min (Vibromix®, Sartorius Stedim Biotech, Aubagne Cedex, France). After addition of H_2O (0.5 mL), the mixture was shaken again for 1 min and finally separated into two phases by centrifugation for 10 min at 1000 min^{-1} (Jouan KR25i). The bottom phase (chloroform) containing the PLFA was evaporated under a gentle stream of N_2 prior to derivatisation

The PLFA were extracted from a soil using also the PB/MeOH/chloroform mixture (2 mL/5 mL/2.5 mL; BLIGH AND DYER, 1959). The soil suspended in the PB/MeOH/chloroform mixture was shaken for 2 h on a rotary shaker (Infors HT, Bottmingen, Switzerland). After shaking, 2.5 mL of each H_2O and chloroform was added to the mixture in order to separate into two phases (overnight). The bottom chloroform phase was dried using sodium sulfate and evaporated under N_2. This phase was then fractionated into neutral lipids, glycolipids and PLFA according to MILTNER ET AL. (2005) by chromatography over silica columns (Unisil) impregnated previously with ammonium acetate (0.02 M). To elute neutral lipids, glycolipids and PLFA chloroform (5 mL), ACN (5 mL) and MeOH (15 mL) were used, respectively. The MeOH was evaporated under N_2 and the PLFA were then derivatised.

The dried samples containing PLFA extracted from either *C. necator* or the soil were resuspended in the mixture of MeOH/Trimethylchlorosilane [(TMCS); 9:1; v:v] and methylated at 60°C in a oven (Memmert, Schwabach, Germany) for 2 h as described by THIEL ET AL., (2001). After cooling to room temperature, the excess of derivatisation reagent

was removed in the N_2-gasstream. Finally, the dried sample was redissolved in 50 µL of hexane prior to further gas chromatographic analysis.

The tFA in the soil were analysed by methylation with the same derivatisation reagent as for PLFA, (MeOH/TMCS; 1.8 mL/0.2 mL) added directly to the dry soil (THIEL ET AL., 2001). After derivatisaton, methylated tFA were extracted from soil according to MILTNER, ET AL. (2005) with diethyl ether and purified over silica gel columns (Baker). The eluate was then evaporated under N_2 and dried sample was finally dissolved in 100 µL of hexane prior to GC/MS and GC-C-irMS analyses.

The Fatty Acid Methyl Esters (FAME) in PLFA and tFA fractions were quantified and identified by GC/MS equipped with BPX-5 column as described in section 5.4.2. FA are designated as A:BωC where A is the number of C atoms, B is the number of double bonds and C is the position of the double bond relative to the aliphatic end of the molecule. The prefix "cy" assigns a cyclopropyl fatty acid, and branched fatty acids are indicated by i-, a- or 10Me.

For quantification, heneicosanoate methyl ester (FAME 21:0) was added to each sample as an internal standard prior to injection into the GC/MS. The FAME were separated using the following temperature program: initial temperature 50°C (hold for 1 min), heat to 250°C (0 min) at 4°C/min and finally to 300°C (10 min) at 2°C/min. The injector was set at 280°C, the He flow was 2.5 mL/min. FAME were identified by comparison of the retention times and the mass spectra with those of a standard mixture BAME (Bacterial Acid Methyl Esters). The isotopic composition of the FAME was determined GC-C-irMS (Finnigan MAT 253 coupled to Trace GC, Thermo Electron, Bremen, Germany) equipped with BPX-5 column (50 m × 0.32 m × 0.5 µm, SGE International, Darmstadt, Germany). The FAME were separated with the following temperature program for GC-C-irMS: initial temperature 70°C (1 min), heat to 130°C (0 min) at 20°C/min, to 150°C (5 min) at 2°C/min, to 165°C (5 min) at 2°C/min, to 230°C (0 min) at 2°C/min and finally to 300°C (5 min) at 20°C/min. The injector was set at 250°C and the He flow was 2 mL/min.

With the methylation, one additional C is introduced resulting in a shift of the isotopic composition. This shift was corrected for the original isotopic composition of the respective FA and calculated according to BOSCHKER (2004) as follows:

$$ICcorr = \frac{(n_{FAME} + n_{MeOH}) \times IC_{meas} - IC_{MeOH}}{n_{FAME}}$$

where:

n_{FAME} – number of C in respective FAME without the methyl group

n_{MeOH} – number of C of MeOH

IC_{corr} – corrected isotopic composition of FAME without the methyl group

IC_{meas} – measured isotopic composition of FAME with the methyl group

IC_{MeOH} – C isotopic composition of MeOH

5.4.5 Amino acids (AA) analyses

^{13}C-amino acids (AA) hydrolysed from proteins were analysed in soil in two fractions: in the total AA fraction (tAA) and in the living microbial biomass AA fraction (bioAA). As considered for the fatty acids, the difference between the total and the biomass AA fractions represents the refractory AA in the non-living SOM. AA in *C. necator* were hydrolysed directly from the biomass similarly as the tAA from the total soil, whereas bioAA in the soil were determined in the extracted living microbial biomass.

Prior to hydrolysis, bacterial cells were harvested from MM by centrifugation (3300 min^{-1} for 15 min at 4°C; Jouan KR25i). AA in the biomass of *C. necator* and in the total soil (tAA) were hydrolysed with 6 M HCl for 22 hours at 110°C as described by MACKO AND UHLE (1997) and SILFER ET AL. (1991). After hydrolysis, biomass pellets and soil particles were discarded by filtering through glass-fibre membrane filters. The HCl was then evaporated in an N_2-gasstream and AA from biomass of *C. necator* were further derivatised, whereas the soil hydrolysate was purified.

After evaporation of the HCl excess, soil sample was resuspended in 4 mL of 0.1 M HCl and purified over cation exchange resin (DOWEX 50W-X8) according to AMELUNG AND ZHANG (2001). Cation exchange resin was prepared according to BOAS ET AL. (1953), by pre-rinsing with 2 M NaOH, conditioning with 2 M HCl, and washing with de-ionized H_2O until the pH of the eluate was neutral. The sample containing the AA was pipetted on top of the column. The column then was washed with oxalic acid (25 mL, pH 1.6-1.8) followed by 0.01 M HCl (5 mL) and de-ionised H_2O (5 mL). Finally the AA were eluted with 2.5 M NH_4OH (30 mL) and NH_4OH was evaporated. The dried samples were re-dissolved in 4 mL 0.1 M HCl and centrifuged for 15 min at 4200 min^{-1} (Jouan KR25i). After centrifugation, the 0.1 M HCl excess was evaporated under N_2 prior to derivatisation.

The carboxyl groups of AA in dried sample were esterified with a mixture of Isopropanol/Acetylchloride (IP/AC; 1 mL/250 µL) at 110°C for 1 h and the amino groups trifluoroacetylated with a mixture of Dichloromethane/Trifluroacetic Acid Anhydride (DCM/TFAA; 500 µL/500 µL) at 60°C for 1 h as described previously by MILTNER ET AL. (2005). After derivatisation, the samples were dried under N_2 and purified using a mixture of PB (1 mL) and chloroform (0.5 mL) as described previously by UEDA ET AL. (1989). The

mixture was shaken, centrifuged (Centrifuge 5415R eppendorf; Eppendorf, Hamburg, Germany) at 600 min^{-1} for 10 min and the chloroform phase was finally evaporated. After drying, the samples containing the derivatised AA were re-dissolved in 100 µL DCM for injection into the GC-MS.

For the determination of the AA in the living biomass (bioAA), the biomass was first extracted from soil as described by JACOBSEN AND RASMUSSEN (1992). The efficiency of the bacterial biomass extraction has been reported to be in the range of 40% by independent studies (JACOBSEN AND RASMUSSEN, 1992; MILTNER ET AL., 2009). Hence, bioAA are underestimated but the relative trends of bio-AA are correct because of the relatively constant recoveries even in different soils. The cells were extracted from soil by shaking using an end-over-end shaker (2h; GFL 3040, Fachhandel für Labor- und Medizintechnik GmbH, Kothen, Germany) according to MILTNER ET AL. (2009) with 1 g Chelex 100 (chelating cation exchange resin) and 10 mL of sodium deoxycholate/Polyethylenglycol 600 solution (0.1%/2.5%). After shaking, soil particles were removed using low speed centrifugation (960 min^{-1}, 15 min; Jouan KR25i) and finally the biomass in supernatant was harvested using high-speed centrifugation (11,000 min^{-1}, 1h, 4°C; Jouan KR25i). Biomass pellets containing AA were further hydrolysed, purified and derivatised in the same way as tAA.

The identity, quantity and isotopic composition of the AA were determined by GC/MS and GC-C-irMS. The temperature program of GC/MS was as follows: 70°C (5 min) to 100°C (30 min) at 30°C/min, to 175°C (5 min) at 10°C/min, to 250°C (5 min) at 10°C/min, to 340°C (15 min) at 30°C/min. The injector was set at 280°C; the He flow was 1.7 mL/min. For quantification and identification of respective AA in samples, an external standard containing all detectable AA in the samples [alanine (Ala), glycine (Gly), threonine (Thr), serine (Ser), valine (Val), leucine (Leu), isoleucine (Ile), proline (Pro), aspartate/asparagine (Asp), glutamate/glutamine (Glu), phenylalanine (Phe), and lysine (Lys)] was used. Asparagine and glutamine are desaminated during the acid hydrolysis, resulting in aspartate and glutamate, respectively. Therefore they are shown as Asp and Glu. The internal standard L-norleucine was added to each sample before hydrolysis to estimate the losses of AA in soil during the two purification steps. The isotopic composition of each AA was determined using GC-C-irMS with the same column as for the FAME and the following temperature program: 50°C (0 min) to 100°C (10 min) at 15°C/min, to 130°C (0 min) at 2°C/min, to 220°C (0 min) at 10°C/min, to 250°C (10 min) at 30°C/min. The injector was set at 250°C; the He flow was 2 mL/min.

The original isotopic enrichment of respective AA in samples, after shifting their isotopic composition during two derivatisation steps, was corrected using the following equation (SILFER ET AL., 1991):

$$IC_{corr} = \frac{IC_{meas} \times (n_{AA} + n_{TFAA} + n_{IP}) - IC_{TFAA} \times n_{TFAA} - IC_{IP} \times n_{IP}}{n_{AA}}$$

where:

n_{AA}– number of C in respective AA

n_{TFAA} - number of C of TFAA

n_{IP} - number of C of IP

IC_{corr} – corrected isotopic composition of AA

IC_{meas} – measured isotopic composition of AA

IC_{TFAA} – C isotopic composition of TFAA

IC_{IP} – C isotopic composition of IP

5.5 Data analyses

A stable isotope tracer (^{13}C) was used to investigate the formation and the fate of biogenic residues during biodegradation of a target contaminant in a system. The application of ^{13}C-labelled compounds in biodegradation studies allows the analysis of their structural assignments using more sophisticated analytical techniques such as GC-MS (RICHNOW ET AL., 1999). By extraction of known ^{13}C-labelled biogenic residues representatives (FA and AA) formed during ^{13}C-labelled compound biodegradation and inspection of their chemical structures using GC-MS, we can thus prove the microbial origin of NER in the soil.

However, the soil is also naturally abundant in ^{13}C (~ 1%), therefore blank (without compound application) and control (^{12}C-labelled compound) samples were used for the correction of ^{13}C abundance in a soil amended with the tested ^{13}C-labelled compound. Similarly to the soil, ^{13}C abundance in the liquid culture experiments was also corrected accordingly due to addition of ^{12}C-2,4-D to ^{13}C-2,4-D incubation experiments .

The contents of ^{13}C-FA, ^{13}C-AA, evolved $^{13}CO_2$ during mineralisation, ^{13}C-parent compound and its ^{13}C-metabolites and ^{13}C in either total biomass or NER were presented as a percentage of the initially applied ^{13}C-labelled compound to an experiment. In addition, a mass balance of ^{13}C in the system was taken into consideration when interpreting the data on the ^{13}C label incorporation into biogenic residues.

All incubation experiments were prepared in triplicates and results are shown as averages. The error bars represent the standard deviation of these triplicates.

6 RESULTS

The incorporation of ^{13}C label into biogenic residue representatives (FA and AA) during microbial degradation of ^{13}C-labelled compounds was studied in two different systems: simple liquid pure culture experiments and complex soil systems.

The main objective of liquid culture experiments was to trace the fate of ^{13}C label derived from $^{13}C_6$-2,4-D in the simple biological system with a particular focus on ^{13}C-incorporation into the biomass of the 2,4-D degrader. Due to simplicity, these experiments were also designed to estimate the ratio of ^{13}C-FA and ^{13}C-AA to the total biomass ^{13}C content, which are necessary for a proper quantification of the total amount of biogenic residues in the soil systems.

The formation and the fate of biogenic residues during biodegradation of two different model compounds ($^{13}C_6$-2,4-D and $^{13}C_6$-ibu) were investigated in complex soil experiments. The general objective was to estimate the extent of biogenic residue formation and their amount in relation to total NER and finally to assess the risk for the environment related to NER formation from readily degradable organic contaminants in soil.

6.1 Liquid culture experiments

Cupriavidus necator JMP 134 was selected for the biodegradation studies, because it is a well-described 2,4-D soil degrader. *C. necator* grows on 2,4-D as C source. The ^{13}C label incorporation into FA and AA of *C. necator* during microbial degradation of 2,4-D was investigated directly from $^{13}C_6$-2,4-D and indirectly via CO_2 fixation from $^{13}CO_2$

The detailed description of PLFA classes typical for specific groups of microorganisms, their designation and AA abbreviations used for data interpretation have already been presented in the sections 2.6.2.1, 5.4.4 and 5.4.5, respectively.

6.1.1 Incubation experiment with $^{13}C_6$-2,4-D

The ^{13}C incorporation into the biomass of *C. necator* during biodegradation of $^{13}C_6$-2,4-D was studied over 14 days. The ^{13}C content in FA, AA, in total biomass and the ^{13}C mass balance in the system were analysed after 1, 2, 3, 7 and 14 days of incubation. The abiotic experiment with *C. necator* and $^{13}C_6$-2,4-D showed neither $^{13}C_6$-2,4-D biodegradation nor ^{13}C incorporation into FA and AA of *C. necator* after 14 days, indicating the importance of bacterial activity in the degradation of this compound and in the formation of ^{13}C-labelled FA and AA.

6.1.1.1 Mass balance of $^{13}C_6$-2,4-D in the system

The Duran glass bottle (500 mL) was aerated with humidified air prior to CO_2 measurement in order to trap the evolved CO_2 in a headspace into NaOH solution. However, due to reported high overpressure in the small headspace volume of about 250 mL and the need to connect the bottle to a pump, some CO_2 was lost before aeration. The experiment with *C. necator* JMP134 grown on unlabelled 2,4-D and under $^{13}CO_2$ atmosphere, in which the initial $^{13}CO_2$ concentration was known, revealed that at each sampling date ~ 70% of the initial $^{13}CO_2$ was lost (data not shown). Therefore, the data on the mineralisation kinetics presented in **Figure 16** are highly underestimated.

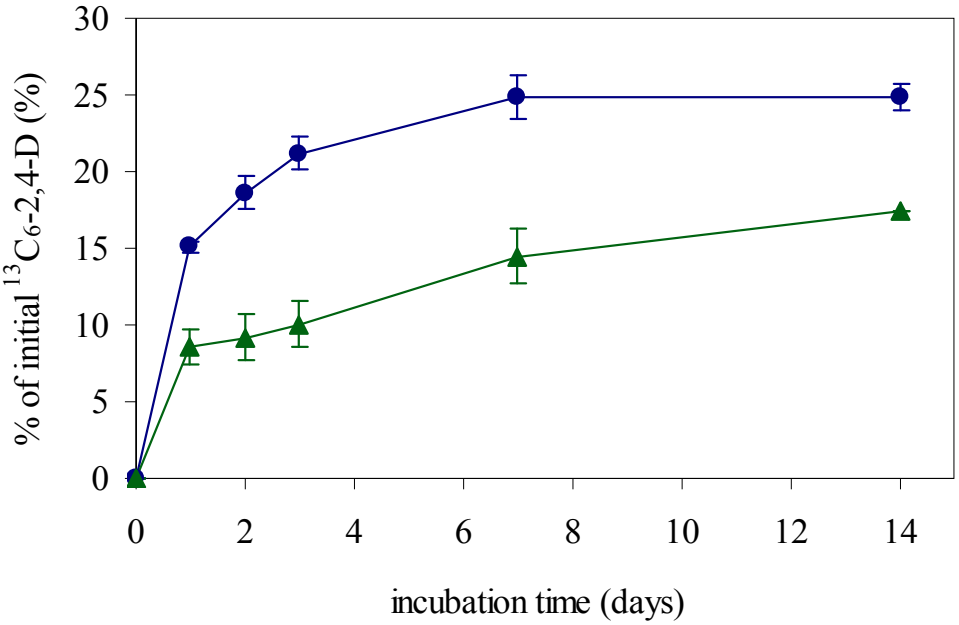

Figure 16. Distribution of ^{13}C label in an incubation experiment with A *C. necator* JMP 134 grown on $^{13}C_6$-2,4-D medium. Mineralisation (●) and biomass (▲). Extractable $^{13}C_6$-2,4-D and metabolites not detectable since day 1

After the first day of incubation, 15% of ^{13}C derived from $^{13}C_6$-2,4-D was already mineralised (50% of $^{13}C_6$-2,4-D equivalents after correction for 70% loss). At that time, neither $^{13}C_6$-2,4-D nor its known ^{13}C-aromatic ring metabolites were detected, indicating their complete utilisation. After day 1, the content of $^{13}CO_2$ evolved during 2,4-D mineralisation increased slowly and finally on the day 7 reached a plateau (~ 83% of $^{13}C_6$-2,4-D equivalents after correction for 70% loss). The incorporation of ^{13}C from $^{13}C_6$-2,4-D into the biomass started immediately (after 1 day) and their contents remained constant until day 3 (~ 10% of $^{13}C_6$-2,4-D equivalents; see **Table 6**). Thereafter, the amount of ^{13}C found in the total biomass increased slowly reaching finally 17.4% of the added $^{13}C_6$-2,4-D equivalents. However, based on the OD measurement of a culture suspension, the biomass growth of *C. necator* JMP134

reached a plateau already after 1 day of incubation (see **Figure 17**), demonstrating clearly that ^{13}C-incorporation into the biomass was not growth-depending.

Table 6. Incorporation of ^{13}C into the biomass of *C. necator* JMP 134 grown on $^{13}C_6$-2,4-D

Incubation time (days)	Label incorporation [% of $^{13}C_6$-2,4-D]				
	1	**2**	**3**	**7**	**14**
biomass	9.5 (± 1.1)	9.2 (±1.5)	10.0 (± 1.5)	14.5 (± 1.8)	17.4 (± 0.06)
PLFA	0.3 (± 0.05)	0.5 (± 0.02)	0.6 (± 0.01)	0.8 (± 0.05)	0.6 (± 0.04)
AA	6.0 (± 0.07)	4.7(± 0.2)	6.1 (± 0.3)	7.3 (± 0.2)	8.1 (± 0.4)
PLFA/Biomass	**3.1**	**5.4**	**6**	**5.5**	**3.4**
AA/Biomass	**63.2**	**51.1**	**61**	**54.3**	**46.6**

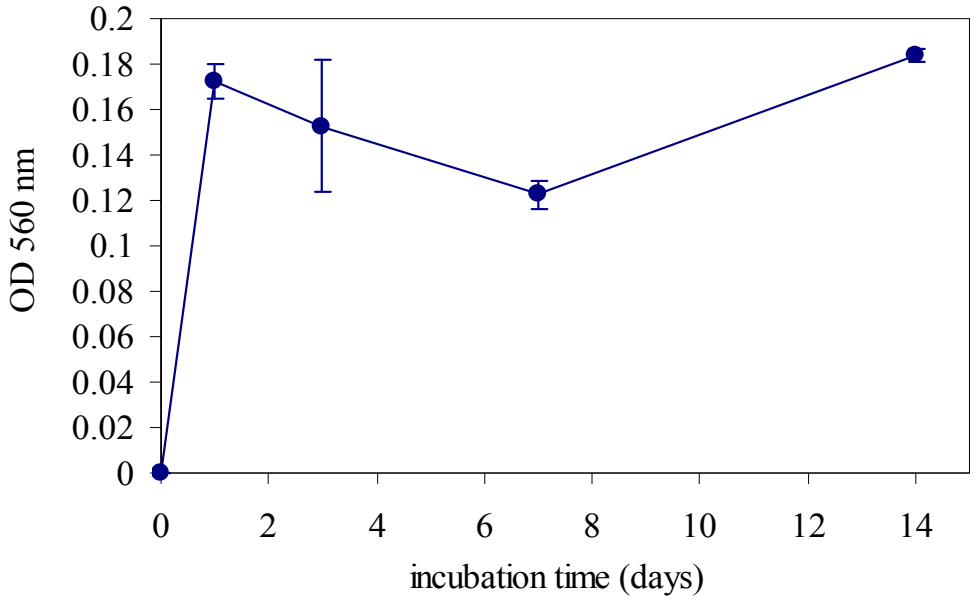

Figure 17. Time course of the biomass growth of *C. necator* JMP 134 on $^{13}C_6$-2,4-D medium based on the OD measurement (560 nm)

^{13}C incorporation into FA and AA of *C. necator* JMP 134 was observed in the $^{13}C_6$-2,4-D incubation experiment. The contents of ^{13}C-PLFA in *C. necator* represented about 3–6% of the total ^{13}C in the biomass (see **Table 6**), which is in a good accordance to ~ 5% in FA in the microbial biomass reported by BAS ET AL. (2003). The amounts of ^{13}C-AA of *C. necator* were in the range of 46–63% of the total amount of ^{13}C in the biomass, which is consistent with the protein content in microbial biomass (50–55%) reported by MADIGAN AND MARTINKO (2006).

6.1.1.2 Incorporation of ^{13}C into PLFA of *C. necator* JMP 134 grown on $^{13}C_6$-2,4-D

The ^{13}C label was rapidly incorporated into PLFA (on day 1), all classes of PLFA received ^{13}C label and remained enriched until the end of the experiment (see **Figure 18**). The amounts of ^{13}C in the PLFA increased strongly reaching a maximum on day 7 (0.8% of $^{13}C_6$-2,4-D equivalents). Thereafter, their contents decreased until day 14 (0.6% of the initially $^{13}C_6$-2,4-D added). At the beginning (day 1), the monounsaturated PLFA contained slightly more ^{13}C label than the saturated straight chain PLFA and the saturated cyclopropyl PLFA. On the day 2, the contents of ^{13}C in the saturated cyclopropyl PLFA increased strongly reaching a maximum on the day 7 (~ 0.2% of $^{13}C_6$-2,4-D equivalents) and this class of PLFA remained dominant until the end of the incubation period.

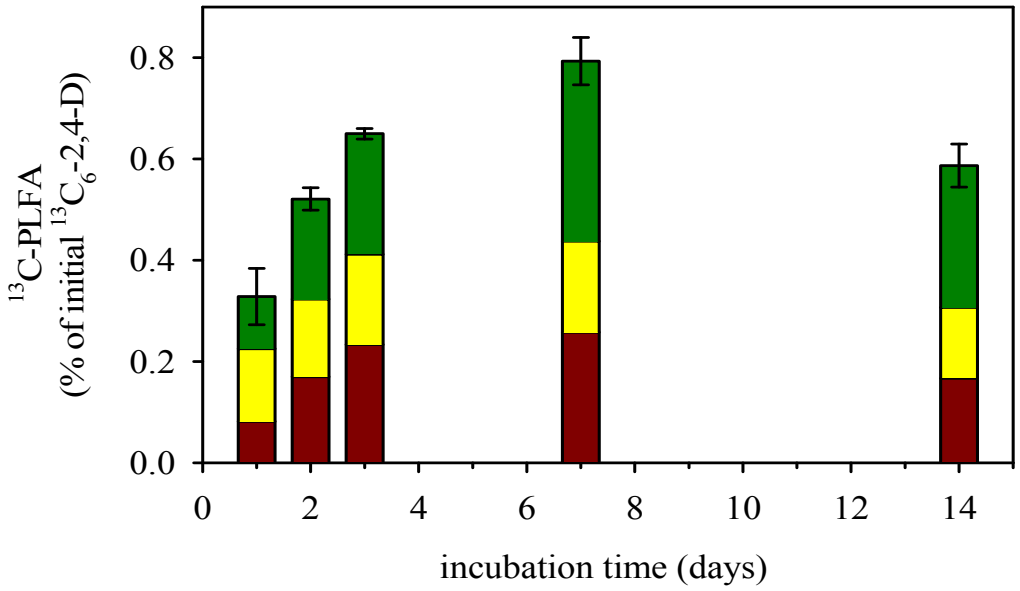

Figure 18. Distribution of ^{13}C label in lipid classes of PLFA of *C. necator* JMP 134 grown on $^{13}C_6$-2,4-D medium. [saturated straight-chain (■), monounsaturated (□), and saturated cyclopropyl PLFA (■)]

6.1.1.3 Incorporation of ^{13}C into AA of *C. necator* JMP 134 grown on $^{13}C_6$-2,4-D

The incorporation of ^{13}C label into AA started already on the first day (**Table 7**) and the contents of ^{13}C-AA were constant until day 3 (excluding the ^{13}C-AA on day 2). Thereafter, the amount of ^{13}C in AA increased slightly, finally reaching 8.13% of $^{13}C_6$-2,4-D equivalents. The ^{13}C label was distributed within all AA and all ^{13}C-AA contained ^{13}C label at the end of the incubation. Both ^{13}C-Asp and ^{13}C-Glu were dominant AA throughout the incubation period. After 3 days, the amount of ^{13}C in Glu and Asp decreased slightly, whereas ^{13}C-Thr, ^{13}C-Val, ^{13}C-Leu, ^{13}C-Ile and ^{13}C-Pro increased strongly. After 7 days, the contents of ^{13}C in

Threo and Val increased again, while ^{13}C-Phe decreased. At the end, ^{13}C-Thr and ^{13}C-Gly decreased (~ 2 times and 4 times, respectively), while the amount of ^{13}C in Leu, Ile, Pro, Asp, Glu and Phe increased. At the same time, a very low content of ^{13}C label was also detected in Ser.

Table 7. Distribution of the ^{13}C label in various ^{13}C-AA of *C. necator* JMP 134 grown on $^{13}C_6$-2,4-D medium

	^{13}C-AA [% of $^{13}C_6$-2,4-D]				
Incubation time (days)	**1**	**2**	**3**	**7**	**14**
Alanine	n.d.	n.d.	0.43 (± 0.6)	0.46 (± 0.07)	0.28 (± 0.08)
Glycine	0.07 (± 0.01)	0.16 (± 0.02)	n.d.	0.53 (± 0.04)	**0.13**↓ (± 0.02)
Threonine	0.25 (± 0.004)	0.24 (± 0.11)	**0.65**↑ (± 0.08)	**0.86**↑ (± 0.002)	**0.46**↓ (± 0.05)
Valine	0.09 (± 0.09)	0.12 (± 0.06)	**0.27**↑ (± 0.03)	**0.6**↑ (± 0.004)	0.56 (± 0.09)
Serine	n.d.	n.d.	n.d.	n.d.	0.003 (± 0.11)
Leucine	0.61 (± 0.003)	0.62 (± 0.04)	**0.94**↑ (± 0.02)	0.82 (± 0.1)	**1.2**↑ (± 0.04)
Isoleucine	0.14 (± 0.02)	0.13 (± 0.01)	**0.30**↑ (± 0.009)	0.32 (± 0.01)	**0.38**↑ (± 0.01)
Proline	0.55 (± 0.02)	0.39 (± 0.03)	**0.51**↑ (± 0.07)	0.59 (± 0.02)	**0.73**↑ (± 0.03)
Aspartate[a]	**1.19** (± 0.03)	**1.17** (± 0.24)	**1.01**↓ (± 0.07)	**1.07** (± 0.02)	**1.56**↑ (± 0.32)
Glutamate[b]	**1.52** (± 0.08)	**0.98**↓ (± 0.06)	**0.89**↓ (± 0.18)	**1.07** (± 0.004)	**1.53**↑ (± 0.33)
Phenylanine	0.77 (± 0.02)	0.59 (± 0.04)	0.76 (± 0.14)	**0.58**↓ (± 0.03)	**0.77**↑ (± 0.04)
Lysine	0.85 (± 0.005)	0.25 (± 0.02)	0.35 (± 0.17)	0.44 (± 0.12)	0.49 (± 0.01)
TOTAL	**6.06 (± 0.07)**	**4.67 (± 0.17)**	**6.13 (± 0.34)**	**7.34 (± 0.19)**	**8.13 (± 0.41)**

[a] incl. asparagine; [b] incl. glutamine; n.d. - not detectable; values are shown as averages ± standard deviation values printed in bold represent characteristic values, arrows visualise increase or decrease of the respective AA compared to the preceeding sampling time

6.1.2 Incubation experiment under $^{13}CO_2$ atmosphere

The incorporation of ^{13}C label from $^{13}CO_2$ into the biomass of *C. necator* JMP 134 grown on unlabelled 2,4-D, but in the presence of $^{13}CO_2$ was studied over 14 days. The content and the isotopic composition of ^{13}C in PLFA, AA and in total biomass were analysed after 2, 3, 7 and 14 days of incubation. The main objective of this experiment was to study the relevance of heterotrophic CO_2 fixation in ^{13}C label incorporation into the biomass of *C. necator* during 2,4-D biodegradation. However, the first sampling date (day 1) considered in the incubation experiment with $^{13}C_6$-2,4-D was excluded and not analysed, because CO_2 fixation plays usually a minor role in the initial phase of biodegradation experiment. One triplicate set of the biotic incubation experiment with $^{13}CO_2$ and without 2,4-D showed no incorporation of ^{13}C into the biomass of *C. necator*, demonstrating strong dependence of $^{13}CO_2$ fixation on the presence 2,4-D in the medium. The abiotic incubation experiment also revealed no ^{13}C incorporation into the PLFA and AA of *C. necator* after 7 days, indicating the importance of biological activity for CO_2 fixation.

6.1.2.1 Incorporation of ^{13}C into the biomass of *C. necator* JMP 134 grown under $^{13}CO_2$

A very low ^{13}C incorporation into the biomass of *C. necator* JMP 134 from $^{13}CO_2$ was observed already after 2 days of incubation (0.07% of the initially $^{13}CO_2$ added; see **Table 8**). Thereafter, the amount of ^{13}C in the total biomass was increasing progressively throughout the incubation time, finally reaching 4.2% of the added $^{13}CO_2$ equivalents. The biomass grew rapidly from day 0 until day 3 (see **Figure 19**), indicating the high importance of CO_2 fixation in the later phase of the incubation experiment. Thereafter, the biomass growth reached a plateau.

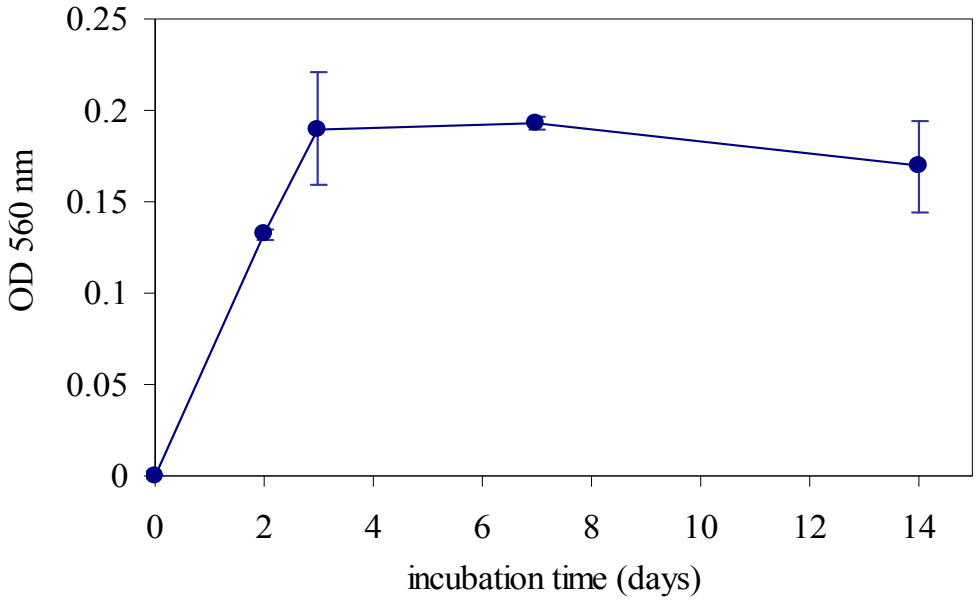

Figure 19. Time course of the biomass growth of *C. necator* JMP 134 on unlabelled 2,4-D medium and under $^{13}CO_2$ based on the OD measurement (560 nm)

The ^{13}C label was incorporated into PLFA and AA of *C. necator* JMP 134 in the $^{13}CO_2$ fixation experiment. The amount of ^{13}C-PLFA in *C. necator* JMP 134 was in the range of 3–10% of the total ^{13}C in the biomass (excluding the day 2 where the amount of PLFA was very low; see **Table 8**), what is in good agreement with the data of BAS ET AL. (2003) and with those of the $^{13}C_6$-2,4-D experiment with *C. necator*. ^{13}C-AA in *C. necator* JMP 134 represented 42–62% of the total ^{13}C in the biomass, which is close to the percentage of proteins in biomass reported by MADIGAN AND MARTINKO (2006) and to the $^{13}C_6$-2,4-D experiment with *C. necator*.

Table 8. Incorporation of ^{13}C into biomass of *C. necator* JMP 134 grown on unlabelled 2,4-D medium and under $^{13}CO_2$

Incubation time (days)	^{13}C-AA [% of $^{13}CO_2$]			
	2	**3**	**7**	**14**
biomass	0.07 (± 0.5)	1.6 (±0.4)	3.5 (± 0.2)	4.2 (± 0.2)
PLFA	0.01 (± 0.004)	0.17 (± 0.02)	0.15 (± 0.008)	0.12 (± 0.003)
AA	0.02 (± 0.07)	1.0 (± 0.4)	1.5 (± 0.3)	1.8 (± 0.08)
PLFA/Biomass	**14.3**	**10.5**	**4.3**	**2.9**
AA/Biomass	**28.6**	**62.5**	**42.9**	**42.9**

6.1.2.2 Incorporation of ^{13}C into PLFA of *C. necator* JMP 134 grown under $^{13}CO_2$

The ^{13}C derived from $^{13}CO_2$ was incorporated into the PLFA immediately on day 2, only the monounsaturated PLFA and the saturated straight chain PLFA carried ^{13}C label (see **Figure 20**).

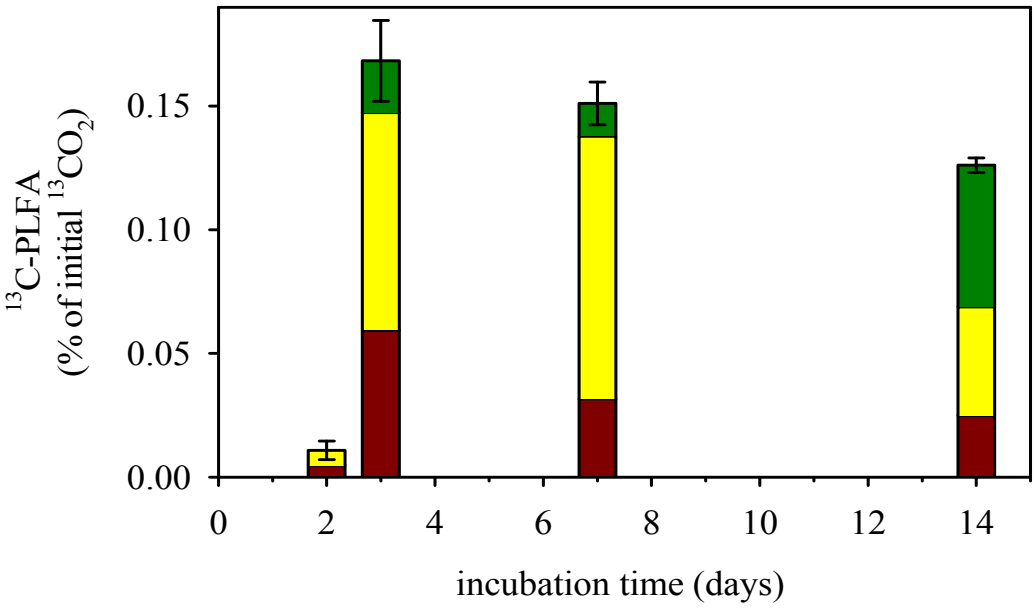

Figure 20. Distribution of ^{13}C-label in lipid classes of PLFA of *C. necator* JMP 134 grown on unlabelled 2,4-D medium and under $^{13}CO_2$.
[saturated straight-chain (■), monounsaturated (■), and saturated cyclopropyl PLFA (■)]

The contents of ^{13}C-PLFA increased rapidly, reaching their maximum on the day 3 (0.17% of the initially $^{13}CO_2$ added) and remained constant until day 7. At the end, the ^{13}C-PLFA decreased to 0.12% of the initially $^{13}CO_2$ added. From day 3 onwards, the ^{13}C label was distributed within all classes of PLFA and on both day 3 and 7, the monounsaturated PLFA dominated over other classes of PLFA. Similarly as observed in the $^{13}C_6$-2,4-D experiment,

the amount of ^{13}C in the cyclopropyl PLFA increased strongly at the end of experiment, indicating the onset of starvation (KAUR ET AL., 2005). At the same time, the content of ^{13}C-monounsaturated PLFA decreased to about half of the value at day 3.

6.1.2.3 Incorporation of ^{13}C into AA of *C. necator* JMP 134 grown under $^{13}CO_2$

The incorporation of ^{13}C started on the second day of incubation and this label was detected only in Asp (see **Table 9**). After 2 days, a continuous increase of ^{13}C in AA until the end of incubation was observed and finally on day 14, ^{13}C-AA reached 1.85% of the initially added $^{13}CO_2$. After 3 days, Thr contained most ^{13}C and dominated over all other AA until the end, whereas ^{13}C-Glu and ^{13}C-Asp were also significantly enriched. After 7 days, the amounts of ^{13}C in Thr, Asp, Ile increased, but the ^{13}C in Glu and Phe decreased. At the end, an increase of ^{13}C in Val and Leu was observed, while the amount of thelabel in Thr decreased.

Table 9. Distribution of the ^{13}C label in various ^{13}C-AA of *C. necator* JMP 134 grown on unlabelled 2,4-D medium and under $^{13}CO_2$

	^{13}C-AA [% of $^{13}C_6$-2,4-D]			
Incubation time (days)	**2**	**3**	**7**	**14**
Alanine	n.d.	n.d.	n.d.	0.07 (± 0.04)
Glycine	n.d.	0.02 (± 0.01)	0.04 (± 0.04)	n.d.
Threonine	n.d.	**0.32**↑ (± 0.13)	**0.66**↑ (± 0.03)	**0.46**↓ (± 0.03)
Valine	n.d.	n.d.	0.03 (± 0.01)	**0.26**↑ (± 0.01)
Serine	n.d.	n.d.	n.d.	n.d.
Leucine	n.d.	0.07 (± 0.02)	0.08 (± 0.06)	**0.16**↑ (± 0.004)
Isoleucine	n.d.	0.01 (± 0.002)	**0.12**↑ (± 0.01)	0.12 (± 0.01)
Proline	n.d.	0.05 (± 0.02)	0.05 (± 0.009)	n.d.
Aspartate[a]	0.02 (± 0.07)	**0.19**↑ (± 0.05)	**0.29**↑ (± 0.005)	0.26 (± 0.02)
Glutamate[b]	n.d.	**0.19** (± 0.02)	**0.14**↓ (± 0.02)	0.13 (± 0.0008)
Phenylanine	n.d.	0.13 (± 0.09)	**0.07**↓ (± 0.002)	0.11 (± 0.007)
Lysine	n.d.	n.d.	n.d.	0.25 (± 0.04)
TOTAL	**0.02 (± 0.07)**	**0.97 (± 0.42)**	**1.49 (± 0.29)**	**1.85 (± 0.08)**

[a] incl. asparagine; [b] incl. glutamine; n.d. - not detectable; values are shown as averages ± standard deviation
values printed in bold represent characteristic values, arrows visualise increase or decrease of the respective AA compared to the preceeding sampling time

6.2 Soil experiments

The formation and the fate of biogenic residues in soil systems were investigated with two various organic contaminants, $^{13}C_6$-2,4-D and $^{13}C_6$-ibu. Soil samples were analysed for the amounts and isotopic compositions of FA and AA. The supplemental data on the ^{13}C mass balance in the soil, which includes mineralisation, solvent-extractable $^{13}C_6$-2,4-D or $^{13}C_6$-ibu and their metabolites, and total NER were kindly provided by Cristobal Girardi (Dept. Environmental Biotechnology, UFZ, Leipzig; for details see also GIRARDI ET AL., submitted).

The labelled biomarkers were extracted from the living part of SOM as ^{13}C-PLFA or as ^{13}C-bioAA, whereas the total fractions were directly analysed from soil (^{13}C-tFA and ^{13}C-tAA). The data presented in this section as FA or AA in the non-living SOM fraction were calculated by subtracting the amounts of the living fractions (PLFA or bioAA) from the amounts of total fraction (tFA or tAA). The detailed description of FA classes typical for specific groups of microorganisms, their designations and AA abbreviations used for the data interpretation have already been presented in the sections 2.6.2.1, 5.4.4 and 5.4.5, respectively.

6.2.1 Soil incubation experiment with $^{13}C_6$-2,4-D

The formation and the fate of FA and AA during microbial degradation of 2,4-D in the soil system were studied in two different soil experiments. In the first experiment, which focused on tracing ^{13}C label incorporation into FA and AA directly from the labelled herbicide, the soil was amended with $^{13}C_6$-2,4-D. In the second experiment, the soil was treated with unlabelled 2,4-D and incubated under $^{13}CO_2$ in order to study the indirect ^{13}C incorporation into PLFA via heterotrophic CO_2 fixation.

The formation and fate of FA and AA in the soil experiment with $^{13}C_6$-2,4-D was studied over 64 days. These microbial biomarkers were analysed for their ^{13}C content and isotopic composition after 2, 4, 8, 16, 32 and 64 days of incubation. No incorporation of ^{13}C into FA and AA after 32 and 64 days was observed in sterile soil experiments incubated with $^{13}C_6$-2,4-D, indicating their biotic formation. The results on the formation of FA and AA and their fate in the soil incubated with $^{13}C_6$-2,4-D were published in NOWAK ET AL. (in press).

6.2.1.1 Mass balance of $^{13}C_6$-2,4-D in the soil

The kinetic of ^{13}C distribution within the soil fractions during microbial degradation of $^{13}C_6$-2,4-D is presented in **Figure 21** (GIRARDI ET AL., submitted). The degradation of $^{13}C_6$-2,4-D in soil started immediately without an apparent lag phase, and the readily available 2,4-D was present at high concentrations until day 4 (30.4% of the $^{13}C_6$-2,4-D added). From day 8 onwards, only residual amounts of $^{13}C_6$-2,4-D were detected in soil (0.5–2.3% of the initially $^{13}C_6$-2,4-D added). At the end of incubation, ~ 58% of the initial ^{13}C label in the soil was evolved as $^{13}CO_2$, ~ 36% was measured as NER and only 0.5% constituted the solvent extractable $^{13}C_6$-2,4-D (after purification by SPE). The total amounts of unidentified solvent extractable (before purification) were relatively high until day 8 (40–90% of $^{13}C_6$-2,4-D equivalents) and decreased to ~ 8% of $^{13}C_6$-2,4-D equivalents on the day 64. The solvent extractable 2,4-D residues were extracted from soil samples with a MeOH/de-ionised H_2O

mixture (1:1, v/v) using ASE, which is a relatively harsh extraction method (NORTHCOTT AND JONES, 2000). Therefore, the high solvent extractable 2,4-D residues could be assigned to easily extractable biogenic residues before their stabilisation in SOM. The total recovery of ^{13}C from $^{13}C_6$-2,4-D on day 64 was ~ 102.5%.

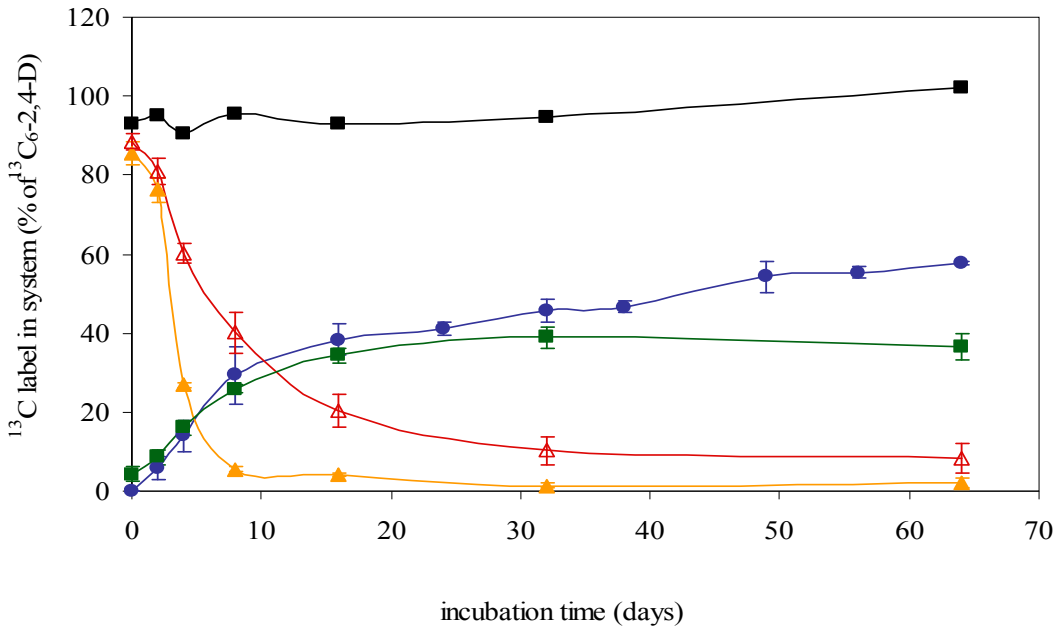

Figure 21. Distribution of the initial ^{13}C label from $^{13}C_6$-2,4-D (%) in the system within 64 days of incubation experiment (GIRARDI ET AL., submitted). Mineralisation (●), extractable amount before purification (△), extractable amount after purification (▲), NER (■) and recovery (■)

No mineralisation was found in the abiotic soil experiments after 64 days, the solvent extractable $^{13}C_6$-2,4-D and $^{13}C_6$-2,4-DCP accounted for about of 60% of $^{13}C_6$-2,4-D equivalents (GIRARDI ET AL., submitted). At the same time, NER amounted to about 19% of the $^{13}C_6$-2,4-D equivalents, indicating for their formation even in absence of living microrganisms.

6.2.1.2 Formation of FA and their fate in soil incubated with $^{13}C_6$-2,4-D

The ^{13}C label was found only in short chain FA (C_{14}-C_{20}), which are typical for bacteria (CRANWELL, 1974). These organisms were thus involved in 2,4-D degradation in soil. The incorporation of ^{13}C label into the PLFA during the degradation of $^{13}C_6$-2,4-D started already after 2 days of incubation and the PLFA remained enriched in ^{13}C until the end of the incubation experiment (**Figure 22A**).

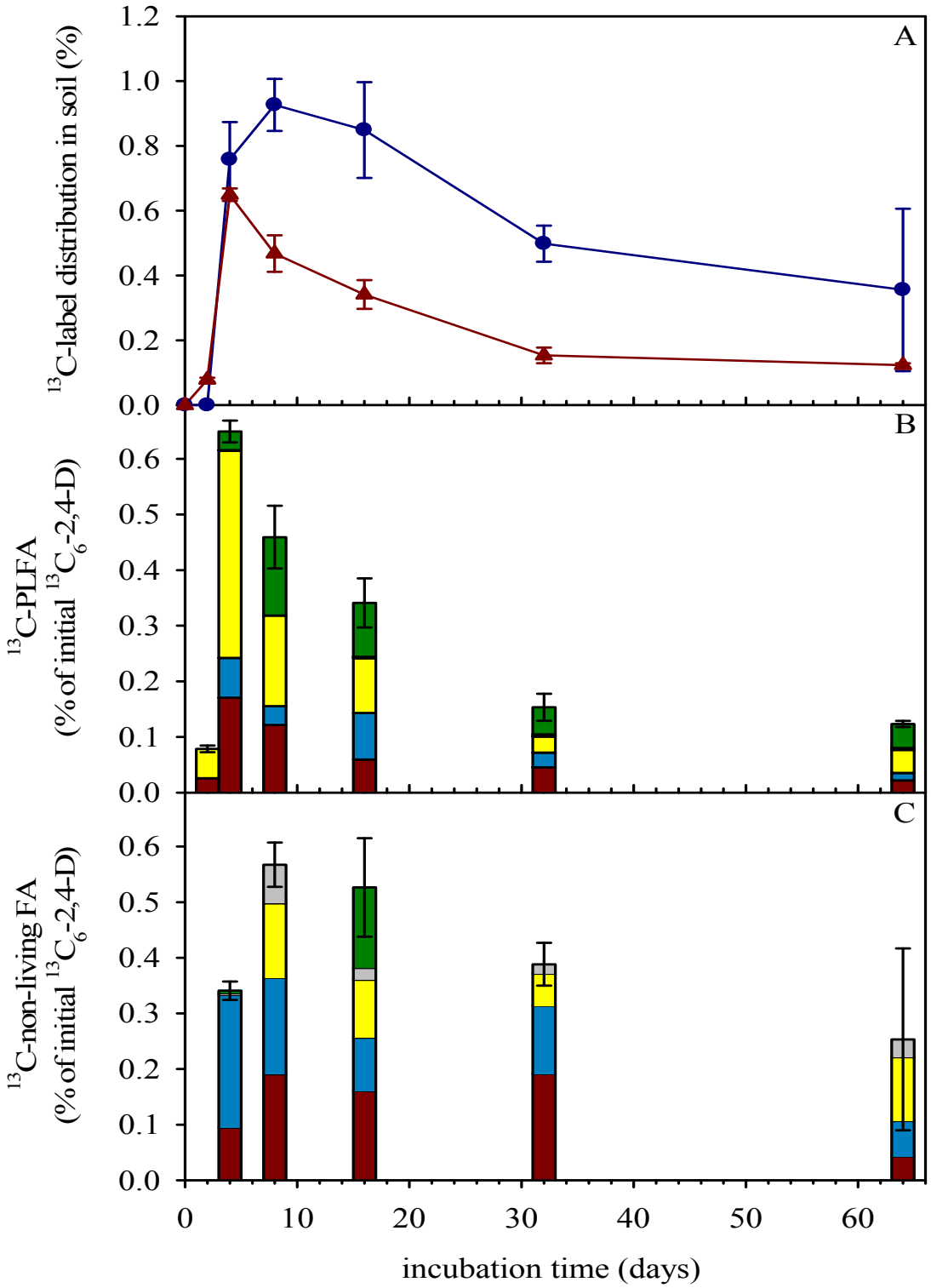

Figure 22. Time course of ^{13}C label incorporation during $^{13}C_6$-2,4-D microbial degradation in soil (**A**) within tFA (●) and PLFA (▲). Distribution of ^{13}C-label in lipid classes of PLFA (**B**) and t-FA in non-living SOM (**C**).

[saturated straight-chain (■), saturated methyl-branched (■), monounsaturated (■), polyunsaturated (■) and saturated cyclopropyl FA (■)]

The maximum content of ^{13}C in PLFA (~ 0.7% of the initially added $^{13}C_6$-2,4-D equivalents) was detected already on day 4. Thereafter, the amounts of ^{13}C-labelled PLFA from membranes of the living microbial biomass decreased continuously until day 32 (~ 0.2% of $^{13}C_6$-2,4-D equivalents). After 4 days, a simultaneous flux of ^{13}C from the living biomass to the non-living fraction of SOM was observed. On days 8 and 16, the contents of ^{13}C-FA in the non-living fraction of SOM reached a maximum (~ 0.5% of the initial $^{13}C_6$-2,4-D amount in soil). The ^{13}C-FA in the non-living SOM fraction started to decline after 16 days. After day 32, already about of 70% of the label found in the ^{13}C-tFA could be assigned to the non-living SOM. At the same time, the contents of both ^{13}C-PLFA and ^{13}C-FA in the non-living SOM fraction remained nearly constant until the end of incubation. At the end of the experiment, the ^{13}C-FA in the non-living fraction declined to 50% and the ^{13}C-PLFA to 20% of their maximum, indicating clearly the degradation of biomass-derived residues in SOM.

During the initial phase of $^{13}C_6$-2,4-D degradation, the ^{13}C label was found only in the saturated straight chain and in the monounsaturated PLFA, indicating that Gram-negative bacteria were the initial degraders of this herbicide (**Figure 22B**; see also **Table 10**). On day 4, when the content of ^{13}C-PLFA was highest, the monounsaturated PLFA contained most of the label in comparison to other classes of PLFA, and 16:1ω7c PLFA was the dominant PLFA. On day 4, when a continuous decrease of ^{13}C-PLFA in soil was observed, the incorporation of ^{13}C label into the saturated cyclopropyl PLFA started. After 8 days, when the amounts of ^{13}C-monounsaturated PLFA (e.g. 16:1ω7c, 16:1ω5c, 18:1ω7c and 18:1ω5c) declined strongly, the ^{13}C-cy 17:0 carried most of the label (except for 16:0) and dominated until the end of incubation period. At the same time, the labelled saturated straight-chain PLFA, saturated methyl-branched PLFA and the monounsaturated PLFA from the living biomass were already partly incorporated into the non-living SOM (**Figure 22C**). However, the ^{13}C cyclopropyl PLFA were not incorporated into the non-living SOM (apart from day 16), what might be explained by the fact that this class of FA became labelled later than other FA.

Table 10. The classification and composition of ^{13}C label in respective PLFA during microbial degradation of $^{13}C_6$-2,4-D in soil

Incubation time (days)	^{13}C-PLFA [10^{-4}; % of $^{13}C_6$-2,4-D]					
	2	**4**	**8**	**16**	**32**	**64**
Saturated straight chain						
14:0	23.6 (± 2.4)	25.0 (± 25.3)	n.d.	n.d.	n.d.	n.d.
15:0	n.d.[a]	n.d.	3.6 (± 2.7)	n.d.	n.d.	n.d.
16:0	232 (± 1.7)	1615 (± 19.6)	1185.3 (± 161.5)	552.6 (± 41.4)	403.6 (± 23.9)	220.4 (± 11.7)
18:0	n.d.	57 (± 31.9)	25.9 (± 42.5)	32.2 (± 6.5)	31.6 (± 29.7)	n.d.
20:0	n.d.	6.8 (± 10.5)	n.d.	6.6 (± 9.3)	19.7 (± 9.3)	n.d.
Saturated methyl branched						
i [b] 14:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
i 15:0	n.d.	227.8 (± 58.6)	42.7 (± 42.6)	25.6 (± 10.5)	23.7 (± 37.5)	51.7 (± 5.1)
a [c] 15:0	n.d.	142.1 (± 46.7)	12.2 (± 38.6)	43.4 (± 3.3)	7.8 (± 22.9)	28.7 (± 5.7)
i 16:0	n.d.	64.8 (± 12.7)	4.9 (± 49)	n.d.	n.d.	n.d.
i 17:0	n.d.	99.6 (± 5.2)	20.2 (± 4.2)	22.3 (± 0.13)	7.2 (± 15.6)	26.2 (± 9.6)
a 17:0	n.d.	33.9 (± 11.1)	13.6 (± 0.8)	n.d.	n.d.	26.3 (± 29.4)
10 Me [d] 16:0	n.d.	n.d.	158.8 (± 2)	n.d.	19.4 (± 30.6)	n.d.
10 Me 17:0	n.d.	n.d.	28.5 (± 6)	32.9 (± 10.4)	2.5 (± 0.2)	n.d.
10 Me 18:0	n.d.	77.4 (± 18)	32.4 (± 28.9)	39.7 (± 3.5)	2.5 (± 6.8)	n.d.
18:0 br [f]	n.d	71.6 (± 0.07)	25.6 (± 3.2)	674.2 (± 531.4)	199.9 (± 24.9)	n.d.
Monounsaturated						
16:1ω7c[c]	139.3 (± 38.4)	**2100.6** (± 72)	**671.9** ↓ (± 70.4)	215.9 (± 9.0)	115 (± 22)	136.3 (± 7.7)
16:1ω5c	56.9 (± 43)	292.2 (± 5.1)	**73.1** ↓ (± 15.4)	n.d.	n.d.	30.5 (± 2.2)
18:1ω9c	59.7 (± 10.2)	201.9 (± 39.6)	42.4 (± 4.3)	143 (± 29.7)	n.d.	n.d.
18:1ω7c	252.6 (± 2)	988.7 (± 18.9)	**779.1** ↓ (± 89.8)	589.5 (± 30.2)	173.2 (± 70.2)	232.8 (± 23.6)
18:1ω5c	20.1 (± 5)	63.2 (± 9.3)	**9.1** ↓ (± 3.5)	n.d.	n.d.	n.d.
Monounsaturated branched						
15:1 br	n.d.	27.7 (± 11.5)	16.2 (± 6.8)	10.4 (± 8.2)	0.6 (± 5.4)	4.4 (± 2.1)
17:1 br	n.d.	51.0 (± 5.5)	36.0 (± 2.7)	28.1 (± 10)	n.d.	10.7 (± 18)
Polyunsaturated						
18:2	n.d.	1.5 (± 12)	1.6 (± 0.02)	7.1 (± 0.8)	n.d.	n.d.
18:2ω6,9	n.d.	10.3 (± 12.5)	n.d.	15.7 (± 23)	39.9 (± 12.9)	35.2 (± 73.7)
Saturated cyclopropyl						
cy 17:0	n.d.	**239** (± 59.2)	**1292** ↑ (± 80)	893.8 (± 49.2)	457.8 (± 11.4)	335.7 (± 6.4)
cy 19:0	n.d.	94.2 (± 24.8)	118.8 (± 27.5)	78.7 (± 4.0)	30.2 (± 26.9)	94.7 (± 33.2)
TOTAL	**784 (± 102.8)**	**6486 (± 511)**	**4677 (± 683.7)**	**3412 (± 775.2)**	**1535 (± 350.3)**	**1234 (± 228.6)**

[a]a- anteiso; [b]br – branched; [c]c - cis isomer; [d]i – iso; [e] n.d. - not detectable; [f]Me - methyl; values in brackets (±) represent the standard deviation of the average of triplicate; values printed in bold represent characteristic values, arrows visualise increase or decrease of the respective FA compared to the preceeding sampling time

6.2.1.3 Formation of AA and their fate in soil incubated with $^{13}C_6$-2,4-D

The incorporation of the ^{13}C label into the living biomass AA fraction (bioAA) during microbial degradation of 2,4-D in soil started 2 days later than into the PLFA (**Figure 23**).

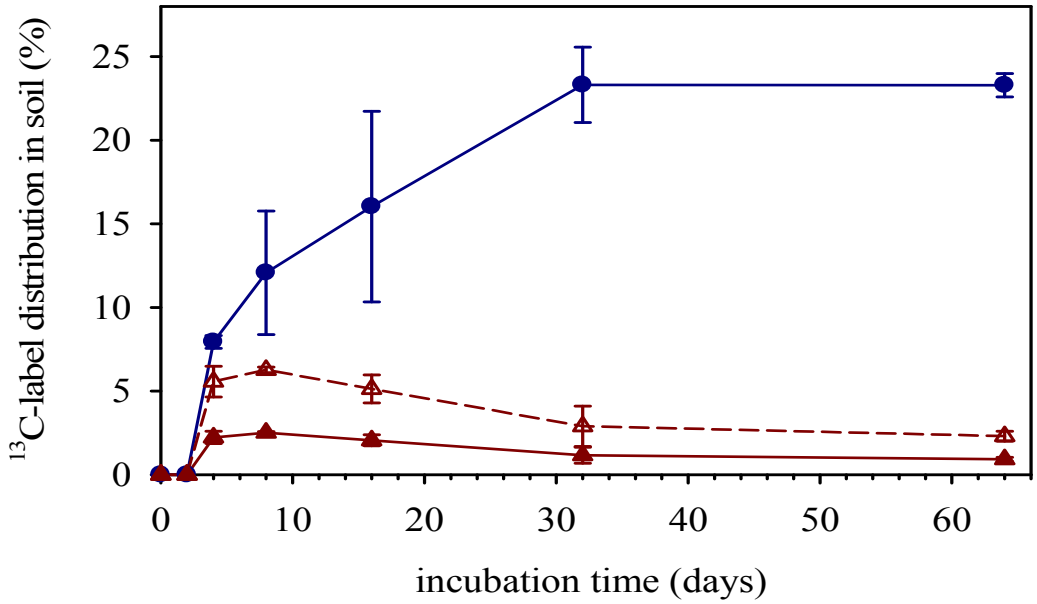

Figure 23. Time course of ^{13}C label incorporation within tAA (●), bioAA (▲) and bioAA (△) recalculated based on 40% extraction efficiency (for details see section 5.4.5) during microbial degradation of $^{13}C_6$-2,4-D in soil

The amounts of ^{13}C-bioAA were highest on days 4 and 8 (2.2% and 2.5%, respectively, of the initially $^{13}C_6$-2,4-D equivalents added). From day 16, a decay of ^{13}C-bioAA was observed and at that time the majority (~ 70%) of the ^{13}C-tAA was already detected in the non-living part of SOM, although it clearly derived from the living biomass. A ^{13}C flux of AA from the living to the non-living fraction of SOM started after 4 days as it was also observed for PLFA. After 32 days, the contents of both ^{13}C-bioAA and ^{13}C-AA in the non-living SOM did not change significantly until the end of the experiment. At the end of the incubation (day 64), the contents of ^{13}C-AA in the living biomass fraction of SOM were 64% lower (0.9% of $^{13}C_6$-2,4-D equivalents) than their maximum detected in this experiment. Finally, 90% of the ^{13}C-tAA in soil was stabilised in the non-living SOM pool, reaching 22% of the initial content of $^{13}C_6$-2,4-D equivalents added to soil. On day 4, when the incorporation of ^{13}C label into bioAA fraction was initiated, Glu carried most of this label (1.9% of the initial amount $^{13}C_6$-2,4-D in soil; see **Table 11A**) among the ^{13}C-AA. At the same time, much lower amounts of ^{13}C were found in Asp and Val (each ~ 0.2% of the initially $^{13}C_6$-2,4-D added).

Table 11. Distribution of the ^{13}C label in various ^{13}C-bioAA (**A**) and ^{13}C-AA in the non-living SOM (**B**) during microbial degradation of $^{13}C_6$-2,4-D in soil

A

	^{13}C-AA [% of $^{13}C_6$-2,4-D]					
Incubation time (days)	**2**	**4**	**8**	**16**	**32**	**64**
Alanine	n.d.	n.d.	n.d.	0.10 (± 0.05)	0.04 (± 0.10)	0.05 (± 0.03)
Glycine	n.d.	n.d.	n.d.	0.19 (± 0.09)	0.02 (± 0.14)	n.d.
Threonine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Valine	n.d.	0.16 (± 0.04)	0.13 (± 0.04)	0.34 (± 0)	0.10 (± 0.11)	0.04 (± 0.01)
ß-alanine	n.d.	n.d.	n.d.	n.d.	0.002 (± 0.01)	0.004 (± 0.007)
Leucine	n.d.	n.d.	0.38 (± 0.03)	0.15 (± 0.03)	0.10 (± 0.93)	0.15 (± 0.01)
Isoleucine	n.d.	0.02 (± 0.05)	0.08 (± 0.04)	0.04 (± 0)	0.12 (± 0.44)	0.07 (± 0.03)
Proline	n.d.	n.d.	0.35 (± 0.03)	0.26 (± 0.1)	0.21 (± 0.66)	0.18 (± 0.05)
Aspartate[a]	n.d.	0.14 (± 0.14)	**0.60** ↑ (± 0.17)	0.27 (± 0.47)	n.d.	n.d.
Glutamate[b]	n.d.	**1.90** (± 0.50)	**0.23** ↓ (± 0.06)	0.25 (± 0.58)	0.11 (± 0.17)	n.d.
Phenylanine	n.d.	n.d.	0.31 (± 0.04)	0.16 (± 0.17)	0.15 (± 0.50)	0.34 (± 0.09)
Lysine	n.d.	n.d.	0.42 (± 0.01)	0.30 (± 0.14)	0.31 (± 0.29)	0.08 (± 0.03)
TOTAL	**n.d.**	**2.22 (± 0.37)**	**2.50 (± 0.07)**	**2.06 (± 0.33)**	**1.16 (± 0.48)**	**0.91 (± 0.12)**

B

	^{13}C-AA [% of $^{13}C_6$-2,4-D]					
Incubation time (days)	**2**	**4**	**8**	**16**	**32**	**64**
Alanine	n.d.	0.5 (± 4.4)	3.9 (± 2.7)	1.0 (± 7.4)	1.0 (± 1.3)	1.6 (± 4.0)
Glycine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Threonine	n.d.	n.d.	n.d.	n.d.	0.01 (± 0.01)	0.3 (± 0.1)
Valine	n.d.	2.6 (± 1.8)	n.d.	0.7 (± 2.1)	n.d.	0.9 (± 0.7)
ß-alanine	n.d.	n.d.	n.d.	0.3 (± 2.0)	0.2 (± 0.9)	0.3 (± 0.2)
Leucine	n.d.	n.d.	n.d.	3.4 (± 0.4)	1.8 (± 5.5)	3.7 (± 0.5)
Isoleucine	n.d.	2.3 (± 1.5)	n.d.	0.5 (± 1.7)	0.6 (± 1.7)	1.8 (± 0.3)
Proline	n.d.	n.d.	n.d.	n.d.	3.8 (± 5.1)	2.5 (± 0.02)
Aspartate[a]	n.d.	1.6 (± 3.7)	0.5 (± 0.8)	1.3 (± 0.1)	1.6 (± 0.7)	1.3 (± 0,5)
Glutamate[b]	n.d.	n.d.	n.d.	3.6 (± 0.03)	2.7 (± 1.1)	1.4 (± 0.2)
Phenylanine	n.d.	n.d.	0.2 (± 0.6)	0.5 (± 0.2)	5.4 (± 1.6)	4.8 (± 0.2)
Lysine	n.d.	n.d.	1.5 (± 2.3)	0.02 (± 0.01)	1.5 (± 2.9)	1.2 (± 0.3)
TOTAL	**n.d.**	**7.0 (± 0.3)**	**6.1 (± 1.9)**	**11.3 (± 4.1)**	**18.6 (± 1.8)**	**19.8 (± 0.6)**

[a] incl. asparagine; [b] incl. glutamine; n.d. - not detectable; values are shown as averages ± standard deviation
values printed in bold represent characteristic values, arrows visualise increase or decrease of the respective AA compared to the preceeding sampling time

After 8 days, when the content of ^{13}C in Glu decreased strongly, the ^{13}C-Asp increased rapidly and this bioAA was dominant over the other ^{13}C-AA, indicating heterotrophic CO_2 fixation (MILTNER ET AL., 2005). In addition, on day 8, the ^{13}C label was also distributed to lesser extent within Val, Leu, Ile, Pro, Phe and Lys. On day 16, when the ^{13}C-bioAA decreased, the incorporation of ^{13}C into two additional AA Ala and Gly, was observed. The ^{13}C-AA from the living biomass fraction were also transferred into the non-living SOM pool (see **Table 11B**). After 32 days of incubation, when the contents of both ^{13}C-bioAA and ^{13}C-AA in the non-living SOM did not change significantly, the distribution of the ^{13}C label in the individual AA in the non-living and living SOM fraction remained nearly constant until the end of the experiment.

6.2.1.4 Biogenic residues in soil incubated with $^{13}C_6$-2,4-D

The FA represent about 5% of the microbial biomass (BAS ET AL., 2003); this was also proved in the liquid experiments with *C. necator* JMP 134 grown on 2,4-D, which clearly showed that ^{13}C-labelled FA represented ~ 5% of the total ^{13}C in the biomass (see sections 6.1.1.2 and 6.1.2.1). Therefore, considering this content of FA in bacterial cells, we can conclude from the highest amounts of the ^{13}C-tFA (about 1%) determined on day 8 that at least 20% biomass in $^{13}C_6$-2,4-D experiment had been formed. The proteins containing AA are the most abundant components (50–55% of the microbial biomass) of bacterial cells (MADIGAN AND MARTINKO, 2006), which is also consistent with the results obtained in the experiment with *C. necator* (sections 6.1.1.1 and 6.1.2.1). Therefore, taking into consideration this conversion factor of 2, we can conclude from the amounts of 22% found in the ^{13}C-tAA at the end of soil incubation, that there were 44% biogenic residues in this experiment.

6.2.2 Soil incubation experiment under $^{13}CO_2$ atmosphere

The $^{13}CO_2$ evolved during mineralisation of $^{13}C_6$-2,4-D in the soil may be used as an additional C source by microorganisms for the synthesis of their cellular components (KREBS, 1941), which are also finally incorporated into SOM. Therefore, the objective of this soil experiment was to study the contribution of heterotrophic CO_2 fixation to the formation of biogenic residues during 2,4-D biodegradation and to distinguish from ^{13}C incorporation into these residues directly from the labelled 2,4-D.

The formation and the fate of ^{13}C-PLFA in soil spiked with non-labelled 2,4-D and incubated under $^{13}CO_2$ was studied over 64 days. The labelled CO_2 was added in an amount equivalent to the final release of CO_2 evolved during biodegradation of labelled 2,4-D in the soil. One triplicate set of abiotic incubation experiments with $^{13}CO_2$ and with unlabelled 2,4-D showed no incorporation of the label into the PLFA, indicating the relevance of microorganisms in the CO_2 fixation process in the soil.

6.2.2.1 Formation of PLFA and their fate in soil incubated under $^{13}CO_2$ atmosphere

GIRARDI ET AL. (submitted; see also section 6.2.1.1) reported that the readily available $^{13}C_6$-2,4-D was at high concentrations in soils until day 4; thereafter, only traces of this herbicide were detected. The CO_2 fixation usually plays a minor role during first days of incubation, when the readily available C source is still present at high concentrations. Therefore, two sampling dates (days 2 and 4), considered in the investigation of the formation and fate of PLFA in soils incubated with $^{13}C_6$-2,4-D were excluded and not analysed in the $^{13}CO_2$ fixation experiments. The incorporation of ^{13}C label into PLFA during heterotrophic

CO_2 fixation was compared with the amounts of ^{13}C-PLFA found in the $^{13}C_6$-2,4-D experiment. The distribution of this label derived from $^{13}C_6$-2,4-D or $^{13}CO_2$ during 2,4-D degradation in these two experiments is presented in **Figure 24A**.

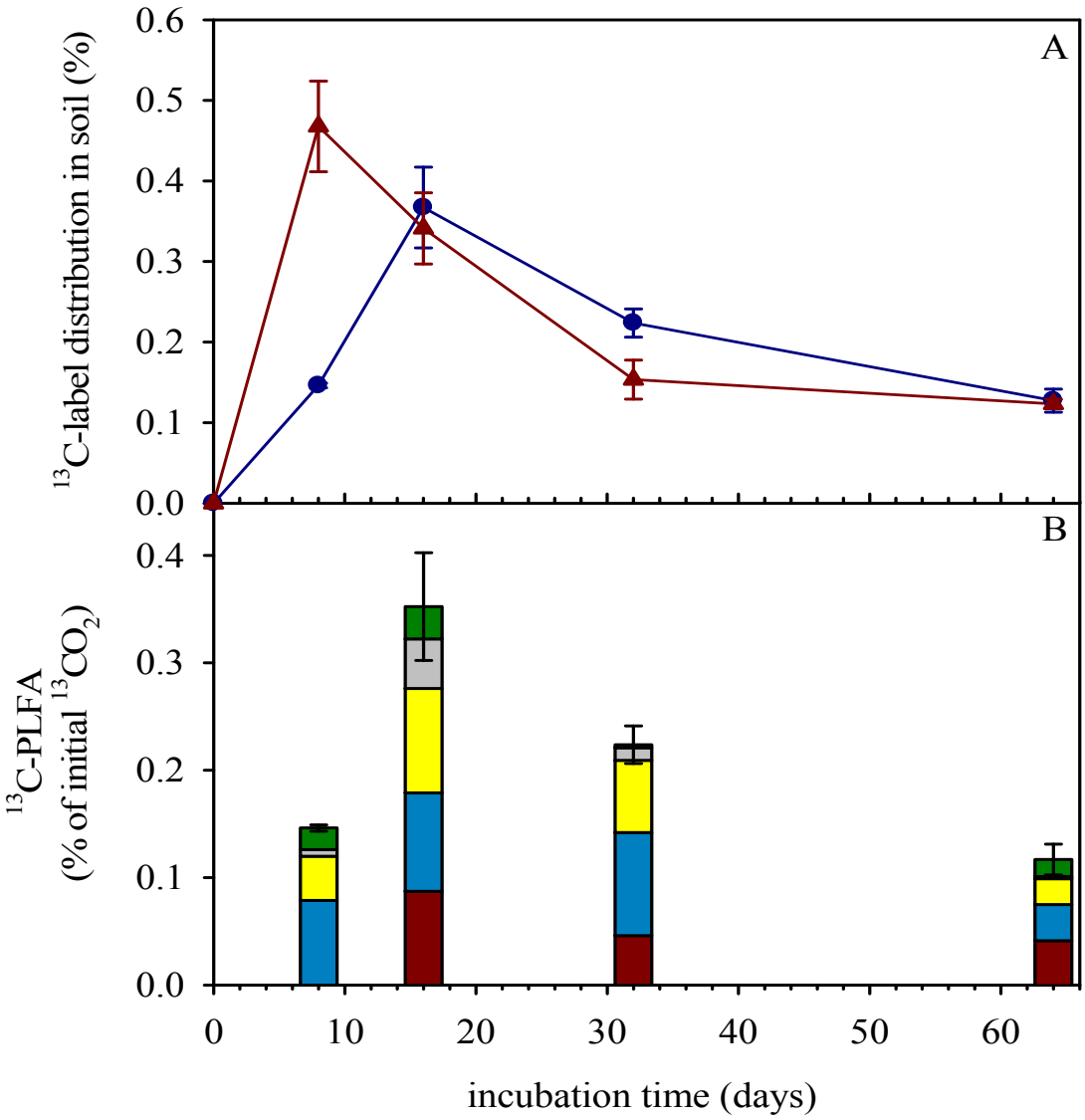

Figure 24. Comparison of the distribution of the ^{13}C label within PLFA (**A**) from biodegradation of $^{13}C_6$-2,4-D (▲) and from $^{13}CO_2$ fixation (●) and within classes of PLFA after $^{13}CO_2$ fixation (**B**).
[saturated straight-chain (■), saturated methyl-branched (■), monounsaturated (■), polyunsaturated (■) and saturated cyclopropyl FA (■)]

After 8 days of incubation, the incorporation of ^{13}C into the PLFA from $^{13}C_6$-2,4-D was still three times higher (~ 0.5% of the initial $^{13}C_6$-2,4-D equivalents added) than that from $^{13}CO_2$ fixation (0.15% of the initial $^{13}CO_2$ in system). The highest incorporation of ^{13}C into the

PLFA via heterotrophic CO_2 fixation was observed on day 16 (~ 0.4% of the initial $^{13}CO_2$) and was in the same range as the ^{13}C incorporation into PLFA from $^{13}C_6$-2,4-D at that time. On day 16, a decrease of both ^{13}C-PLFA and ^{13}C-FA in the non-living SOM fraction was observed in the experiment with $^{13}C_6$-2,4-D. This indicates that in the absence of the readily degradable $^{13}C_6$-2,4-D in soil, the ^{13}C derived from these FA was recycled via heterotrophic CO_2 fixation, which reached a maximum on that day. From day 32, the CO_2 fixation decreased slowly until the end of the incubation period.

The ^{13}C label derived from $^{13}CO_2$ was incorporated to all classes of PLFA (**Figure 24B**). After 8 days, the saturated methyl-branched PLFA (e.g. i:15:0, a:15:0, i:17:0 and 10Me16:0; see **Table 12**) contained more ^{13}C label than the monounsaturated PLFA (except for 18:1ω7c), in particular in comparison to 16:1ω7c, which was the dominant PLFA detected in the soil incubated with $^{13}C_6$-2,4-D (**Table 10**). After 16 days of incubation, the amounts of ^{13}C in the monounsaturated PLFA and saturated methyl-branched PLFA were nearly similar, indicating the contribution of both Gram-negative and Gram-positive bacteria to CO_2 fixation. At that time, however, more ^{13}C from $^{13}CO_2$ was incorporated into the monounsaturated 18:1ω7c and 18:1ω9c and into the polyunsaturated 18:2ω6,9 in comparison to the other ^{13}C-PLFA. From day 32, the methyl-branched PLFA contained slightly more ^{13}C from $^{13}CO_2$ than the monounsaturated PLFA until the end of experiment.

The presence of a readily available organic substrate, e.g. acetic acid, stimulates CO_2 fixation (MILTNER ET AL., 2005). In order to estimate this effect on the fixation process, we also incubated one triplicate set of soil systems with $^{13}CO_2$ without the herbicide for 64 days. After 64 days of incubation, the incorporation of ^{13}C into the PLFA from CO_2 in the absence of 2,4-D was 10 times lower (0.013% of the initial $^{13}CO_2$) than the amounts fixed by soil microorganisms grown in the presence of this readily degradable compound (0.13% of the initial $^{13}C_6$-2,4-D).

Table 12. The classification and composition of ^{13}C label in respective PLFA during $^{13}CO_2$ fixation in soil.

Incubation time (days)	^{13}C-PLFA [10^{-4}; % of $^{13}CO_2$]			
	8	**16**	**32**	**64**
Saturated straight chain				
14:0	n.d.[e]	n.d.	37.0 (± 35.5)	25.3 (± 7)
15:0	n.d.	62.8 (± 45.9)	n.d.	n.d.
16:0	n.d.	459.6 (± 209.1)	348.5 (± 14.6)	209.1 (±19.3)
18:0	n.d.	200.9 (± 99.3)	10.1 (± 15.2)	122.1 (±22.3)
20:0	8.5 (± 1.4)	149.8 (± 84.2)	64 (± 12.4)	55.9 (±18.6)
Saturated methyl branched				
[d]i 14:0	n.d.	n.d.	n.d.	n.d.
i 15:0	**231.9** (± 31.7)	132.8 (± 146.2)	167 (± 33.3)	33.3 (± 5.5)
[a]a 15:0	**136.2** (± 19.4)	78.8 (± 200.1)	90.3 (± 20.5)	41.1 (± 2.2)
i 16:0	n.d.	110.2 (± 64.8)	190.7 (± 34.1)	8.5 (± 7)
i 17:0	**107.6** (± 23.9)	n.d.	97 (± 4.8)	9.4 (± 1.4)
a 17:0	68.3 (± 15.6)	189.8 (± 121.9)	n.d.	1.5 (± 23.9)
10 Me[f] 16:0	**105.3** (± 33.8)	217.5 (± 89.6)	247.2 (± 61.7)	39.7 (± 6)
10 Me 17:0	28.6 (± 26.3)	34.0 (± 4.9)	48.9 (± 0.9)	56.1 (± 0.8)
10 Me 18:0	65.3 (± 3.9)	70.1 (± 99.8)	54 (± 3.8)	101.9 (± 29.2)
18:0 br[b]	36.1 (± 25.9)	84.2 (± 69.5)	64.2 (± 0.9)	45.1 (± 13.3)
Monounsaturated				
16:1ω7c[c]	56.6 (± 25.1)	52.4 (± 104.7)	75 (± 25.7)	46.7 (± 39.8)
16:1ω5c	41.4 (± 19.7)	63.2 (± 72.7)	69.2 (± 0.6)	27.9 (± 12.3)
18:1ω9c	n.d.	**326.3** (± 164.3)	113.3 (± 65.4)	n.d.
18:1ω7c	**261.2** (± 35.2)	**347.5** (± 70.2)	237.1 (± 118.2)	87.7 (± 138.9)
18:1ω5c	n.d.	22.31 (± 18.8)	109.6 (± 0.5)	62.9 (± 13.2)
Monounsaturated branched				
15:1br	12.8 **(**± 8.1)	80.4 (± 16.6)	1.6 (± 4.8)	6.0 (± 9.7)
17:1br	38.9 **(**± 14.1)	77.8 (± 55.7)	66.4 (± 13.4)	10.1 (± 3.9)
Polyunsaturated				
18:2	18.3 (± 19.9)	n.d.	118.3 (± 13.3)	19.3 (± 17.0)
18:2ω6,9	45 (± 48.8)	**462.4** (± 92.9)	n.d.	n.d.
Saturated cyclopropyl				
cy 17:0	91.5 (± 13.4)	121.6 (± 18.8)	1.6 (± 14.6)	159.7 (± 18.5)
cy 19:0	107.8 (± 50.9)	179 (± 202.6)	25.8 (± 7.5)	n.d.
TOTAL	**1462 (± 29.7)**	**3524 (± 501.4)**	**2237 (± 175.3)**	**1169 (± 143.1)**

[a]a- anteiso; [b]br – branched; [c]c - cis isomer; [d]i – iso; [e]n.d. - not detectable; [f]Me - methyl; ; values in brackets (±) represent the standard deviation of the average of triplicates; values printed in bold represent characteristic values

6.2.3 Soil incubation experiment $^{13}C_6$-ibu

The formation and fate of biogenic residues during microbial degradation of $^{13}C_6$-ibu in the soil were studied over 90 days. The content and the isotopic composition of FA and AA were determined after 2, 7, 14, 28, 59 and 90 days of incubation. The supporting data on the mass balance of ^{13}C derived from $^{13}C_6$-ibu in the soil system were provided by Cristobal Girardi (see also GIRARDI ET AL., submitted). Similar to the $^{13}C_6$-2,4-D experiment, no ^{13}C incorporation into FA and AA was observed in the abiotic soil experiments with $^{13}C_6$-ibu.

6.2.3.1 Mass balance of $^{13}C_6$-ibu in the soil

The distribution of the ^{13}C in the soil system during microbial degradation of $^{13}C_6$-ibu is shown in **Figure 25** (GIRARDI ET AL., submitted).

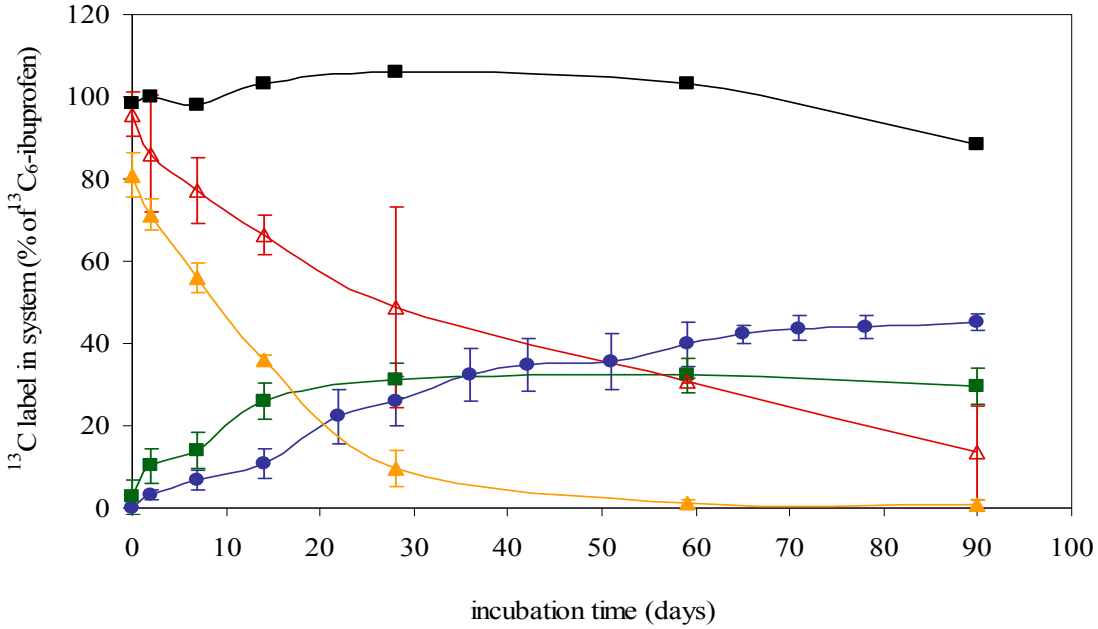

Figure 25. Distribution of the initial ^{13}C label from $^{13}C_6$-ibu (%) in the system over 90 days of incubation experiment (GIRARDI ET AL., submitted). Mineralisation (●), extractable amount before purification (△), extractable amount after purification (▲), NER (■) and recovery (■)

The degradation of $^{13}C_6$-ibu started later than $^{13}C_6$-2,4-D degradation. The solvent extractable $^{13}C_6$-ibu and its metabolites were detected until day 59 (~ 1% of $^{13}C_6$-ibu equivalents). At the end, 45% of the initial ^{13}C label in soil was mineralised, 30% was found as NER and only 0.5% was identified as $^{13}C_6$-ibu (after purification by SPE). The content of solvent bulk extractable ^{13}C (before purification) was high until day 59 (48% of $^{13}C_6$-ibu equivalents) and its decrease was observed on day 90 (13% of $^{13}C_6$-ibu equivalents). The solvent extractable Ibu residues were extracted from soil using the same method as used for $^{13}C_6$-2,4-D. The total recovery of ^{13}C at the end was much lower (88% of the initial ^{13}C in the system) than in the $^{13}C_6$-2,4-D soil experiment.

No mineralisation of $^{13}C_6$-ibu after 90 days was seen in the abiotic soil experiments, the solvent extractable fraction accounted for 65% of the initially added,$^{13}C_6$-ibu and ~ 16% of $^{13}C_6$-ibu equivalents was found as NER (GIRARDI ET AL., submitted).

6.2.3.2 Formation of FA and their fate in soil incubated with $^{13}C_6$-ibu

The incorporation of ^{13}C label into PLFA started immediately and they were enriched until the end of the incubation time. During the first 28 days of incubation, the ^{13}C label was detected only in the membranes of living biomass (PLFA, see **Figure 26A**).

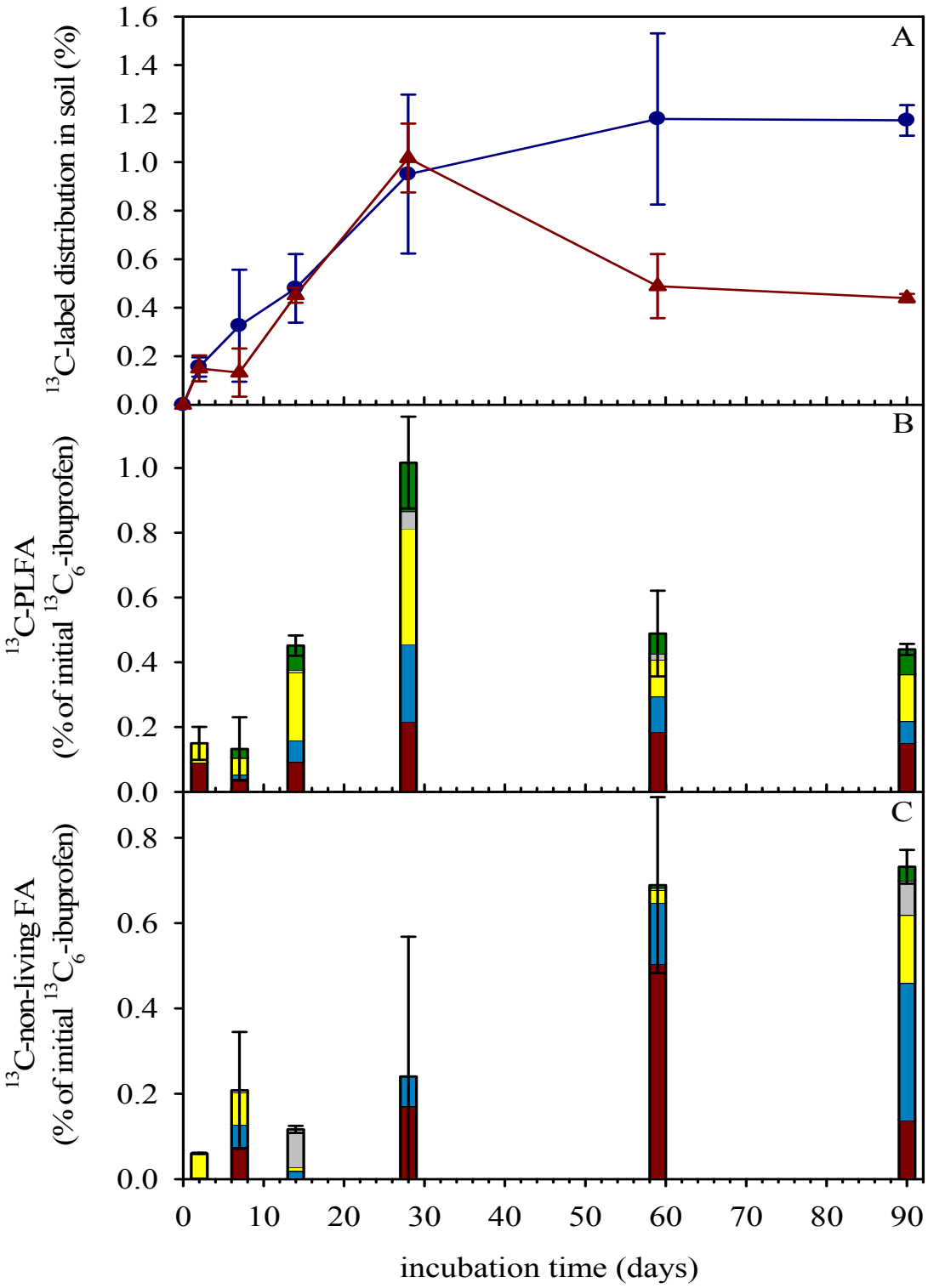

Figure 26. Time course of ^{13}C label incorporation during microbial degradation of $^{13}C_6$-ibu in soil (**A**) within tFA (●) and PLFA (▲). Distribution of ^{13}C label in lipid classes of PLFA (**B**) and tFA in non-living SOM (**C**).

[saturated straight-chain (■), saturated methyl-branched (■), monounsaturated (■), polyunsaturated (■) and saturated cyclopropyl FA (■)]

On day 28, the ^{13}C-PLFA reached their maximum (1% of $^{13}C_6$-ibu equivalents). After 28 days, the ^{13}C-PLFA decreased significantly and a ^{13}C flux from the living part to the non-living fraction of SOM was observed. After 59 days, the content of ^{13}C-PLFA decreased to 50% of their maximum (~ 0.5% of $^{13}C_6$-ibu equivalents), whereas the ^{13}C-FA in the non-living fraction were highest and reached 0.7% of the initial $^{13}C_6$-ibu amounts in soil. After day 59, about of 60% of the label in the t-FA could be already assigned to the non-living SOM. From this time, the amounts of both ^{13}C-PLFA and ^{13}C-FA in the non-living SOM were nearly constant until the end of the experiment.

At the beginning of the incubation, the ^{13}C label was found in the saturated straight chain and in the monounsaturated PLFA (16:1ω7c, 16:1ω5c, 18:1ω9c and 18:1ω7c, **Figure 26B**, see also **Table 13**). From day 7 until day 28, the monounsaturated PLFA carried more ^{13}C label than the other PLFA classes. During that time the amounts of ^{13}C-monounsaturated (in particular 16:1ω7c 18:1ω9c and 18:1ω7c) and also the ^{13}C-saturated cyclopropyl (cy 17:0) increased rapidly. However, incorporation of ^{13}C into the saturated methyl branched PLFA started already on day 7 and their contents increased strongly until day 28. On day 28, when the amount of ^{13}C-PLFA reached its maximum, the highest amount (except for 16:0) of the ^{13}C label was found in the monounsaturated 16:1ω7c and 18:1ω7c and the cyclopropyl cy 17:0. In addition, a high incorporation of ^{13}C into the polyunsaturated 18:2ω6,9 and the saturated methyl branched i:16:0 was also observed. After 59 days, the label was distributed nearly equally within the monounsaturated PLFA and the saturated methyl branched PLFA, whereas at the end of experiment, the monounsaturated PLFA contained more ^{13}C than saturated branched PLFA.

On day 59, since when ^{13}C-FA were detected in the non-living SOM, saturated strain chain FA carried most of ^{13}C label (**Figure 26C**). From that time onwards, the saturated methyl branched contained more ^{13}C than monounsaturated FA. Finally, ^{13}C-cyclopropyl FA and ^{13}C-polyunsaturated FA were also incorporated into the non-living SOM.

Table 13. The classification and composition of ^{13}C label in respective PLFA during microbial degradation of $^{13}C_6$-ibu in soil

Incubation time (days)	^{13}C-PLFA [10^{-4}; % of $^{13}C_6$-ibu]					
	2	**7**	**14**	**28**	**59**	**90**
Saturated straight chain						
14:0	n.d.	29.6 (± 98.6)	32.3 (± 8.4)	301.4 (± 12.5)	81.7 (± 94.7)	4.7 (± 0.8)
15:0	24.7 (± 12.4)	3,2 (± 24.2)	38.8 (± 4.3)	51.2 (± 30.6)	104.2 (± 85.3)	n.d.
16:0	74.1 (± 138.1)	377.6 (± 655.4)	728.9 (± 82.5)	1361.3 (± 130)	1499 (± 719.4)	934.8 (± 171.2)
18:0	141.8 (± 345.1)	n.d.	135.2 (± 47.7)	470.6 (± 14.9)	116.9 (± 158.7)	560.6 (± 126.6)
20:0	n.d.	n.d.	n.d.	n.d.	70.2 (± 33.5)	22.6 (± 6.5)
Saturated methyl branched						
i [b] 14:0	n.d.[e]	n.d.	7.4 (± 0.8)	8,3 (± 0)	21.7 (± 13.0)	5.4 (± 2)
i 15:0	n.d.	n.d.	61.1 (± 5.4)	387 (± 64)	287.5 (± 188.3)	41 (± 136.4)
a [c] 15:0	n.d.	2.8 (± 109.9)	160.8 (± 8.3)	190 (± 51.2)	15.1 (± 100.2)	12 (± 84.7)
i 16:0	n.d.	n.d.	74.5 (± 9.2)	**523.9** (± 48)	57.9 (± 98.6)	48.8 (± 55.6)
i 17:0	n.d.	20.4 (± 125.7)	41.7 (± 19.4)	148.2 (± 27.7)	99.4 (± 96.8)	61.5 (± 52)
a 17:0	3.2 (± 3.4)	31.1 (± 116)	19.2 (± 30.9)	110.5 (± 30.4)	253.4 (± 249.6)	141 (± 16.7)
10 Me [d] 16:0	n.d.	n.d.	120.5 (± 11.8)	382.3 (± 48.6)	191.1 (± 164.5)	278.5 (± 54.5)
10 Me 17:0	n.d.	n.d.	n.d.	n.d.	n.d.	16.4 (± 4)
10 Me 18:0	n.d.	54.7 (± 98.9)	76.8 (± 0.9)	231.6 (± 26)	95.5 (± 57.6)	n.d.
18:0 br [f]	n.d.	18.5 (± 140.7)	72.5 (± 33.9)	193.8 (± 8)	62 (± 46.4)	55.6 (± 24.4)
Monounsaturated						
16:1ω7c[c]	359 (± 28.4)	196.7 (± 10.1)	**855.7** ↑ (± 41.9)	**1041** ↑ (± 98.7)	232.5 (± 162.2)	49 (± 83.6)
16:1ω5c	66.2 (± 9.7)	n.d.	25.1 (± 13.8)	242.3 (± 63.3)	n.d.	39.3 (± 57.4)
18:1ω9c	89.5 (± 181.5)	136 (± 371.8)	**189.3** ↑ (± 92.5)	**539.6** ↑ (± 76.9)	28.1 (± 219)	473.8 (± 104.1)
18:1ω7c	67.6 (± 243.3)	179.4 (± 358.9)	**971.8** ↑ (± 47)	**1679** ↑ (± 377.3)	872.2 (± 378)	861 (± 64.8)
18:1ω5c	n.d.	n.d.	57.7 (± 0)	68.5 (± 45)	n.d.	18.7 (± 3.9)
Monounsaturated branched						
17:1 br	n.d.	n.d.	29.7 (± 13.1)	217.7 (± 13.1)	n.d.	10.9 (± 72.7)
Polyunsaturated						
18:2	n.d.	4.4 (± 209.4)	n.d.	n.d.	n.d.	n.d.
18:2ω6,9	n.d.	18 (± 481.9)	78.3 (± 79.1)	**525.1** (± 3.7)	188.2 (± 204.3)	n.d.
Saturated cyclopropyl						
cy 17:0	n.d.	153.5 (± 169.4)	**649** ↑ (± 36.4)	**944.5** ↑ (± 238.8)	317.3 (± 153)	616.7 (± 107.5)
cy 19:0	n.d.	96.4 (± 249.9)	89 (± 45.8)	550.7 (± 75)	294.1 (± 245.7)	145.2 (± 75.8)
TOTAL	**1498.2 (± 509)**	**1325 (± 980.1)**	**4515.7 (± 312.4)**	**10168 (± 1416.6)**	**4887.5 (± 1322.2)**	**4397.6 (± 168.7)**

[a]a- anteiso; [b]br – branched; [c]c - cis isomer; [d]i – iso; [e]n.d. - not detectable; [f]Me - methyl; values in brackets (±) represent the standard deviation of the average of triplicates; values printed in bold represent characteristic values, arrows visualise increase or decrease of the respective FA compared to the preceeding sampling time

6.2.3.3 Formation of AA and their fate in soil incubated with $^{13}C_6$-ibu

The incorporation of ^{13}C into the living biomass AA fraction (bioAA) started later (day 7, see **Figure 27**) than into PLFA (day 2). A flux of ^{13}C-bioAA to the non-living SOM fraction was already detected on day 7 and continued throughout the incubation. The amounts of ^{13}C-bioAA reached a maximum on day 28 (~ 3.2% of $^{13}C_6$-ibu equivalents) and then declined gradually until the end of the experiment, whereas the contents of ^{13}C-AA in the non-living fraction remained stable already after 58 days. The content of ^{13}C-bioAA decreased to 23% of its maximum, reaching on day 90 about 0.7% of the initially added $^{13}C_6$-ibu. At the end (day 90), 93% of the total ^{13}C-AA in soil was stabilised in the non-living SOM pool, reaching finally 27% of $^{13}C_6$-ibu equivalents.

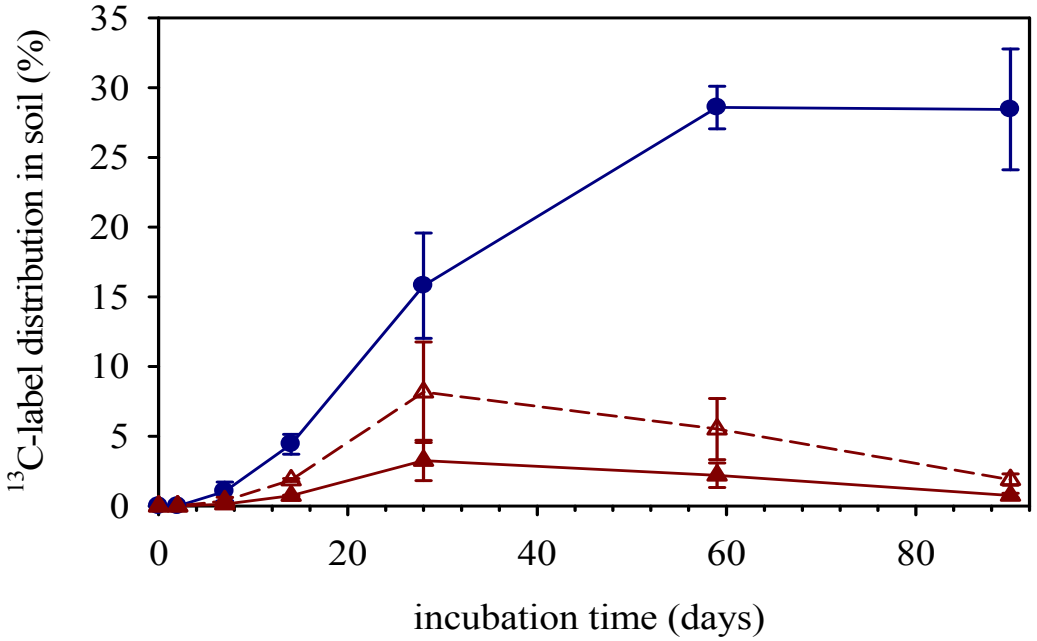

Figure 27. Time course of ^{13}C label incorporation within tAA (●), bioAA (▲) and bioAA (△) recalculated based on 40% extraction efficiency during microbial degradation of $^{13}C_6$-ibu in soil

On day 7, only Asp carried ^{13}C (see **Table 14A**). After 14 days, the ^{13}C-Asp dominated (0.4% of $^{13}C_6$-ibu equivalents) over other bioAA. However, Gly and Lys (each ~ 0.2% of $^{13}C_6$-ibu equivalents) also contained high amount of ^{13}C. Only traces of ^{13}C were detected in Thr and Ile. When the amounts of ^{13}C-bioAA were highest (day 28), both ^{13}C-Asp (0.9% of $^{13}C_6$-ibu equivalents) and ^{13}C-Glu (0.8% of $^{13}C_6$-ibu equivalents) dominated, but the label was also incorporated to a lesser extent into other AA (e.g. Gly, Thr, Leu, Ile, Pro, Glu, Phe and Lys). Interestingly, at that time, a very low amount of ^{13}C in the nonprotein amino acid ß-Ala (0.008% of $^{13}C_6$-ibu equivalents) was found. On day 59, when the contents of two

dominant ^{13}C-bioAA (^{13}C-Glu and ^{13}C-Asp) decreased strongly, the label was incorporated into all microbial AA. In addition, at that time, ^{13}C in ß-Ala increased significantly (0.05% of $^{13}C_6$-ibu equivalents). At the end, ^{13}C-Asp carried most of the label (0.3% of the initial $^{13}C_6$-ibu added). In addition to Asp, the label was also detected in five other AA (Leu, Ile, Pro, Phe and Lys).

Table 14. Distribution of the ^{13}C label in various ^{13}C-bioAA (**A**) and ^{13}C-AA in the non-living SOM (**B**) during microbial degradation of $^{13}C_6$-ibu in soil

A

Name of AA	Incubation time (days) [% of $^{13}C_6$-ibu]					
	2	**7**	**14**	**28**	**59**	**90**
Alanine	n.d.	n.d.	n.d.	n.d.	0.27 (± 0.08)	n.d.
Glycine	n.d.	n.d.	0.16 (± 0.04)	0.16 (± 0.02)	0.27 (± 0.04)	n.d.
Threonine	n.d.	n.d.	0.02 (± 0.006)	0.12 (± 0.04)	0.11 (± 0.01)	n.d.
Valine	n.d.	n.d.	n.d.	n.d.	0.17 (± 0.006)	n.d.
ß-alanine	n.d.	n.d.	n.d.	**0.008** (± 0.03)	**0.05**↑ (± 0.01)	n.d.
Leucine	n.d.	n.d.	n.d.	0.15 (± 0.03)	0.21 (± 0.04)	0.06 (± 0.03)
Isoleucine	n.d.	n.d.	0.0009 (± 0.01)	0.04 (± 0.003)	0.11 (± 0.007)	0.04 (± 0.01)
Proline	n.d.	n.d.	n.d.	0.21 (± 0.02)	0.11 (± 0.04)	0.10 (± 0.007)
Aspartate[a]	n.d.	0.15 (± 0.11)	**0.42** ↑ (± 0.002)	**0.92** ↑ (± 0.14)	**0.26** ↓ (± 0.34)	**0.30** (± 0.12)
Glutamate[b]	n.d.	n.d.	n.d.	**0.81** ↑ (± 0.43)	**0.50** ↓ (± 0.33)	n.d.
Phenylanine	n.d.	n.d.	n.d.	0.33 (± 0.29)	0.09 (± 0.06)	0.18 (± 0.06)
Lysine	n.d.	n.d.	0.16 (± 0.02)	0.5 (± 0.5)	0.06 (± 0.16)	0.08 (± 0.09)
TOTAL	**n.d.**	**0.15 (± 0.11)**	**0.76 (± 0.04)**	**3.25 (± 1.5)**	**2.20 (± 0.88)**	**0.75 (± 0.16)**

B

Name of AA	Incubation time (days) [% of $^{13}C_6$-ibu]					
	2	**7**	**14**	**28**	**59**	**90**
Alanine	n.d.	n.d.	n.d.	n.d.	n.d.	2.5 (± 1.9)
Glycine	n.d.	n.d.	n.d.	2.5 (± 0.5)	0.6 (± 0.5)	0.6 (± 4.8)
Threonine	n.d.	n.d.	n.d.	0.3 (± 1.2)	n.d.	n.d.
Valine	n.d.	n.d.	n.d.	n.d.	n.d.	3.5 (± 1.7)
ß-alanine	n.d.	n.d.	n.d.	n.d.	n.d.	0.2 (± 0.2)
Leucine	n.d.	n.d.	n.d.	0.8 (± 0.6)	0.9 (± 0.1)	3.1 (± 1.5)
Isoleucine	n.d.	n.d.	0.3 (± 0.035)	0.6 (± 0.6)	0.4 (± 0.8)	1.7 (± 0.3)
Proline	n.d.	n.d.	n.d.	0.3 (± 0.3)	0.1 (± 0.2)	1.8 (± 3.4)
Aspartate[a]	n.d.	**0.3** (± 0.3)	**3.0** (± 0.5)	**3.2** (± 0.3)	**9.9** (± 1.0)	4.0 (± 10.3)
Glutamate[b]	n.d.	n.d.	n.d.	0.6 (± 0.6)	**5.9** (± 1.0)	3.4 (± 16.0)
Phenylanine	n.d.	n.d.	n.d.	0.6 (± 0.1)	1.9 (±1.0)	1.5 (± 1.6)
Lysine	n.d.	n.d.	n.d.	n.d.	4.3 (± 0.5)	4.1 (± 0.7)
TOTAL	**n.d.**	**0.3 (± 0.3)**	**3.3 (± 0.5)**	**8.9 (± 3.8)**	**24.0 (± 1.3)**	**26.4 (± 4.0)**

[a] incl. asparagine; [b] incl. glutamine; n.d. - not detectable; values are shown as averages ± standard deviation
values printed in bold represent characteristic values, arrows visualise increase or decrease of the respective AA compared to the preceeding sampling time

The microbial AA were also incorporated into the non-living SOM fraction (see **Table 14B**). On day 14, when ^{13}C flux to the non-living SOM was observed, the label was found only in the dominant ^{13}C-Asp (3.0% of $^{13}C_6$-ibu equivalents) and in ^{13}C-Ile (0.3% of $^{13}C_6$-ibu equivalents). After 59 days, ^{13}C-Asp still dominated (9.9% of $^{13}C_6$-ibu equivalents), but Glu was also highly enriched in ^{13}C (5.9% of $^{13}C_6$-ibu equivalents). At that time, the ^{13}C label was incorporated to lesser extent into other AA (Gly, Leu, Ile, Pro, Phe and

Lys). At the end, all ^{13}C-microbial AA except for Thr were partly stabilised in the non-living part of SOM and the label was distributed within these AA nearly equally.

6.2.3.4 Biogenic residues in soil incubated with $^{13}C_6$-ibu

The content of ^{13}C-tFA reached 1.2% of $^{13}C_6$-ibu equivalents at the end of the experiment; considering a conversion factor of 20 we can conclude that at least 24% of ^{13}C label from $^{13}C_6$-ibu went through the microbial biomass. From the amount of ^{13}C-tAA of 27% (of $^{13}C_6$-ibu equivalents) at the last day, we can estimate that there were 54% biogenic residues in this experiment (using the conversion factor of 2).

7 DISCUSSION

The soil biodegradation studies with $^{13}C_6$-2,4-D and $^{13}C_6$-ibu proved that microorganisms used C directly from these contaminants or indirectly via CO_2 fixation to form their biomass components. After the death of microorganisms, their biomass components were incorporated into SOM and finally formed biogenic residues, which lead to the overestimation of the risk related to NER formed during biodegradation of these contaminants in soils.

The simple liquid culture experiment with *C. necator* JMP 134 provided a general view on the processes of ^{13}C-FA and ^{13}C-AA formation during 2,4-D biodegradation. In addition, this experiment showed the relative abundance of FA and AA in the total biomass of this strain, necessary for an estimation of the total biogenic residues content in the soil experiments.

7.1 Liquid culture experiments

Two liquid culture experiments with $^{13}C_6$-2,4-D and with $^{13}CO_2$ showed that the bacterial strain *C. necator* JMP 134 used the ^{13}C derived either from this herbicide or from $^{13}CO_2$ for its PLFA and AA synthesis. In addition, the abiotic systems clearly showed that ^{13}C label incorporation from labelled 2.4-D or from CO_2 were relevant only for biotic incubation experiments. $^{13}C_6$-2,4-D was degraded rapidly and dissipated already after the first day of incubation. Therefore, ^{13}C label incorporation into PLFA and AA directly from the labelled 2,4-D was observed only until day 1. In the later phase of experiment, when neither 2,4-D nor its known aromatic metabolites were detected in the medium, ^{13}C was incorporated into PLFA and AA only via CO_2 fixation.

Incorporation of ^{13}C into biomass of *C. necator* JMP 134 grown on 2,4-D medium

The ^{13}C label incorporation into the biomass of *C. necator* JMP 134 in the $^{13}C_6$-2,4-D experiment started immediately and remained constant until day 3. Thereafter, the ^{13}C content in the biomass still increased, although already after the first day of incubation $^{13}C_6$-2,4-D was completely depleted and no biomass growth was observed (see results in section 5.1.1.1 and 5.1.2.1). This is contrary to the results in the $^{13}C_6$-2,4-D experiment presented by LERCH ET AL. (2007), which indicated a low content of ^{13}C in the biomass of *C. necator* JMP 134 at the beginning and its strong increase in the later phase of incubation. This biomass growth was also consistent with the OD measurement of the culture (LERCH ET AL., 2007). However, in their experiment the initial concentration of 2,4-D was much higher (250 mg/L) than in the present study (77 mg/L) and this herbicide was still detectable after the first day of incubation.

Although the amounts of ^{13}C label in PLFA increased continuously until day 7, this however, was not related to growth. At the beginning of incubation, the ^{13}C-monounsaturated PLFA were dominant over other PLFA, their amounts then decreased strongly, whereas the content of ^{13}C in saturated cyclopropyl PLFA increased rapidly throughout the incubation time. The cyclopropyl PLFA are known starvation biomarkers, formed by the methylation of the double bond in monounsaturated PLFA (KAUR ET AL., 2005). This modification makes these FA more stable and minimizes the membrane lipid losses under stress conditions (KAUR ET AL., 2005). Therefore, this mechanism was employed by *C. necator* JMP 134 in the absence of 2,4-D in the medium. The continuous increase of the $^{13}CO_2$ evolution observed until day 7 in the $^{13}C_6$-2,4-D study and of the ^{13}C incorporation into PLFA in the $^{13}CO_2$ fixation experiment until day 3 clearly indicates the continuous metabolisation of ^{13}C incorporated into biomass residues via CO_2 fixation. The presence of CO_2 is reported to be essential for the growth of many microorganisms (KREBS, 1941), since CO_2 plays a key role in the synthesis of the tricarboxylic acid cycle (TCC) intermediates malate and oxalacetate from pyruvate or phosphoenolpyruvate (MILTNER ET AL., 2004; FEISTHAUER ET AL., 2008; see **Figure 28**). At the end of the incubation, the contents of ^{13}C in PLFA decreased strongly in both experiments and the ^{13}C-saturated cyclopropyl PLFA were dominant over other PLFA (in particular in the $^{13}CO_2$ experiment), indicating the decreasing contribution of CO_2 fixation.

At the beginning of the $^{13}C_6$-2,4-D experiment ^{13}C was highest in Glu. This AA is synthesised from 2-oxoglutarate formed in the TCC (see **Figure 28**). At that time, Asp also contained significant amounts of ^{13}C in comparison to other ^{13}C-AA. Asp is derived from oxaloacetate, which is the acceptor molecule for acetate in the TCC (MICHAL, 1999). When oxaloacetate is removed from the TCC for Asp synthesis, it has to be replenished by various anaplerotic reactions, which include the carboxylation of phosphoenolpyruvate; thus Asp is a direct product of heterotrophic CO_2 fixation (FEISTHAUER ET AL., 2008; MILTNER ET AL., 2004). This is in good accordance to the $^{13}CO_2$ experiment, in which the ^{13}C label was found only in Asp at the beginning (day 2), when the CO_2 fixation started. Both Glu and Asp formed within the TCC are important precursors for other AA, Pro and Thr, Ile, Lys, respectively (*arginine and methionine were not detected in experiments*). Therefore, these ^{13}C-labelled AA were found in the later phase of incubation period. Interestingly, the contents of ^{13}C in Thr increased strongly from day 3 until day 7 in both experiments and then decreased rapidly at the end. The simultaneous decline of ^{13}C in both Thr and PLFA at the end thus indicates the decrease of CO_2 fixation contribution to ^{13}C incorporation into the biomass of *C. necator*. Other AA, such as Ala, Val and Leu formed from pyruvate and Phe from

phosphoenolpyruvate were also enriched in the later phase of incubation period. However, the trends of both decline and increase of their ^{13}C contents could be assigned to the different food demands of this bacterial strain throughout the incubation time. The low incorporation of ^{13}C into Ser found only at the end of $^{13}C_6$-2,4-D experiment demonstrates the formation of this AA from ^{13}C-Gly. The ^{13}C-Gly was presumably converted directly from glyoxylate, which is a cleavage product of isocitrate (MICHAL, 1999) or of 2,4-D metabolite (see **Figure 10** in section 4.1).

At the end of the $^{13}C_6$-2,4-D incubation experiment, ~ 17% of initially added $^{13}C_6$-2,4-D was detected in the total biomass of *C. necator* JMP 134, 8% was found in AA and 0.6% was incorporated into the PLFA. However, also a relatively high amount of ^{13}C was incorporated into the biomass of *C. necator* via CO_2 fixation (~ 4.2% of the inital $^{13}CO_2$ amount). Therefore, it should be kept in mind that CO_2 fixation can contribute significantly to the incorporation of C into biomass components and thus to the NER formation in soils. In addition, the results from both $^{13}C_6$-2,4-D and $^{13}CO_2$ incubation experiments showed that ^{13}C-labelled AA represented ~ 50% of the total ^{13}C in the biomass, demonstrating their relatively high abundance in microbial cells. Therefore, this microbial biomarker can be helpful in the quantification of biogenic residues in complex soil systems.

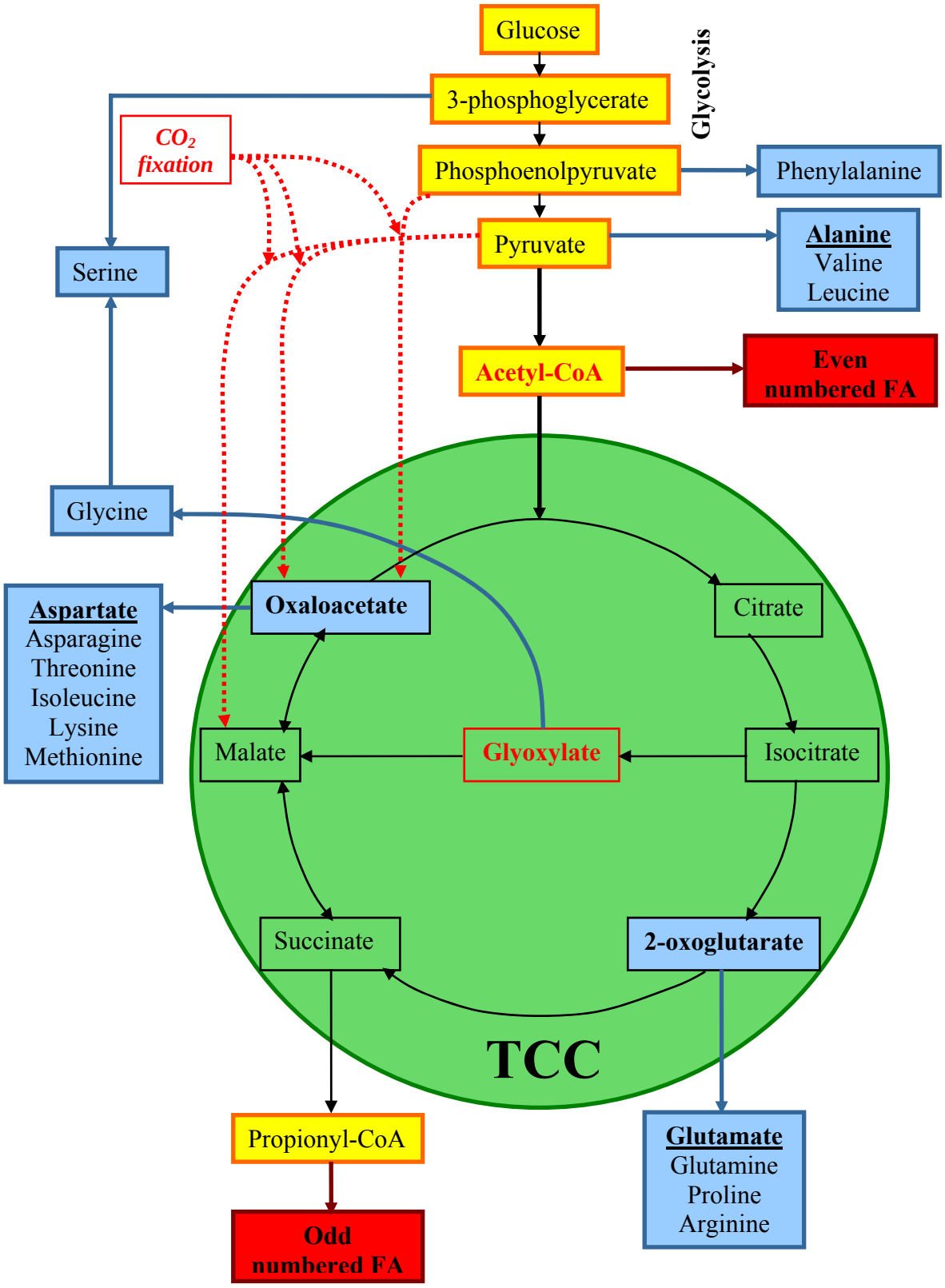

Figure 28. Scheme of the relevant pathways for AA and FA synthesis in TCC and glycolysis; and the anaplerotic reactions (····) replenishing the TCC (adapted from MICHAL, 1999; FEISTHAUER ET AL., 2008)

7.2 Soil experiments

Two soil degradation experiments with $^{13}C_6$-2,4-D and $^{13}C_6$-2,4-ibu prove for the first time that biogenic residues formation can be a relevant process during microbial degradation of many organic contaminants in soil. In case of the readily biodegradable 2,4-D and Ibu, the majority of NER are formed from biomass components. However, the kinetics of biogenic residue formation in the biodegradation experiments with these various organic contaminants was different and depended strictly on the degradation kinetics in soil.

7.2.1 Soil incubation experiment with $^{13}C_6$-2,4-D

The results from the soil incubation experiment with $^{13}C_6$-2,4-D provide detailed insight into the C flux from the ^{13}C-labelled pollutant 2,4-D or from labelled CO_2 (evolved during 2,4-D mineralisation) via microbial biomass to non-living SOM. It has been clearly shown that microorganisms use the readily available C derived from 2,4-D for their biomass synthesis, which was proven by identifying the ^{13}C label within the biomarkers FA and AA. After cell lysis and the decay of microbial biomass, both ^{13}C-labelled PLFA and bioAA were distributed within the microbial food web and incorporated into the non-living fraction of SOM, which was finally stabilised. These results are also in good agreement with recent studies on the fate of ^{13}C labelled *E. coli* in soil (KINDLER ET AL., 2006, 2009; MILTNER ET AL., 2009), which showed that biomass-derived C was distributed within the microbial food web and finally contributed to the formation of refractory non-living SOM (KINDLER ET AL., 2006, 2009; LÜDERS ET AL., 2006). The majority of NER formed during microbial degradation of 2,4-D in soil was composed of biomass-derived components.

7.2.1.1 Biogenic residues as NER in soil incubated with $^{13}C_6$-2,4-D

At the end of the incubation experiment, the amount of ^{13}C found in tAA (non-living SOM + bioAA fraction) was high and reached 22% of the initially added $^{13}C_6$-2,4-D equivalents corresponding to 61% of the NER based on isotope mass balance. It should be kept in mind that these AA do not represent free AA, which are reported to be degraded very rapidly in soil (JONES, 1999; VINOLAS ET AL., 2001), but they were hydrolysed from peptides and proteins. However, it is impossible to extract all biomolecules from soil microbial biomass, therefore only part of them could be analysed. By the extraction of some known microbial biomolecules e.g. FA and AA, we can estimate the actual amounts of biogenic residues in soil. The particular focus however, has been laid on AA from proteins and peptides due to their high abundance in microbial biomass. Two experiments with *C. necator* JMP 134 grown on 2,4-D medium showed that ^{13}C-labelled AA represented ~ 50% of the total ^{13}C in the

biomass (see results in sections 6.1.1.1 and 6.1.2.1). In addition, the ratio of ^{13}C-AA to total ^{13}C in biomass was relatively stable at the different sampling dates; thus AA are relevant for estimating the NER content in soil. Therefore, both the results from this experiment and a conservative conversion factor of 2 (MADIGAN AND MARTINKO, 2006) was considered for quantification of the actual incorporation of C derived from 2,4-D into the biomass, which resulted in a total amount of 44% (of $^{13}C_6$-2,4-D equivalents) for biogenic residues. The amount of these residues is close to the total NER amount of ~ 36% (of $^{13}C_6$-2,4-D equivalents) in soil measured by GIRARDI ET AL. (submitted; see also results in section 6.2.1.1). This small discrepancy in the NER contents could be related to the different methods used for NER determination. NER are generally quantified as CO_2 released from combusted soil after the pollutant residues extraction (BARRIUSO ET AL., 2008; WAIS, 1998). This analytical approach was also implemented by GIRARDI ET AL. (submitted). 2,4-D residues were extracted from soil by ASE, which is a harsh extraction method (NORTHCOTT AND JONES, 2000). At the end of experiment, about 8% of the initial $^{13}C_6$-2,4-D equivalents was extracted from soil by ASE, but from that amount only 0.5% was identified as 2,4-D. Therefore, a small part of biogenic residues stabilised in SOM could be still extracted from soil and thus affected the lower total NER content measured by GIRARDI ET AL. (submitted). It should be noted that the hydrolysis of AA from proteins is a very efficient extraction method, which enables also the extraction of AA stabilised in SOM; this could thus affect the higher biogenic NER content than the total NER content. However, the estimated amounts of biogenic residues indicate that the biomass compounds were finally converted into the biogenic residues already after 32 days and represented the majority of 2,4-D-derived NER in the soil (see **Figure 29**).

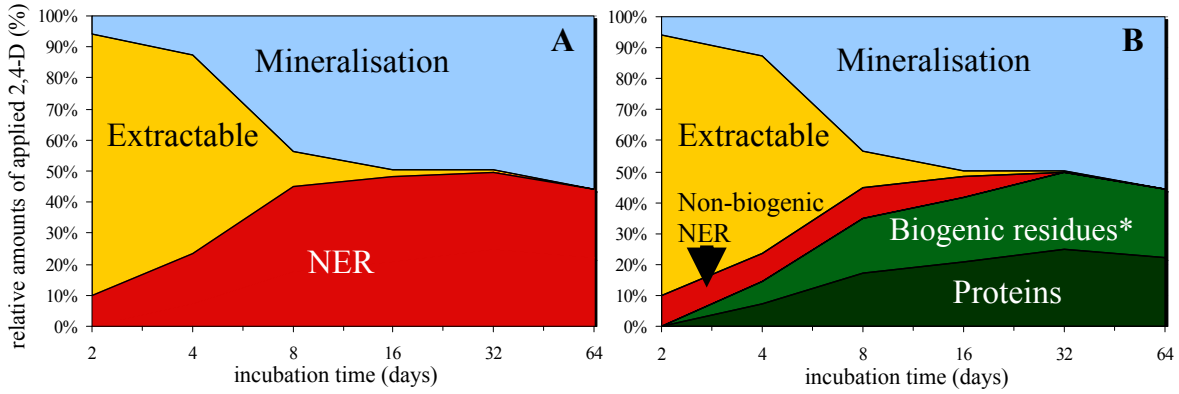

Figure 29. Scheme of the ^{13}C conversion over microbial degradation of $^{13}C_6$-2,4-D in soil. **A: conventional mass balance and B: new mass balance considering biogenic residues formation.** *The biogenic residues were estimated from AA using a conversion factor of 2 (for details see text).

Studies with anthracene (KÄSTNER ET AL., 1999), phenanthrene (RICHNOW ET AL., 2000) and TNT (WEISS ET AL., 2004) proved that C or N from these contaminants was incorporated into soil microbial biomass, which contributed to the NER formation in soils. The NER amounts formed during degradation of [ring-^{14}C-U]-labelled 2,4-D or 2,4-DCP in non-sterile composted straws were much higher than in sterile ones, where the parent compounds remained unchanged (BENOIT AND BARRIUSO, 1997). The high NER content found in the present study, where the $^{13}C_6$-2,4-D was nearly mineralised already after 16 days (GIRARDI ET AL., submitted) in comparison with the negligible mineralisation in sterile soils is in a good accordance to those in non-sterile straws (BENOIT AND BARRIUSO, 1997). Therefore, high contents of NER in non-sterile composted straw or soil underlines the key role of soil microorganisms in the formation of residues from 2,4-D in native soil. Another soil biodegradation experiment with [ring-^{14}C-U] 2,4-D showed that the amount of ^{14}C label from this herbicide detected in the microbial biomass was similar to that observed for [1-^{14}C] glucose, suggesting the utilisation of 2,4-D as a C source (ROBERTSON AND ALEXANDER, 1994). In addition, the well-known 2,4-D soil degrader *C. necator* JMP 134 also used C from 2,4-D for their biomass components synthesis in the liquid culture experiments (see discussion in section 7.1.1).

There is also evidence for abiotic residue formation, because 18.6% of $^{13}C_6$-2,4-D equivalents were recovered as NER in sterile soils at the end of incubation. However, in the sterile soils, the biogenic residue representatives ^{13}C-FA and ^{13}C-AA were not formed, indicating that these NER were exclusively formed by the reported abiotic sequestration mechanisms (PIGNATELLO AND XING, 1996; ALEXANDER, 2000; GEVAO ET AL., 2000; KATAYAMA ET AL., 2010). It was reported that autoclaving changes the soil physico-chemical properties (SHAW ET AL., 1999; BERNS ET AL., 2008), therefore the contaminant behaviour in sterile soils can be different than in native soils. In addition, the rapid mineralisation of $^{13}C_6$-2,4-D in the biotic soil experiment, will reduce the extent of abiotic NER formation because rapid 2,4-D degradation competes with abiotic residue formation. The fact that the non-living AA and FA can explain most of the NER formation strongly suggests a high contribution of biotic NER formation. Several studies demonstrated that microorganisms can also mediate the enzymatic covalent oxidative coupling of 2,4-DCP to humic substances (BOLLAG, 1991; PALOMO AND BHANDARI, 2005, 2006). However, it should be noted that these studies were limited to simple (BOLLAG, 1991; HATCHER ET AL., 1993), or abiotic systems (PALOMO AND BHANDARI, 2005; 2006) and covalently bound 2,4-DCP in SOM has never been detected.

In addition, all available studies on NER formation during microbial degradation of 2,4-D in soil are limited to quantitative analyses with radioactive tracers (BENOIT AND BARRIUSO, 1997; BOIVIN ET AL., 2005). In contrast to these available results, where no information on the chemical nature of NER in SOM was presented, the present study shows that nearly all NER formed during biodegradation of $^{13}C_6$-2,4-D in soil were composed from microbial components stabilised in the SOM pool during the later phase of experiment (aging process).

Finally, the high content of ^{13}C-AA detected in the present study contradicts the generally accepted view that the parent compounds or their primary metabolites) are the main precursors of phenoxyacetic acid herbicides derived NER, in particular via sorption processes (BOLLAG ET AL., 1991; BOLLAG ET AL., 1992; PALOMO AND BHANDARI, 2005, 2006).

7.2.1.2 Fate and stability of FA in soil incubated with $^{13}C_6$-2,4-D

The formation of ^{13}C-PLFA during microbial degradation of $^{13}C_6$-2,4-D in soil was rapid during the first four days of incubation, since the amount of the readily available $^{13}C_6$-2,4-D in soil was still high (GIRARDI ET AL., submitted). Taking into consideration a relatively rapid turnover of FA in bacterial cells and a total FA content of ~ 5% (see results in section 6.2.1.4), for the maximum of about 1% of $^{13}C_6$-2,4-D equivalents detected in the tFA fraction on the day 8, it can be concluded that more than 20% of the ^{13}C initially added must have passed through the microbial biomass. The high amount of ^{13}C found in the monounsaturated PLFA indicates that Gram-negative bacteria were initially involved in 2,4-D transformation. This is in good accordance with the previous study reported by LERCH ET AL. (2009A), in which the content of ^{13}C in monounsaturated tFA fraction (including the PLFA) was highest. The high incorporation of ^{13}C label into even-numbered PLFA (such as: 16:1ω7c; 16:0; 18:1ω7c; 18:1ω9c; 16:1ω5c) in the initial phase of incubation experiment clearly indicates for their direct synthesis from Acetyl-CoA (see **Figure 28** in section 7.6.1), which is the end product of 2,4-D degradation (see **Figure 10** in section 4.1). In addition, a lower incorporation of ^{13}C within the saturated methyl-branched PLFA, which are typical for Gram-positive bacteria, was also observed in this experiment. This indicates that this group of bacteria took indirectly part in the transformation of 2,4-D via fixation of the CO_2 evolved during mineralization of this pollutant or by uptake of metabolites formed by Gram-negative bacteria. When the amounts of ^{13}C in monounsaturated PLFA started to decrease after 8 days, the amount of ^{13}C in saturated cyclopropyl PLFA was highest. This high of ^{13}C content in the cyclopropyl PLFA indicates that bacteria employed starvation mechanism (KAUR ET AL., 2005), which agrees with the low residual concetrations of 2,4-D at that time (GIRARDI ET AL., submitted). In the later phase of the experiment, when the microbial biomass started to decay,

a flux of ^{13}C from living cells to non-living SOM was observed. After 16 days, besides ^{13}C-PLFA of the decaying biomass, the ^{13}C-FA of the non-living SOM were also continuously metabolised at a low level and distributed within the food web. Therefore, when $^{13}C_6$-2,4-D was nearly depleted, the ^{13}C released from these ^{13}C-FA was thus rapidly recycled via CO_2 fixation by Gram-negative and Gram-positive bacteria and by fungi.

Two independent studies showed that the NER contents formed during 2,4-D biodegradation in soil (BOIVIN ET AL., 2005) and 15 years-old NER after fresh soil amendment (LERCH ET AL., 2009B) decreased slightly over time. BOIVIN ET AL. (2005) suggested that this decline could be related to the temporal immobilisation of ^{14}C from 2,4-D in the protoplasm of some soil microorganisms. The decrease of NER in these above-mentioned studies thus could be assigned to the release of FA from both the decaying biomass and biomass residues in the non-living SOM, as it was observed in the present study.

At the end of the $^{13}C_6$-2,4-D soil incubation experiment, the amount of ^{13}C-PLFA decreased to 20% of its maximum value, indicating the metabolisation of cell components of the initial 2,4-D degraders via the microbial food web. However, the ^{13}C-FA in the non-living SOM decreased to 50% of their maximum value, demonstrating that the compounds in non-living fraction were partly stabilised in SOM against microbial attack. KINDLER ET AL. (2009) also reported the decline of ^{13}C-tFA derived from *E. coli* to 24% (of the initial amount of *^{13}C-E. coli*) in soil after eight months of incubation. In spite of the long incubation time, still about 50% of microbial biomass C remained in soil (KINDLER ET AL., 2006), demonstrating the high stability of microbial biomass residues in soil.

7.2.1.3 Fate and stability of AA in soil incubated with $^{13}C_6$-2,4-D

Compared to PLFA, the incorporation of ^{13}C into the bioAA fraction started 2 days later, indicating that PLFA as components of microbial cell membranes are turned over faster and thus received the ^{13}C label earlier than the AA. However, the flux of ^{13}C from the decaying biomass AA fraction into the non-living SOM was higher than that observed for the PLFA. At the beginning of the incubation, when the readily degradable $^{13}C_6$-2,4-D was still present at high amounts (GIRARDI ET AL., submitted), Glu carried highest label of all ^{13}C-AA detected in this experiment. However, on day 8, the content of ^{13}C in Asp was highest compared to the other ^{13}C-AA. Glu is formed from 2-oxoglutarate derived from the TCC; see **Figure 28** in section 7.1.1. The dominant incorporation of ^{13}C into Glu thus clearly indicates for a different route of the label into AA in first days of incubation, which was followed by CO_2 fixation from mineralised CO_2 and from decaying microbial biomass, as it was shown by the high amount of ^{13}C-Asp. Asp has been reported to be a direct product of heterotrophic CO_2

fixation in soil (MILTNER ET AL., 2005), which is also in good accordance to the pure culture experiment with *C. necator* JMP 134 grown in presence of $^{13}CO_2$ atmosphere (see section 6.1.1). In addition, CO_2 fixation as a relevant process for biogenic residue formation in the later phase of 2,4-D degradation experiment was also proven by the detection of ^{13}C label in the PLFA of the $^{13}CO_2$ soil experiment. Overall, both Asp and Glu are important precursors for other AA, e.g. Glu for Pro and Asp for Lys and Ile (see **Figure 28** in section 7.1.1). Therefore, lower amounts of ^{13}C-Lys, ^{13}C-Ile and ^{13}C-Pro were also detected later in this experiment. On day 16, when only traces of readily available 2,4-D residues were left and ^{13}C-bioAA started to decline, the ^{13}C label also was distributed within Ala, Gly and Phe, which would point to their biosynthesis via different intermediates of glycolysis (MADIGAN AND MARTINKO, 2006; MICHAL, 1999).

At the end of the incubation, the content of the ^{13}C-bioAA fraction decreased to 36% of its maximum found in the experiment, indicating the decrease of the contribution of the living biomass fraction to SOM pool over the incubation time. Surprisingly, the amounts of ^{13}C-AA in the non-living SOM from day 32 onwards remained stable and finally reached 19.8% of the initial amount of $^{13}C_6$-2,4-D in soil, demonstrating the stabilisation of proteins from the decaying microbial biomass. MILTNER ET AL. (2009) also demonstrated the high stability of ^{13}C-labelled bacterial proteins in the non-living part of SOM; their contents decreased only marginally even over a period of several months. Proteins have been reported to be stabilised in SOM by various sorption processes (KLEBER ET AL., 2007), which protect them from the microbial degradation (RILLIG ET AL., 2007) and affect their stability for a very long period of time (KNICKER ET AL., 1993; KÖGEL-KNABNER, 2002). These results thus underline the high importance of AA in NER formation from 2,4-D due to their high abundance in the microbial biomass and the stability in contrast to FA, which were turned over relatively fast.

In a study on the fractionation of [U-ring-^{14}C] 2,4-D residues XIE ET AL. (1997) showed that most of the radioactivity was associated with humic acids. It should be noted that proteins can bind to humic acids by hydrophobic and ionic interactions, which protect them from chemical and microbial attack (ZANG ET AL., 2000). Therefore, the dominance of ^{14}C label in the bound-humic acid fraction could be assigned to AA, as observed in the present study. Moreover, in the soil experiments with $^{13}C_6$-2,4-D, the amount of labelled biogenic residues did not change after 32 days of incubation, which was caused by their stabilisation in SOM. This could also explain the constant mineralisation rate of 90-years-old NER after addition of the fresh soil reported by LERCH ET AL. (2009B), when taking into consideration the fact that these NER are mostly biogenic.

7.2.2 Soil incubation experiment with $^{13}C_6$-ibu

This part of the study provides insight into the C flux from $^{13}C_6$-ibu during its biodegradation via microbial biomass components to the non-living part of SOM. The microorganisms used the C derived from this contaminant to synthesise their biomass, as it was shown by ^{13}C incorporation into the microbial biomarkers FA and AA. After cell death, biomass constituents such as ^{13}C-PLFA and ^{13}C-bioAA from the decaying cells were distributed into the food web and finally incorporated into the non-living SOM fraction. These results are in good agreement with the studies on the fate of both labelled *E. coli* in soil (KINDLER ET AL., 2006, 2009; MILTNER ET AL., 2009) and ^{13}C-biogenic residue in the soil incubated with $^{13}C_6$-2,4-D (NOWAK ET AL., in press). However, the kinetics of biogenic residues formation in soil incubated with $^{13}C_6$-ibu was initially slower than that observed in the $^{13}C_6$-2,4-D experiment. Nevertheless, biomass-derived compounds such as FA and AA also contributed significantly to NER formation from$^{13}C_6$-ibu in soil. Biomass residues represented the majority of $^{13}C_6$-ibu-derived NER, which is also consistent with the $^{13}C_6$-2,4-D experiment.

7.2.2.1 Biogenic residues as NER in soil incubated with $^{13}C_6$-ibu

The content of ^{13}C-tAA detected at the end of the $^{13}C_6$-ibu experiment was high, reaching 27% of the initial $^{13}C_6$-ibu equivalents and corresponding to 90% of the NER based on isotope mass balance. Considering a conservative conversion factor of 2 (for details on the conversion factors see section 6.1.1.1), these 27% in ^{13}C-tAA corresponds to a total amount of 54% (of $^{13}C_6$-ibu equivalents) for biogenic residues. However, the total ^{13}C-NER content measured by GIRARDI ET AL (submitted; see also section 6.2.3.1) was much lower (30% of $^{13}C_6$-ibu equivalents) than the estimated amount of biogenic residues. It should be noted that the total NER content was determined by EA-C-irMS after the removal of Ibu residues using a harsh extraction method (ASE). The estimated amount of NER strongly depends on the extraction method used for the bioavailability measurement of a target compound (KHAN 1991; NORTHCOTT AND JONES, 2000; MORDAUNT ET AL., 2005). GIRARDI ET AL. (submitted; see also results in section 6.2.3.1) reported that the solvent bulk extractable amount prior to purification by SPE accounted for ~ 14% of $^{13}C_6$-ibu equivalents; and from that only 0.5% was identified as Ibu. In addition, the contents of the bulk solvent extractable varied over time. At the beginning of biogenic residue formation, a high content of the solvent bulk extractable (~ 50% of $^{13}C_6$-ibu equivalents on the day 28) was observed, which decreased strongly at the end; this could be assigned to the aging processes of NER. Therefore, the ~ 14% extracted at the end from soil prior to quantitative NER analyses could still contain a

certain part of living biomass residues (e.g. bioAA and PLFA), which are much easier to extract before their stabilisation in SOM. In addition, the hydrolysis of AA from proteins is definitely a more efficient extraction method than ASE and enables the extraction of all AA, also those stabilised in SOM, which results in the higher NER estimation in the present study. Nevertheless, the high content of biogenic residues derived from $^{13}C_6$-ibu detected at the end of the incubation period clearly indicates that nearly all $^{13}C_6$-ibu-derived NER were based on the stabilised compounds of microbial origin (see **Figure 30**), which is also in good accordance to the data obtained in the $^{13}C_6$-2,4-D experiment (NOWAK ET AL., in press).

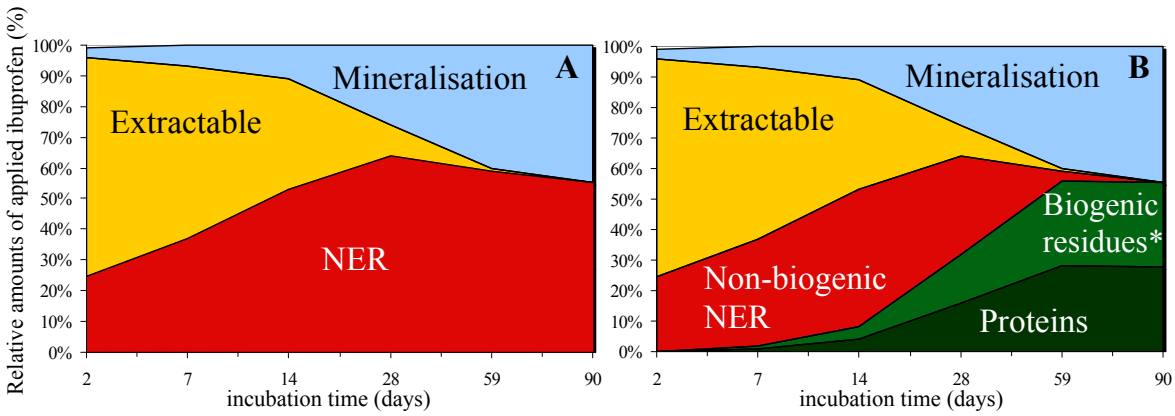

Figure 30. Scheme of the ^{13}C conversion over microbial degradation of $^{13}C_6$-ibu in soil. **A: conventional mass balance** and **B: new mass balance considering biogenic residues formation.** *The biogenic residues were estimated from AA using a conversion factor of 2 (for details see text).

GIRARDI ET AL (submitted) clearly presented that - in contrast to the biologically active soils - NER formation and mineralisation in the sterile soils incubated with $^{13}C_6$-ibu were negligible during the first 28 days of incubation. However, after 90 days, the NER contents in the inactive soils reached about 16% of $^{13}C_6$-ibu equivalents (GIRARDI ET AL., submitted), indicating abiotic NER formation by ibu entrapment into SOM after prolonged incubation times (PIGNATELLO AND XING, 1996; ALEXANDER, 2000; GEVAO ET AL., 2000; KATAYAMA ET AL., 2010). However, no ^{13}C incorporation into FA, AA and thus into microbial biomass was observed, clearly indicating the crucial role of microorganisms in biogenic NER formation in non-sterile soils.

The $^{14}C_3$-ibu-derived NER content after 100 days of incubation (~ 50% of $^{14}C_3$-ibu equivalents; RICHTER ET AL., 2007) was in the same range as the biogenic NER amount detected in the present study (54% of $^{13}C_6$-ibu equivalents). However, $^{14}C_3$-ibu-derived NER were formed rapidly and reached a maximum already after 4 days; their contents then decreased only slightly (RICHTER ET AL., 2007). Contrary to these results, in the present

$^{13}C_6$-ibu experiment, the NER reached a maximum after 59 days and their contents remained stable until the end of the incubation period. This is related to the different labelling positions of the compound used in these two studies. In the present study, the compound was labelled in the aromatic ring, which is cleaved much later during biodegradation than the methyl group labelled in the $^{14}C_3$-ibu experiment is removed (RICHTER ET AL., 2007), which is reflected by different kinetics of NER formation. NER in the $^{13}C_6$-2,4-D experiment were also formed very quickly (NOWAK ET AL., in press; see also section 7.2.1.1), but this contaminant was degraded much faster than $^{13}C_6$-ibu (GIRARDI ET AL., submitted). This was caused by the ability of the present soil microorganisms to degrade 2,4-D after previous 2-methyl-4-chloro phenoxyacetic acid (MCPA) application to the soil (INES MERBACH, personal communication), which also belongs to the group of phenoxy compounds as 2,4-D.

7.2.2.2 Fate and stability of FA in soil incubated with $^{13}C_6$-ibu

Incorporation of the ^{13}C-label into PLFA started immediately and increased rapidly until day 28, when the readily available $^{13}C_6$-ibu was still present at relatively high concentrations in the soil (GIRARDI ET AL., submitted; see also section 6.2.3.1). The monounsaturated PLFA carried most of the ^{13}C label and dominated over other ^{13}C-labelled PLFA until day 28, indicating that Gram-negative bacteria were important initial Ibu degraders in soil. This is in good accordance to the study on NER formation from $^{13}C_6$-2,4-D in soil (NOWAK ET AL., in press; see section 7.2.1.2). As the same soil was used in these two experiments, the same groups of microorganisms might have been involved in the degradation of both acidic aromatic molecules $^{13}C_6$-ibu and $^{13}C_6$-2,4-D. In the later phase of incubation period, also the saturated methyl branched PLFA were highly enriched in ^{13}C and their importance increased over time, in particular when only traces of $^{13}C_6$-ibu were left. This class of PLFA is typical for Gram-positive bacteria, which indirectly take part in the degradation of $^{13}C_6$-ibu, as it was also demonstrated previously in the $^{13}C_6$-2,4-D soil experiment. The observed continuous increase of ^{13}C incorporation into the cyclopropyl PLFA throughout the incubation time indicates starvation of the microorganisms (KAUR ET AL., 2005). In addition, the continuous decline of ^{13}C-PLFA in the living biomass, the distribution of ^{13}C within all classes of PLFA and the contaminant depletion in soil over the incubation period indicated that the Ibu-derived C was distributed within the food web, as found in the $^{13}C_6$-2,4-D study. In addition to bacteria, fungi also took part in the turnover of ^{13}C-labelled PLFA from dead cells, as proven by the ^{13}C found in the fungal biomarker PLFA 18:2ω6,9. From the content of ^{13}C-tFA of 1.2% (of $^{13}C_6$-ibu equivalents) detected at the end of experiment and considering a conversion factor of 20 (for details see 6.1.1.1) we can conclude that at least 24% of biomass must have

been formed. This is close to the amount found in the 2,4-D soil incubation experiment (22% of $^{13}C_6$-2,4-D equivalents).

At the end of the experiment, ^{13}C-PLFA decreased to 50% of their maximum, whereas ^{13}C-FA in the non-living SOM fraction remained unchanged. This is contradictive to the study with $^{13}C_6$-2,4-D, where apart from the observed decline of ^{13}C-labelled PLFA to 20% (of $^{13}C_6$-2,4-D equivalents), the contents of ^{13}C-FA in the non-living fraction also decreased (50% of $^{13}C_6$-2,4-D equivalents; see section 6.2.1.2). However, $^{13}C_6$-ibu dissipated slower than $^{13}C_6$-2,4-D. The PLFA thus received the ^{13}C label much later. Therefore, both their decline and ^{13}C flux to the non-living fraction were also observed later than in the $^{13}C_6$-2,4-D experiment. Additionally, both the lower decrease of ^{13}C-PLFA and the stability of ^{13}C-FA in the non-living SOM until the end of $^{13}C_6$-ibu experiment may be related to the duration of the incubation, which might have been stopped before destabilisation of part of Ibu residues started, as it was seen in the 2,4-D study. In addition, RICHTER ET AL. (2007) also reported a slight release of rapidly formed ^{14}C-NER derived from $^{14}C_3$-ibu over the incubation time (~ 5% of $^{14}C_3$-ibu equivalents after 100 days).

7.2.2.3 Fate and stability of AA in soil incubated with $^{13}C_6$-ibu

AA in biomass-living fraction received ^{13}C label later than PLFA, which is in good agreement with the data obtained in the experiment with $^{13}C_6$-2,4-D (NOWAK ET AL., in press; see section 7.2.1.3). However, ^{13}C-bioAA derived from $^{13}C_6$-ibu started to decline at the same time as ^{13}C-PLFA, what is different from the soil experiment with $^{13}C_6$-2,4-D, where the decrease of ^{13}C-bioAA content was observed later than that of ^{13}C-PLFA. This discrepancy in the decline of bioAA is difficult to explain and could be related to the different dissipation kinetics of the used contaminants and also to timing of the sampling events relative to proceses in soil in these two soil incubation experiments. After 58 days, the contents of ^{13}C-AA in the non-living fraction derived from $^{13}C_6$-ibu did not change and remained stable until the end, whereas the ^{13}C-bioAA decreased to 23% of their maximum after 90 days (of $^{13}C_6$-ibu equivalents). This is in good accordance to the $^{13}C_6$-2,4-D soil experiment, where ^{13}C-AA in the non-living SOM were also constant, indicating their high importance in the NER formation.

In the initial phase of $^{13}C_6$-ibu degradation, the label was incorporated only into Asp. Thereafter, the content of ^{13}C-Asp increased significantly and this AA was dominant until day 28, when $^{13}C_6$-ibu was present at a low concentration in soil (GIRARDI ET AL., submitted; see also 6.2.3.1). The fast appearance of the label in Asp and its later dominance over the other ^{13}C-bioAA suggest that ibu-derived ^{13}C is incorporated into the bioAA via heterotrophic CO_2 fixation (MILTNER ET AL., 2004; FEISTHAUER ET AL., 2008). After 14 days, a lower amount of

^{13}C was found also in Lys and Ile, for which Asp is an important precursor. This however is, in contrast to the study with $^{13}C_6$-2,4-D, where the label was first incorporated into Glu, at the time when the amount of readily available $^{13}C_6$-2,4-D in soil was still high. Although the molecular structures of $^{13}C_6$-ibu and $^{13}C_6$-2,4-D are similar, these compounds are degraded by soil microorganisms via different pathways. In the soil incubated with $^{13}C_6$-ibu, ^{13}C-Glu was also formed, but later, when the ^{13}C-bioAA started to decline and $^{13}C_6$-ibu was nearly depleted. Interestingly, on days 28 and 58, the non-protein AA ^{13}C-ß-Ala was found. The ratio of Asp to its decomposition product ß-Ala informs about the intensity of SOM decomposition (DAUWE AND MIDDELBURG, 1998). The ratio of Asp/ß-Ala on day 28 was 121, whereas on day 58 it decreased to 5.4, which clearly shows that the ^{13}C-AA in the microbial biomass were decaying. The high contents of ^{13}C-Glu found in the later phase of the experiment would thus point to a different degradation pathway of ibu-derived ^{13}C. Furthermore, similar to the $^{13}C_6$-2,4-D study, in the later phase of $^{13}C_6$-ibu-amended soil incubation, a lower label incorporation into other AA (Pro, Ala, Gly, Thr, Leu, Phe, and Val) was also observed.

8 CONCLUSIONS

In the present study, the incorporation of ^{13}C into biomass constituents (FA and AA) and their contribution to NER formation in soil during microbial degradation of $^{13}C_6$-2,4-D and $^{13}C_6$-ibu were studied and discussed. Both experiments were carried out with stable isotope labelled compounds, which enabled a detailed analysis of the chemical structure of the extracts containing either AA or FA. The detection of the ^{13}C label in the biomass components (PLFA and bioAA) and the comparison of their amounts to the contents of ^{13}C in the non-living SOM part (tFA and tAA) in soil clearly showed that these biomass components, which were stabilised in SOM pool, contributed to NER formation in soil. Special attention was given to the AA due to their high abundance (~ 50%) in microbial cells, as it was proven in the simple pure culture system with *C. necator* JMP 34.

Two soil experiments with $^{13}C_6$-2,4-D and $^{13}C_6$-ibu proved for the first time that biogenic residue formation contributes to a major extent to the formation of NER from these contaminants. For both 2,4-D and Ibu, nearly all of the NER derived from microbial biomass. The incorporation of the label into the biomass components was very fast in the 2,4-D experiment and slower at the beginning in the case of Ibu study due to a prolonged phase of microorganisms adaptation. However, in both soil experiments in the later phase of incubation, the biomolecules derived from the decaying biomass, in particular AA, were stabilised in SOM for longer periods.

In general, major contributions of biogenic residues in NER formation are to be expected if the respective organic contaminant is readily degraded by microorganisms under significant formation of CO_2. The exact pathways of biogenic residue formation during microbial degradation of organic contaminants in soil are summarised in **Figure 31**. Depending on the yield coefficients of C conversion into biomass, we expect ratios of biomass plus biogenic residues to CO_2 of about 0.2 to 1. In the $^{13}C_6$-2,4-D study, the ratio was ~ 0.8 and in the $^{13}C_6$-ibu was 1.2. However, in case of highly reduced C-containing organic compounds, this ratio may be higher as it was seen in the $^{13}C_6$-ibu biodegradation experiment. However, the position of the label in the parent molecule requires consideration in terms of incorporation into biomass. For instance, due to the oxidation state of the C atoms in the triazine ring of atrazine they will not be incorporated into microbial biomass (STRUTHERS ET AL., 1998) but will be released as CO_2, which then might be assimilated by microorganisms. Similar effects are observed for the C at the 9 position of anthracene (KÄSTNER ET AL., 1999) or phenanthrene (RICHNOW ET AL., 2000). CO_2 fixation has also been reported to be a relevant process in soils (MILTNER ET AL., 2004), which also contributes to

NER formation, as it was shown in two experiments, in the $^{13}CO_2$ experiment with unlabelled 2,4-D and *C. necator* JMP 134 experiment, where ~ 4% of ^{13}C assimilated in the biomass was derived from $^{13}CO_2$.

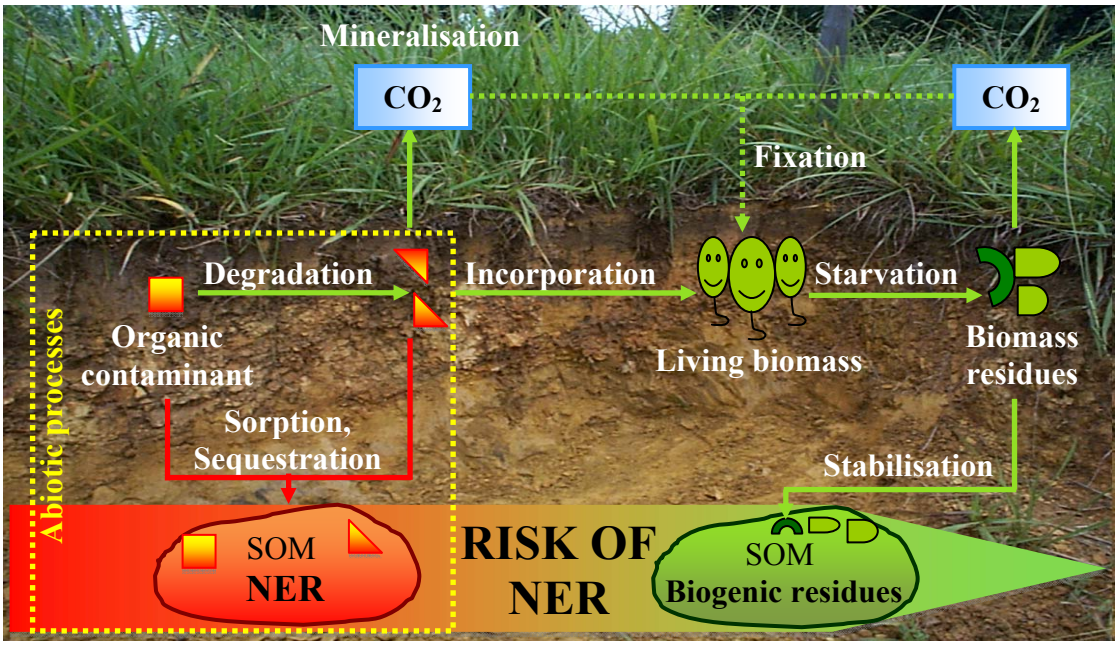

Figure 31. Scheme of the abiotic and the biotic NER formation during microbial degradation of organic contaminants in soil

The results from the two soil experiments indicate that biogenic residues formation can be relevant for many biodegradable pesticides and chemicals of environmental concern. These residues in soil contain only microbial components, which are natural products and explicitly excluded from the IUPAC definition of NER (ROBERTS, 1984). This is contrary to the generally accepted view that NER originating from the microbial degradation of organic contaminants consist mainly of the parent compounds or their metabolites, which are sorbed or sequestered within SOM components (BOLLAG ET AL., 1992; ALEXANDER, 2000; BARRACLOUGH, ET AL., 2005; BARRIUSO ET AL., 2008; see also abiotic processes in **Figure 31**). However, abiotic NER formation and biogenic residues formation are the competitive processes and do not occur together in a similar extent, because in the biotic treatment, the rapid mineralisation of an organic compound, reduces the extent of abiotic NER formation. The physico-chemical interactions of a contaminant with the SOM were reported mainly in sterile soils (PIGNATELLO AND XING, 1996; ALEXANDER, 2000; PALOMO AND BHANDARI, 2005; 2006), and were also observed in the abiotic treatments with either $^{13}C_6$-2,4-D or $^{13}C_6$-ibu (GIRARDI ET AL., submitted). In addition, this abiotic NER formation can also occur, when a target compound is toxic for soil microorganisms, and thus inhibits the biotic NER formation.

The difficulties in proper identification of NER in soils are caused by the limitations of radiotracers, which only enable proper quantitative analyses. In addition, other factors may have led to the fact that the formation of biogenic residues has not been observed for decades. Firstly, AA in soil are generally bound in peptides or proteins, which are difficult to extract using organic solvents or water (even when harsh methods like ASE are used), in particular if they are stabilised in SOM. Secondly, native proteins in soil are surprisingly resistant to microbial degradation (RILLIG ET AL., 2007) and remain there for long period of time (KNICKER ET AL., 1993; KÖGEL-KNABNER, 2002), because they are stabilised in SOM by sorption to both organic and mineral surfaces (KLEBER ET AL., 2007).

Therefore, it is essential to distinguish the formation of xenobiotic-derived bound residues via various abiotic processes according to IUPAC from its non-toxic biogenic counterpart when assessing the risks of organic contaminants in soil. This differentiation is of utmost importance for assessing the risk of easily biodegradable active compounds, as shown by the results on the degradation of 2,4-D and Ibu in soil.

LITERATURE

ABDELHAFID, R.H., HOUOT, S., BARRIUSO, E. 2000A. Dependence of atrazine degradation on C and N availability in adapted and non-adapted soils. *Soil Biol. Biochem.* 32, 389–401.

ABDELHAFID, R.H., HOUOT, S., BARRIUSO, E. 2000B. How increasing availabilities of carbon and nitrogen affect atrazine behaviour in soils. *Biol. Fert. Soils* 30, 333–340.

ADRIANO, D.C.; BOLLAG, J.-M.; FRANKENBERGER, J.R.; SIMS, R.C. 1999. Bioremeditation of contaminated soils, Agronomy monograph 37, American Society of Agronomy. eds. (1999).

AGA, D.S.; THURMAN, E. M. 2001. Formation and transport of the sulfonic acid metabolites of alachlor and metolachlor in soil. *Environ. Sci. Technol.* 35, 2455–2460.

ALEXANDER, M. 2000. Aging, bioavailability, and overestimation of risk from environmental pollutants. *Environ. Sci. Technol.* 34, 4259–4265.

ALLARD, B. 2006. A comparative study on the chemical composition of humic acids from forest soil, agricultural soil and lignite deposit, bound lipid, carbohydrate and amino acids distributions. *Geoderma* 130, 77–96.

AMELUNG, W.; ZHANG, X. 2001. Determination of amino acids enantiomers in soil. *Soil Biol. Biochem.* 33, 553–562.

AMY, P.S.; SCHULKE, J.W.; FRAZIER, L.M.; SEIDLER, R.J. 1985. Characterization of aquatic bacteria and cloning of genes specifying partial degradation of 2,4-dichlorophenoxyacetic acid. *Appl. Environ. Microbiol.* 49, 1237–1245.

ANDREU, V.; PICÓ. Y. 2004. Determination of pesticides and their degradation products in soil: critical review and comparison of methods. *Trends in Anal. Chem.* 23, 772–789

ATLAS, R.M.; BARTHA, R. 1997. Microbial Ecology, Fundamentals and Applications 4th Edn., The Benjamin/Cummings Publishing Company, Redwood City, CA.

BALDOCK, J.A.; OADES, J. M.; VASSALLO, A. M.; WILSON, M. A. 1989. Incorporation of uniformly labelled ^{13}C-glucose carbon into the organic fraction of a soil. Carbon balance and CP/MAS ^{13}C NMR measurements. *Soil Biol. Biochem.* 27, 725–746.

BALUCH, H.U.; SOMASUNDARAM, L.; KANWAR, R.S.; COATS, J.R. 1993. Fate of major degradation products of atrazine in Iowa soils. *J Environ Sci. Health*, Part B28, 127–149.

BARRACLOUGH, D.; KEARNEY, T.; CROXFORD, A. 2005. Bound residues: environmental solution or future problem? *Environ. Pollut.* 133, 85–90.

BARRIUSO, E.; BENOIT, P.; DUBUS, I.G. 2008. Formation of pesticide nonextractable (bound) residues in soil: magnitude, controlling factors and reversibility. *Environ. Sci. Technol.* 42, 1845–1854.

BARRIUSO, E.; HOUOT, S.; SERRA-WITTLING, C. 1997. Influence of compost addition to soil on the behaviour of herbicides. *Pest. Sci.* 49, 65–75.

BAS, P.; ARCHIMEDE, H.; ROUZEAU, A.; SAUVANT, D. 2003. Fatty acid composition of mixed-rumen bacteria: effect of concentration and type of forage. *J Dairy Sci.* 86, 2940–2948.

BEADLE, C.A.; SMITH, A.R.W. 1982. The purification and properties of 2,4-dichlorophenol hydroxylase from a strain Acinetobacter species. *Eur. J Biochem.* 123, 323–332.

BENOIT, P.; BARRIUSO, E. 1997. Fate of ^{14}C-ring labeled 2,4-D, 2,4-dichlorophenol and 4-chlorophenol during straw composting. *Biol. Fert. Soils* 25, 53-59.

BENOIT, P.; BARRIUSO, E.; HOUOT, S.; CALVET, R. 1996. Influence of the nature of soil organic matter on the sorption-desorption of 4-chlorophenol, 2,4-dichlorophenol and the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D). *Eur. J Soil Sci.* 47, 567–578.

BERNS, A.E.; PHILIPP, H.; NARRES, H.-D.; BURAUEL, P.; VEREECKEN, H.; TAPPE, W. 2008. Effect of gamma-sterilization and autoclaving on soil organic matter structure as studied by solid state NMR, UV and fluorescence spectroscopy. *Eur. J. Soil Sci.* 59, 540–550.

BERNS, A.E.; VINKEN, R.; BERTMER, M.; BREITSCHWERDT, A.; SCHÄFFER, A. 2005. Use of ^{15}N-depleted compost in bound residue studies. Chemosphere 59, 649–658.

BERRY, D.F., BOYD, S.A. 1985. Decontamination of soil through enhanced formation of bound residues. *Environ. Sci. Technol.* 19, 1132–1133.

BLAIR, N.; FAULKNER, R.D.; TILL, A.R.; KORSCHEN, M.; SCHULZ, E. 2006. Long-term management impacts on soil C, N and physical fertility Part II: Bad Lauchstadt static and extreme FYM experiments. *Soil Till. Res.* 91, 39–47.

BLASCHETTE, A., 1974. Allgemeine Chemie, Band I: Atome, Molekule, Kristalle Akademische Verlagsgesellschaft, Frankfurt/Main.

BLIGH, E.G.; DYER, W.J. 1959. A rapid method of total lipid extraction and purification. Can J *Biochem. Physiol.* 37, 911–917.

BOAS, N., 1953. Method for the determination of hexosamines in tissues. J Biol Chem 204, 553-563.

BOIVIN, A.; AMELLAL, S.; SCHIAVON, M.; VAN GENUCHTEN, M.T. 2005. 2,4-Dichlorophenoxyacetic acid (2,4-D) sorption and degradation dynamics in three agricultural soils. *Environ. Pollut.* 138, 92–99.

BOL, R.; POIRIER, N.; BALESDENT, J.; GLEIXNER, G. 2009. Molecular turnover time of soil organic matter in particle-size fractions of an arable soil. *Rapid Com. in Mass Spectr.* 23, 2551–2558.

BOLLAG, J.-M. (ED.). 1991. Enzymatic binding of pesticide degradation products to soil organic matter and their possible release. ACS, Washington.

BOLLAG, J.M.; LIU, S.Y. 1990. Biological transformation processes of pesticides, in: Cheng, H.H. (Ed.), Pesticides in the Soil Environment: Processes, Impacts and Modeling. SSSA, Masison WI, pp. 169–211.

BOLLAG, J.M.; MYERS, C.J.; MINARD, R.D. 1992. Biological and chemical interactions of pesticides with soil organic matter. *Sci. Total Environ.* 123-124, 205–217.

BOSCHKER, H.T.S; MIDDELBURG, J.J. 2002. Stable isotopes and biomarkers in microbial ecology. *FEMS Microbiol. Ecol.* 40, 85–95.

BOSCHKER, H.T.S. 2004. Linking microbial community structure and functioning: stable isotope (^{13}C) labeling in combination with PLFA analysis, in: Kowalchuk, G.A.B.; F. J. D.; Head, I. M.; Akkermans, A. D. L.; Elsas, J. D. V. (Ed.), Molecular Microbial Ecology Manual, Second Edition. Kluwer Academic Publisher, pp. 1673–1688.

BRONICK, C.J.; LAL, R. 2005. Soil structure and management: a review. *Geoderma* 124, 3–22

BRUSSEAU, M.L.; JESSUP, R.E.; RAO, S.C. 1991. Nonequilibrium sorption of organic chemicals: elucidation of rate-limiting processes. *Environ. Sci. Technol.* 25, 134–142.

BUNDT, M.,;WIDMER, F.; PESARO, M.; ZEYER, J.; BLASER, P. 1995. Preferential flow paths: biological 'hot spots' in soils *Soil Biol. Biochem.* 33, 729–738.

BURAUEL, P.; FÜHR, F., 2000. Formation and long-term fate of non-extractable residues in outdoor lysimeter studies. *Environ. Pollut.* 108, 45–52.

BUSER, H.-R.; POIGER, T.; MÜLLER, M.D. 1999. Occurrence and environmental behavior of the chiral pharmaceutical drug ibuprofen in surface waters and in wastewater. *Environ. Sci. Technol.* 33, 2529–2535.

BÜYÜKSÖNMEZ , F.; RINK, R.; HESS, T.; BECHINSKI, E. 1999. Occurrence, degradation and fate of pesticides during composting, Part I, Composting, pesticides, and pesticides degradation. *Compost Sci. Util.* 7, 66–82.

CALDERBANK, A. 1989. The occurrence and significance of bound pesticide residues in soil. *Rev. Environ. Contam. Toxicol.* 108, 71–102.

CASTIGLIONI, S.; BAGNATI, R.; FANELLI, R.; POMATI, F.; CALAMARI, D.; ZUCCATO, E. 2006. Removal of pharmaceuticals in sewage treatment plants in Italy. *Environ. Sci. Technol.* 40, 357–363.

CHAUDHRY, G.R.; HUANG, G.H. 1988. Isolation and characterisation of a new plasmid from a Flavobacterium sp. which carries the genes for degradation of 2,4-dichlorophenoxyacetic acid. *J Bacteriol.* 170, 3897–3902.

CHEFETZ, B.T.; TARCHITZKY, J.; DESHMUKH, A. P.; HATCHER, P. G.; CHEN, Y. 2002. Structural characterization of soil organic matter and humic acids in particle-size fractions of an agricultural soil. *Soil Sci. Soc. Am. J.* 66, 129–141.

CHRISTENSEN, B.T. 2001. Physical fractionation of soil and structural and functional complexity in organic matter turnover. *Eur J Soil Sci.* 52, 345–353.

CHUNG, N.; ALEXANDER, M. 1998. Differences in sequestration and bioavailability of organic compounds aged in dissimilar soils. *Environ. Sci. Technol.* 32, 855–860.

CLARA, M.; KREUZINGER, N.; STRENN, B.; GANS, O.; KROISS, H. 2005. The solids retention time-a suitable design parameter to evaluate the capacity of wastewater treatment plants to remove micropollutants. *Water Res.* 39, 97–106.

COLEMAN, D.C.; REID, C.P.P.; COLE, C.V. 1983. Biological strategies of nurient cycling in soil systems. *Adv. in Ecol. Res.* 13, 1–55.

CORNELISSEN, G.; RIGTERINK, H.; FERDINANDY, M.M.A; VAN NOORT, P.C.M. 1998. Rapidly desorbing fractions of PAHs in contaminated sediments as a predictor of the extent of bioremediation. *Environ. Sci. Technol.* 32, 966–970.

CRANWELL, P.A. 1974. Monocarboxylic acids in lake sediments: indicators derived from terrestrial and aquatic biota of paleoenvironmental trophic levels. *Chem. Geol.* 14, 1–14.

CRAVEN, A. 2000. Bound residues of organic compounds in the soil: the significance of pesticide persistence in soil and water: a European regulatory view. *Environ. Pollut.* 108, 15–18.

CRESPÍN, M.; GALLEGO, M.; VALCÁRCEL 2001. Study of the degradation of the herbicides 2,4-D and MCPA at different depths in contaminated agricultural soil. *Environ. Sci. Technol.* 35, 4265–4270.

DAUWE, B.; MIDDELBURG, J.J. 1998. Amino acids and hexosamines as indicators of organic matter degradation state in North Sea sediments. *Limnol. Oceanogr.* 43, 782–798

DEC, J.; BOLLAG, J.-M. 1997. Determination of covalent and non- covalent binding interactions between xenobiotic chemicals and soil. *Soil Sci*. 162, 858–874.

DEJONGHE, W.; GORIS, J.; EL FANTROUSSI, S.; HOFTE, M.; DE VOS, P.; VERSTRAETE, W.; TOP, E.M. 2000. Effect of dissemination of 2,4-D degradation and on bacterial community structure in two different soil horizons. *Appl. Environ. Microbiol*. 66, 3297–3304.

DIGIOVANNI, G.D.;NEILSON, J.W.; PEPPER, I.L.; SINCLAIR, N.A. 1996. Gene transfer of Alcaligenes eutrophus JMP 134 plasmid pJP4 to indigenous recipients. *Appl. Environ. Microbiol.* 62, 2521–2526.

DON, R.H.; PEMBERTON, J.M. 1981. Related properties of six pesticide degradation plasmids isolated from Alcaligenes paradoxus and Alcaligenes eutrophus. *J Bacteriol*. 145, 681–686.

DOYLE, R.C.; KAUFMANN, D.D.; BURT, G.W. 1978. Effect of dairy manure and sewage on ^{14}C-pesticide degradation in soil. *J Agric. Food Chem.* 26, 987–989.

DRENOVSKY, R. E.; ELLIOTT, G. N.; GRAHAM, K. J.; SCOW, K. M. 2004. Comparison of phospholipid fatty acid (PLFA) and total soil fatty acid methyl esters (TSFAME) for characterizing soil microbial communities. *Soil Biol. Biochem.*, 36 ,1793–1800.

EDGEHILL, R.U; FIN, R.K. 1983. Microbial treatment of soil to remove pentachlorophenol. *Appl. Environ. Microbiol.* 45, 1122–1125.

EDWARDS, M.; TOPP, E.; METCALFE, C.D.; LI, H.; GOTTSCHALL, N.; BOLTON, P.; CURNOE, W.; PAYNE, M.; BECK, A.; KLEYWEGT, S.; LAPEN, D.R. 2009. Pharmaceutical and personal care products in tile drainage following surface spreading and injection of dewatered municipal biosolids to an agricultural field. *Sci. Total Environ.* 407, 4220–4230.

EHLERS, L.J.; LUTHY, R.G. 2003. Contaminant bioavailability in soil and sediment. *Environ. Sci. Technolnol.* 37, 295A–302A.

EKSCHMITT, K.; LIU, M.; VETTER, S.; FOX, O.; WOLTERS, V. 2005. Strategies used by soil biota to overcome soil organic matter stability-why is dead organic matter left over in the soil? . *Geoderma* 128, 167–176.

ESCHENBACH, A.; WIENBERG, R.; MAHRO, B. 1998. Fate and stability of non-extractable residues of ^{14}C-PAH in contaminated soils under environmental stress conditions. *Environ. Sci. Technol.* 32, 2585–2590.

FABBRI, D.C.; CHIAVARI, G.; GALLETTI, G. C. 1996. Characterization of soil humin by pyrolysis(methylation)-gas chromatography/mass spectrometry: Structural relationships with humic acids. *J. Anal. Appl. Pyrolysis* 37, 161–172.

FEISTHAUER, S.; WICK, L.Y.; KÄSTNER, M.; KASCHABEK, S.R.; SCHLOMANN, M.; RICHNOW, H.-H. 2008. Differences of heterotrophic $^{13}CO_2$ assimilation by Pseudomonas knackmussii strain B13 and Rhodococcus opacus 1CP and potential impact on biomarker stable isotope probing. *Environ. Microbiol.* 10, 1641–1651.

FOGARTHY, A.; TUOVINEN, O.H. 1991. Microbiological degradation of pesticides in yard waste composting. *Microbiol. Mol. Biol. Rev.* 55, 225–233.

FORSTER, R.K.; MCKERCHER, R.B. 1973. Laboratory incubation studies of chlorophenoxyacetic acids in chernozemic soils. *Soil Biol. Biochem.* 5, 333–337.

FÜHR, F.; OPHOFF, H.; BURAUEL, P.; WANNER, U.; HAIDER, K. 1998. Modification of definition of bound residues, in: Fuhr, F., Ophoff (Ed.), Pesticide Bound Residues in Soil. Wiley-VCH, Weinheim, pp. 175–176.

FULTHORPE R.R.; RHODES, A.N.; TIEDJE J.M. 1996. Pristine soils mineralize 3-chlorobenzoate and 2,4-dichlorophenoxyacetic acid via different microbial populations. *Appl. Environ. Microbiol.* 62, 1159–1166.

GAULTIER, J.; FARENHORST, A.; CATHCART, J.; GODDARDB, T. 2008. Degradation of [carboxyl-^{14}C] 2,4-D and [ring-U-^{14}C] 2,4-D in 114 agricultural soils as affected by soil organic carbon content *Soil Biol. Biochem.* 40, 217–227.

GAVRILESCU, M. 2005. Fate of pesticides in the environment and its bioremediation. *Eng. Life Sci.* 5, 497–526.

GERSTL, Z.; KLIGER, L. 1990. Fractionation of the organic matter in soils and sediments and their contribution to the sorption of pesticides. *J Environ. Sci. Health* 25, 729–741

GETENGA, Z.M.; MADADI, V.; WANDIGA, S.O. 2004. Studies on biodegradation of 2,4-D and metribuzin in soil under controlled conditions. *Bull. Environ. Contam. Toxicol.* 72, 504–513.

GEVAO, B.; SEMPLE, K.T.; JONES, K.C. 2000. Bound pesticide residues in soils: a review. *Environ. Pollut.* 108, 3–14.

GEVAO, B.; JONES, K.C.; SEMPLE, K.T.; CRAVEN, A.; BURAUEL, P. 2003. Nonextractable pesticide residues in soil. *Environ. Sci. Technol.* 37, 138A–144A.

GEVAO, B.; JONES, K.C.; SEMPLE, K.T. 2005. Formation and release of non-extractable ^{14}C-Dicamba residues in soil under sterile and non-sterile regimes. *Environ. Pollut.* 133, 17–24.

GOMEZ, M.J.; MARTINEZ BUENO, M.J.; LACORTE, S.; FERNANDEZ-ALBA, A.R.; AGUERA, A. 2007. Pilot survey monitoring pharmaceuticals and related compounds in a sewage treatment plant located on the Mediterranean coast. *Chemosphere* 66, 993–1002.

GOVLOVEVA, L.A.; AHARONSON, N.; GREENHALGH, R.; SETHUNATHAN, N.; VONK, J.W. 1990. The role and limitations of microorganisms, in the conversion of xenobiotics. *Pure Appl. Chem.* 62, 351–364.

GRASSET, L.G.; GUIGNARD, C.; AMBLES, A. 2002. Free and esterified aliphatic carboxylic acids in humin and humic acids from a peat sample as revealed by pyrolysis with tetramethylammonium hydroxide or tetraethylammonium acetate. *Org. Geochem.* 33, 181–188.

GREEN, C.T.; SCOW, K.M. 2000. Analysis of phospholipid fatty acids (PLFA) to characterize microbial communities in aquifers. *Hydrogeol. J* 8, 126–141.

HÄGGBLOM, M. 1992. Microbial breakdown of halogenated aromatic pesticides and related compounds. *FEMS Microbiol. Rev.* 103, 29–71.

HAIDER, K. 1998. Von der toten organischen Substanz zum Humus. *Pflanzernährung Bodenkunde* 162, 363–317.

HAIDER, K.; SCHÄFFER, A. 2009. Soil biochemistry. science Publishers, Enfield, Jersey, Plymouth.

HARMSEN, J. 2007. Measuring bioavailability: from a scientific approach to standard methods. *J Environ. Qual.* 36, 1420–1428.

HATCHER, P.G.; BORTIATYNSKI, J.M; MINARD, R.D.; DEC, J.; BOLLAG, J.-M. 1993. Use of high-resolution ^{13}C-NMR to examine the enzymatic covalent binding of ^{13}C-labeled 2,4-dichlorophenol to humic substances. *Environ. Sci. Technol.*, 27, 2098–2103.

HATZINGER, P.B.; ALEXANDER, M. 1995. Effect of aging of chemicals in soil on their biodegradability and extractability. *Environ. Sci. Technol.* 29, 537–545.

HAWTHORNE, S.B.; GRABANSKI, C.B.; MARTIN, E.; MILLER, D.J. 2000. Comparisons of soxhlet extraction, pressurized liquid extraction, supercritical fluid extraction and subcritical water extraction for environmental solids: recovery, selectivity and effects on sample matrix. *J Chromatogr.* A 892, 421–433.

HAYES, M.H.B.; MACCARTHY, P.; MALCOLM, R.L.; SWIFT, R.S. 1989. Humic Substances II, Search of Structure. Chichester John Wiley&Sons.

HLADY, V.; BUIJS, J. 1996. Protein adsorption on solid surfaces. *Cur. Opin. Biotechnol.* 7, 72–77.

HSU, T.-S.; BARTHA, R. 1974. Interaction of pesticide-derived chloroaniline residues with soil organic matter. *Soil Sci.* 116, 444–452.

HUANG, W.; PENG, P.; YU, Z.; FU, J. 2003. Effects of organic matter heterogeneity on sorption and desorption of organic contaminants by soils and sediments. *Appl. Geochem.* 18, 955–972.

ISO/DIS, 2006. Soil quality. Guidance for the selection and application of methods for the assessment of bioavailability of contaminants in soil and soil materials. ISO, Geneva, Switzerland.

JACOBSEN, C.S.; RASMUSSEN, O.F. 1992. Development and application of a new method to extract bacterial DNA from soil based on separation of bacteria from soil with cation-exchange resin. *Appl. Environ. Microbiol.* 58, 2458–2462.

JENKINSON, D.S.; LADD, J.N. 1981. Microbial biomass in soil: measurement and turnover, in: Paul, E.A., Ladd, J.N. (Ed.), Soil Biochemistry. Marcel Dekker, New York, pp. 415–471.

JONES, D.L. 1999. Amino acid biodegradation and its potential effects on organic nitrogen capture by plants *Soil Biol. Biochem.* 31, 613–622 .

JONES, D.L.; HEALEY, J.R.; WILLET, V.B.; FARRAR, J.F.; HODGE, A. 2005. Dissolved organic nitrogen uptake by plants - an important N uptake pathway? *Soil Biol. Biochem.* 37, 413–423.

JONES, O.A.H.; VOULVOULIS, N.; LESTER, J.N. 2007. The occurrence and removal of selected pharmaceutical compounds in a sewage treatment works utilising activated sludge treatment. *Environ. Pollut* .145, 738–744.

JONES-LEPP, T.L.; STEVENS R. 2007. Pharmaceuticals and personal care products in biosolids/sewage sludge - the interface between analytical chemistry and regulation. *Anal. Bioanal. Chem.* 387, 1173–1183.

JOSS, A.; KELLER, E.; ALDER, A.C.; GOBEL, A.; MCARDELL, C.S.; TERNES, T.; SIEGRIST, H. 2005. Removal of pharmaceuticals and fragrances in biological wastewater treatment. *Water. Res.* 39, 3139–3152.

KÄSTNER, M.; STREIBICH, S.; BEYRER, M.; RICHNOW, H.-H.; FRITSCHE, W. 1999. Formation of bound residues during microbial degradation of [^{14}C]anthracene in soil. *Appl. Environ. Microbiol.* 65, 1834–1842.

KÄSTNER, M. 2000. "Humification" Process or formation of refractory soil organic matter, in: Rehm, H.J.; Reed, G.; Pühler, A.; Stadler, P. (Ed.), Biotechnology, 2nd Edition. Wiley-VCH, Weinheim, pp. 89–125.

KÄSTNER, M.; HOFRICHTER, M. 2001. Biodegradation of humic substances, in: Steinbüchel, A.; Hofrichter, M. (Ed.), Biopolymers Vol. 1 - Lignin, humic substances and coal. Wiley-VCH, Weinheim, pp. 350–378.

KÄSTNER, M.; RICHNOW, H.-H. 2001. Formation of residues of organic pollutants within the soil matrix – mechanisms and stability, in: Stegmann, R.; Brunner, G., Calmano; W., Matz, G. (Ed.), Treatment of Contaminated Soil. Springer, Berlin, Heidelberg, New York, pp. 219–251.

KAMAGATA, Y.; FULTHORPE, R.R.; TAMURA, K.; TAKAMI, H.; FORNEY, L.J.; TIEDJE, J.M. 1997. Pristine environments harbor a new group of oligotrophic 2,4-dichlorophenoxyacetic acid-degrading bacteria. *Appl. Environ. Microbiol*. 63, 2266–2272.

KATAYAMA A., B.R.; BURNS G.R.; CARAZO E.; FELSOT A.; HAMILTON D.; HARRIS C.; KIM Y.H.; KLETER G.; KÖDEL W.; LINDERS J.; PEIJNENBURG J.G.; SABLJIC A.; STEPHENSON R.G.; RACKE D.K.; RUBIN B.; TANAKA K.; UNSWORTH J.; WAUCHOPE R.D. 2010. Bioavailability of xenobiotics in the soil environment., in: Whitacre, D.M. (Ed.), Rev Environ Contam Toxicol. Springer, New York , Dordrecht, Heidelberg, London, pp. 1–86.

KAUFMANN, D.D.; BLAKE, J. 1973. Microbial degradation of several acetamide, acylanilide, carbetamide, toluidine and urea pesticides. *Soil Microbiol. Biochem.* 5, 297–308.

KAUR, A.; CHAUDHARY, A.; KAUR, A.; CHOUDHARY, R.; KAUSHIK, R. 2005. Phospholipid fatty acid – A bioindicator of environment monitoring and assessment in soil ecosystem. *Cur. Sci.* 89, 1103–1112.

KELLEHER, B.R.; SIMPSON, A.J. 2006. Humic substances in soils: are they really chemically distinct?. *Environ. Sci. Technol.* 40, 4605–4611.

KELSEY, J.W.; KOTTLER, B.D.; ALEXANDER, M. 1997. Selective chemical extractants to predict bioavailability of soil-aged organic chemicals. *Environ. Sci. Technol*. 31, 214–217.

KHAN, S.U.; IVARSON, K.C. 1981. Microbiological release of unextracted (bound) residues from an organic soil treated with prometryn. *J Agric. Food Chem.* 29, 1301–1303.

KHAN, S.U.; DUPONT, S. 1987. Bound pesticide residues and their bioavailability, in: Greenhalgh, R., Roberts, T.R. (Ed.), Pesticide Science and Technology. Blackwell Scientific Publications, Oxford, pp. 417–420.

KHAN , S.U. 1991. Bound residues. In: Grover, R., Cessna, A.J. Eds. Environmental Chemistry of herbicides, Vol. 2., CRC, Boca Raton, FL, pp. 265–279.

KILPI, S.; BACKSTROM, V.; KORHOLA, M. 1980. Degradation of 2-methyl-4-chlorophenoxyacetic acid (MCPA), 2,4-dichlorophenoxyacetic acid (2,4-D), benzoic acid and salicylic acid by Pseudomonas sp. HV3. *FEMS Microbiol. Lett.* 8, 177–182.

KINDLER, R.; MILTNER, A.; RICHNOW, H.-H.; KÄSTNER, M. 2006. Fate of gram-negative bacterial biomass in soil-mineralization and contribution to SOM. *Soil Biol. Biochem.* 38, 2860–2870.

KINDLER, R.; MILTNER, A.; THULLNER, M.; RICHNOW, H.-H.; KÄSTNER, M. 2009. Fate of bacterial biomass derived fatty acids in soil and their contribution to soil organic matter. *Org. Geochem.* 40, 29–37.

KLEBER, M.; SOLLINS, P.; SUTTON, R. 2007. A conceptual model of organo-mineral interactions in soils: self-assembly of organic molecular fragments into zonal structures on mineral surfaces. *Biogeochem.* 85, 9–24.

KLEBER, M.; HONG, J.-P.; K. STAHR. 1998. Microbial biomass C- and N-dynamics in grassland soils amended with liquid manure. *Zeitschrift für Pflanzenernaehrung und Bodenkunde* 161, 87–92.

KLIBANOV, A.M.; TU, T.-M.; SCOTT, K.P. 1983. Peroxidase-catalyzed removal of phenols from coal-conversion wastewater. *Sci* 21, 259–260.

KNICKER, H.; FRÜND, R.; LÜDEMANN, H.-D. 1993. The chemical nature of nitrogen in native soil organic matter. *Naturwissenschaften* 80, 219–221.

KÖGEL-KNABNER, I. 2002. The macromolecular organic composition of plant and microbial residues as inputs to soil organic matter. *Soil Biol. Biochem.* 34, 139–162.

KÖRSCHENS, M; MERBACH, I.; SCHULZ, E. 2000. 100 Jahre Statischer Düngungsversuch Bad Lauchstädt. Herausgegeben anlässlich des Internationalen Symposiums vom. 5. bis 7. Juni 2000.

KOSJEK, T.; HEATH, E.; KOMPARE, B. 2007. Removal of pharmaceutical residues in a pilot wastewater treatment plant. *Anal. Bioanal. Chem.* 387, 1379–1387.

KRAMER, R.W.K.; E. B.; ZANG, X.; GREEN-CHURCH, K. B.; JONES, R. B.; FREITAS, M. A.; HATCHER, P. G. 2001. Studies of the structure of humic substances by electrospray ionization coupled to a quadrupole-time of flight (Qq-TOF) mass spectrometer, in: Ghabbour, E.A., Davies, G. (Ed.), Humic Substances: Structures, Models and Functions. Royal Society of Chemistry, Cambridge, UK, pp. 95–107.

KREBS, H.A. 1941. Carbon dioxide assimilation in heterotrophic organisms. *Nature* 147, 560–563.

KUBIAK, R.; FÜHR, F.; MITTELSTAEDT, W. 1990. Comparative studies on the formation of bound residues in soil in outdoor and laboratory experiments. *International J Environ. Anal. Chem.* 39, 47–57.

LAPEN, D.R.; TOPP, E.; METCALFE, C.D.; LI, H.; EDWARDS, M.; GOTTSCHALL, N.; BOLTON, P.; CURNOE, W.; PAYNE, M.; BECK, A. 2008. Pharmaceutical and personal care products in tile drainage following land application of municipal biosolids. *Sci. Total Environ.* 399, 50–65.

LEADBETTER, E.R. 1997. Procaryotic diversity: form, ecophysiology, and habitat, in: Hurst, C.J., Knudsen, G.R., McInerney, M.J., Stetzenbach, L.D., Walter, M.V. (Ed.), Manual of Environmental Microbiology. Washington, DC: ASM Press, pp. 14–24.

LERCH, T.Z.; DIGNAC, M.-F.; NUNAN, N.; BARDOUX, G.; BARRIUSO, E.; MARIOTTI, A. 2009A. Dynamics of soil microbial populations involved in 2,4-D biodegradation revealed by FAME-based Stable Isotope Probing. *Soil Biol. Biochem.* 41, 77–85.

LERCH, T.Z.; DIGNAC, M.F.; NUNAN, N.; BARRIUSO, E.; MARIOTTI, A. 2009B. Ageing processes and soil microbial community effects on the biodegradation of soil ^{13}C-2,4-D nonextractable residues. *Environ. Pollut.*157, 2985–2993.

LERCH, T.Z.; DIGNAC, M.F.; BARRIUSO, E.; BARDOUX, G.; MARIOTTI, A. 2007. Tracing 2,4-D metabolism in Cupriavidus necator JMP134 with ^{13}C-labelling technique and fatty acid profiling. *J Microbiol. Methods* 71, 162–174.

LISTE, H.-H.; ALEXANDER, M. 2002. Butanol extraction to predict bioavailability of PAHs in soil. *Chemosphere* 46, 1011–1017.

LOFFLER, D.; ROMBKE, J.; MELLER, M.; TERNES, T.A. 2005. Environmental fate of pharmaceuticals in water/sediment systems. *Environ. Sci. Technol.* 39, 5209–5218.

LOISEAU, L.,;BARRIUSO, E. 2002. Characterization of the Atrazine's Bound (Nonextractable) Residues Using Fractionation Techniques for Soil Organic Matter. *Environ. Sci. Technol.* 36, 683–689.

LÜDERS, T.; KINDLER, R.; MILTNER, A.; FRIEDRICH, M.W.; KÄSTNER, M. 2006. Identification of bacterial micropredators distinctively active in a soil microbial food web. *Appl. Environ. Microbiol.* 72, 5342–5348.

MACKO, S.A.; UHLE, M.E. 1997. Stable nitrogen isotope analysis of amino acid enantiomers by gas chromatography/combustion/isotope ratio mass spectrometry. *Anal. Chem.* 69, 926–929.

MADIGAN, M.; MARTINKO, J. 2006. Brock Biology of Microorganisms. Pearson Prentice Hall.

MANZANO, M.; MORÁN, A.C.;TESSER, B.; GONZÁLEZ, B. 2007. Role of eukaryotic microbiota in soil survival and catabolic performance of the 2,4-D herbicide degrading bacteria Cupriavidus necator JMP134. *Antonie van Leeuwenhoek* 91, 115–126.

MARSCHNER, B.; BRODOWSKI, S.; DREVES, A.; GLEIXNER, G.; GUDE, A.; GROOTES, P.M.; HAMER, U.; HEIM, A.; JANDL, G.; JI, R.; KAISER, K.; KALBITZ, K.; KRAMER, C.; LEINWEBER, P.; RETHEMEYER, J.; SCHÄFFER, A.; SCHMIDT, M.W.I.; SCHWARK, L.; WIESENBERG, G.L.B. How relevant is recalcitrance for the stabilization of organic matter in soils? *J. Plant Nutr. Soil Sci.* 171, 91–110.

MATAMOROS, V.; GARCIA, J.; BAYONA, J.M. 2008. Organic micropollutant removal in a full-scale surface flow constructed wetland fed with secondary effluent. *Water Res.* 42, 653–660.

MCCLELLAN, K.; HALDEN, R.U. 2010. Pharmaceuticals and personal care products in archived U.S. biosolids from the 2001 EPA National Sewage Sludge Survey. *Water Res.* 44, 658–668.

MCGHEE, I.; BURNS, R.G. 1995. Biodegradation of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2-methyl-4-chlorophenoxyacetic acid (MCPA) in contaminated soil. *Appl. Soil Ecol.* 2, 143–154.

MERINI, L.J.; CUADRADO, V.; GIULIETTI, A.M. 2008. Spiking solvent, humidity and their impact on 2,4-D and 2,4-DCP extractability from high humic matter content soils. *Chemosphere* 71, 2168–2172.

MICHAL, G. 1999. Biochemical Pathways. Spektrum Akademischer Verlag GmbH, Heidelberg.

MIEGE, C.; CHOUBERT, J.M.; RIBEIRO, L.; EUSEBE, M.; COQUERY, M. 2009. Fate of pharmaceuticals and personal care products in wastewater treatment plants-conception of a database and first results. *Environ. Pollut.* 157, 1721–1726.

MILTNER, A.; RICHNOW, H.-H.; KOPINKE, F.-D.; KÄSTNER, M. 2004. Assimilation of CO_2 by soil microorganisms and transformation into soil organic matter. *Org. Geochem.* 35, 1015–1024.

MILTNER, A.K.; F.-D.; KINDLER, R.; SELESI, D.; HARTMANN, A.; KÄSTNER, M. 2005. Non-phototrophic CO_2 fixation by soil microorganisms. *Plant Soil* 269, 193–203.

MILTNER, A.; KINDLER, R.; KNICKER, H.; RICHNOW, H.-H.; KÄSTNER, M. 2009. Fate of microbial biomass-derived amino acids in soil and their contribution to soil organic matter. *Org. Geochem.* 40, 978–985.

MORDAUNT, C.J.; GEVAO, B.; JONES, K.C.; SEMPLE, K.T. 2005. Formation of non-extractable pesticide residues: observations on compound differences, measurement and regulatory issues. *Environ. Pollut.* 133, 25–34.

MORTLAND, M.M. 1986. Mechanisms of adsorption of non-humic organic species by clays, in: Huang, W., Schnitzer, M. (Ed.), Interaction of Soil Minerals with Natural Organics and Microbes. SSSA Special Publication 17, pp. 59–75.

MUGO, S.M.B., BOTTARO, C. S. 2004. Characterization of humic substances by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Comm. Mass Spec*. 18, 2375–2382.

MÜLLER, K.; MAGESAN, G.N.; BOLAN, N.S. 2007. A critical review of the influence of effluent irrigation on the fate of pesticides in soil. *Agric. Ecos. Environ.* 120, 93–116.

NOORKAMP, E.R.J.; GROTENHUIS, T.C.; RULKENS, W.H. 1997. Selection of an efficient extraction method for the determination of polycyclic aromatic hydrocarbons in contaminated soil and sediment. *Chemosphere* 35, 1907–1917

NORTHCOTT, G.L.; JONES, K.C. 2000. Experimental approaches and analytical techniques for determining organic compound bound residues in soil and sediment. *Environ. Pollut.* 108, 19–43.

NRC (NATIONAL RESEARCH COUNCIL) 2002. Bioavailability of Contaminants in Soils and Sedminents: Processes, Tools and Applications, National Academies Press, Washington DC.

OADES, J.M. (ED.) 1995. An Overview of Processes Affecting the Cycling of Organic Carbon in Soils. Wiley, New York.

OECD, 2002. Guideline For Testing of Chemicals. Aerobic and Anaerobic Transformation in Soil. 307.

PALOMO, M.; BHANDARI, A. 2005. Time-Dependent Sorption−Desorption Behavior of 2,4-Dichlorophenol and Its Polymerization Products in Surface Soils. *Environ. Sci. Technol*. 39, 2143–2151.

PALOMO, M.; BHANDARI, A. 2006. Impact of aging on the formation of bound residues after peroxidase-mediated treatment of 2,4-DCP contaminated soils. *Environ. Sci. Technol.* 40, 3402–3408.

PAUL, E.A.; CLARK, F.E. 1989. Soil Microbiology and Biochemistry. Academic Press, Inc., San Diego, CA.

PAUL, E.A., CLARK, F.E., 1996. Soil Microbiology and Biochemistry. Academic Press, San Diego, CA.

PAULI, F.W. 1967. Soil Fertility. London Adam Hilger.

PELZ, O.; CIFUENTES, L.A.; HAMMER, B.T.; KELLEY, C.A.; COFFIN, R.B. 1998. Tracing the assimilation of organic compopounds using ^{13}C analysis of unique amino acids in the bacterial peptidoglycan cell wall. *FEMS Microbiol. Ecol.* 25, 229–240.

PEREZ, R.C.; MATIN, A. 1982. Carbon dioxide assimilation by Thiobacillus Novellus under nutrient-limited mixotrophic conditions *J. Bacteriol.* 150, 46–51.

PICCOLO, A. 1996. Humic Substances In Terrestrial Ecosystems, in: Piccolo, A. (Ed.), Humus and soil conservation. Elsevier, Amsterdam, pp. 225–264.

PICCOLO, A.; CONTE, P.; COZZOLINO, A.; PACI, M. 2001. Combined effects of an oxidative enzyme and dissolved humic substances on ^{13}C-labelled 2,4-D herbicide as revealed by high-resolution ^{13}C NMR spectroscopy. *J Ind. Microbiol. Biotechnol*. 26, 70–76.

PICKUP, R.W. 1991. Development of molecular methods for the detection of specific bacteriain the environment. *J. Gen. Microbiol.* 137, 1009–1019.

PIEPER, D.M.; REINEKE, W.; ENGESSER, K.-H.; KNACKMUSS, H.-J. 1988. Metabolism of 2,4-dichlorophenoxyacetic acid, 4-chloro-2-methylphenoxyacetic acid and 2-methylphenoxyacetic acid by Alcaligenes eutrophus JMP 134. *Arch. Microbiol.* 150, 95–102.

PIGNATELLO, J.J. 1989. Sorption dynamics of organic compounds in soils and sediments, in: Sawhney, B.L., Brown, B.K. (Ed.), Reactions and Movements of Organic Chemicals in Soil. SSSA and ASA Publisher, Madison USA, pp. 45–79.

PIGNATELLO, J.J.; XING, B. 1996. Mechanisms of slow sorption of organic chemicals to natural particles. *Environ. Sci. Technol.* 31, 1–11.

PRINTZ, H.; BURAUEL, P.; FÜHR, F. 1995. Effect of organic amendment on degradation and formation of bound residues of Methabenthiazuron in soil under constant climatic conditions. *J Environ. Sci. Health* B30, 435–456.

RACKE, K.D.; LICHTENSTEIN, E.P. 1985. Effects of soil microorganisms in the release of bound ^{14}C residues from soils previously treated with [^{14}C]Parathion. *J Agric. Food Chem.* 33, 938–943.

RADJENOVIĆ, J.; JELIĆ, A.; PETROVIĆ, M.; BARCELÓ, D. 2009. Determination of pharmaceuticals in sewage sludge by pressurized liquid extraction (PLE) coupled to liquid chromatography-tandem mass spectrometry (LC-MS/MS). *Anal. Bioanal. Chem.* 393, 1685–1695.

REICHENBERG, F.; MAYER, P. 2006. Two complementary sides of bioavailability: accessibility and chemical activity of organic contaminants in sediments and soils. *Environ. Toxicol. Chem.* 25, 1239–1245.

REID, B.J.; JONES, K.C.; SEMPLE K.T. 2000. Bioavailability of persistent organic pollutants in soils and sediments--a perspective on mechanisms, consequences and assessment. *Environ. Pollut.*108, 103–112.

RICE, P.J.; ANDERSON, T.A.; COATS, J.R. 2002. Degradation and persistence of metolachlor in soil: effects of concentration, soil moistrure, soil depth, and sterilization. *Environ. Toxicol. Chem.* 21, 2640–2648.

RICHNOW, H.-H.; SEIFERT, R.; HEFTER, J.; LINK, M.; FRANCKE, W.; SCHÄFER, G.; MICHAELIS, W. 1997. Organic pollutants associated with macromolecular soil organic matter: Mode of binding. *Org. Geochem.* 26, 745–758.

RICHNOW H.-H; SEIFERT, R.; HEFTER, J.; KÄSTNER, M.; MAHRO, B.; MICHAELIS, W. 1994. Metabolites of xenobiotica and mineral oil constituents linked to macromolecular organic matter in polluted environments. *Org. Geochem.* 22, 671–681.

RICHNOW, H.-H.; ANNWEILER, E.; KONING, M.; LUTH, J.C.; STEGMANN, R.; GARMS, C.; FRANCKE, W.; MICHAELIS, W. 2000. Tracing the transformation of labelled [1-^{13}C]phenanthrene in a soil bioreactor. *Environ. Pollut* .108, 91–101.

RICHNOW, H.-H.; ESCHENBACH, A.; MAHRO, B.; KÄSTNER, M.; ANNWEILER, E.; SEIFERT, R.; MICHAELIS, W. 1999. Formation of nonextractable soil residues: a stable isotope approach. *Environ. Sci. Technol.* 33, 3761–3767.

RICHNOW, H.-H.; ESCHENBACH, A.; MAHRO, B.; SEIFERT, R.; WEHRUNG, P.; ALBRECHT, P.; MICHAELIS, P. 1998. The use of ^{13}C-labelled polycyclic aromatic hydrocarbons for the analysis of their transformation in soil. *Chemosphere* 36, 1477–1483.

RICHTER, O.; KULLMER, C.; KREUZIG, R. 2007. Metabolic fate modeling of selected human pharmaceuticals in soils. *Clean* 35, 495–503.

RILLIG, M.C.; CALDWELL, B.A.; WOESTEN, H.A.B.; SOLLINS, P. 2007. Role of proteins in soil carbon and nitrogen storage: controls on persistence. *Biogeochem*. 85, 25–44.

ROBERTS, T.R. 1984. Non-extractable pesticide residues in soils and plants. *Pure Appl. Chem.* 56, 945–995.

ROBERTS, T.R.; HUTSON, D.H.; LEE, P.W.; NICHOLLS, P.H.; PLIMMER, J.R. (Ed.), 1998. Metabolic Pathways of Agrochemical Part 1. The Royal Society of Chemistry, Cambridge, UK, pp. 66–74.

ROBERTSON, B.K.; ALEXANDER, M. 1998. Sequestration of DDT and dieldrin in soil: Disappearance of acute toxicity but not the compounds. *Environ. Toxicol. Chem.* 17, 1034–1038.

SANDMANN, E.R.I.C.; LOOS, M.A.; VAN DYK, L.P. 1988. The microbial degradation of 2,4-dichlorophenoxyacetic acid in soil. *Rev. Environ. Contam. Toxicol.* 101, 1–51.

SAXENA, A., BARTHA, R., 1983. Microbial mineralization of humic acid-3,4-dichloroaniline complexes. Soil Biol Biochem 15, 59–62.

SHAW, L.J.; BEATON, Y.; GLOVER, L.A.; KILLHAM, K.; MEHARG, A.A. 1999. Re-inoculation of autoclaved soil as a non-sterile treatment for xenobiotic sorption and biodegradation studies. *Appl. Soil Ecol.*, 11, 217–226.

SCHIAVON, M. 1988. Studies of the movement and the formation of bound residues of atrazine, of its chlorinated derivatives, and of hydroxyatrazine in soil using ^{14}C-ring-labelled compounds under outdoor conditions. *Ecotoxicol. Environ. Saf.* 10, 347–354.

SCHNITZER, M. 1978. Humic Substances: Chemistry and Reactions., in: Schnitzer, M., Khan, S.U. (Ed.), Soil Organic Matter. Elsevier, Amsterdam.

SCHNITZER, M.; HINDLE, C.A.; MEGLIC, M. 1986. Supercritical gas extraction of alkanes and alkanoic acids from soil and humic material. *Soil Sci. Soc. Am. J* 50, 913–919.

SCHNITZER, M.A. 2000. Lifetime perspective on the chemistry of soil organic matter, in: Sparks, D.L. (Ed.), Adv Agron. Academic Press San Diego, CA, pp. 1–58.

SCHOLES, R.J.; SCHOLES, M.C. 1995. The effect of land use on nonliving organic matter in the soil, in: Zepp, R.G., Sonntag, C. (Ed.), Role of Nonliving Organic Matter in the Earth's Carbon Cycle. John Wiley&Sons, Chichester, pp. 209–226.

SCHULTEN, H.-R. 1999. Analytical pyrolysis and computational chemistry of aquatic humic substances and dissolved organic matter. *J. Anal. Appl. Pyrolysis* 49, 385–415.

SEMPLE, K.T.; DOICK, K.J.; JONES, K.C.; BURAUEL, P.; CRAVEN, A.; HARMS, H. 2004. Defining bioavailability and bioaccessibility of contaminated soil and sediment is complicated. *Environ. Sci. Technol.* 38, 228A–231A.

SEMPLE, K.T.; DOICK, K.J.; WICK, L.Y.; HARMS, H. 2007. Microbial interactions with organic contaminants in soil: definitions, processes and measurement. *Environ. Pollut.* 150, 166–176.

SEMPLE, K.T.; MORRISS, A.W.J.; PATON, G.I. 2003. Bioavailability of hydrophobic organic contaminants in soils: fundamental concepts and techniques for analysis. *European J Soil Sci*. 54, 809–818.

SENESI, N. 1992. Binding mechanisms of pesticides to soil humic substances. *Sci. Total Environ.* 123-124, 63–76.

SILFER, J.A.; ENGEL, M.H.; MACKO, S.A.; JUMEAU, E.J. 1991. Stable carbon isotope analysis of amino acid enantiomers by conventional isotope ratio mass spectrometry and combined gas chromatography/isotope ratio mass spectrometry. *Anal. Chem.* 63, 370–374.

SIMS, J.L.; SIMS, R.C.; MATTHEWS, J.E. 1990. Approach to bioremediation of contaminated soil. *Hazardous Waste Hazardous Materials* 7, 117–149.

SIMPSON, A.; SIMPSON, M.; SMITH, E.; KELLEHER, B. 2007. Microbially derived inputs to soil organic matter: are current estimates too low? *Environ. Sci. Technol.* 41, 8070–8076.

SINGH, B.K.; WALKER, A. 2006. Microbial degradation of organophosphorus compounds. *FEMS Microbiol. Rev.* 30, 428–471.

SMITH, A.E.; AUBIN, A.J. 1991. Metabolites of [^{14}C]-2,4-dichlorophenoxyacetic acid in Saskatchewan soils. *J Agric. Food Chem.* 39, 2019–2021.

SMITH, A.E.; LAFOND, G.P. 1990. Effect of long-term phenoxyacetic acid field applications on the rate of microbial degradation. ACS Symp Ser, pp. 14–22.

SOLLINS, P.; HOMANN, P.; CALDWELL, B.A. 1996. Stabilization and destabilization of soil organic matter: mechanisms and controls. *Geoderma* 74, 65–105

STENSON, A.C.; LANDING, W. M.; MARSHALL, A. G.; COOPER, W. T. 2002. Ionization and fragmentation of humic substances in electrospray ionization Fourier transform-ion cyclotron resonance mass spectrometry. *Anal. Chem.* 74, 4397–4409.

STEVENSON, F.J. 1994. Humus Chemistry: Genesis, Composition, Reaction. John Viley&Sons, New York.

STOKES, J.D.; PATON, G.I.; SEMPLE, K.T. 2006. Behaviour and assessment of bioavailability of organic contaminants in soil: relevance for risk assessment and remediation. *Soil Use Managem.* 21, 475–486.

STOLPE, N.B.; SHEA, P.J. 1995. Alachlor and atrazine degradation in a Nebraska soil and underlying sediments. *Soil Sci.* 160, 359–370.

STOTT, D.E.; KASSIM, G.; JARREL, W.M.; MARTIN, J.P.; HAIDER, K., 1983. Stabilisation and incorporation into biomass of specific plant carbons during biodegradation in soil. *Plant Soil* 70, 15–26.

STRUTHERS, J.K.; JAYACHANDRAN, K.; MOORMAN, T.B. 1998. Biodegradation of atrazine by Agrobacterium radiobacter J14a and use of this strain in bioremediation of contaminated soil. *Appl. Environ. Microbiol.* 64, 3368–3375.

STUER-LAURIDSEN, F.; BIRKVED, M.; HANSEN, L.P.; LUTZHOFT, H.C.; HALLING-SORENSEN, B. 2000. Environmental risk assessment of human pharmaceuticals in Denmark after normal therapeutic use. *Chemosphere* 40, 783–793.

SUTTON, R.; SPOSITO, G. 2005. Molecular structure in soil humic substances: the new view. *Environ. Sci. Technol.* 39, 9009–9015.

THENG, B.K.G. 1982. Clay activated organic interactions. *Dev. Sedimentol* 35, 197–238.

THIEL, V.; PECKMANNB, J.; RICHNOW, H.H.; LUTH, U.; REITNER, J.; MICHAELIS, W. 2001. Molecular signals for anaerobic methane oxidation in Black Sea seep carbonates and a microbial mat. *Mar. Chem.* 73, 97–112.

TISDALL, J.M.,1996. Formation of soil aggregates and accumulation of soil organic matter. In. Carter, M.R., Stewart, B.A. (Eds.), Structure and organic matter storage in agricultural soils. CRC Press, Boca Raton, FL, pp. 57–96

UEDA, K.; MORGAN, S.L.; FOX, A.; GILBART, J.; SONESSON, A.; LARSSON, L.; ODHAM, G. 1989. D-Alanine as a chemical marker for the determination of streptococcal cell wall levels in mammalian tissues by gas chromatography/negative ion chemical ionization mass spectrometry. *Anal. Chem.* 61, 265–270.

VAN HAMME, J.D. 2004. Bioavailability and biodegradation of organic pollutants - A microbial perspective, in: Soil Biology, Vol. 2: Biodegradation and Bioremendiation, Eds. Singh, A.; Ward, O.P., Springer Verlag, Berlin, Heidelberg, pp. 37–56.

VERSTRAETE, W.; DEVLIEGHER, W. 1996. Formation of non-bioavailable organic residues in soil: Perspectives for site remediation *Biodegradation* 7, 471–485.

VILLAVERDE, J.; KAH, M.; BROWN, C.D. 2008. Adsorption and degradation of four acidic herbicides in soils from southern Spain. *Pest. Manag. Sci.* 64, 703–710.

VESTAL, J.R.; WHITE, D.C. 1989. Lipid Analysis in Microbial Ecology. *Biosci.* 39, 535–541.

VINOLAS, L.C.; HEALEY, J.R.; JONES, D.L. 2001. Kinetics of soil microbial uptake of free amino acids *Biol. Fert. Soils* 33, 67–74.

VINTHER, F.P.; EILAND, F.; LAIND, A.-M.; ELSGAARD, L. 1999. Microbial biomass and numbers of denitrifiers related to macropore channels in agricultural and forest soils *Soil Biol. Biochem.* 31, 603–611.

VON LÜTZOW, M.; KÖGEL-KNABNER, I.; LUDWIG, B.; MATZNER, E.; FLESSA, H.; EKSCHMITT, K.; GUGGENBERGER, G.; MARSCHNER, B.; KALBITZ, K. 2008. Stabilization mechanisms of organic matter in four temperate soils: Development and application of a conceptual model. J. *Plant Nutr. Soil Sci.* 171, 111–124.

VOOS, G.; GROFFMAN, P.M. 1997A. Dissipation of 2,4-D and dicamba in a heterogeneous landscape. *Appl. Soil Ecol.* 5, 181–187.

VOOS, G.; GROFFMAN, P.M. 1997B. Relationships between microbial biomass and dissipation of 2,4-D and dicamba in soil. *Biol. Fert. Soils* 24, 106–110.

WAIS, A. 1998. Non-extractable Residues of Organic Xenobiotics in Soils - A Review, in: Fuhr, F., Ophoff, H. (Ed.), Pesticide Bound Residues in Soil. Wiley-VCH, Weinheim, pp. 5–17.

WALDMAN, M.; SHEVAH, Y. 1993. Biodegradation and leaching of pollutants: monitoring aspects. *Pure Appl. Chem.* 65, 1595–1603.

WEISS, M.; GEYER, R.; RUSSOW, R.; RICHNOW, H.H.; KÄSTNER, M. 2004. Fate and metabolism of [^{15}N]2,4,6-trinitrotoluene in soil. *Environ. Toxicol. Chem.* 23, 1852–1860.

WEISS, M.; GEYER, R.; GÜNTHER, T.; KÄSTNER, M. 2004. Fate and stability of ^{14}C-labeled 2,4,6-trinitrotoluene in contaminated soil following microbial bioremediation processes. *Environ. Toxicol. Chem.* 23, 2049–2060.

WHITE, J.L. 1976. Clay-pesticide interaction, in: Kaufmann, D.D., Still, G.G, Paulson, G.D., Bandal, S.K. (Ed.), Bound and Conjugated Pesticide Residues. ACS Symposium Series 29, pp. 208–218.

XIE, H.; GUETZLOFF, T.F.; RICE, J.A. 1997. Fractionation of pesticide residues bound to humin. *Soil Sci.* 162, 421–429.

XU, F.; BHANDARI, A. 2003A. Retention and distribution of 1-naphthol and naphthol polymerization products on surface soils. *J Environ. Eng.* 129, 1041–1050.

XU, F.; BHANDARI, A. 2003B. Retention and extractability of phenol, cresol, and dichlorophenol exposed to two surfaces soil in the presence of horseradish peroxidase. *J Agric. Food Chem.* 51, 183–188.

XU, J.; WU, L.; CHANG, A.C. 2009. Degradation and adsorption of selected pharmaceuticals and personal care products (PPCPs) in agricultural soils. *Chemosphere* 77, 1299–1305.

YALKOWSKY, S.H.; DANNENFELSER, R.M. 1992. Aquasol database of aqueous solubility. College of Pharmacy, University of Arizona, Tuscon, AZ.

YEE, D.; WEINBERGER, P.; KHAN, S.U. 1985. Release of of soil-bound prometryne residues under different soil pH and nitrogen fertilizer regimes. *Weed Sci*. 33, 882–887.

YOUNG, E. http://umbbd.msi.umn.edu/2,4-d/2,4-d_map.html.

YOUNG, J.M.; CRAWFORD, J.W. 2004. Interactions and self-organization in the soil-microbe complex. *Sci.* 304, 1634–1637.

ZANG, X.; VAN HEEMST, J.D.H; DRIA, K.J.; HATCHER, P.G. 2000. Encapsulation of protein in humic acid from a histosol as an explanation for the occurrence of organic nitrogen in soil and sediment *Org.Geochem.* 31, 679–695.

ZECH, W.; SENESI, N.; GUGGENBERGER, G.; KAISER, K.; LEHMANN, J.; MIANO, T.M.; MILTNER, A.; SCHROTH, G. 1997. Factors controlling humification and mineralization of soil organic matter in the tropics. *Geoderma* 79, 117–161.

ZELLES, L. 1997. Phospholipid fatty acid profiles in selected members of soil microbial communities. *Chemosphere* 35, 275–294.

ZELLES, L. 1999. Fatty acid patterns of phospholipids and lipopolysaccharides in the characterisation of microbial communities in soil: a review. *Biol. Fert. Soils* 29, 111–129.

ZIECHMANN, W. 1994. Humic Substances. BI Wiss Verlag, Mannheim, Wien, Zürich.

ZIELKE, R.C.; PINNAVAIA, T.J.; MORTLAND, M.M. 1989. Adsorption reactions of selected organic molecules on clay mineral surfaces, in: Sawhney, B.L., Brown, B.K. (Ed.), Reactions and Movement of Organic Chemicals in Soils. SSSA Special Publication 22, pp. 81–97.

ZORITA S; MÅRTENSSON, L.; MATHIASSON L. 2009. Occurrence and removal of pharmaceuticals in a municipal sewage treatment system in the south of Sweden. *Sci. Total Environ*. 407, 2760–2770.

ZWIENER, C.; SEEGER, S.; GLAUNER, T.; FRIMMEL, F.H. 2002. Metabolites from the biodegradation of pharmaceutical residues of ibuprofen in biofilm reactors and batch experiments. *Anal. Bioanal. Chem.* 372, 569–575.

ACKNOWLEDGEMENTS

This work has been carried out at the Department of Environmental Biotechnology of Helmholtz Centre for Environmental Research – UFZ, Leipzig, in the frame of RAISEBIO Marie Curie Early Stage Research Training. The European Commission is acknowledged for funding of the RAISEBIO Project (Contract: MEST-CT-2005-020984) under the Human Resources and Mobility Activity within the 6th Framework Programme.

My sincerest gratitude and greatest thanks to my supervisor Prof. Dr. Matthias Kästner for giving me the chance to carry out the research work within RAISEBIO project.at his Institute.

I would like to thank Prof. Dr. Andreas Schäffer for his willingness to be supervisor at RWTH Aachen University and for his strong interest in disussions concerning my PhD work.

Special thanks to Prof. Dr. Matthias Kästner and Dr. Anja Miltner for their encouragement, supervision and scientific support from the preliminary to the concluding level of my PhD project.

Many thanks to Dr. Herman Heipieper for his valuable scientific advices, Dr. Matthias Gehre and Ursula Günther for their great help in support in GC-C-irMS measurements and all instructions.

A special thank to Annette Schmidt, the manager of RAISEBIO project for her unconditional help on any social problems during the the project time and her willingness for discussion.

Thanks also to my colleague Cristobal Girardi for the cooperation in mass balances of organic contaminants experiments.

I would like to thank also all my colleagues from RAISEBIO project, from UBT and ISOBIO departments for very nice and warm atmosphere in workingplace, which is very important, especially during the bad moments in our life.

I would to express my gratitude to Prof. Hans Harms and Annemarie Harms, without them my scientific carrer would not have been started.

Finally, I would like to thank my family, in particular my mother, for her strong belief in me and her efforts in enabling me to live in the world of sounds. I thank also my sister and her husband for their sharing my time abroad.

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NOWAK, K.M.; MILTNER, A.; GEHRE, M.; SCHÄFFER, A; KÄSTNER, M. Formation and fate of bound residues from microbial biomass during 2,4-D degradation in soil. *Environ. Sci. Technol.* in press;

NOWAK, K.M.; GIRARDI, C.; MILTNER, A.; GEHRE, M.; SCHÄFFER, A.; KÄSTNER, M. Formation and fate of biogenic non-extractable residues during biodegradation of $^{13}C_6$-ibuprofen in soil", in preparation;

GIRARDI, C.; NOWAK, K.M.; LEWKOW, B.; MILTNER, A.; GEHRE, M.; KÄSTNER, M. Comparison of microbial degradation of C-isotope-labelled pharmaceutical ibuprofen and the herbicide 2,4-D in water and soil, submitted to *Environ. Pollut*.

Conferences & Proceedings:

NOWAK, K.M.; MILTNER, A.; SCHÄFFER, A.; KÄSTNER, M. Formation and fate of "non-extractable" residues from biomass and CO_2 during the biotic degradation of pollutant in soil. Oral presentation at SETAC Europe 20th Annual Meeting, Seville, Spain, 23–27.05.2010;

NOWAK, K.M.; MILTNER, A.; SCHÄFFER, A.; KÄSTNER, M. Formation and fate of "non-extractable" residues from biomass and CO_2 during the biotic degradation of pollutant in soil. Oral presentation at International Symposium Microbial contaminant degradation at biogeochemical interfaces, Leipzig, 2–4.03.2010;

NOWAK, K.M.; KÄSTNER, M.; MILTNER, A. Formation of "bound" residues from biomass during the biotic degradation of an herbicide in soil. Oral presentation at EGU, General Assembly, Vienna, Austria, 19–24.04.2009;

NOWAK, K.M.; KÄSTNER, M.; MILTNER, A. "Bound" residues from biomass and CO_2 in soils – formation, fate and stability during biotic incubation. Poster presentation at SETAC Europe 18th Annual Meeting, Warsaw, Poland, 25–29.05.2007;

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