Organic-Geochemical Investigations on the Mode of Incorporation of a Defined Nonylphenol Isomer and the Herbicide MCPA in Soil Derived Organo-Clay Complexes

Von der Fakultät für Georessourcen und Materialtechnik der
Rheinisch-Westfälischen Technischen Hochschule Aachen

zur Erlangung des akademischen Grades eines
Doktors der Naturwissenschaften

genehmigte Dissertation
vorgelegt von

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Tag der mündlichen Prüfung: 28. September 2011

In loving memory of my mother

Acknowledgments

This dissertation was conducted at the Institute of Geology and Geochemistry of Petroleum and Coal (LEK) at the RWTH Aachen University. This work was done in the context of the Priority Program SPP 1315 "Biogeochemical Interfaces in Soil" of the German Research Foundation (DFG). I am grateful to the DFG for financial support. Results of this thesis were presented at national and international scientific conferences e.g. in Frankfurt, Dresden, Aachen, Girona (Spain), Brisbane (Australia) and have been submitted to international scientific journals.

Though only my name appears on the cover of this dissertation, I could never have reached the heights or explored the depths without the help, support, and efforts of a lot of people. Foremost, I want to express my special gratitude to my supervisor Prof. Dr. Jan Schwarzbauer for giving me the opportunity to work in his research group and for many stimulating discussions guiding me through various scientific up´s and down´s resulting in the present dissertation. I thankfully appreciate the co-supervision of Prof. Dr. Andreas Schäffer and Dr. Burkhard Schmidt at the Institute for Environmental Research at the RWTH Aachen University. Their experiences, comments and constructive reviews were of inestimable value in answering more or less complex issues and improving the quality of the published results. I sincerely thank Prof. Dr. Ralf Littke as head of the LEK for introducing to me the wonderful world of geology. A very special thank you to my comrade-in-arms Timm Klausmeyer. Beyond our professional cooperation he had become a very good friend of mine. The present results based on the excellent work of this "charming" biologist. Helping me with all kinds of laboratory concerns, my gratitude is dedicated to Annette Schneiderwind, Yvonne Esser, Brigitte Thiede, and Hilde Patti. For all the people at both institutes who are dear to me in course of my stay in Aachen, representative I would like to thank Philipp Weniger. Albert Einstein once said: "Only the "stupid" need organization, the genius controls the chaos!". By performing her bachelor thesis within the framework of this study, Katharina Albert contributed considerable acknowledgement for their work with model organo-clay complexes. For supporting me in administrational affairs, I am very grateful to Olga Schefler, Dorothee Degen, Kristine Holz and hopefully she is enjoying her retirement Dorit Kanellis.

Last but not least my deep gratitude to my family, my lovely girlfriend Saskia Hähn and her family for supporting and encouraging me to proceed with this dissertation. Their love had brightened even the darkest days. Thank you!

Zusammenfassung

Im Laufe der letzten Jahrzehnte wuchs das wissenschaftliche Interesse, die Wechselwirkungen von organischen Kontaminanten mit natürlich vorkommendem organischem Material näher zu untersuchen und zu verstehen, speziell mit dem Hinblick auf Toxizität, Bioverfügbarkeit und Transport von Xenobiotika in der Umwelt. Zahlreiche Veröffentlichungen haben gezeigt, daß sowohl in Böden wie auch in Sedimenten hohe Anteile an anthropogenen Substanzen mit herkömmlichen Extraktionsverfahren nicht freisetzbar sind. Es wird daher vermutet, daß solche nicht-extrahierbaren Rückstände, bei denen es sich sowohl um die Ausgangssubstanz als auch um deren Transformationsprodukte handeln kann, in das organische Material bzw. Bestandteile von Böden und Sedimenten eingebunden sind. Die Einbindung sogenannter "gebundener Rückstände" ist dabei von verschiedensten Faktoren wie Systemcharakteristik (z.B. Bodenzusammensetzung, pH-Wert, Wassergehalt, aerob/anaerobe Bedingungen), der mikrobiellen Aktivität aber auch dem durchgeführten Extraktionsverfahren abhängig. Bisherige veröffentliche Arbeiten beschäftigten sich größtenteils mit extrahierbaren oder hydrolytisch freisetzbaren Kontaminanten, wobei es zum Verständnis des Einbindungsverhaltens unabdingbar ist, die genauen Bindungszustände der nicht-extrahierbaren Substanzen zu kennen. Mit Hinblick auf die hier durchgeführte Arbeit konnte vielfach gezeigt werden, daß gerade in Böden, Organo-Ton-Komplexe eine der wichtigsten Kompartimente für die Bildung nicht-extrahierbarer Rückstände darstellen, was sich aus der großen Oberfläche, dem hohen Anlagerungspotentials des organischen Materials sowie daraus resultierend, einer enormen Anzahl funktioneller Gruppen (potentielle Bindungsstellen) ergibt.
Um das Verständnis der Formierung nicht-extrahierbarer (gebundener) Rückstände zu erweitern, und damit das Remobilisierungspotential und Umweltverhalten niedermolekularer, polarer organischer Kontaminanten besser verstehen und erklären zu können, wurden verschiedene Experimente mit ^{13}C- und ^{14}C-markierten Verbindungen durchgeführt. Die beiden applizierten Substanzen wiesen dabei Unterschiede in ihren chemisch-biologischen Eigenschaften wie auch dem Abbauverhalten im Boden auf. Im ersten Versuch konnte gezeigt werden, daß sowohl 353-Nonylphenol (4-(3,5-dimethylhept-3-yl)phenol) als auch MCPA (2-Methyl-4-chlorphenoxyessigsäure) innerhalb kürzester Zeit (48 h) nicht-extrahierbare Rückstände bilden, besonders in der Tonfraktion. Eine Auftrennung der Organo-Ton-Komplexe in verschiedene Unterfraktionen mittels MIBK-Methode (HCl Fraktion, Humin-, Fulvinsäuren, gebundene Huminsäuren und gebundene Lipide) zeigte, daß sich Nonylphenol (NP) bevorzugt in Huminsäuren und MCPA in Fulvin-

säuren einlagerte. In den folgenden Versuchsansätzen wurde das Einbindungsverhalten beider Xenobiotika in Organo-Ton Komplexe näher untersucht. Die Anwendung eines sequentiellen chemischen Degradationsverfahrens (1. basische Hydrolyse, 2. BBr_3, 3. RuO_4 Oxidation, 4. TMAH Thermochemolyse) ermöglichte es, gezielt Bindungen zu spalten und damit Informationen über die Einbindungsart und daraus resultierend die Umweltrelevanz der nicht-extrahierbaren Rückstände zu erhalten. Im Falle des NP-Ansatzes, brachte die ^{13}C-Markierung eines Ringkohlenstoffs zusätzliche Informationen aus der Messung mittels nicht-invasiver ^{13}C-CP/MAS NMR. Im Verlauf der Inkubationszeit zeigte sich eine Einbindung des aromatischen Rings in die Huminsäuren, wobei der Ring nach 180 Tagen immer noch intakt vorlag und damit nicht vollständig abgebaut wurde. Komplementäre Untersuchungen durch invasive chemische Degradation der einzelnen Huminstofffraktionen (Humin-, Fulvinsäuren und Humin) und Identifizierung sowie Quantifizierung mittels GC-MS ergaben ausschließlich NP als nicht-extrahierbare Substanz. Darüber hinaus konnten Ester (Amid)-Bindungen als die vorherrschende Einbindungsart festgestellt werden, wohingegen Ether- und C-C-Bindungen kaum ein Rolle spielten. Im Verlauf der Inkubation kam es zu einem Rückgang chemisch freisetzbarer Rückstände, was auf Veränderungsprozesse (ageing) entweder der eingebundenen Substanz, der Huminstoffe oder der Einbindungsart schließen läßt. Im Falle des MCPA-Ansatzes wurden separat zwei unterschiedliche Konzentrationen des MCPAs auf Bodenproben appliziert. Die hohe Konzentration (1.000 mg MCPA/kg soil) führte zu einem erheblichen Rückgang der mikrobiellen Aktivität im Vergleich zur Kontrolle ohne Applikation und der niedrig dosierten Probe (8,5 mg MCPA/kg soil). Mit steigender mikrobieller Aktivität nahm die freisetzbare Menge an eingebundenen Rückständen ab was erkennen läßt, daß mikrobiell bedingt es zu einer Einbindungsart kommt, die mit den von uns durchgeführten Verfahren nicht erfaßt werden konnte. Im Vergleich zum NP-Ansatz war auch hier der höchste Anteil an freisetzbarer Radioaktivität nach der Spaltung von Ester (Amid)-Bindungen (basische Hydrolyse) zu finden. Weiterhin konnte ebenfalls nur die Ausgangssubstanz, MCPA, als nicht-extrahierbare Substanz identifiziert werden.
Zusammenfassend konnten wir zeigen, daß Ester (Amid)-Bindungen maßgeblich an der Bildung nicht-extrahierbarer NP- und MCPA-Rückstände beteiligt waren, wobei wir zwischen den einzelnen Huminstofffraktionen (Humin-, Fulvinsäuren und Humin) Unterschiede bzgl. der Einbindungsart feststellen konnten. Für beide Substanzen ist sowohl eine direkte kovalente Bindung mit funktionellen Gruppen innerhalb der Huminstoffe wie auch ein Einschluß der Rückstände in Zwischenräume des makromolekularen Materials denkbar. Hinsichtlich des Umweltverhaltens ist zu sagen, daß reversible Ester (Amid)-Bindungen relativ leicht unter natürlichen Bedingungen gespalten werden können (z.B. enzymatisch katalysierte Hydrolyse). Daraus ergibt sich ein potentielles Risiko der Remobilisierung und damit Relevanz für die Umwelt von sowohl den nicht-extrahierbaren Ausgansverbindungen als auch deren Transformationsprodukten.

Zusätzlich zu den bereits genannten Ergebnissen des NP-Ansatzes, zeigte sich in biotischen und abiotischen Versuchen eine stereoselektive Auftrennung der beiden NP-Diastereomere beim Übergang vom extrahierbaren in den nicht-extrahierbaren (gebundenen) Zustand. Neben bereits bekannten Prozessen wie z.B. stereoselektive mikrobielle Transformation, sind diese Ergebnisse der erste Hinweis einer stereoselektiven Bildung nicht-extrahierbarer Rückstände.

Abstract

During the last decades the scientific interest to understand the interactions of organic contaminates with naturally occurring organic material increased. Emphasis was laid on the toxicity, bioavailability and transport of xenobiotics within the environment. Numerous publications have shown, that high portions of anthropogenic substances could not be released by conventional extraction procedures from soils or sediments. It was assumed, that these non-extractable residues are incorporated in the organic matter or constituents of soils and sediments as both, the applied parent compound or a transformation product. Incorporation of the so called "bound residues" depends on several factors such as system characteristics (e.g. soil composition, pH-value, water content, aerob/anaerob conditions), microbial activity and even the performed extraction method. So far, publications had predominantly dealed with extractable and hydrolytically cleavable contaminants. However, for the understanding of the incorporation behavior, knowledge of the binding mode of non-extractable substances is required. In terms of the performed work, numerous authors revealed organo-clay complexes as one of the major compartments in soil responsible for the formation of non-extractable residues due to their huge surface area, the high potential of the organic matter to be adsorbed and the resulting enormous amount of functional groups (reacting sites).

To enhance the knowledge of the formation of non-extractable (bound) residues and to understand and explain the re-mobilization potential as well as the environmental behavior of low molecular weight, polar organic contaminates, distinct experiments were performed with ^{13}C and ^{14}C-labeled substances. Both applied compounds differed in their biochemical properties and degradation behavior in soil. The first approach showed, that both 353-Nonylphenol (4-(3,5-dimethylhept-3-yl)phenol) and MCPA (4-chloro-2-methylphenoxyacetic acid) rapidly (48 h) formed non-extractable residues, especially in the clay fraction. A separation of organo-clay complexes in distinct sub-fractions by means of the MIBK method (HCl fraction, humic acids, fulvic acids, bound humic acids, bound lipids) showed, that nonylphenol (NP) was predominantly incorporated into humic acids and MCPA into fulvic acids. In the following experiments, the mode of incorporation of both xenobiotics into organo-clay complexes was investigated in more detail. The performance of a sequential chemical degradation procedure (1. alkaline hydrolysis, 2. BBr_3, 3. RuO_4 oxidation, 4. TMAH thermochemolysis) facilitated the selective cleavage of chemical bonds and thus, revealed information on the mode of incorporation and the resulting relevance of non-extractable residues for the environment. In case of

the NP-approach, additional information were obtained from the ^{13}C-labeling of an aromatic ring carbon and the non-invasive ^{13}C-CP/MAS NMR measurements. In course of the experimental time, the aromatic ring was incorporated into humic acids and still intact after 180 days of incubation. Complementary investigations by means of an invasive chemical degradation of humic sub-fractions (humic acids, fulvic acids, and humin) and the identification and quantitation of liberated compounds by GC-MS, revealed only NP as non-extractable substance. Moreover, the preferred mode of incorporation was determined as ester (amide) linkages, whereas ether and C-C-bonds were only of minor importance for the incorporation. During the incubation period, portions of chemically releasable residues decreased, which pointed to ageing processes either of the incorporated compounds, the humic material or the incorporation mode.

In case of the MCPA-approach, two different concentrations of MCPA were separately applied to soil samples. The high concentration (1,000 mg MCPA/kg soil) led to a considerable decrease of the soil microbial activity as compared to the control samples without application and the low level assay (8.5 mg MCPA/kg soil). With increasing microbial activity, the amount of releasable non-extractable residues decreased, indicating a microbially induced mode of incorporation which could not be traced by the executed degradation methods. Equal to the NP-approach, the highest amount of radioactivity was liberated after cleavage of ester (amide) bonds (alkaline hydrolysis) and only the parent compound, MCPA, was found as non-extractable substance.

In summary, we showed that ester (amide) bonds were of significant importance in the formation of non-extractable NP and MCPA residues. However, among the distinct humic sub-fractions (humic acids, fulvic acids, and humin), radioactive balancing indicated different modes of incorporation. It could be assumed that both xenobiotics were on the one hand directly linked to functional groups of the humic matter via covalent bonds or on the other hand sequestrated (entrapped) in cavities of the macromolecular structure. In terms of the behavior in the environment, reversible ester (amide) bonds can be cleaved rather easily under natural conditions (e.g. enzymatically catalyzed hydrolysis) resulting in a potential risk to be re-mobilized and thus both the non-extractable parent compound and their transformation products are still of great importance for the environment.

Additionally to the results of the NP-approach, incubation experiments under biotic and abiotic conditions revealed a stereoselective separation of both NP-diastereomers when shifting from the extractable to the non-extractable (bound) state. Besides already know stereoselective processes e.g. microbial transformation, this is the first evidence of such a process induced by the formation of non-extractable residues.

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Abbreviations

APEO	Alkylphenol polyethoxylate
BHA	Bound humic acids
BL	Bound lipids
BBr_3	Boron tribromide
CP/MAS-NMR	Cross-polarization/magic angle spinning-nuclear magnetic resonance spectroscopy
ESR	Electron spin resonance spectroscopy
EU	European Union
FA	Fulvic acid
FT-IR	Fourier transform infrared spectroscopy
GC-MS	Gas chromatography mass spectrometry
HA	Humic acid
HPLC	High performance liquid chromatography
HS	Humic substance
HU	Humin
IUPAC	International Union of Pure and Applied Chemistry
LOD / LOQ	Limit of detection / quantification
LSC	Liquid scintillation counting
MCP	4-Chloro-2-methylphenol
MCPA	2-Methyl-4-chlorophenoxyacetic acid
MIBK	Methyl isobutyl ketone
NER	Non-extractable residues
NP	Nonylphenol
NPEO	Nonylphenol polyethoxylate
REACH	Registration, evaluation, authorization, and restriction of chemicals (European Union)
RuO_4	Ruthenium tetroxide
SPM	Simultaneous pyrolysis methylation
TMAH	Tetramethylammonium hydroxide
TOC	Total organic carbon
US EPA	United States Environmental Protection Agency
WHC	Water holding capacity of soil
WHO	World Health Organization
XRD	X-ray diffractometry

1 Introduction

1.1 Xenobiotics in the Environment

The production of xenobiotics is closely related to a raising industrialization, increasing living standards, growing world population and higher demand of (renewable) energy. Figure 1.1 shows the production of chemicals in the European Union over the last eight years. More than half of the produced compounds were classified in the range of harmful to cancerogenic, mutagenic and reprotoxic [EU, 2010]. Major industries responsible for a high emission of anthropogenic substances are chemical industries, mineral oil processing, iron- and steel production, battery producers or paper producing industries [Heinrich and Hergt, 2002]. Additionally, environmental relevant compounds are produced in power plants and motor vehicles. In the fifties and sixties, people became aware of the fact, that chemicals released into the environment could cause harmful effects. For instance, organochlorine insecticides (e.g. DDT, pentachlorophenol) used in agriculture led to a decrease of several wild living animals. Since that time, xenobiotics have been intensely investigated in terms of their mode of action, their toxicity, the behavior in the environment and their risks resulting from the production and application [Parlar and Angerhöfer, 1995]. Nowadays, numerous organizations (e.g. WHO, REACH (EU), EPA (USA)) all over the world monitoring the complying of legal guidelines and regulation with respect to risks of xenobiotics for humans, animals and the environment. Despite these high regulation standards, ongoing fundamental research will still be required to gain profound knowledge of the behavior and fate of anthropogenic compounds.

A xenobiotic (Greek, xenos "foreign"; bios "life") is a compound that is foreign to a living organism. Principal xenobiotics include drugs, carcinogens, and various compounds that have been introduced into the environment by artificial means (IUPAC). Xenobiotics could also be of natural origin. For example, plants synthesize and release a large number of "secondary compounds". These products are used to defend the plant from some counter animal predation; some protect against microbial pathogens; and others suppress competition from rival plant species [Coleman et al., 1997].
Each dumping of material either by natural ways or by human activities, leads to a change of the substantial environmental quality. Whether this change shows desired or undesired effects depends on the structure, behavior and fate of the released compounds. To estimate and predict local and global changes and to evaluate the environmental

relevance, the following aspects have to be taken into account [Parlar and Angerhöfer, 1995]:

1. Amount of produced substance
2. Usage patterns
3. Tendency of dispersion
4. Persistence
5. Impact to organisms and systems (toxicity and eco-toxicity)
6. Transformation under environmental conditions

The present work addresses the above mentioned aspects 3-6. Two xenobiotics, a defined nonylphenol isomer and the herbicide MCPA, were applied to natural soil samples and incubated up to 180 or 120 days. Fate (metabolism) and incorporation behavior of the compounds were investigated with a closer look at the formation of non-extractable (bound) residues within organo-clay complexes.

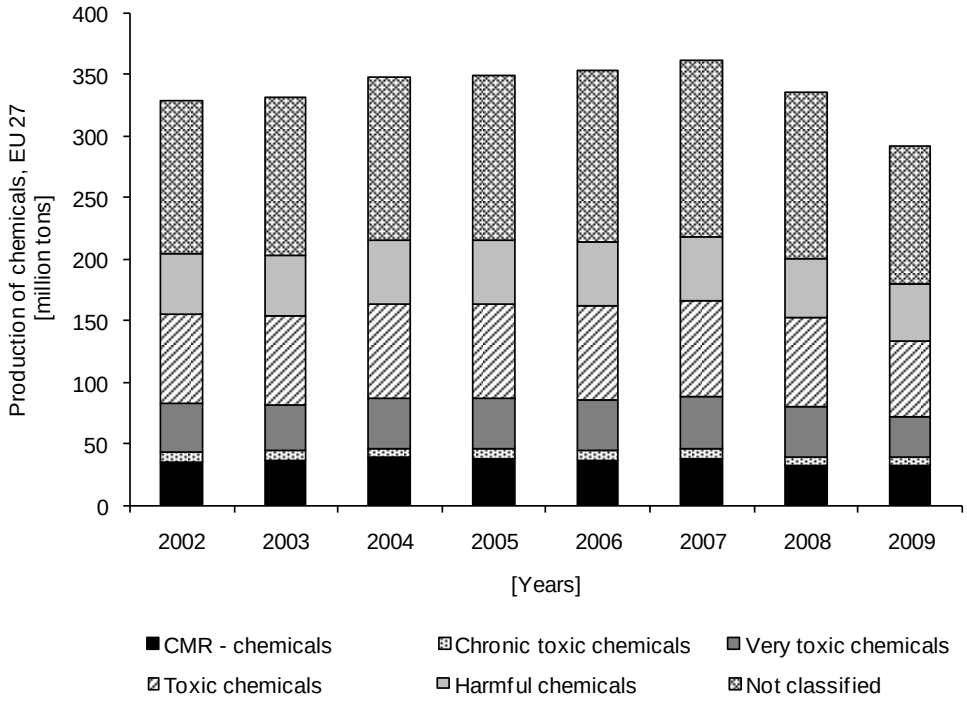

Figure 1.1: Aggregated volumes of toxic chemicals produced in the 27 countries of the European Union, divided into toxicity and not classified classes. The most dangerous ones are the CMR chemicals (carcinogenic, mutagenic and reprotoxic), followed by chronic toxic, very toxic, toxic and harmful chemicals [EU, 2010].

1.2 Non-extractable (bound) residues (NER)

Non-extractable (bound) residues in plants, soils and animals are defined as chemical species (parent substance, active ingredients, metabolites) originating from xenobiotics that are unextracted by methods which do not significantly change the chemical nature of these residues or the structure of the matrix. These non-extractable residues are considered to exclude fragments recycled through metabolic pathways leading to naturally occurring products [Roberts, 1984, Fuehr et al., 1998]. The term "bound" is, to a large extent, defined by the extraction method used [Bollag and Loll, 1983]. The American Institute of Biological Sciences (AIBS) - Environmental Task Group stated the first definition in 1975: "Bound pesticide residues in the soil are unextractable and chemically unidentifiable pesticide residues remaining in the fulvic acids, humic acids and humin fractions after exhaustive sequential extraction with nonpolar organic and polar solvents". "Bound" in connection with xenobiotic residues was first mentioned in literature by Bailey and White [Bailey and White, 1964]. Before 1962, both analytical methodology and knowledge about the metabolism of many xenobiotics were limited. Besides the analysis of the applied parent compound, only easily extractable residues were investigated. Compounds which could not be detected were classified as "persistent", "disappeared", "lost" or "volatilized". The introduction of radiolabeling (^{14}C) made it possible to obtain a "balance" and to account for the fate of the applied xenobiotics. Treatment of the extracted soils by combustion or strong hydrolysis revealed, that non-extractable or bound residues could be released and detected but with the difficulties of possible altering the chemical structure. Because of formerly "unseen", non-extracted residues, the concept of persistence or instability of pesticides had to be reconsidered from scratch [Katan et al., 1976]. Now and then in common is that bound residues are thought to be reduced in their mobility, bioavailability and toxicity [Helling and Krivonak, 1978, Senesi, 1992]. Furthermore, it is assumed that the formation of bound residues is affected by microorganisms and that humic substances play a major role as reaction partners [Wais, 1996]. Khan discussed potential risk in terms of bound residues [Khan, 1982a, Wais, 1996]:

1. Type and/or identity of the bound residues are not sufficiently established.
2. Little is known about the significance of bound residues pertaining to bioavailability, toxicity and accumulation.
3. Since bound residues cannot be detected by conventional analytical methods, an assessment of the impact on plants and soils is difficult.
4. Nothing is known about the environmental behavior of bound residues.

Because of the already existing difficulties in the research of the risks and the associated lack of knowledge, methodologies presented in this work may help partly answer some of the mentioned aspects. Table 1.1 summarizes the most important parameters influencing degradation and transport of xenobiotics [Wais, 1996].

Table 1.1: Parameters influencing degradation and transport of xenobiotics [Wais, 1996].

Climate parameters	Soil parameters	Biological parameters
temperature	texture	microbial biomass
humidity	humic substances	biological diversity
aeration	clay minerals	plant cover
	pH-value	
	micro nutrients	

The formation of non-extractable residues (NER) can be explained as a sequence of three steps:

(1) Rapid ("flash") formation $\longmapsto$ (2) Formation $\longmapsto$ (3) Maturation

The first (flash) step depends on the extractability of compounds at the beginning of the soil incubation (usually 24 h after application). Difficulties of the solvent to compete with the soil–pesticide interactions or to access hidden sites in organo–mineral colloids protecting diffusing xenobiotics. The second step leads to the formation of NER. It is characterized by its kinetics rate. High portions of NER are usually generated if the rate is high, resulting in a quickly reached plateau. The maturation steps correspond to the fate of NER when their formation rate decreases. Roughly, three situations can be found: (a) a "plateau" is reached and the NER portion remains "stable" during time; (b) the NER formation carries on at a lower rate indication continuous "incorporation" of new residues in the NER pool; or (c) the NER proportion decreases with a "release" rate. It should be noted that the shape of kinetic curves is influenced also by the type of soil, incubation conditions and the time range [Barriuso et al., 2008].

1.3 Incorporation of xenobiotics into soil organic matter

An incorporation of xenobiotics into soil is closely related to the chemical structure of the compound, the type of soil and humic matter as well as the transformation by microorganisms and abiotic factors. In some ways produced metabolites are more likely to bind to soil organic matter than the parent chemicals. Microorganism could on the one hand catalyze the incorporation by producing extracellular enzymes for example, or on the other hand influence the incorporation by changing the physical and chemical environment (e.g. pH-value, redox conditions, oxygen content) of soil through their metabolic activities. Besides these biotic factors, clay minerals and metal-ions could show catalytic properties leading also to the formation of non-extractable residues [Bollag and Loll, 1983].

1.3.1 Soil organic matter

A key role in the incorporation processes of xenobiotics plays the organic matter. It refers to the whole of the organic material in soils, including litter, light fraction (i.e. plant residues in varies stages of decomposition), microbial biomass, water-soluble organics, and stabilized organic matter (humus). In most agricultural soils, predominate organic matter occurs as stable humus [Stevenson, 1994]. Humic material is classically divided into three fractions by first separating the organic matter from the inorganic matrix using 0.1 to 0.5 N NaOH and secondly changing the pH-value of the solution:

1. *Fulvic acids* are soluble in acid and base.
2. *Humic acids* are soluble in dilute alkali and precipitates upon acidification of the alkaline extract.
3. *Humin* is insoluble in both acid and dilute alkali.

The humin fraction as mentioned in the following chapters consists of insoluble organic material and inorganic matrix (e.g. clay minerals). To investigate the humin fraction in more detail, Rice and MacCarthy introduced the MIBK method in 1990. Before that time, researchers digested the inorganic matrix with a mixture of concentrated hydrofluoric and hydrochloric acid. Both the inorganic and the organic fraction was thought to be degraded by this abrasive method [Rice and Maccarthy, 1990]. Besides the mentioned methods, which all could alter the organic material, milder but less efficient extractants have been used with variable success. Some of them are neutral salts ($Na_4P_2O_7$), anhydrous formic acid, organic chelating agents (e.g. acetylacetone, 8-hydroxychinoline) or dilute mineral acids [Stevenson, 1994].

Regarding the structure of the classical humic fractions, they are thought to be large aromatic polymers which occur as micelles in the nature. The polymers are made up of nitrogen heterocycles, quinones, phenols, and benzoic acids (Figure 1.2). They contain carboxyl, hydroxyl, carbonyl, and thiol groups which may act as binding sites for carbohydrates, amino acids and xenobiotics. Humic fractions also have aliphatic moieties, some of which are hydrophobic [Bollag and Loll, 1983]. Humic fractions differ from each other in their constitution [Stevenson, 1994, Bollag and Loll, 1983]:

1. *Fulvic acids*: more oxygen than humic acids but less carbon; most of the oxygen in functional groups (e.g. carbonyl); more carboxyl groups (more acidic) than humic acids; molecular weight ranges from 270–2100 Dalton.
2. *Humic acids*: a large portion of oxygen is found in the "core" structure in ether and ester bonds; molecular weight ranges from 1400–1000000 Dalton.
3. *Humin*: lowest portion of oxygen and exchangeable acidity and highest of carbon as compared to fulvic and humic acids.

For the formation of humic material (humification) four hypotheses have been stated [Bollag and Loll, 1983]:

1. *Plant alteration hypothesis*: Plant material like lignin and others undergo only

slight changes and form high molecular weight humic acids.

2. *Microbial synthesis hypothesis*: Bacteria and fungi synthesize intracellular high molecular weight humic polymers during metabolism to derive energy from plants. After lysis, these polymers are released into soil.
3. *Chemical polymerization hypophesis*: Monomers (e.g. amino compounds, phenol) are produced by soil microbes intracellular and transfered into soil where these monomers polymerize.
4. *Cell autolysis hypothesis*: Both plants and microbes contribute to humification. After autolysis monomers (e.g. sugars, phenols, amino acids) polymerize in soil.

One of the most important mechanisms for the humification process is oxidative coupling. It is known, that biogenic (microorganisms) as well as abiotic (clay minerals) processes could lead to the formation of polymers out of phenols, anilines or amino acids [Bollag and Loll, 1983]. However, despite a comprehensive research of the structure and formation of humic matter over the last decades, the complexity and heterogeneity complicated the investigations, and thus up to know only little is known about the above mentioned facts.

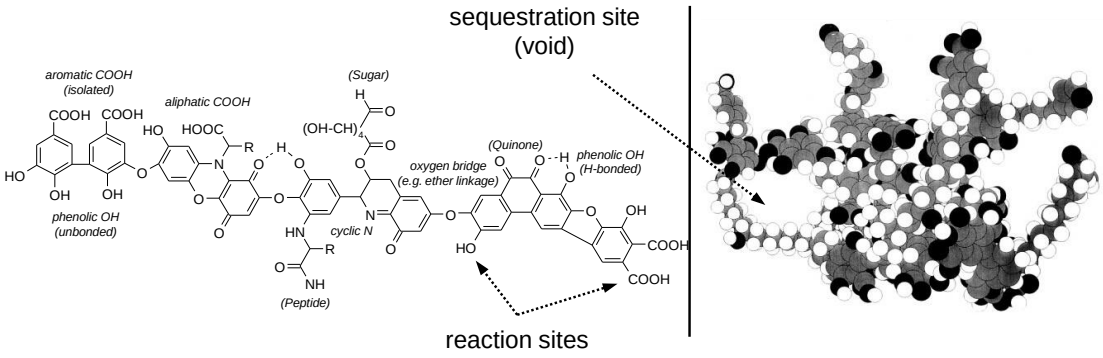

Figure 1.2: Chemical structure of humic acid. Left: hypothetical structure consisting of several sub-units (e.g. phenols, sugars, peptides, quinones). Phenolic and carboxylic groups act as reaction sites for chemical linkage (e.g. ester, ether) [Stevenson, 1994]; right: 3D model of a humic acid according to Schulten and Schnitzer. White: hydrogen; grey: carbon; black: oxygen (nitrogen) [Schulten and Schnitzer, 1995].

1.3.2 Incorporation mechanism

Incorporation of xenobiotics and their metabolites occurs predominantly via reactions with functional groups of soil organic matter. Besides adsorption and sequestration processes, incorporation ranges from reversible (e.g. van-der-Waals forces) to irreversible (e.g. covalent linkages) depending on the properties of both humic material and xenobiotics. This includes size, shape, configuration, molecular structure, chemical functions, solubility, polarity, polarizability and charge distribution of interacting species, as well

as acid and basic character of the residues. Under natural conditions, types of bindings may rarely appear separately. It is more likely, that xenobiotics are incorporated via simultaneously occurring mechanisms [Senesi, 1992]. Nevertheless, Figure 1.3 shows, that bioavailability of xenobiotics and their metabolites decreases due to an incorporation into humic substances. Depending on the strength and mode of linkage, not only bioaccessiblity could be reduced but also an irreversible incorporation may occur. In the following, most of the conceivable interactions are discussed in detail according to Senesi and Wais [Senesi, 1992, Wais, 1996].

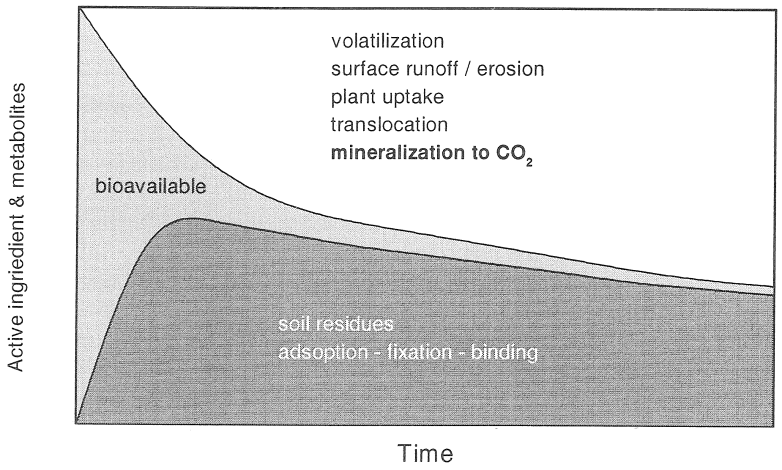

Figure 1.3: Fate of residues in soil in the course of time [Wais, 1996].

Ionic bonds (Ion exchange)

Ionic bonds occur with xenobiotics or metabolites only if these compounds exist in cationic form or act as proton acceptors (e.g. amines). Atrazine for instance, can react directly via the cationic amino groups with anionic moieties of the humic matter (e.g. carboxylates, phenolates). In some cases, bonded compounds could only be extracted with strong bases, whereas commonly used solvent extraction methods are ineffective. The strength of ionic linkages is in a similar range as covalent bonds and thus, under optimum conditions (e.g. pH-value, steric conformation of the compound and humic matter) ionic bonds could be a strong fixation of xenobiotics. However, this type of linkage is closely related to the pH-value of the soil.

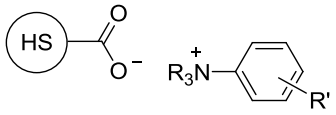

Figure 1.4: Incorporation of xenobiotics into humic substances (HS) via ionic bonds.

Hydrogen bonds

The presence of a large number of oxygen- and nitrogen-containing functional groups on humic substances could lead to the formation of hydrogen bonds with compounds containing suitable complementary groups. In this context, residues have to compete with already bonded ligands, e.g. water molecules. Acidic and anionic xenobiotics (e.g. chlorophenoxyalkanoic acids) can be linked via hydrogen bonds if the pH-value is below their pK_a. The so existing non-ionized form could interact with carboxyl, carbonyl, hydroxyl or similar groups of the humic matter.

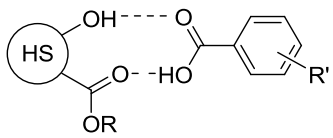

Figure 1.5: Incorporation of xenobiotics into humic substances (HS) via hydrogen bonds.

Charge-transfer complexes

Charge-transfer or electron-donor-electron-acceptor complexes are characterized by electronic transitions to an excited state in which there is a partial transfer of electronic charge from the donor (high electronic density, π-electrons e.g. aromatic systems) to the acceptor moieties (e.g. quinones) (IUPAC). The electrostatic attraction between both species results in a stabilization of these complexes. Investigations of charge-transfer complexes can be done by IR spectroscopy due to a shift of the absorption band of C-H-"out-of-plane" oscillation of the aromatic compounds or by ESR spectroscopy due to an increase of the amount of free radicals in humic acids.

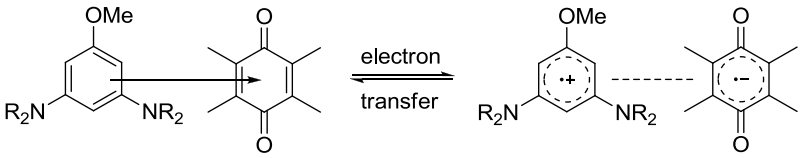

Figure 1.6: Interaction of xenobiotics (electron-donor) with humic quinones (electron-acceptor) via charge-transfer complex.

Covalent bonds

Covalent bonds between xenobiotics, their metabolites and humic matter are induced by chemicals, photochemicals or enzymatic catalysts. Due to strong bond forces ($\rangle$ 300 kJ/mol) compounds are incorporated in a stable way. Among the several types of covalent bonds, one can distinguish between reversible and irreversible incorporation

processes. These processes have a great impact on the bioaccessiblity of NER. Ester bonds are comparatively weak and reversible covalent bonds which can be cleaved easily in nature and thus, a remobilization of compounds could be possible. In contrast, *C*-*C* bonds are hardly cleavable. Residues incorporated via that type of bond are irreversible linked and thus, no longer accessible.
Functional groups (e.g. phenolic, carboxylic moieties) of the humic matter have a distinct influence on the formation of covalent bonds. Due to an integration of xenobiotics into humic substances, they are subjected to further transformation processes involved in humification. Besides the classical bond formation (e.g. ester, ether, *C*-*C*), it was stated that cross-coupling reactions between free xenobiotic radicals and humic acid radical structures could also lead to the formation of bound residues.

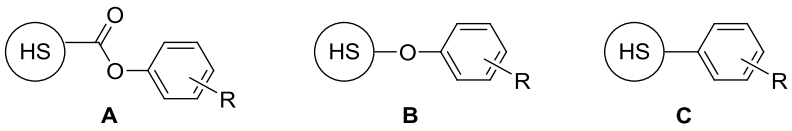

Figure 1.7: Incorporation of xenobiotics into humic substances (HS) via covalent bonds: (A) ester bond, (B) ether bond, (C) *C*-*C* bond.

Other types of bonds

Additionally to the presented incorporation mechanisms, there are still other types of linkages which should be noted briefly. Ligand exchange occurs if water molecules associated with metal ions complexed by humic acids are substituted by xenobiotic molecules. Van-der-Waals forces are relatively weak as compared to chemical bonds. These interactions arise due to short-range dipolar or induced-dipolar attractions. While van-der-Waals forces only play a minor role in the formation of bond residues, their influence is more likely during adsorption processes of xenobiotics to soil components. Hydrophobic sorption could be assumed as an interaction of non-polar xenobiotics with hydophobic groups (e.g. aliphatic side chains, lignin components) of humic substances.

Besides the above mentioned incorporation processes where non-extractable residues are regarded as xenobiotic residues, soil biota could degrade xenobiotics to small fragments or CO_2 [Roberts, 1984]. Both may be assimilated by microorganisms (biomass) or incorporated into humic matter in course of humification (Figure 1.8).

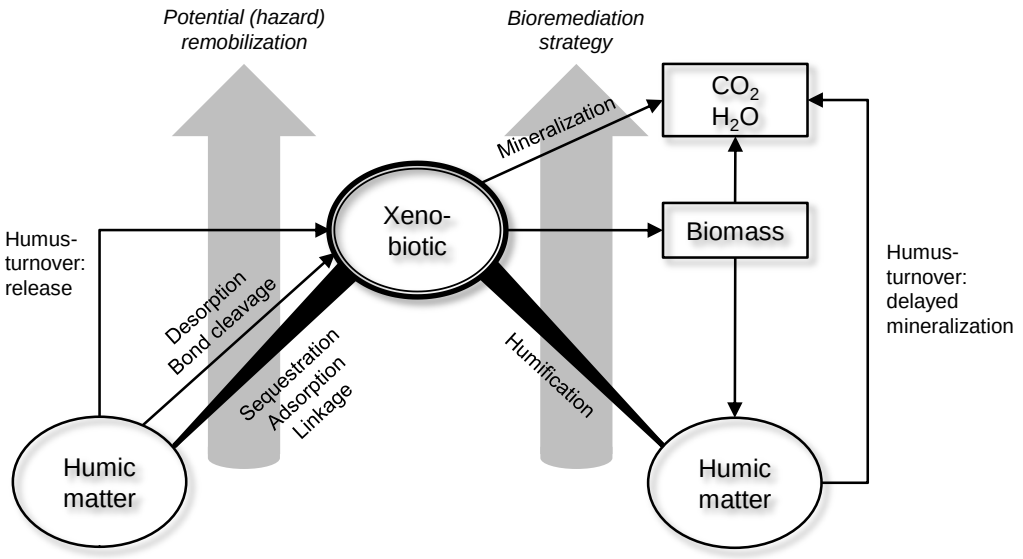

Figure 1.8: Long term behavior of NER in soil, adapted from Hartlieb *et al.* [Hartlieb et al., 2003].

1.4 Methodologies for the characterization of NER

Processes leading to the formation of non-extractable residues are highly complex and there is still a lack of deep going knowledge about the interactions of xenobiotics to organic substances. Much of the uncertainty in terms of the elucidation of bound residue formation arises from our poor understanding of the structure of soil organic matter. To assess the fate and risk of non-extractable residues, it is indispensable to understand the formation processes in detail. Methods executed to characterize these residues are presented in the following chapters [Northcott and Jones, 2000].

1.4.1 Non-invasive methods

Most of the used non-invasive techniques are spectroscopic methods. In recent years, immunochemical analysis was developed as another non-destructive method. Within this approach, distinct antibodies selectively bind to a restricted part of an antigen. These antigens are the non-extractable residues derived from the applied xenobiotics. Antibodies could be fluorescent labeled and thus, after antigen-antibody reaction, bound residues could be determined by means of optical methods (e.g. fluorescence microscopy). Immunochemical analysis may obtain information on the occurrence, distribution and binding of the residues [Dankwardt and Hock, 2001].

NMR spectroscopy

With the development of superconducting magnets, Fourier transform (FT) techniques for data acquisition and manipulation as well as CPMAS (Cross-polarization Magic-Angle Spinning) methods, NMR became a routine application to samples which are structurally complex, heterogeneous, very diluted, solids or intermediate states such as colloids or gels. The implementation of less sensitive or less abundant nuclei (e.g. ^{13}C, ^{15}N) have further enhanced the potential of NMR spectroscopy. NMR in soil science was used for the investigation of the structure of organic material, incorporation processes of residues into humic substances, decomposition processes and the characterization of biopolymers [Preston, 1996]. Development of CPMAS-NMR allowed researchers to investigate samples in solid state. With the so obtained spectra, it was possible to distinguish between different types of functional groups. For instance, Kögel-Knabner *et al.* showed increasing rigidity in the alkyl chains with depth in the forest floor [Koegel-Knabner et al., 1992].
Because of the low sensitivity, NMR is unlike to replace analytical techniques such as GC or HPLC. Nevertheless, the method could be used to understand the behavior of xenobiotics in soil or under special conditions where conventional separation or detection techniques do not work well [Preston, 1996]. For example, Dec *et al.* used NMR spectroscopy after executing a silylation procedure to characterize bound and sequestered residues of cyprodinil in soil [Dec et al., 1997b].

Other spectroscopic methods

Other methods include FT-IR (Fourier-Transform Infrared), fluorescence and ESR (Electron Spin Resonance) spectroscopy. ESR spectroscopy is a sensitive method for detecting paramagnetic species and free radicals in samples. It is useful to study charge-transfer complexes but rather limited in the application of bound residue characterization.
Fluorescence spectroscopy could be used to determine equilibrium constants for the association or fluorescing hydrophobic organic compounds with dissolved organic matter. Advantages are, that partition coefficients could be measured *in situ* and that the obtained results could be extrapolated to non-polar compounds with similar chemical properties. Limitations are the availability of fluorescing compounds and the fact, that fluorescence is completely quenched upon association of the compounds to dissolved organic matter.
FT-IR is a powerful technique to identify and determine the structure of organic, inorganic and biochemical species. Almost all molecular species are able to absorb IR radiation. The obtained complex spectra lead to an unequivocal characterization of the measured compounds. By means of FT-IR spectroscopy binding mechanisms could be investigated. Shifts in specific functional group IR adsorption bands (e.g. hydrogen bonding or proton transfer ability) can be measured and compared to pure and artificial conjugated compounds to provide information on specific functional group interactions

with sorbing solids [Northcott and Jones, 2000]. Martin-Neto *et al.* studied the mechanism of atrazine sorption to humic acids. They found that at a pH ‹ 4 soption occured via hydrogen bonds or proton-transfer processes [Martin-Neto et al., 1994].

1.4.2 Invasive methods

In contrast to the above mentioned methods, samples are destroyed and could not be used further after executing invasive techniques. However, the information content of the obtained results is usually much higher as compared to non-invasive methods. Destructive methods are used in geoscience as well as soil science to investigate the structure of organic macromolecules or to elucidate the incorporation behavior and structure of non-extractable xenobiotics and their metabolites.

Chemical degradation

The term "chemical degradation" includes numerous chemical reactions which are used to cleave different kinds of linkages and thus, released compounds could be investigated in detail. Due to possible altering effects of the interested substances as a consequence of chemical treatment, interpretation of the obtained results could be difficult and in many cases, conclusions on the origin sample could only be drawn indirectly from reaction artifacts.
Schaeffer-Reis *et al.* investigated a sulfur rich kerogen derived from Italy [Schaeffer-Reiss et al., 1998]. The kerogen was treated sequentially by a saponification, desulfurization and oxidation step. 80% of the kerogen has been converted to soluble organic matter, whereas onyl 1% was GC-amenable. Sulfur rich kerogen and macromolecular oil fractions were also investigated by Richnow *et al.* [Richnow et al., 1992]. Sequential chemical degradation (Ni(0)cene/$LiAlD_4$; BCl_3/$LiAlH_4$; RuO_4) led to a cleavage of S- and O-bonds and a degradation of aromatic moieties. The determined sulphur positions in the macromolecule suggested an early diagenetic sulphur incorporation into the biological precursor compounds which led to the formation of a cross-linked network. Investigations of the structure of soil humin fractions was done by Almendros *et al.* [Almendros and González-Vila, 1987]. By means of sequential performed oxidation steps ($K_2S_2O_8$, $KMnO_4$) they could distiguish between loosely and strongly associated compounds. Metabolites of xenobiotics (PAHs, PCBs) linked to macromolecular organic matter (soil and sediment) were investigated by Richnow *et al.* [Richnow et al., 1993]. The authors used NaOH, BCl_3 and Rh/C as chemicals for the sequential degradation process. Hydrolysis reactions with $Na^{18}OH$ showed, that some metabolites formed stable ester bonds via condensation processes with functional groups of humic substances. Schwarzbauer *et al.* investigated the incorporation processes of DDT in particulate matter of the Teltow Canal (Germany) [Schwarzbauer et al., 2003]. Results of the sequential degradation (KOH, BBr_3, RuO_4,

pyrolysis) implied a weak association to the non-extractable particular matter for the observed DDT-related contaminants, which could be released due to modification and degradation of the organic macromolecular matrix.

Pyrolysis and TMAH-Thermochemolysis

Pyrolysis and TMAH-thermochemolysis were multiple utilized for the characterization of polymeric compounds (e.g. kerogen, humic substances, coals, proteins, polysaccharides, plastics, synthetic resins). On the one hand, the analytical method can provide important clues for understanding the chemical structure of complex macromolecules. On the other hand, pyrolysis can cause drastic modifications of the original building blocks which may lead to incorrect conclusions on the structure.
Additionally to the established pyrolysis techniques (e.g. Curie-point, flash pyrolysis), pyrolysis in the presence of tetraalkylammonium hydroxide (TMAH) has been developed as alternative pyrolytical method. This procedures termed as "thermally assisted hydrolysis and methylation" (THM) or "TMAH thermochemolysis" (formely "simultaneous pyrolysis methylation" (SPM)) provide complementary information to those obtained by conventional pyrolysis. It is suggested, that previously stated structural models of e.g. humic compounds, must be revised to conform with the new insight offered by pyrolysis/methylation.
However, analytical pyrolysis is a well-established method for the chemical structure characterization of intractable materials [Challinor, 2001, Saiz-Jimenez, 1994].

1.4.3 Overview of the applied degradation methods

In this study, degradation of contaminated humic sub-fractions was executed sequentially. Methods closely follow those published by Richnow *et al.*, Schwarzbauer *et al.* and Kronimus *et al.* [Richnow et al., 1993, Schwarzbauer et al., 2003, Kronimus and Schwarzbauer, 2007].

Alkaline hydrolysis

Alkaline hydrolysis facilitate cleavage of ester and amide bonds. Reaction products in case of esters are carboxylates and alcohols, and in case of amides, carboxylates and amines.

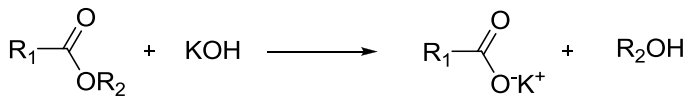

Figure 1.9: Chemical degradation procedure: Alkaline hydrolysis.

BBr_3-treatment

Boron tribromide as Lewis acid cleaves via a so called "electron pair donor-acceptor complex" ether bonds, resulting in the formation of bromides and alcohols. Furthermore, ester bonds are affected by BBr_3 as well, resulting in cleavage to alcohols and carboxylic acids [Bhatt and Kulkarni, 1983]. As the by-product of ether cleavage (organic bromides) could be identified, it is possible to distinguish between ether and ester incorporation. In this study, ester cleavage via alkaline hydrolysis was executed prior BBr_3-treatment and thus, ester cleavage by means of BBr_3 could be precluded.

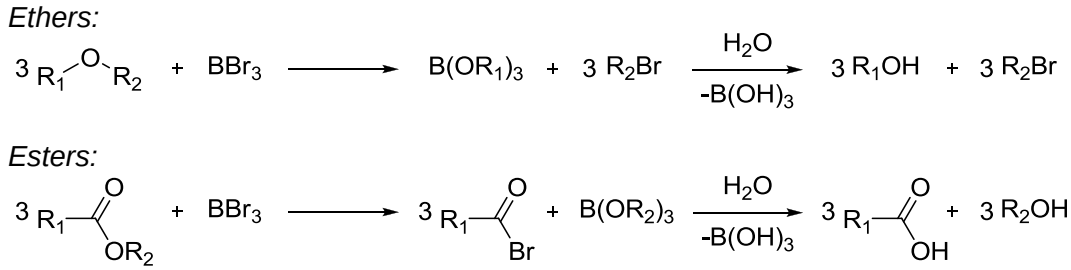

Figure 1.10: Chemical degradation procedure: BBr_3-treatment.

RuO_4 oxidation

Ruthenium tetroxide (RuO_4) as degradation method has been used for the characterization of aliphatic and alicyclic portions of coals, kerogen, humic substances and the macromolecular structure of meteorites [Stock and Tse, 1983, Boucher et al., 1991, Gonzales-Vila et al., 1994, Remusat et al., 2005]. RuO_4 is a mild and selective oxidizing reagent, destroys aromatic rings and convert them into CO_2. Depending on the attached functional groups, conversation is slow, or absent, when efficient electron withdrawing groups are present; it is very quick when aromatic rings are activated by electron-donating groups. Moreover, RuO_4 converts oxidatively alcohols into ketones or aldehydes, aldehydes into acids, and ethers into esters or lactones [Mallya and Zingaro, 1984, Ilsley and Zingaro, 1986]. All in all, RuO_4 oxidizes aromatic rings and functionalized carbon atoms with a high specificity.

Aliphatic and acyclic products released, appear as carboxylic acids. The carboxylic functional groups indicating points of attachment in the organic macromolecule and positions of labile functional groups, such as carbon-carbon double bonds and ether links [Stock and Tse, 1983]. An aliphatic or acyclic moiety linked to an aromatic ring will thus yield a compound with one more carbon bearing the carboxylic functional group [Stock and Tse, 1983]. RuO_4 is only required in a catalytic amount, because once reduced by the organic matter, it may be converted back to the active form by sodium periodate (co-oxidant) [Sharpless et al., 1981].

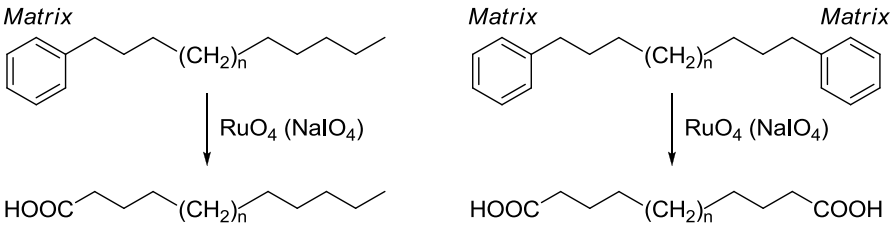

Figure 1.11: Chemical degradation procedure: RuO_4 oxidation; formation of carboxylic acids by ruthemium tetroxide oxidation of matrix derived aromatic compounds [Richnow et al., 1992].

TMAH thermochemolysis

TMAH thermochemolysis combines both, efficient thermochemolytical cleavage of macromolecular structures and subsequent methylation of carboxylic acids and hydroxy moieties, into GC-amenable (less polar) compounds (e.g. methyl esters and methyl ethers). It has been demonstrated that pyrolysis in the presence of TMAH does not only involve the *in situ* methylation of volatile pyrolysis products, but is also a high temperature saponification/transesterification reaction, via intermediate TMAH salts. Thermochemolysis, was proven to be effective at sub-pyrolysis temperatures of 250-310°C, and thus this method could be executed as fast, sensitive, one-step procedure for the analysis of geomacromolecules containing functional groups and for xenobiotics incorporated in humic substances [Garcette-Lepecq et al., 2001, Challinor, 2001, Ishida et al., 1995]. However, reaction processes during TMAH thermochemolysis are not well understood until now and so, further research is required to elucidate obtaining thermochemolysis products.

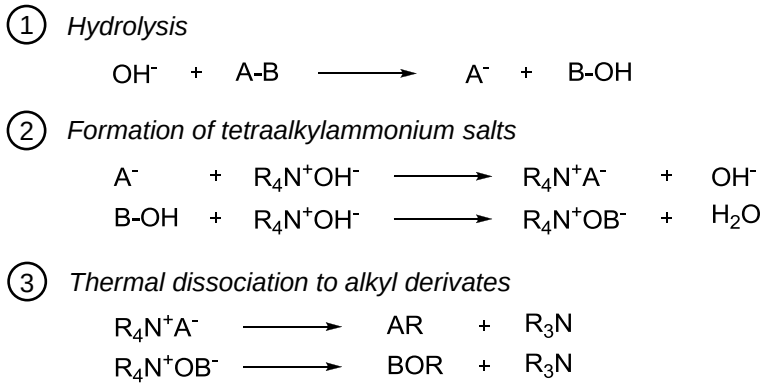

Figure 1.12: Chemical degradation procedure: TMAH thermochemolysis; A-B represents the hydrolysable analyte molecule (e.g. humic matrix) [Challinor, 2001].

1.5 Nonylphenol

4-Alkylphenols and 4-alkylphenol polyethoxylates (APEO) are used worldwide as non-ionic surfactants. Among the numerous alkyphenols showing different chain length, those with a nonyl chain are mainly produced. In North America nonylphenol (NP) making up approximately 85% of the alkylphenol market and nonylphenol ethoxylate (NPEO) making up more than 80% of the alkylphenol ethoxylate market [Lani, 2010]. In 2004 the U.S. demand of NPs was about 180,000 tons [APERC, 2000]. Guenther *et al.* reported in 2002 a global production of APEOs of approximately 650,000 tons/year [Guenther et al., 2002]. In 1997 the EU industry produced 73,500 tons NP [EU, 2002]. Figure 1.13 shows the NP mass balance of Germany in 2000 and 2005. As a consequence of the European Guideline (2003/53/EG) in 2003, the industry was urged not to place products on the market containing concentrations of NP or NPEO equal or higher than 0.1% of mass. This led to a reduction of the NP production in germany from 37,000 tons in 2000 to 19,000 tons in 2005 [Hillenbrand et al., 2006, EU, 2003].

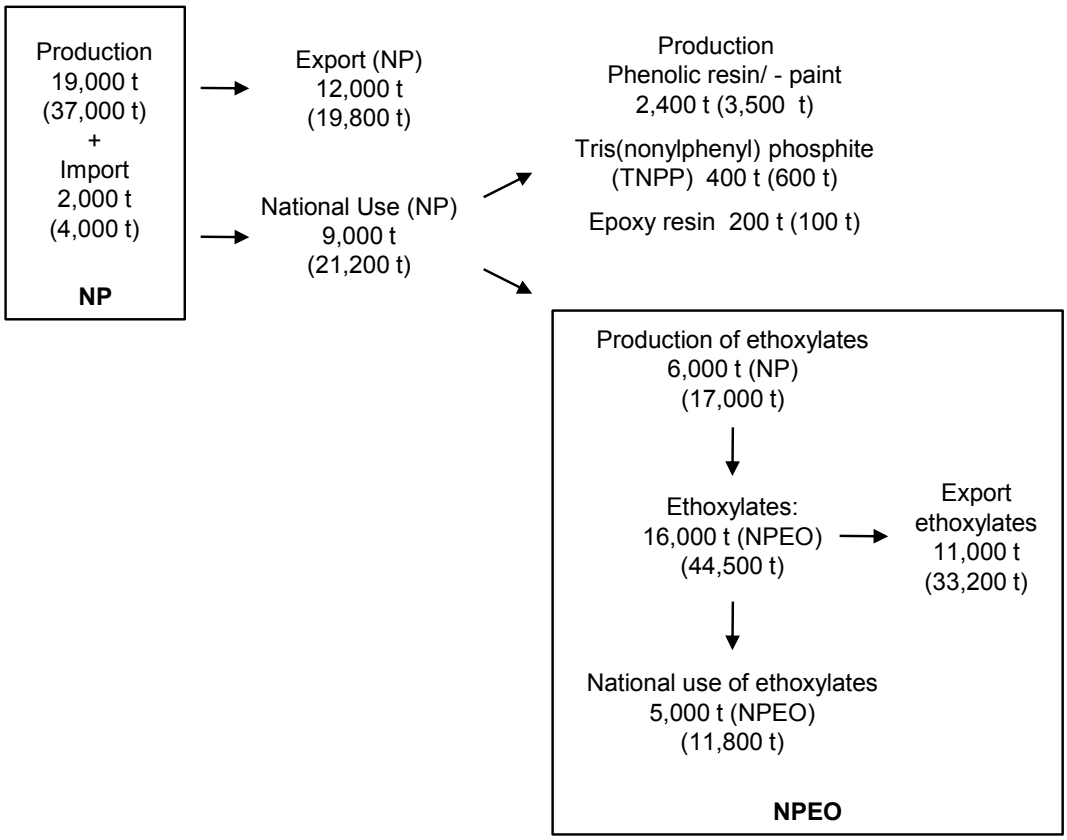

Figure 1.13: Mass balance of NP in Germany in the year 2005 (in parenthesis: year 2000) [Hillenbrand et al., 2006].

1.5.1 Properties

Nonylphenol consists of an aromatic ring with a hydroxyl moiety and a nonly chain. The different polarities of both substituents result in an amphiphile character of the molecule. For the synthesis of NP, nonanol and phenol reacts via a Friedel-Crafts-Alkylation under acid catalysis, predominantly to the para substituted product. Side reactions lead to the formation of the ortho- and dialkylated NP [Vinken et al., 2002, Russ et al., 2005].
Due to rearrangement processes of the occurring carbenium ions during the synthesis, technical produced NPs consists of a mixture of branched isomers [Telscher, 2006]. Eganhouse *et al.* identified more than 60 4-NP isomers within commercially available technical NP. They found significant differences in the compositions between two samples from the same and from different suppliers [Eganhouse et al., 2009].

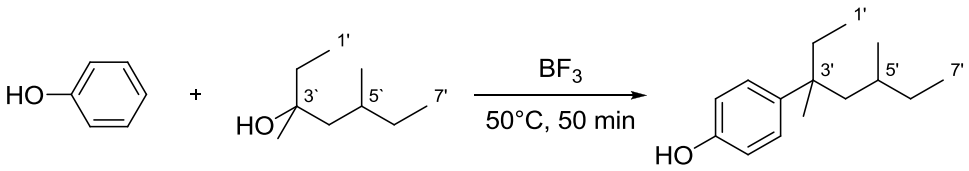

Figure 1.14: Synthesis of the 353-NP isomer.

Properties of technical NP are summerized in Table 1.2.

1.5.2 Application

NPs are used for the production of resins and plastics or as stabilizer (1997: 37% of EU production). A small amount is used for the production of phenolic oximes to extract copper from ore (3% of EU production). Above all this, the predominant type of application of NP is the synthesis of NPEOs (60% of EU production). These are used e.g. in the

- textile processing industry (1994: 11.9% of EU produced NPEO)
- leather processing industry (9.6%)
- polymer industry (7.2%)
- paints, lacquers and varnishes industry (6.2%)
- chemical industry (7.0%)
- personal domestic (5.6%)
- public domain (e.g. cleaning products) (29.7%)
- agricultural industry (4.9%)

Commercial NPEO is a complex mixture of isomers and oligomers because of the different structures of the alkyl side chain. The lipophilic chain may contain 2 to 20 ethoxy units [DiCorcia et al., 1998]. Regarding the functional use of NPEO, 46.1% are used as surface active agents (related to the total amount in the EU in 1994). 44.7% are incredients

Table 1.2: Properties of NP [Hillenbrand et al., 2006, EU, 2002, GESTIS, 2010, Leisewitz and Schwarz, 1997, Ahel et al., 1993, BUA, 1988, Nordic-Council, 1996, Chang et al., 2007].

Properties	Nonylphenol
Molecular formular	$C_{15}H_{24}O$
Molar mass	220.34 g/mol
Appearance	Liquid, colorless to yellowish
Water solubility	6 mg/L (20°C)
Density	0.949 g/cm^3 (20°C)
Vapor pressure	10 Pa (20°C)
Melting point	-8°C - +2°C
Boiling point	295 - 304°C
Sorption behavior (K_{OC})	60,000 L/kg (estimated) 4-NP: 32,400 L/kg (estimated)
Partition behavior ($logK_{OW}$)	4.48 ± 0.12; 3.28 (pH=7)
Flammability	Hardly inflammable (flashpoint: 155°C)
Degradability	Half-life in surface water: 150 days Half-life in soil: 300 days Microbial degradation under aerob and anaerob conditions.
Bioaccumulation	Highly toxic for fish, invertabrate and agea. LC_{50} and EC_{50} are in the range of 0.13 - 1.40 mg/L (fish), 0.18 - 3.00 mg/L (invertebrate) and 0.03 - 1.50 mg/L (algea). Could inhibit the growth of soil bacteria. PNEC (water): 0.33 µg/L
R/S sentences	R: 22-34-50/53-62-63; S: 26-36/37/39-45-46-60-61

in cleaning and washing agents, whereas small portions are used for cosmetics, foaming agents or dust binding agents [EU, 2002].

The high consumption of NPEO in several kinds of industries and products leads to an entry of these compounds into the environment either directly via e.g. agricultural products or indirectly via e.g. surface active substances or cleaning products resulting in an accumulation in wastewater, soil and sewage sludge. However, released NPEOs are biodegraded rather quickly to metabolites like short chain ethoxylates, nonylphenoxy ethoxy acetic acids or nonylphenols themselves, which are more toxic and persistent in the environment than the parent compounds [Field and Reed, 1996, Wang et al., 2006, HLUG, 2003].

1.5.3 Environmental behavior

Due to the amphiphile character, NP tends to sorb to various materials. In technical NP more than 85% of the isomers possess a quarternary α-carbon on the branched alkyl chain. This constitution is resistant to β- and ω-oxidation of the alkyl-chain and leads to the persistent behavior in the environment [Corvini et al., 2006]. Biodegradation of NP was investigated under aerob conditions. In wastewater treatment plants 56% (after 20 days) and 78% (after 40 days) of NP was mineralized. In sediments 80% of NP was transformed after 70 days of incubation. In soil a transformation (ring cleavage) of 95% after 48 days was investigated with an application rate of 280 mg/kg (iso-NP). By adding digested sludge to soil and 25 mg NP/kg soil, 92% was transformed after 40 days, whereas by applying 250 mg/kg only 60% was transformed [HLUG, 2003]. Recently published results showing that 90% of a single NP-isomer was transformed in soil after 30 days of incubation [Zhang et al., 2009]. With an application rate of 0.2-3.0 mg NP/kg soil an impact on the microorganisms was observed. Under anaerob conditions Chang *et al.* investigated the behavior of NP in sludge. They found after 84 days a degradation of 85% in petrochemical sludge and an almost complete degradation in sewage sludge [Chang et al., 2005].
The degradation rates suggested a rather low persistence of NP in the environment. Nevertheless, NP are ubiquitously detectable [Sharma et al., 2009, Soares et al., 2008, Guenther et al., 2002] which may be a result of a continuous entry of NP in the environment (e.g. industry, households, agriculture) or the performed lab experiments reflecting not the natural conditions in its entirety.
Besides the amphiphile and sorption properties, NP was proven as endocrine disrupter. The hormonal impact was tested with fishes [Schwaiger et al., 2002, Kazeto et al., 2004] or rats [Karrow et al., 2004, Gong and Han, 2006]. The effect of NP on humans was investigated by applying ^{13}C-labeled NP. Elimination half-life time in blood was found to be rather short (2-3 h) [Mueller et al., 1998]. Preuss *et al.* investigated the estrogenic effect of single NP isomers with standardized test systems. Among the six tested isomers, 353-NP showed almost the same relative potency as the technical mixture, whereas the others were of lower relative potency. However, the technical mixture of NP showed an average of 10,000 fold lower estrogenic potency as compared to estradiol [Telscher, 2006].

1.5.4 Metabolism of NP

Investigations on the microbial degradation and metabolic pathway of either technical NP or single isomers including the linear 4n-NP are limited, especially in soil. Most studies consider the degradation of NPEOs in sewage sludge, wastewater or sediments [Yong et al., 2009, Ferguson et al., 2003, Ahel et al., 2000].
As a consequence of the complexity of technical NP and thus a complex metabolic pat-

tern could be expected, many studies on NP degradation are dealing with single isomers of the mixture. Telscher *et al.* and Zhang *et al.* investigated the behavior of single NP isomers in soil and soil/sludge mixtures. DeWeert *et al.* used sediments polluted with branched NPs for their investigations. Common to all was that the only metabolite was found to be a nitro-NP [Telscher et al., 2005, Zhang et al., 2009, DeWeert et al., 2010]. It was concluded that ammonium in the matrix led to the formation of the nitro-group [DeWeert et al., 2010].

To overcome difficulties resulting from the environmental matrix and to investigate the degradation pathway of NP in more detail, bacteria able to transform NP were extracted from wastewater or sediments [Corvini et al., 2006]. Moreover, studies dealing with the metabolic pathway of NP in plant cells (e.g. tabacco, soybean) or fungi were published recently [Schmidt et al., 2003, Berger et al., 2005, Vallini et al., 2001]. There are several ways to degrade alkylphenols. A degradation of the alkyl chain without attacking the aromatic ring, the cleavage of the aromatic ring without attacking the alkyl chain or a combination of both methods. Figure 1.15 shows possible metabolic pathways of alkylphenols according to Gabriel *et al.* [Gabriel et al., 2005].

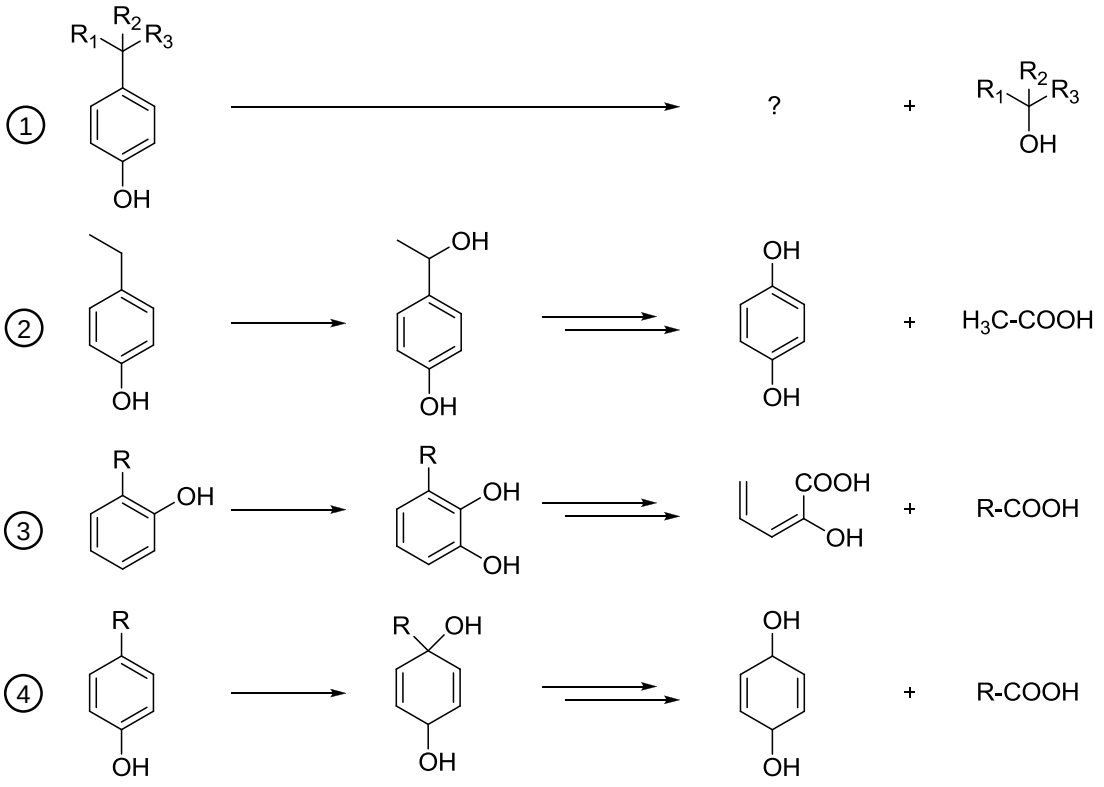

Figure 1.15: Metabolic pathways of alkylphenols. 1) Degradation of a α-quarternary NP by Sphingomonas strains, 2) transformation of the α-methylen group to a keto group, Bayer-Villiger reaction, and hydrolysis, 3) meta-cleavage pathway, 4) ipso-substituation [Gabriel et al., 2005].

1.6 MCPA

The Food and Agriculture Organization of the United Nations published a definition of pesticide in 2002: "Pesticide means any substance or mixture of substances intended for preventing, destroying or controlling any pest, including vectors of human or animal disease, unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport or marketing of food, agricultural commodities, wood and wood products or animal feedstuffs, or substances which may be administered to animals for the control of insects, arachnids or other pests in or on their bodies. The term includes substances intended for use as a plant growth regulator, defoliant, desiccant or agent for thinning fruit or preventing the premature fall of fruit, and substances applied to crops either before or after harvest to protect the commodity from deterioration during storage and transport" [UN, 2002].
Each year approximately 2.5 million tons of pesticides are used worldwide predominantly in agriculture [Alavanja, 2009]. The US EPA estimated the world pesticide expenditures of approximately 31.7 billion $ in 2001 [Kiely et al., 2004]. In Germany around 250 different active ingredients are components of around 900 commercial available products. In 2004, 35,000 out of 115,000 tons of pesticides were produced for national use and 80,000 tons were exported [LfU, 2008]. Most frequently used pesticides are herbicides followed by insecticides, fungicides and others (e.g. rodenticides, molluscicides, fumigants) (Figure 1.16).

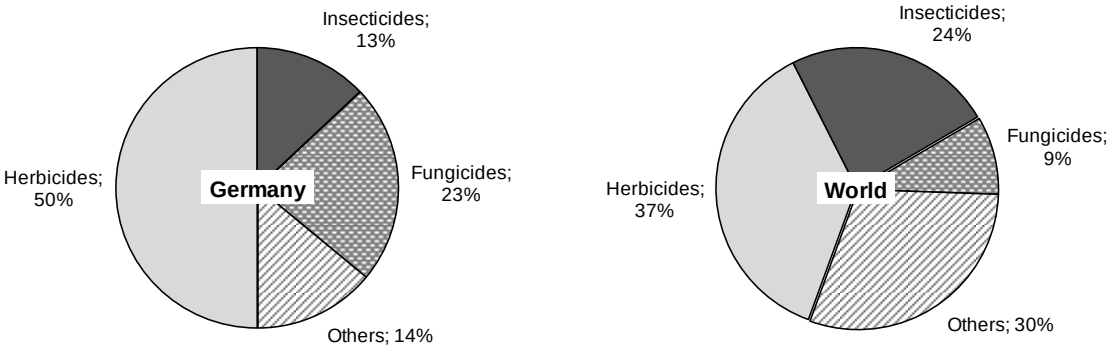

Figure 1.16: Percentage of used pesticides in Germany (2004) and worldwide (2001, estimated) [LfU, 2008, Kiely et al., 2004].

1.6.1 Properties and Application

MCPA bases on investigations of Dr. Bill Templeman. In 1940 he discovered the selective properties of α-naphthyl acid at ICI (Plant protection devision). These investigations led to the synthesis of MCPA and 2,4-D in 1955 [Syngenta, 2010]. MCPA is a herbicide which acts as plant growth regulator and is used for post-emergence control of annual and perennial broad-leaved weeds (e.g. cereals, crops, grassland or trees), as well as

aquatic broad-leaved weeds. MCPA is adsorbed through both leaves and roots and is translocated throughout the plant. It inhibits growth in the same manner as natural auxin (3-indoleacetic acid). At the molecular level, it influences levels of RNA and DNA polymerase and levels of enzymes involved in normal growth and development processes [Roberts et al., 1998, Health-Canada, 2009].

1.6.2 Environmental behavior in soil

MCPA is rapidly degradated in soil. The rate of degradation depends on the type of soil, soil pH, soil moisture, climatic conditions, concentration of MCPA and the organic content of the soil [Sattar and Paasivirta, 1980]. Half-life in soil varying between 7-41 days [EU, 2008]. In acidic soil, degradation of MPCA occured within 5-9 weeks, whereas under neutral conditions (pH 6.3 and above) degradation happened much faster (1 week) [Sattar and Paasivirta, 1980].
MPCA has been detected in surface- and groundwater. The migration from soil to water could be a result of direct (e.g. spraying) or indirect transport mechanisms (e.g. leaching, erosion). However, the leaching potential was found to be rather low below a soil depth of 15 cm. A relation of the organic soil content and the mobility of MCPA in soil was indicated [Health-Canada, 2009]. Haberhauer *et al.* showed in soil column experiments, that the transport or retention of MCPA in soil depends strongly on the constitution of the dissolved organic matter [Haberhauer et al., 2002].
In commercial products MCPA is commonly formulated as salts or esters. Field studies with MCPA-Ethylhexylester showed a rapid degradation to the free acid after one day of application and an almost complete conversion within 3 days [Health-Canada, 2009]. In soils showing a field moisture content of 15% MCPA-Isooctylester persists longer ($>$ 90% after 48 hours) as compared to a soil moisture content of 50% ($<$ 5% after 48 hours) [Smith and Hayden, 1980].
MCPA is moderately toxic to birds and fishes and practically non-toxic to freshwater invertebrates, estuarine and marine organisms as well as bees. For humans, MCPA shows only slight toxic effects. Symptoms of acute intoxication are slurred speech, twitching, jerking and spasms, drooling, low blood pressure, and unconsciousness. After application of MCPA to test persons, half of a 5 mg dose were excreted after a few days in the urine and no residues were found after five days. Test on carcinogenesis and mutagenesis revealed no or only litte effects [EXTOXNET, 1993]. In contrast to MCPA, the main metabolite in soil 4-chloro-2-methylphenol shows a high toxic effect to aquatic organisms. The acute toxicity to fish expressed as LC_{50}-value (96 h) was observed to be 2.3-6.6 mg/L and the EC_{50} (48 h) to daphnids was 0.3-1.0 mg/L. Toxic effects to rats (LD_{50} 2,650-3,196 mg/kg, oral) were lower than those of MCPA [OECD-SIDS, 1998].

Table 1.3: Properties of MCPA [EU, 2008, Sigma-Aldrich, 2010, Health-Canada, 2009].

Properties	MCPA (acidic form)
Molecular formular	$C_9H_9ClO_3$
Molar mass	200.60 g/mol
Appearance	Colourless crystalline solid White to light brown solid flakes or powder
Water solubility	pH 1: 0.395 g/L (unbuffered, 25°C) pH 5: 26.2 g/L (25°C) pH 7: 293.9 g/L (25°C) pH 9: 320.1 g/L (25°C)
Density	1.18 - 1.21 g/cm^3 (20°C)
Vapor pressure	4 x 10^{-4} Pa (32°C)
Melting point	115 - 116°C
Boiling point	Decomposition is observed at approx. 290°C
Sorption behavior (K_{OC})	10 - 157 (mean: 74)
Partition behavior (K_D)	0.05 - 1.99 L/kg (pH=7)
Partition behavior ($\log K_{OW}$)	pH 1: 2.70 (0.001 mol/L) pH 7: -0.71 (0.001 mol/L)
Dissociation constant	pK_a=3.37 (25°C)
Flammability	Hardly inflammable
Degradability	DT_{50} in soil: 7 - 41 d (aerobic, 20°C, lab) DT_{90} in soil: 79 d (aerobic, 25°C, lab) DT_{50} in water: 14 d DT_{50} in water/sediment: 17 d No degradation under anaerobic conditions (lab)
Bioaccumulation	LD_{50} rat: 962 mg/kg bw (oral) LC_{50} rainbow trout: 91 mg/L (96 h) EC_{50} daphnia magna: 180 mg/L (48 h)
Application rates	0.7 - 3.5 L/ha depending on the weed (MCPA equivalent: 500 g/L)*
R/S sentences	R: 22/38/41/50/53; S: 26/37/39/60/61

* Nufarm, MCPA Ester 500 (2-ethylhexyl ester of MCPA)

1.6.3 Metabolism of MCPA in soil

Figure 1.17 shows the degradation pathway of MCPA in soil. Microbial activity is the key factor for the degradation process of MCPA.

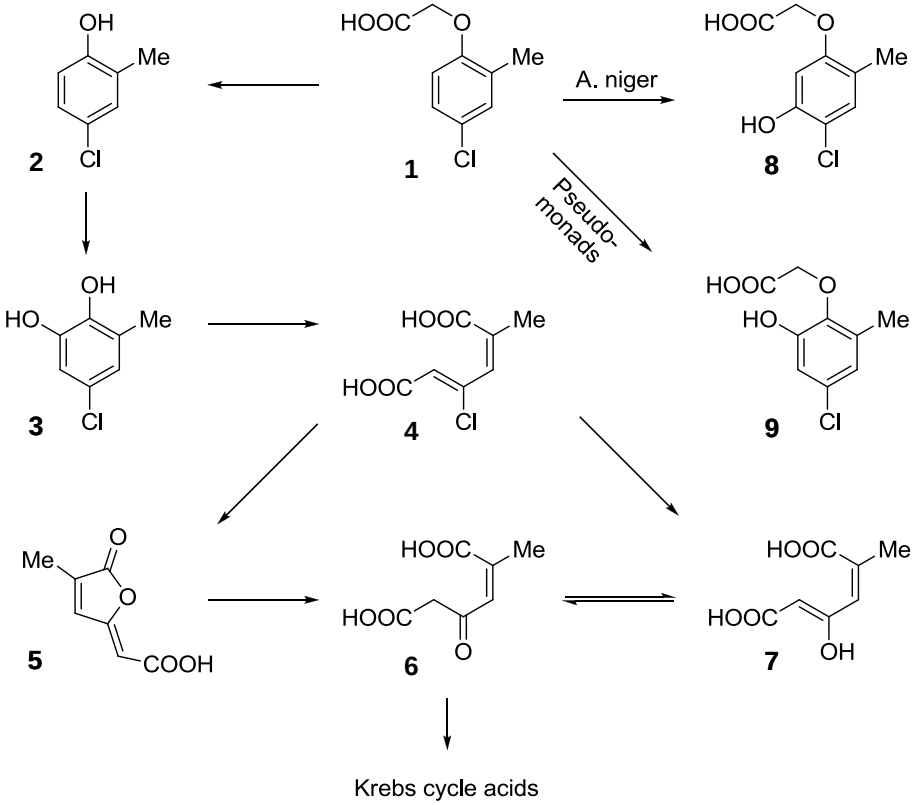

Figure 1.17: Metabolic pathway of MCPA in soil [Roberts et al., 1998].

In a first step, the ether linkage of the side chain is cleaved resulting in **2**. Hydroxylation of **2** gives the catechol **3** which is further converted by *ortho*-ring opening into a chloromuconic acid **4**. A subsequent degradation leads to the acids **5, 6, 7** which can metabolised by way of the Krebs tricarboxylic acid cycle. Investigations of isolated microorganism showed two alternative metabolites. With *Aspergillus niger* and *Pseudomonads* MCPA was hydroxylated before ether cleavage resulting in **8** and **9**, respectively. It should be noted, that most of the indentified products derived from isolated cultures. Due to the reactiveness of degradation intermediates, most of the compounds have rarely been isolated from soils [Roberts et al., 1998].

1.7 Aims and overview of the present study

The aims of this work were to apply the above mentioned methods and techniques to elucidate the mode of incorporation of a single nonylphenol isomer and the herbicide MCPA into soil derived organo-clay complexes. Therefore, ^{14}C- and ^{13}C-labeled compounds were applied to soil samples and incubated up to 180 or 120 days.

1.7.1 Research overview

- CHAPTER 2: The distribution of NP and MCPA was examined in soil and distinct soil sub-fractions over a short time period of 48 hours. The study demonstrated a rapid initial incorporation accompanied by a specific distribution into soil sub-fractions and pointed to a complex interaction of clay associated organic matter with low molecular weight compounds.

- CHAPTER 3: Incorporation processes of the applied NP isomer were elucidated by executing complementary analytical methods (e.g. humic matter fractionation, ^{13}C-CP/MAS-NMR, sequential chemical degradation). Non-extractable residues were found to be preferentially incorporated via ester (amide) bonds. Ageing processes led to a decrease of releaseable residues during the incubation time. Only the parent compound could be identified, no transformation products. A comparatively huge amount of the applied radioactivity remained in organo-clay complexes and could not be traced by the executed degradation methods.

- CHAPTER 4: Incubation experiments in soil were carried out separately with two different concentrations (8.5 and 1,000 mg MCPA/kg soil) of MCPA. Ester (amide) bonds were found to be the preferred mode of incorporation. A possible influence of the microbial activity of the soil on the mode of incorporation was observed. Structure elucidation identified MCPA as the only non-extractable substance, whereas the metabolite 4-chloro-2-methylphenol was additionally found as water extractable (bioavailable) and organic solvent extractable (bioaccessible) compound.

- CHAPTER 5: The used NP isomer contained two chiral carbons and thus, the emerging diastereomers could be separated by means of GC-MS. Results of extractable and non-extractable portions of natural, sterile and model organo-clay complexes indicated that the incorporation of NP was a microbial assisted, stereoselective process.

Methods and results introduced in this study support and complete findings achieved in 2011 by Timm Klausmeyer [Klausmeyer, 2011].

1.7.2 Soil samples

Fuhrberg, Germany

The *Fuhrberger Feld* is located around 30 km north-eastern of Hannover (Germany). With an area of 300 km^2 and an annual water production volume of 40 mio m^3, it delivers 80% of the drinking water demand of Hannover. The soils are Gleyic Podzols and Podzols developed from quaternary sands (*Fuhrberger Feld*) and Histosols (*Totes Moor*). For our studies, soil was taken from the location *Hellern* (Fuhrberger Feld) in the north of Fuhrberg and peat from the location *Totes Moor* close to Neustadt am Rübenberge. The use of the *Fuhrberger Feld* area changed during the time from grassland to agriculture. The main clay mineral was found to be smectite.

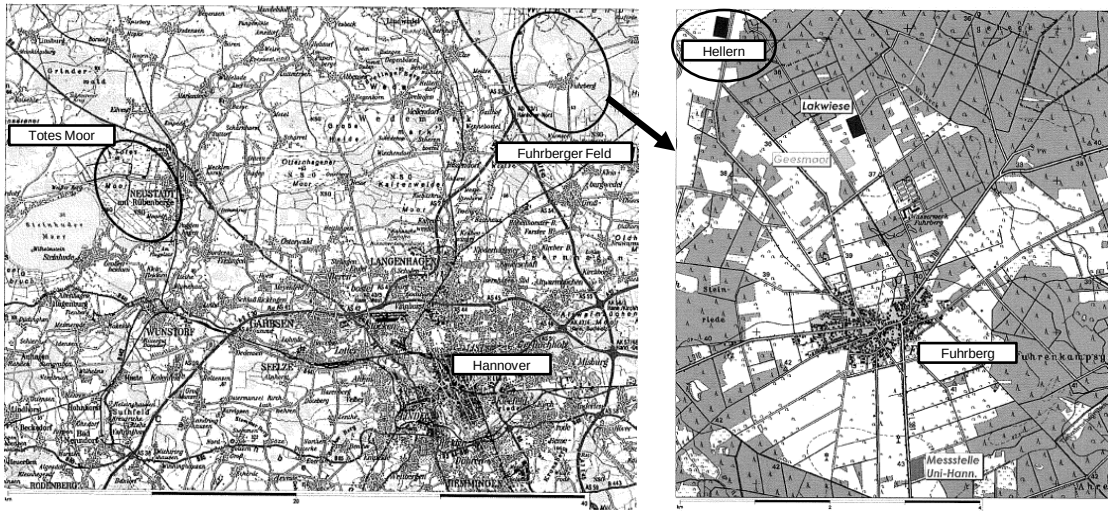

Figure 1.18: Locations of soil sampling: Fuhrberger Feld (Hellern) and Totes Moor, Hannover, Germany (from: Digitale TK50 Niedersachsen/Bremen, LGN).

Ultuna, Sweden

The field experiment site *Ultuna* is located in central Sweden near Uppsala. Eutric Cambisols have developed from postglacial clay (illite as main clay mineral). The soils have been subjected to different organic amendments since 1956.

Characteristics of the soils used for each set of experiments are listed in Table 1.4. Data showing that the location *Hellern* was found to be inhomogeneous regarding the particle size distribution. Despite these variabilities the type of texture for all samples was the same: sandy loam soil.
In case of *Ultuna* the type of soil texture was silty clay loam, due to a relatively high percentage of silt and clay particles.

Table 1.4: Properties of soil samples.

Sample	TOC[(a)] %	TIC[(b)] %	TC[(c)] %	OM[(d)] %	P.D.[(e)] %	pH-value[(f)]	WHC_{max}[(g)] g H_2O/100 g soil
FUHRBERG (Nonylphenol)							
Soil (total)	3.89	0.09	3.98	6.69		5.6	41.6
Sand	0.68	0.04	0.72	1.17	75.40		
Silt	13.47	0.29	13.76	23.17	18.70		
Clay	14.69	0.45	15.13	25.27	5.90		
FUHRBERG (MCPA)							
Soil (total)	5.74	0.16	5.86	9.87		5.4	58.3
Sand	2.09	0.04	2.13	3.59	57.20		
Silt	13.08	0.31	13.39	22.50	30.70		
Clay	14.76	0.41	15.18	25.39	12.10		
FUHRBERG (Sterile soil)							
Soil (total)	9.49	0.12	9.61	16.32		4.5	55.0
Soil (fine)	6.58	0.14	6.72	11.32			
Soil (coarse)	17.35	0.26	17.60	29.84	80.80		
Silt	15.97	0.30	16.27	27.47	13.80		
Clay	16.00	0.45	16.45	27.52	5.30		
ULTUNA (Short term experiments)							
Soil (total)	/	/	/	/		5.8	60.1
Sand	0.38	0.01	0.39	0.65	19.80		
Silt	0.95	0.01	0.97	1.63	51.90		
Clay	2.64	0.08	2.71	4.54	28.30		
MODEL ORGANO-CLAY COMPLEXES							
Organo-clay complexes	4.54	0.32	4.86	7.81			66.6
CLAY MINERALS							
Bentonite	0.02	0.19	0.21	0.04			
Kaolinite	0.03	0.02	0.05	0.05			
Montmorillonite	0.06	0.37	0.43	0.10			

(a) TOC: total organic carbon (b) TIC: total inorganic carbon (c) TC: total carbon
(d) OM: organic matter, calculated as TOC×1.72 [AG-Boden, 1994]
(e) P.D.: particle size distribution (f) $CaCl_2$ method (g) WHC_{max}: maximum water holding capacity

2 Rapid short term distribution of a nonylphenol isomer and the herbicide MCPA in soil derived organo-clay complexes

ABSTRACT. Organo-clay complexes in soil are a major sink for xenobiotics and, thus, enhance their persistence. However, the knowledge on environmental processes of non-extractable residue formation on a short time scale is very restricted. Therefore, this study examined the distribution of 4-(3,5-dimethylhept-3-yl)phenol (NP) and 4-chloro-2-methylphenoxyacetic acid (MCPA), in soil over a short time period of 48 h and in different soil sub-fractions. The overall proportion of organo-clay associated bound residues was not only abundant but also in the same range for both substances (MCPA: 8%; NP: 11% of applied radioactivity). However, a more detailed view revealed two different distribution patterns: a higher proportion of clay associated NP was accompanied by a lower content of bound residues, whereas a smaller fraction of clay associated MCPA was characterized by a higher proportion of non-extractable residues. Further on, a selective accumulation of bound residues among clay-associated humic fractions was observed. NP-residues were linked predominantly to humic acids, whereas MCPA-residues tended to be incorporated more to fulvic acids. It was evident that the overall distribution was influenced primarily by the physico-chemical properties of the contaminants. This study demonstrates very detailed a rapid initial incorporation accompanied by a specific distribution into soil sub-fractions for selected xenobiotics in soil and points to a complex interaction of clay associated organic matter with low molecular weight compounds.

2.1 Introduction

Xenobiotics in agricultural soils originate predominantly from application of pesticides and fertilizers including sewage sludge application. Organic contaminants released into soils exhibit a complex environmental behavior. Besides extractable and, therefore, free available portions chemicals can form non-extractable or bound residues. These processes including ageing have been well investigated for decades, especially for pesticides [MacRae, 1986, Dec et al., 1997a, Barriuso et al., 2008]. The knowledge is restricted to the

determination and description of amounts of free and bound fractions so far. However, information on the short term behavior of chemicals and, in particular, their distribution in diverse sub-fractions of soils are particularly limited [Nieman et al., 1999]. Therefore, the aim of our study was to follow the behavior of anthropogenic contaminants in soils for a short time period concerning different soil sub-fractions. For this purpose the herbicide MCPA and a preselected nonylphenol isomer were investigated as representatives for pesticides and sewage sludge derived xenobiotics, respectively. Besides different emission pathways, they also exhibit different physico-chemical properties which might result in a different environmental behavior [Xie et al., 1997]. Special focus was laid on organo-clay complexes since these are known to play an important role for the formation of bound residues [Wang and Xing, 2005].

2.2 Experimental

2.2.1 Chemicals

^{14}C-labeled 4-(3,5-dimethylhept-3-yl)phenol (NP, 304.14 MBq/mmol) was synthesized via Friedel-Crafts alkylation using 3,5-dimethylheptan-3-ol and a mixture of unlabeled and [ring-U-^{14}C] phenol (2.220 MBq/mmol) according to Russ *et al.* [Russ et al., 2005]. The [ring-U-^{14}C] 4-chloro-2-methylphenoxyacetic acid (MCPA, 59.94 MBq/mmol) was provided by Prof. M. H. Gerzabek (University of Vienna) as a mixture of 92% MCPA methylester and 8% of the free acid.

2.2.2 Spiking experiments

Aliquots of 584 µg (0.18 MBq) of MCPA dissolved in 0.5 mL methanol were applied to approx. 100 g of air-dried, homogenized, and sieved ($<$ 2 mm) soil samples from Ultuna, Sweden. In a second experiment 126 µg (0.17 MBq) of NP dissolved in 0.5 mL petrolether was similarly applied to an identical soil sample. Directly after application, the solvent was evaporated and the flasks were shaken for 15 min in an overhead shaker. Thereafter, the water content of the samples was adjusted to 40% of maximum water holding capacity. The flasks were closed with an absorption device for $^{14}CO_2$ containing soda lime (approx. 15 g). After an incubation period of approx. 48 h at 20°C in the dark, an aliquot of 20 g of the soil samples (referred to dry material) were suspended in 100 mL of water and subjected to ultrasonic assisted disaggregation (total energy input 22 kJ) as described by Morra *et al.* [Morra et al., 1991]. The soda lime was dissolved in conc. HCl and the liberated $^{14}CO_2$ was captured in a suitable scintillation cocktail LumasafeTM plus (Lumac), and was examined by a scintillation counting device (2250CA TRI-CarB®, Canberra-Packard).

2.2.3 Particle size fractionation and isolation of humic substances

Sand, silt and clay fractions were obtained by an initial manual wet sieving for separation the sand from silt and clay. Silt and clay were separated by centrifugation according to Stemmer *et al.* [Stemmer et al., 1998]. Subsequently, the clay fraction containing predominantly the organo-clay complexes was fractionated using a modified methyl-isobutylketone (MIBK) method (Figure 2.1) according to [Rice and Maccarthy, 1990]. Thus, six sub-fractions were obtained: HCl extract (HCl), humic acids (HA), fulvic acids (FA), bound lipids (BL), bound humic acids (BHA) and the extractable fraction.

2.2.4 Radioanalysis

Aliquots of soil fractions (sand, silt and clay) were combusted using a Biological Oxidizer OX500 (R. J. Harvey Instrument Corp.). Emerging $^{14}CO_2$ was captured in Oxysolve C-400 cocktail (Zinnser Analytic). The radioactivity amount contained in liquid samples was determined using LumasafeTM plus (Lumac) scintillation cocktail. Liquid scintillation was executed by means of a 2250CA TRI-CarB® (Canberra-Packard) counter.

2.3 Results and Discussion

In order to study the environmental behavior of 4-(3,5-dimethylhept-3-yl)phenol (NP) and 4-chloro-2-methylphenoxyacetic acid (MCPA) in a short term experiment with respect to the distribution in selected soil sub-fractions ^{14}C-labeled chemicals were used. MCPA is normally used in technical formulation containing its different esters in varying concentrations (e.g. MCPA 2-ethylhexyl ester (2-EHE), Nufarm MCPA Ester 600). Hydrolysis to the corresponding acid occurs in a moistened soil very fast according to Hayden and Smith [Hayden and Smith, 1980]. For the methyl esters as used in our incubation experiments dominant species of MCPA can be assumed to be the free acidic form due to hydrolysis rates lower than 24 h. After 48 h the spiked soil samples were subjected to particle size separation and the organo-clay complexes were further fractionated according to the different subtypes of humic substances. The soil used in the present study derived from Ultuna (Sweden), and represented an Ap-horizon, which has not been treated with MCPA or sewage sludge for the last ten years. The silty clay loam soil consisted of 19% of sand, 50% of silt and 31% of clay with a water holding capacity of 52%. The organic matter content was 4.2% as determined by ignition loss.

2.3.1 Particle size related distribution of radioactivity

The distribution of applied ^{14}C-MCPA and ^{14}C-NP among the different particle size fractions are illustrated in Figure 2.2. The MCPA spiked sample exhibited a high amount of applied radioactivity in the aqueous phase (74%). This finding was thought to be the

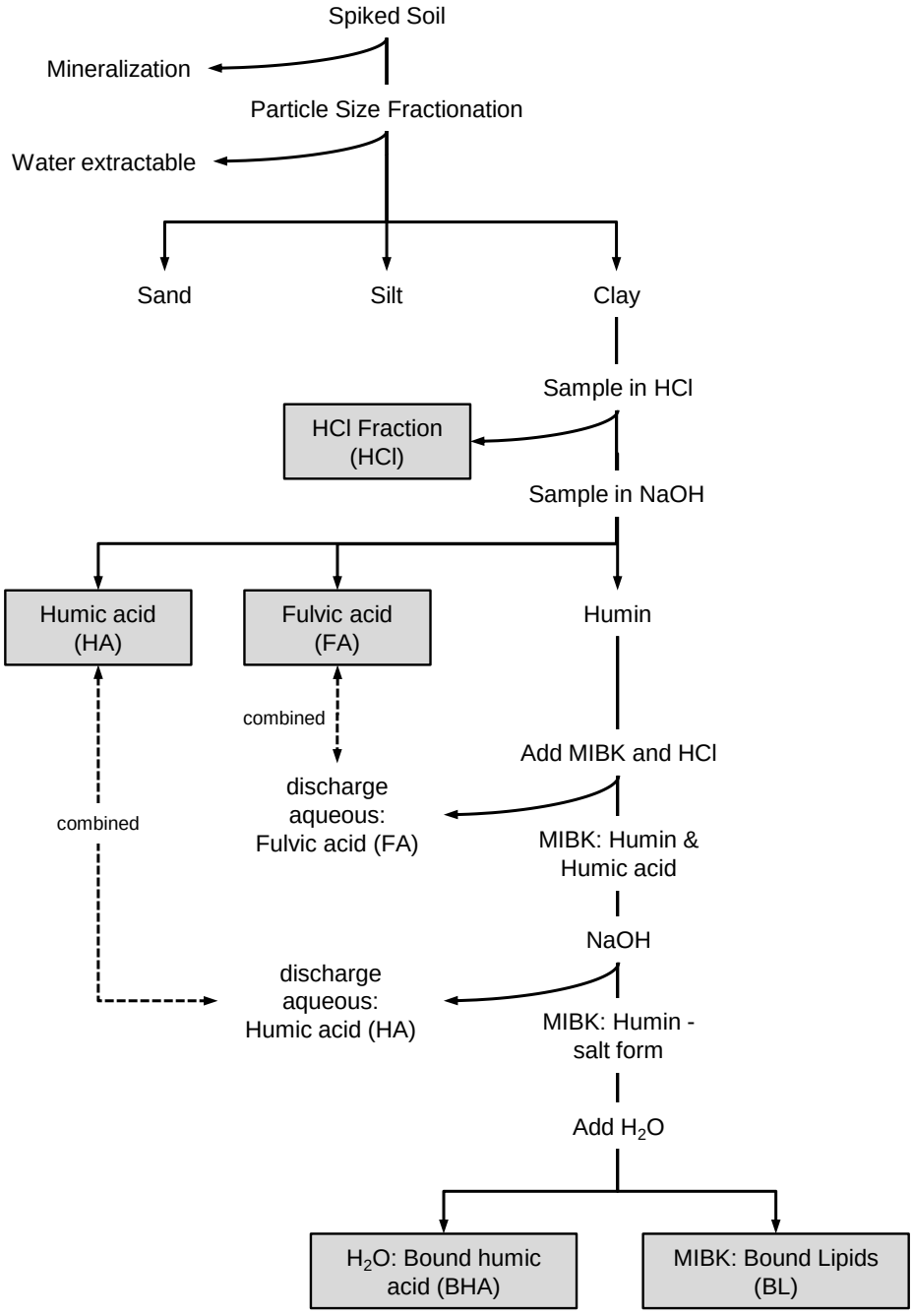

Figure 2.1: Workflow of the short term distribution experiments.

result of MCPA's enhanced water solubility (acid: 1.5 g/L). The particle associated radioactivity was around 22%. On the contrary, 74% of applied radioactivity remained incorporated in the particle fraction in the NP spiked sample with a main accumulation in the clay fraction (43%) and in the silt fraction (27%). The NP isomer tended to accumulate on particles possibly as a result of its minor water solubility (5.0 mg/L).

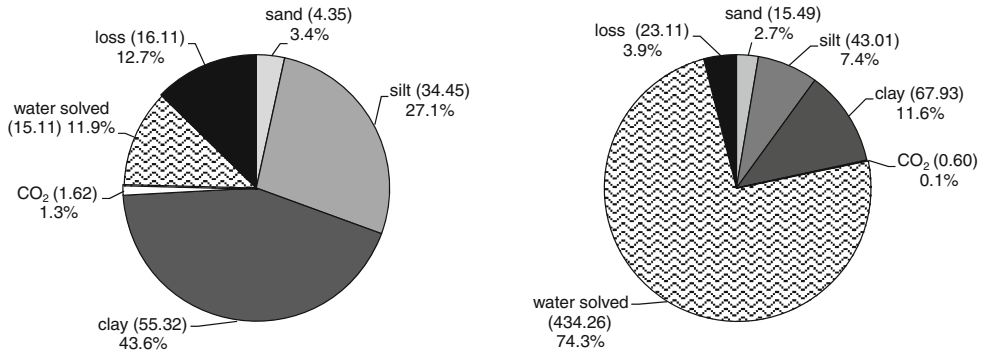

Figure 2.2: Distribution of the radioactivity between individual soil particle size fractions, water phase and CO_2 as mineralization indicator. Percentages refer to the total radioactivity applied to soil,whereas absolute values (in parentheses) are calculated from radioactivity on base of an initial application of 126 µg 4-(3,5-dimethylhept-3-yl)phenol, NP (left), and 584 µg 4-chloro-2-methylphenoxyaceticacid, MCPA (right), to 100 g of soil, respectively.

For both compounds the mineralization was insignificant after 48 h. Furthermore, most of the particle associated radioactivity was detected in the clay fraction. A preferential accumulation in the clay fraction has been described e.g. for PCBs and PAHs [Krauss and Wilcke, 2002] and for sulfonamide [Thiele-Bruhn et al., 2004]. On the one hand, the large particle surface of clay facilitates the interaction with organic contaminants. On the other hand, the clay associated organic material that represent an important proportion of the clay fraction, enhances dominantly the adsorption and incorporation processes. Therefore, the organo-clay complexes play a major role in binding organic pollutants to soil as demonstrated e.g. for pesticides [Xie et al., 1997]. Hence, the partition of NP and MCPA residues in the organo-clay complexes was studied in more detail.

2.3.2 Organo-clay fraction related distribution of radioactivity

Separating the organo-clay fraction into the specific organic sub-fractions leads to a distinct distribution of radioactivity derived from ^{14}C-MCPA and ^{14}C-NP as shown in Figure 2.3. All values are given as percentages of total ^{14}C detected in the clay fraction, respectively. Interestingly, the distribution pattern for ^{14}C-MCPA and that of ^{14}C-NP differed noticeable. After 48 h of incubation, the extractable, free available fraction in case of MCPA application was much lower (18%) as compared to the NP treated sample

(72%). That might be the result of the more reactive carboxyl moiety and the higher polarity of MCPA that both might lead to a faster incorporation in the organic material by adsorption or covalent linkages.

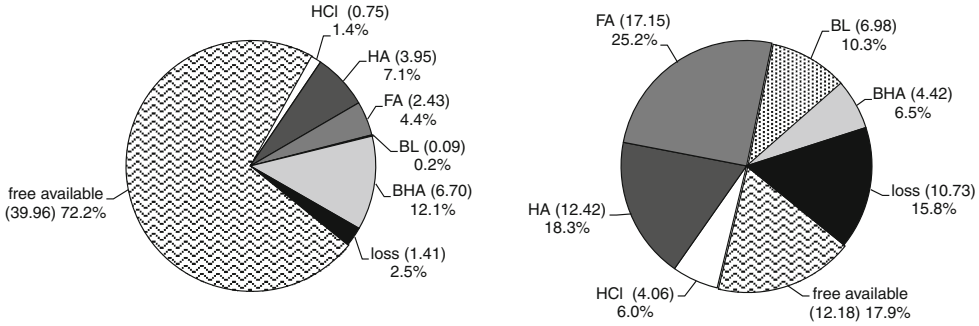

Figure 2.3: Distribution of the radioactivity from ^{14}C-4-(3,5-dimethylhept-3-yl)phenol, ^{14}C-NP (left), and ^{14}C-4-chloro-2-methylphenoxyaceticacid, ^{14}C-MCPA (right), between individual organo-clay fractions and free available fraction (Soxhlet extract). Percentages refer to the total radioactivity detected in the clay fraction, whereas absolute values (in parentheses) are calculated from radioactivity on base of absolute amounts in the clay fraction (see Figure 2.2).

Regarding the distributions of ^{14}C in the bound fraction, main portions of ^{14}C-MCPA derived radioactivity was observed in the humic and fulvic acids, while ^{14}C-NP derived radioactivity accumulated preferentially in the humic acids and bound humic acids. Portions extractable with HCl and bound lipid fractions were of minor importance for both xenobiotics investigated. There is a slight tendency for the more polar xenobiotics to accumulate in the more polar fulvic acid fraction (FA) whereas the more lipophilic pollutant prefers the less polar fraction, the humic acids (HA). These observations are partially supported by former investigations on total soil humic matter. Barriuso *et al.* detected the more polar pesticide atrazine dominantly in the humin and fulvic acid fraction [Barriuso et al., 1991], whereas Nieman *et al.* described a preferential accumulation of the lipophylic pyrene in the humic acid fraction [Nieman et al., 1999]. On the contrary, Khan measured a dominant bound proportion of the more polar prometryn in the humin fraction [Khan, 1982b]. Obviously, the preference to accumulate in distinct fractions of humic substances depends only partially on the polarity of the bound compound. Additionally, a higher metabolism rate as known for MCPA might also result in a preferential association with humic fractions. Further on, the influence of clay minerals on the interaction of humic substances with organic contaminants as realised in organo-clay complexes remain unclear. Generally, for NP and MCPA different incorporation behavior resulting in distinct distributions within soil sub-fractions was observed. Although, MCPA exhibited a lower tendency to interact with particles, a fast formation of bound residues was observed for the particle associated fraction. On the contrary, the high affinity of NP to particles was accompanied by lower formation of non-extractable residues. The overall

distribution seemed to be influenced significantly by the physico-chemical properties of the contaminants. Additionally, a higher metabolism rate might result in a faster formation of non-extractable residues via its metabolites in preferential soil sub-fractions. However, the overall proportion of organo-clay associated bound residues was similar for both substances (MCPA: 8%; NP: 11% of applied ^{14}C). Noteworthy, this significant amount of bound residues and the differentiation between soil sub-fractions has been formed within the short time period of 48 h.

2.4 Conclusion

Xenobiotics used in this study (4-(3,5-dimethylhept-3-yl)phenol (NP) and 4-chloro-2-methylphenoxyacetic acid (MCPA)) differed in their physico-chemical properties, that seem to have a predominant influence on the incorporation process in soil fractions. Comparing the incorporation amount of NP these bound residues have been accumulated in organo-clay sub-fractions as follows: bound humic acids (BHA) › humic acids (HA) › fulvic acids (FA) › acid extractable fraction (HCl) › bound lipids (BL). For MCPA the residues accumulated with a different distribution: fulvic acids (FA) › humic acids (HA) › bound lipids (BL) › bound humic acids (BHA) › acid extractable fraction (HCl). As a major result, this study demonstrates not only a very fast incorporation on a short time scale (lower than 48 h) but points to a complex and selective interaction of clay associated organic matter with low molecular weight compounds.

3 Incorporation mode of a branched nonylphenol isomer in soil derived organo-clay complexes and its temporal behavior

ABSTRACT. Incorporation processes of a defined ^{13}C- and ^{14}C-labeled NP isomer (4-(3,5-dimethylhept-3-yl)phenol) into soil derived organo-clay complexes were investigated in this study. Isolated organo-clay complexes were further separated into humic subfractions (humic acids, fulvic acids and humin) by the commonly used procedure. Non-invasive (^{13}C-CP/MAS NMR) as well as invasive methods (sequential chemical degradation) were applied to humic fractions in order to obtain detailed information about the mode of incorporation, the chemical structure and the change of the incorporation character of non-extractable residues in course of incubation. ^{13}C-CP/MAS NMR measurements of humic acids revealed an increasing incorporation of phenolic compounds during the experimental time which was referred to residues of the introduced ^{13}C-labeled NP isomer containing an intact aromatic ring. Detailed investigations by means of sequential chemical degradation indicated a preferential incorporation of non-extractable NP isomer residues via ester (amide) bonds as released by alkaline hydrolysis. Moreover, the amount of releasable compounds decrease until day 180 indicating a change of the incorporation behavior as a consequence of ageing processes. BBr_3-treatment, RuO_4 oxidation and TMAH assisted pyrolysis released only low portions of non-extractable ^{14}C giving evidence of minor influence of ether and *C-C*-linkages in the incorporation processes. Despite a proofed mineralization only the parent NP isomer could be identified as non-extractable compound.

3.1 Introduction

Soils are important sinks for anthropogenic contaminants in the environment. Release of organic compounds into the soil environment could be a consequence of conscious application as crop protection products, impurities of manure applied to soil, atmospheric deposition or disposal of waste [Northcott and Jones, 2000]. Within soil, interactions of xenobiotics with organic matter are important factors influencing their toxicity, bioavail-

ability, transport and volatility [Stevenson, 1994]. As a consequence of the comparatively huge surface area, high amounts of functional groups (e.g. hydroxyl moieties) and surface charges of clay minerals, these soil components show a high potential to interact with other soil organic matter as well as xenobiotics [Wypych and Satyanarayana, 2004]. Anthropogenic contaminants incorporated in organo-clay complexes can be reduced in their extractability, leading to the formation of non-extractable or so-called "bound" residues. Führ *et al.* [Fuehr et al., 1998] stated that: "Bound residues represent compounds in soil, plant, or animal which persist in the matrix in the form of the parent substance or its metabolite(s) after extraction. The extraction method must not substantially change the compounds themselves or the structure of the matrix". It is evident that the term "bound residues" depends largely on the methods used to extract incorporated compounds. The formation of bound residues depends on the soil characteristics (e.g. organic matter composition, pH value), the properties of the compounds and their metabolites (e.g. polar, non-polar) as well as the influence of microorganisms (e.g. enzymatic induced reactions). The mode of binding ranges from reversible (weak) adsorptive and van-der-Waals forces, over ligand exchange and charge-transfer complexes to reversible or irreversible covalent bonds [Northcott and Jones, 2000, Senesi, 1992].

Generally, there are two principle approaches to elucidate the structure and incorporation mechanism of bound residues. In most cases, a site specific labeling (e.g. ^{14}C, ^{13}C, ^{15}N) of the applied compounds facilitates their characterization. First, there are non-invasive, in-situ spectroscopic methods, e.g. FT-IR or fluorescence spectroscopy. Additionally to these, ^{13}C-NMR as non-invasive method has been used to examine the binding mechanisms of several compounds to humic sub-fractions [Haider et al., 1993, Hatcher et al., 1993]. However, application of non-invasive methods to natural samples is limited, because of interferences of compound specific signals with background noise derived from numerous naturally occurring constituents [Kronimus and Schwarzbauer, 2007]. As a second approach, invasive methods are applicable to distinguish between different types of bonds by selective chemical degradation (e.g. alkaline hydrolysis, oxidation, pyrolysis). A release of xenobiotics occurs if either the macromolecular structure is degraded leading to compounds which remain unaltered in the soil (e.g. by sequestration), or covalent bonds between the soil matrix and the incorporated compounds are cleaved. The latter process can produce artifacts of bound residues and thus, only an indirect elucidation of the chemical structure and binding mechanism is achievable. However, released compounds can be extracted, fractionated, cleaned and measured by trace analysis methods like GC-MS or HPLC-MS, which is a great advantage in terms of sensitivity [Kronimus and Schwarzbauer, 2007].

To understand the environmental fate of xenobiotics and to assess the risk of corresponding non-extractable residues, which can be re-mobilised and thus, become bioavailable, detailed investigations on the binding characteristics and the chemical structures are re-

quired. Within the present study, we examined the incorporation processes of the ^{13}C- and ^{14}C-labeled NP isomer 4-(3,5-dimethylhept-3-yl)phenol in soil derived organo-clay complexes which were demonstrated to be the major sink for this isomer [Riefer et al., 2011]. Incubation experiments up to 180 days were performed and organo-clay complexes were separated from other soil constituents. In order to reveal information on the incorporation processes of NP isomer residues, ^{13}C-CP/MAS-NMR as well as a sequential chemical degradation of humic sub-fractions were executed. Released compounds were extracted and analyzed by means of liquid scintillation counting (LSC), Radio-HPLC and GC-MS. The aim of the present study was to i) balance the releasable portion of the residues during each degradation step, ii) follow the dynamic behavior of specific bonds during the incubation period, iii) elucidate the structure of incorporated compounds, and iv) compare percentages of released compounds calculated from radioactivity measurements with percentages measured via GC-MS in order to obtain information about the mode of incorporation.

3.2 Materials and Methods

3.2.1 Soil

The experiments were conducted using a sandy loam soil obtained from an area close to Fuhrberg (Ap horizon), which is located 30 km north of Hannover, Germany. The soil was air-dried, sieved to pass a 2 mm mesh and immediately used for the incubation experiments. Main soil characteristics were determined comprising particle size distribution, TOC content, pH-value and water holding capacity (Table 3.1). The mineral composition of the clay fraction was determined by means of X-ray diffraction and amounted to: 85.5% smectite, 8.4% kaolinite, 2.7% muscovite and 3.4% quartz.

Table 3.1: Soil parameters.

Particle distribution			TOC[(a)]				WHC_{max}[(b)]	pH (soil)[(c)]
sand	silt	clay	sand	silt	clay	soil		
(%)	(%)	(%)	(%)	(%)	(%)	(%)	(g H_2O/100 g soil)	
75.4	18.7	5.9	0.7	13.5	14.7	3.9	41.6	5.6

(a) TOC: total organic carbon (b) WHC_{max}: soil maximum water holding capacity
(c) $CaCl_2$ method

3.2.2 Chemicals

The ^{13}C- and ^{14}C-labeled NP isomer 4-(3,5-dimethylhept-3-yl)phenol was synthesized according to Russ *et al.* [Russ et al., 2005]. A mixture of non-labeled and [U]-ring-labeled phenol (60 mCi/mmol) was used for preparing the ^{14}C-labeled NP isomer resulting in

a specific activity of 8.22 mCi/mmol, a radiochemical purity of 94% (HPLC), and a chemical purity of 90% (GC-MS), respectively. For the synthesis of the ^{13}C-labeled NP isomer, C1-labeled phenol (99% ^{13}C label, Isotec, Ohio, USA) was used. The NP obtained showed a chemical purity of 90% (GC-MS). Other (non-labeled) chemicals were purchased from Sigma-Aldrich (Taufenkirchen, Germany), Merck (Darmstadt, Germany) and ABCR (Karlsruhe, Germany). Solvents were distilled and purity was checked by gas chromatography before use.

3.2.3 Incubation method

A detailed description of the incubation procedure has been published recently [Riefer et al., 2011]. Briefly, to 100 g of air dried soil, 125 µg (0.167 MBq) ^{14}C-labeled NP isomer dissolved in petrolether was added (1.25 mg NP/kg soil). In case of the ^{13}C-labeled NP isomer, 50 mg dissolved in petrolether was applied to 50 g soil (1 g NP/kg soil). Immediately after application, the solvent was evaporated and the flasks were shaken for 15 min with an overhead shaker for homogenization. All samples were subsequently adjusted to 60% of the maximum water holding capacity (WHC_{max}) using deionized water. The incubation was executed in the dark at 20°C. All incubation experiments were conducted in triplicate. Samples were taken after 1, 7, 14, 30, 90 and 180 days of incubation.

3.2.4 Degradation procedures

For achieving humic sub-fractions, organo-clay complexes were separated from soil by a wet sieving and ultrasonification procedure. The clay fraction was extracted with methanol and dichloromethane by means of Soxhlet apparatus. The remaining organo-clay complexes were fractionated into humic acids, fulvic acids and humin (minerals plus organic) according to the classical alkaline separation method. More details of the former mentioned procedures were described [Riefer et al., 2011]. Chemical degradation of the separated humic sub-fractions was performed sequentially. The workflow is displayed in Figure 3.1.

3.2.5 Alkaline hydrolysis

The pre-separated and dried humic sub-fractions were separately filled into 100 mL glass vessels. 15 mL of an alkaline mixture (2 M) of methanol, water and potassium hydroxide (1:1.2:250 v/v/w) was added. The vessels were closed tightly and the mixture was heated for 24 h at 105°C. After cooling, the solution was acidified with HCl to pH=2. In order to extract the released NP isomer residues, 15 mL diethyl ether was added and the vessels were shaken for 10 min at 190 rpm by means of a horizontal shaker (GFL 3017, Burgwedel, Germany). In case of poor phase separation, samples were centrifuged for

3 min at 1800 rpm using a J20 XPI centrifuge (Beckman Coulter, CA, USA). The organic layer containing NP isomer derived residues was decanted. Remaining precipitate was re-suspended in 15 mL diethyl ether, shaken, and the supernatant was decanted. The extraction procedure was then repeated two times using each 15 mL ethyl acetate. The combined organic layers were dried with anhydrous sodium sulfate, filtered and concentrated to a volume of approx. 0.5 mL by rotary evaporation. The crude extracts were separated into three fractions by column chromatography (Baker, 2 g silica gel 40 µm) using 5 mL n-pentane/dichloromethane (60:40 v/v) (fraction 1), 5 mL dichloromethane (fraction 2), 5 mL methanol (fraction 3). Radioactivity of each fraction was measured by means of a liquid scintillation analyzer.

3.2.6 Boron tribromide treatment

The solid humic sub-fractions, pre-treated by alkaline hydrolysis, were separately suspended in deionized water and neutralized by adding NaOH solution. After drying the samples in an oven at 50°C, 15 mL of a 1 M boron tribromide solution in dichloromethane was added. The suspensions were treated for 15 min in an ultrasonic bath and shaken for 10 min with a horizontal shaker (170 rpm). This procedure was repeated twice. Subsequently, the mixtures were shaken (170 rpm) for 24 hours at room temperature. The suspensions were cooled in an ice bath and the reaction quenched by adding 10 mL deionized water. After adding 10 mL diethyl ether the mixture was shaken (190 rpm) for 10 min. For phase separation, the samples were centrifuged for 3 min at 1800 rpm. The organic layer was decanted and the aqueous suspension extracted another two times with 15 mL diethyl ether. The combined organic layers were dried with anhydrous sodium sulfate, filtered and concentrated to a volume of approx. 0.5 mL by rotary evaporation. The crude extracts were separated into three fractions by column chromatography (Baker, 2 g silica gel 40 µm) using 5 mL n-pentane/dichloromethane (95:5 v/v) (fraction 1), 5 mL dichloromethane (fraction 2), 5 mL methanol (fraction 3). Radioactivity of each fraction was measured by means of liquid scintillation counting (LSC).

3.2.7 Ruthenium tetroxide oxidation

Residual samples remaining from the BBr_3-treatment were dried at 50°C in an oven. 10 mg of RuO_4 and 500 mg of $NaIO_4$, 8 mL CCl_4, 8 mL acetonitrile and 1 mL deionized water were added. The vessels were closed tightly and shaken for 4 hours with a horizontal shaker (170 rpm). For reaction termination, 50 µL MeOH and 2 drops of concentrated H_2SO_4 were added. The samples were centrifuged at 1800 rpm for 3 min and the supernatant decanted. The precipitate was washed using 8 mL of CCl_4, centrifuged and the supernatant was decanted. To the combined organic layers, 5 mL deionized water was added, and the mixture was immediately shaken for 3 min at 190 rpm. Thereafter,

the water layer was removed and extracted three times with 5 mL diethyl ether. The ether extracts were combined with the CCl_4 fraction. The mixture was dried with anhydrous sodium sulfate, filtered and concentrated by means of rotary evaporator to a volume of 0.5 mL. A saturated $Na_2S_2O_3$ solution (0.5 mL) was then added. The organic layer was removed, dried with anhydrous sodium sulfate, filtered and concentrated to a volume of approx. 0.5 mL by rotary evaporation. The crude extracts were separated into two fractions by column chromatography (Baker, 2 g silica gel 40 µm) using 5 mL dichloromethane (fraction 1) and a mixture of 2 mL diethyl ether and 3 mL methanol (fraction 2). Radioactivity of each fraction was measured by means of LSC.

3.2.8 Thermochemolysis (TMAH)

Residual samples from RuO_4-treatment were separately placed in digestion bombs containing a 45 mL teflon tube. 5 mL of a 25% methanolic tetramethylammonium hydroxide (TMAH) solution was added and the mixture was suspended by ultrasonification. Methanol was removed by a gentle stream of nitrogen before closing the bomb. The pasty mixture was heated for 2 h at 270°C and thereafter cooled to -18°C. The bombs were opened and 10 mL diethyl ether was added to the thermo degraded sample residues. The mixture was treated for 5 min in an ultrasonic bath, the extract was decanted into a glass flask and the precipitate sonicated again with 10 mL diethyl ether. After decanting, the latter steps were repeated with dichloromethane and n-hexane. All organic solutions were combined, dried with anhydrous sodium sulfate, filtered and concentrated to a volume of approx. 0.5 mL by rotary evaporation. The crude extracts were separated into three fractions by column chromatography (Baker, 2 g silica gel 40 µm) using 5 mL n-pentane/dichloromethane (95:5 v/v) (fraction 1), 5 mL dichloromethane (fraction 2), 5 mL methanol (fraction 3). Radioactivity of each fraction was measured by means of LSC.

3.2.9 Analytical methods

NMR

The solid state NMR spectra were recorded using a DSX-500 spectrometer (Bruker, Germany) working at a frequency of 500.44 MHz for ^1H and 125.83 MHz for ^{13}C. The cross-polarisation (CP) technique with a contact time of 1 ms, a repetition time of 5 s, and more than 4000 scans was used for all samples. All measurements were performed with dried samples packed in a 7 mm ceramic rotor under magic angle at 5 kHz and at room temperature. The chemical shifts were calibrated based on the chemical shifts of adamantane. The integral intensity of the peak of interest (at 154 ppm) was obtained by deconvolution of each experimental CP data, based on a combination of Lorentzian

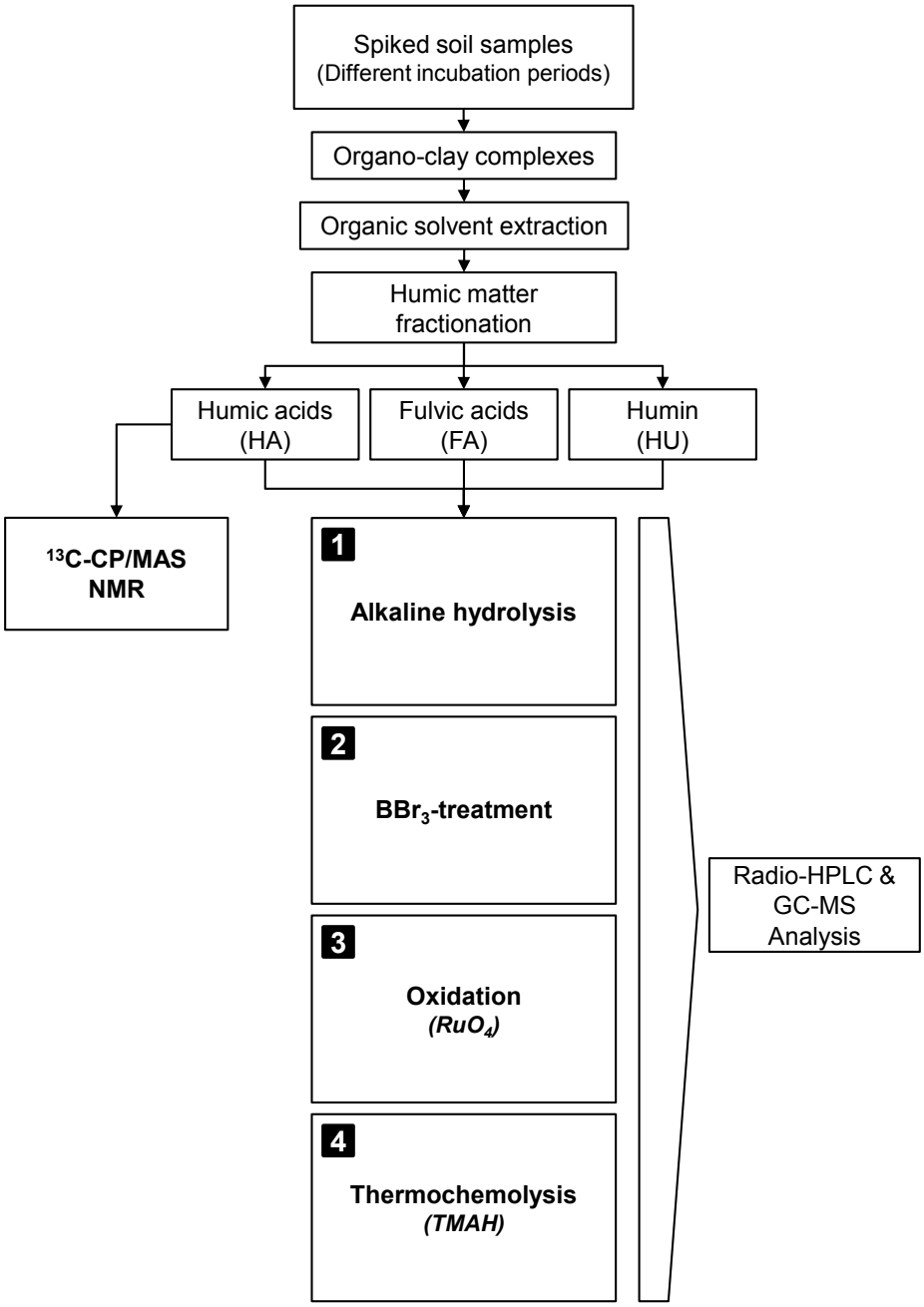

Figure 3.1: Workflow of sample preparation and applied chemical degradation methods.

functions describing the signals present in the spectrum using the DMFIT program [Massiot et al., 2002].

Radioanalysis

Liquid scintillation counting (LSC) of soluble samples was carried out using a LS 6500 TD analyzer (BeckmanCoulter, Krefeld, Germany) and Lumasafe Plus scintillation cocktail (PerkinElmer, Rodgau, Germany). Solid samples were transferred to combustion cones (Canberra-Packard, Rodgau, Germany) and combusted in a biological oxidizer OX 500 (Harvey Instruments/Zinsser, Frankfurt, Germany).

HPLC

HPLC was performed on a HP 1100 (Agilent, Waldbronn, Germany) which includes a solvent delivery system with a binary pump, an autosampler, a diode array detector and a column thermostat. The system was connected to a Ramona Star radiodetector equipped with a 1655 quartz cell (glass, 32-54 µm; internal diameter: 4 mm; volume: 0.4 mL; Raytest, Straubenhardt, Gemany). For detection of radioactivity, Quicksafe Flow 2 (Zinsser Analytic, Frankfurt, Germany) was added with a flow rate of 2 mL/min. Analyses were performed at 35°C on a Synergy Fusion column (250 x 4.6 mm, 4 µm; Phenomenex, Aschaffenburg, Germany); flow rate: 1 mL/min. The nonylphenol isomer was detected at 227 and 279 nm. Elution was carried out with solvents A (water with 0.2% acetic acid) and B (methanol with 0.2% acetic acid): A/B 65:35 (v/v) for 5 min, linear 15 min gradient to 100% B, isocratic B (100%) for 5 min, and return to initial conditions within 5 min.

GC-MS

GC-MS analyses were performed on a Magnum iontrap (FinniganMat, Bremen, Germany) linked to a gas chromatograph 3400 (Varian, Darmstadt, Germany) equipped with a Zebron ZB-1 (Phenomenex, Aschaffenhausen, Germany) capillary column (30 m x 0.25 mm x 0.25 µm) and was programmed at 3 min isothermal time at 60°C, followed by heating with 6 K/min to 300°C. Temperature of the split/splitless injector was 270°C, splitless time was 60 s and helium carrier flow was 1.5 mL/min. The iontrap operated with electron impact ionization (emission current set to 9 µA) and in full scan mode, scanning from 35 to 650 amu. Interface temperature was 270°C and the manifold temperature 220°C. Extracts derived from solvent extraction and chemical degradation procedures were measured by injecting 0.5 or 1 µL. Prior to GC-MS analysis, an internal standard solution of 4-n-nonylphenol (n-NP) was added resulting in a concentration of 80 ng/µL n-NP. Quantification was performed by comparing integrals of two representative ions of the analyte with those of the internal standard. The limit of quan-

tification (signal-to-noise ratio of 10:1) was in the range of 23 ng NP/g clay calculated from GC-MS analysis of the reference compound. Regarding the influence of varying matrices, no attempts were made to quantify the NP isomer at concentrations less than 92 ng NP/g clay. Recoveries for the NP isomer, determined by spiking experiments on pure montmorillonite, were in the range of around 50%.

3.3 Results and Discussion

In preliminary incubation experiments, the distribution of the ^{14}C-labeled NP isomer among soil and organo-clay sub-fractions was studied. Results indicated on the one hand a very fast incorporation of NP isomer residues into sub-fractions of the silty clay loam soil on a short time scale (below 48 h) [Riefer et al., 2010], and on the other hand a predominate incorporation of the residues into organo-clay complexes during an experimental time of 180 days [Riefer et al., 2011].
In this work the non-extractable portion was examined by invasive and non-invasive methods to obtain a detailed insight into the incorporation processes of NP isomer residues into organo-clay sub-fractions Figure 3.1.

3.3.1 Structural characterization of NP isomer residues bound to humic acids derived from organo-clay complexes by ^{13}C-CP/MAS-NMR

To investigate the binding character and the chemical structure of NP isomer residues incorporated into humic matter, the aromatic ring was specifically labeled at the carbon atom directly bond to the hydroxyl moiety (C1). Humic acids were used since these sub-fractions showed the highest portion of incorporated residues [Riefer et al., 2011]. Results obtained from the ^{13}C-NMR measurements are displayed in Figure 3.2 as overlay spectra of humic acids derived from incubation days 1, 30, 90 and 180 including the spectra of the control sample (day 30).
Generally, the entire spectra correlated well with results published by other authors [Fabbri et al., 1998]. The most characteristic peak in all spectra was found for the aliphatic carbons (0-45 ppm). In the chemical shift section of O-alkyl carbons (45-110 ppm), the peak at about 57 ppm could be attributed to methoxyl carbons and that at about 72 ppm to ring carbons of carbohydrates. In the aromatic carbon section (110-160 ppm) the peak around 130 ppm resulted from C- and H-substituted aromatic carbons and around 150 ppm from O-substituted aromatic carbons such as phenols. The carboxyl section (160-220 ppm) showed a peak around 175 ppm which could be traced back to carboxylic, ester or amide carbons. The peak around 220 ppm could be related to carbonyl carbons [Baldock et al., 1992, Drever, 2005].
In the course of the total incubation period, we observed an increase of the peak area at 154 ppm. Due to different concentrations of ^{13}C-atoms in the samples (especially

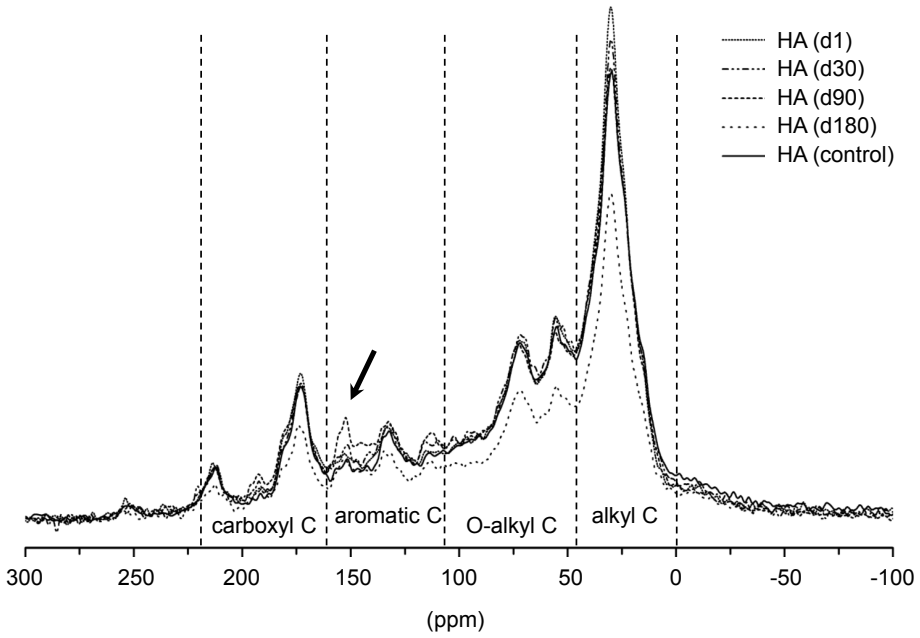

Figure 3.2: Overlay of spectra derived from measurements of the humic acids after 1, 30, 90 and 180 days of incubation (dashed lines) including the spectra of the control humic acid without NP isomer application (solid line) [Klausmeyer, 2011].

at day 180) a ratio of the peak area at 154 ppm to the total peak area of the spectra was calculated. Figure 3.3 shows that the concentration of carbon atoms exhibiting a chemical shift of 154 ppm increased during the entire incubation time. This specific shift is to be expected from the C-1 carbon of phenolic compounds. We thus concluded, that the ^{13}C-NMR peak at 154 ppm was due to residues of the introduced [1-^{13}C]-NP isomer containing an intact aromatic ring. Moreover, after 180 days of incubation the aromatic ring appeared to be still intact and incorporated as such into the humic acid fractions of the organo-clay complexes. Similar results were recently also obtained during incubation experiments with ^{13}C-sulfadiazine (1 g/kg soil) and soil with manure amendment [Junge et al., 2011].

Regarding incorporation processes of phenolic compounds into humic acids, non-covalent mechanisms like adsorption, hydrogen bonds or charge transfer complexes are possible [Senesi, 1992]. In this study, solvent extraction of the organo-clay complexes prior to humic matter fractionation was executed. Thus, concerning the incorporation as NP and its possible transformation products into humic materials, processes producing more stable associations such as sequestration or the formation of covalent linkages were more likely. Covalent coupling of NP isomer residues may be thought to occur mainly via the hydroxyl moiety resulting in the formation of ester or ether bonds. This coupling

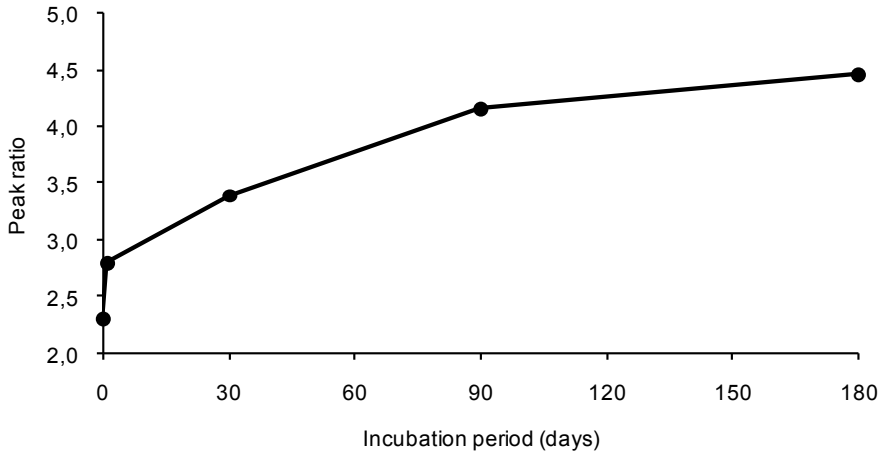

Figure 3.3: Peak ratio calculated from the peak area at 154 ppm and the area of the respective total spectrum [Klausmeyer, 2011].

should lead to an alteration of the electronic environment of the NP ring carbon C1 and thus, to a modified chemical shift in the NMR spectra as compared to the parent substance ^{13}C-NP. However, due to naturally occurring ^{13}C, peaks become comparatively broad in solid state NMR measurements and the sensitivity for the labeled compounds is considerably reduced. A calculation of the chemical shifts of possible binding modes (e.g. ester, ether) additionally showed, that the expected maximum modification of the C1 carbon of the NP isomer amounted to about 10 ppm was consequently too low for an unequivocal assignment to a specific binding mechanism.
Solid state NMR spectra can in some cases provide information on the structure and incorporation processes of xenobiotic bound residues. Because of the limiting factors mentioned above, in the present work, a sequential chemical degradation of the individual humic sub-fractions was performed additionally, to verify the results obtained by the NMR examination on the one hand, and to investigate the incorporation processes and chemical structure of non-extractable NP isomer residues in more detail on the other hand.

3.3.2 Incorporation processes of non-extractable (bound) NP isomer residues as indicated by sequential chemical degradation

Chemical degradation techniques were carried out by several authors to investigate the structure of geomacromolecules or the incorporation of low molecular weight compounds like PAHs or DDT [Richnow et al., 1997, 1992, Schwarzbauer et al., 2003]. In this study four degradation methods were executed sequentially. With each degradation step, the strength of the chemicals used to cleave and release incorporated residues was increased. Alkaline hydrolysis as well as BBr_3-treatment selectively cleave ester (amide) and ether

(ester) bonds, respectively. RuO_4 oxidizes aromatic rings and functionalized carbon atoms with a high specificity under mild conditions [Richnow et al., 1992, Stock and Tse, 1983]. RuO_4 has been applied in catalytic oxidation experiments of coals, oils, algae, lignite, and humic aids [Richnow et al., 1992, Stock and Tse, 1983, Blokker et al., 2000, Válková et al., 2009]. TMAH thermochemolysis combines efficient pyrolytical cleavage with subsequent methylation of functionalized groups. Oxidation and thermochemolysis are both abrasively methods which were carried out to degrade the macromolecular structure and thus, release strongly incorporated residues.

Radioactive balancing of released residues and temporal incorporation behavior within individual organo-clay sub-fractions

Portions of radioactivity released after each treatment are given in Figure 3.4, corresponding numerical values are summarized in Table 3.2. Percentages refer to the initial amount of radioactivity in the individual humic sub-fractions.
In terms of alkaline hydrolysis, the entire radioactivity incorporated in FA and HA associated with the organo-clay complexes was released in case of the samples obtained after one day of incubation. During the following six days of incubation, releasable ^{14}C decreased to 49% (FA) and 70% (HA) of the initial amount of ^{14}C in the individual humic fraction. Thereafter, a slight decrease until 180 days of incubation could be observed. Hydrolysis of the HU fraction released a lower portion of ^{14}C (60%) after one day of incubation. This indicated a qualitatively different initial incorporation as compared to the radioactivity associated with FA and HA fractions. During the entire incubation period, the highest portion of releasable radioactivity was found for the HA fraction (average of 67%). Subsequent cleavage of ether (ester) bonds by BBr_3-treatment [Bhatt and Kulkarni, 1983] released a comparatively lower amount of radioactivity as alkaline hydrolysis. An average portion of 9% of the initial amount of ^{14}C in the individual fraction was released from HU, 7% from the FA and 3% from the HA fraction during the entire incubation period. The lowest releasable portions of radioactivity were found after 180 days of incubation, which could have been a result of ageing (e.g. modification of the incorporation mode, residue transformation). Results of BBr_3-treatment indicated that ether bonds are of minor importance for the formation of non-extractable residues of the NP isomers and that preceding cleavage of ester bonds by means of alkaline hydrolysis was already almost complete. The final oxidation reaction and thermochemolysis released the lowest portions of radioactivity among the executed degradation methods. In the course of incubation time, thermochemolytical cleavage showed no distinct trend in terms of releasable radioactivity. However, oxidative releasable ^{14}C decreased until day 180 indicating also ageing processes of the incorporated residues.
Schwarzbauer *et al.* investigated the linkages of DDT and several metabolites to a sediment taken from the Teltow Canal in Germany. In contrast to our results, they re-

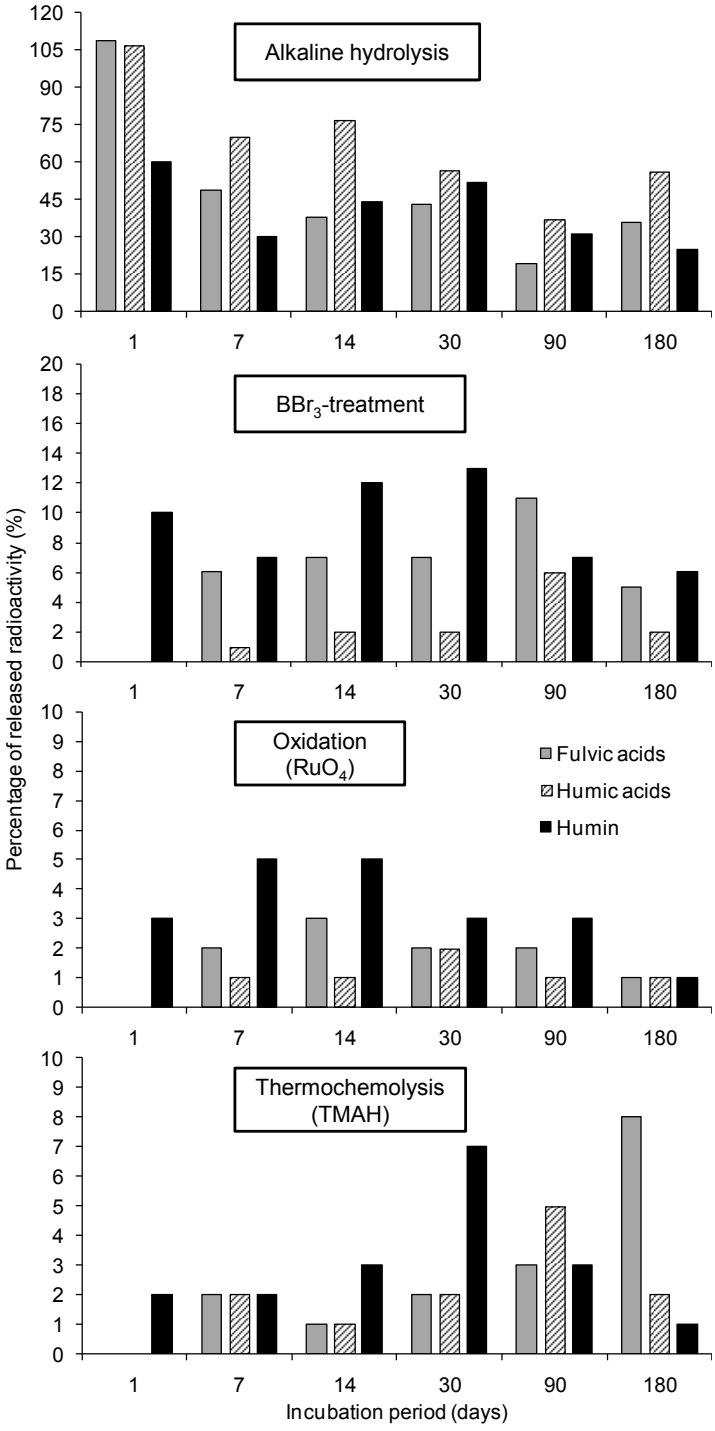

Figure 3.4: Radioactivity extractable in the course of incubation after sequential chemical degradation. Percentages refer to total radioactivity in the individual organo-clay sub-fractions before treatment. Grey bars: ^{14}C released from fulvic acids (FA); striped bars: ^{14}C released from humic acids (HA), black bars: ^{14}C released from humin (HU).

Table 3.2: Percentage of radioactivity released after chemical treatment (Percentages refer to the initial amount of ^{14}C in the individual pre-extracted humic subfractions).

Humic fraction	Incubation period	STEP 1 Hydrolysis KOH/water/ methanol	STEP 2 BBr_3	STEP 3 Oxidation RuO_4	STEP 4 Pyrolysis TMAH	Recovery	Residual $^{14}C^{(a)}$
	(days)	(%)	(%)	(%)	(%)	(%)	(%)
FA	1	109	-	-	-	109	
	7	49	6	2	2	59	
	14	38	7	3	1	49	26
	30	43	7	2	2	54	
	90	19	11	2	3	35	
	180	36	5	1	8	50	18
HA	1	107	-	-	-	107	
	7	70	1	1	2	74	
	14	77	2	1	1	81	10
	30	57	2	2	2	63	
	90	37	6	1	5	49	
	180	56	2	1	2	61	11
HU	1	60	10	3	2	75	
	7	30	7	5	2	44	
	14	44	12	5	3	64	29
	30	52	13	3	7	75	
	90	31	7	3	3	44	
	180	25	6	1	1	33	22

(a) determined by combustion

leased significant portions of DDT and DDT derived metabolites after BBr_3 and RuO_4-treatment [Schwarzbauer et al., 2003]. It should be noted that the incorporation of the xenobiotic into sediments occurred under anoxic conditions, and that these sediments were contaminated with DDT and synthesis byproducts for decades. Compounds within these sediments underwent long ageing processes, which required harsh methods in order to release residues. Our investigations derived from incubation experiments of up to 180 days may indicate that strongly incorporated residues are formed only after long-term ageing. This hypothesis is supported by our study showing a decrease of releasable ^{14}C during the entire incubation time.

In order to elucidate, whether the degradation sequence performed could cover the whole spectrum of possible modes of incorporation of NP isomer derived residues into humic fractions, ^{14}C recoveries for the entire chemical degradation procedure (Table 3.2) were determined by comparing the amount of radioactivity of the individual humic fractions

before treatment with amounts released during the degradation steps. Total recoveries ranged from 33% to 109% with an average of 63%. Disregarding losses of ^{14}C during sample preparation, we found that high percentages of the initial radioactivity were not released by the sequential chemical degradation performed and therefore still remained incorporated into the organo-clay sub-fractions.

Structure elucidation of non-extractable NP isomer residues derived from organo-clay complexes

The use of ^{14}C-labeled compounds facilitates the quantitation of extractable and non-extractable residues. However, the labeling itself provides no information about the structure of the incorporated substances which is a precondition for the assessment of environmental risks. In the solvent extractable fraction, we have shown recently that only the parent compound was detectable [Riefer et al., 2011]. In contrast to this finding, Telscher *et al.* and Zhang *et al.* identified a nitro-metabolite as transformation product after application of a single NP isomer to soil [Telscher et al., 2005, Zhang et al., 2009]. However, they did not differentiate between individual particle and humic fractions or address non-extractable residues.

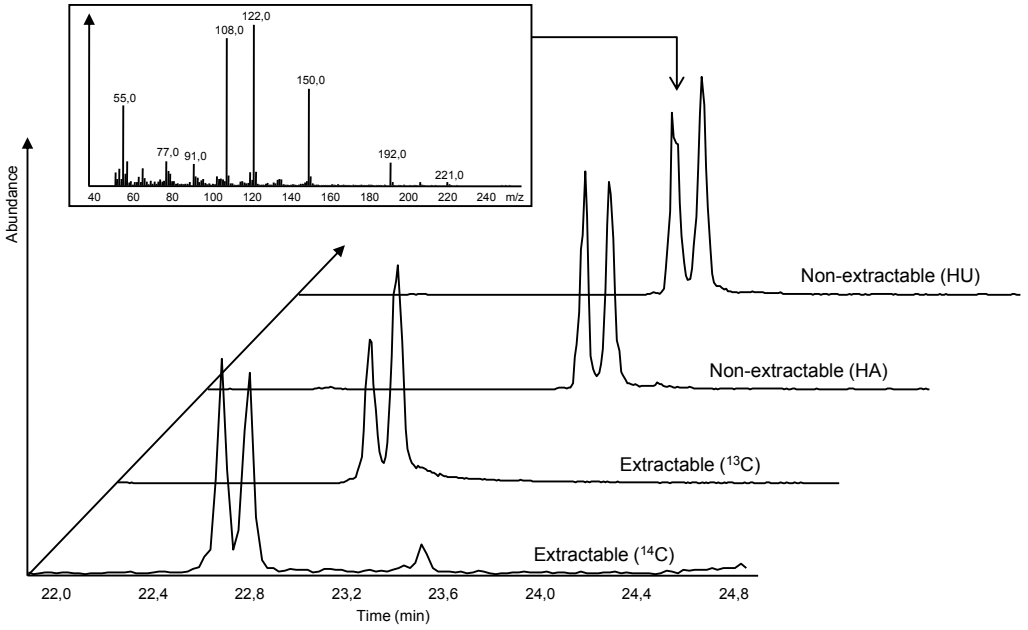

Figure 3.5: Examples of gas chromatograms of the extractable portions after exhaustive Soxhlet extraction (^{14}C: 0.125 g NP/kg soil, ^{13}C: 1 g NP/kg soil) and the non-extractable portion released after alkaline hydrolysis from humic acids (HA) and humin (HU). Peaks were identified by their mass spectra as the applied NP isomer.

In the present study, the radioactivity released after chemical degradation were also exam-

ined by GC-MS and Radio-HPLC. In case of the ester (amide) bond cleavage procedure, only the parent NP isomer was identified in the HA and HU fractions (Figure 3.5). Non-target screening of the GC-MS analysis revealed no liberated transformation products of the NP isomer. Possible metabolites were either not detectable due to structural similarity with natural components of soil organic matter (e.g. phenolic moieties) [Corvini et al., 2006], or their concentration was below the limit of detection. In the extracts derived from ether bond cleavage, oxidation and thermochemolysis, neither the parent compound nor metabolites could be identified.

Mode of incorporation of NP isomer residues into organo-clay sub-fractions and their significance for the environment

As mentioned before, ether bond cleavage (average releasable amount of 7% of the initial ^{14}C within humic fractions), oxidative (3%) and thermochemical degradation (3%) were of minor importance for the release of non-extractable NP isomer derived residues. The highest portion of radioactivity was released after cleavage of ester (amide) bonds with an average amount of 52%. This high amount could be an evidence that besides sequestration the formation of covalent bonds played a major role in the formation of non-extractable residues. An incorporation into the humic fractions was detected already after one day of incubation. In FA and HA, this incorporation proceeded completely via ester bonds. The humin fraction (HU) consists of organic material and clay minerals. X-Ray diffractometry showed that 85% of these clay minerals belonged to the smectite group. Smectites are swellable 2:1 layered silicates. In addition to functional groups on their surfaces, these minerals contain exchangeable cations within the interlayers. Ionic as well as polar, non-ionic xenobiotics can interact with the cations either directly or through water bridges [Cornejo et al., 2008, Kulshrestha et al., 2004, Wang et al., 2009]. The lower portion of ^{14}C released after alkaline hydrolysis from HU after one day of incubation may be traced back to such an intercalation of residues into the clay interlayers. Ageing processes including possible movement of residues into cavities of the organic material not easily accessible, or a change of the character of bonds as well as microbial degradation and incorporation of transformation products could result in the decrease of releasable ^{14}C observed in the further course of incubation. Figure 3.6 displays the most significant incorporation processes.

It was quite obvious that the applied NP isomer was predominantly incorporated via ester (amide) bonds. However, the formation of hydrolysable, especially ester bonds is reversible and cleavage occurs also in nature (e.g. microbially). As consequence of such an incorporation, compounds (e.g. NP isomer or its transformation products) can be released more or less easily and thus, they may be regarded as "bioaccessible" non-extractable residues which may impose a risk for the environment.

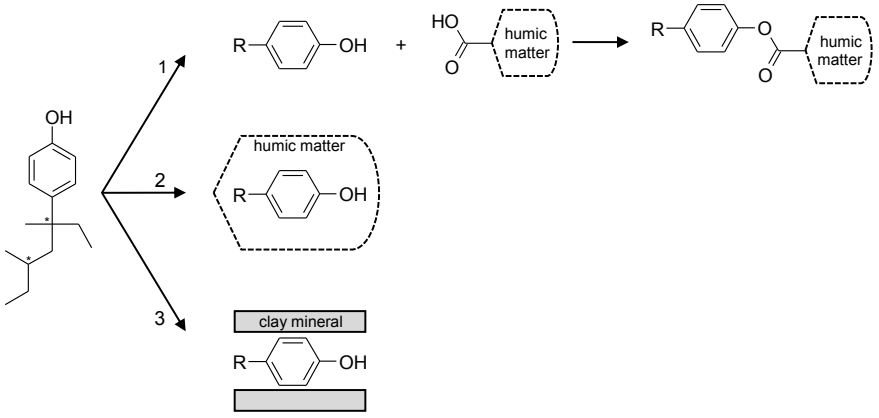

Figure 3.6: Presumed mode of incorporation of the identified NP isomer: 1) Formation of covalent ester bonds between the NP isomer and functional groups of humic matter. Released directly by cleavage of ester bonds 2) Sequestration of the NP isomer in cavities of humic matter. Released indirectly by cleavage of ester bonds of humic matter. 3) Intercalation of the NP isomer in interlayers of the clay minerals. Unaffected by chemical treatment.

Quantitation of extractable and non-extractable residues to verify the incorporation

In order to obtain further information on the incorporation, the percentage of accordance of the actual amount of NP as determined by GC-MS (NP_{GC-MS}) and the amount calculated from radioactive balancing [Riefer et al., 2011] as maximum identifiable NP (NP_{MAX}) in the respective sample was calculated. In the following this accordance will be designated as (NP_{GC-MS}/NP_{MAX})-percentage. Values of about 100% would point to an incorporation of radioactivity mainly via the parent NP isomer, whereas values significantly below would indicate that only a part of the radioactivity was due to the parent. Previous work showed that the concentration of the applied ^{13}C-labeled NP influenced the microbial activity of soil, but as compared to recently published data the activity of these samples were still in range of natural soil samples [Riefer et al., 2011, Sparling and Searle, 1993]. Hence, we supposed a similar behavior of the ^{13}C and ^{14}C-labeled NP during the incubation experiments. Table 3.3, 3.4, and 3.5 show (NP_{GC-MS}/NP_{MAX})-percentages for the extractable and (liberated) non-extractable fractions. Generally, (NP_{GC-MS}/NP_{MAX})-percentages were found to be considerably low. Moreover, values showed a high inconsistency and no distinct trend during incubation. (NP_{GC-MS}/NP_{MAX})-percentages from the ^{14}C-labeled extractable samples (Table 3.3) ranged from 5% to 14% and from the ^{13}C-labeled extractable samples (Table 3.4) from 15% to 71%. The lowest (NP_{GC-MS}/NP_{MAX})-percentages were determined with the ^{13}C-labeled non-extractable samples derived from the alkaline hydrolysis (Table 3.5), ranging from 1% to 4%.

The low (NP_{GC-MS}/NP_{MAX})-percentages in our experiments correspond with results

published by Kaestner *et al.* [Kaestner et al., 1999]. The authors investigated the formation of bound residues of ^{14}C-anthracene in soil. Only small amounts of non-extractable residues could be traced back to the PAH. They suggested different pathways leading to the incorporation of ^{14}C into humic sub-fractions. First, the applied parent compound or identifiable metabolites may have been incorporated biotically or abiotically into the humic matrix either by sequestration or by formation of bound residues, which can be released by chemical treatment. In our experiments, this portion represents the residues clearly identified. Secondly, Kaestner *et al.* suggested an incorporation of unidentified structures as residues complying to the definition of bound residues or as (parts of) structures integrated into humic matter or assimilated into biomass. A third possible incorporation process can be the fixation of microbially produced $^{14}CO_2$ into the soil matrix [Kaestner et al., 1999]. In addition to the above mentioned processes, we assumed that the radioactivity extracted consisted of residues not released as freely available compounds but incorporated into oligomers of the humic matrix as consequence of the cleavage of several binding sites in the organic matter during chemical treatment. This assumption was supported during the necessary sample preparation for GC-MS analysis. Initially dissolved ^{14}C was found in the precipitate after concentration. Further extraction with organic solvents did not release incorporated radioactivity. Besides this precipitation process, which possibly removed bound NP residues from the extracts, the incorporation into oligomeric but soluble matter may have prevented the final detection via GC-MS due to e.g. low volatility. Hence, these NP residues led to the high discrepancies in the amounts of detectable defined compounds and the measured radioactivity. Helling and Krivonak stated that residues remaining fixed to solubilised fulvic or humic acids still have to be considered as bound, since they are not in a discrete and chemically identifiable form [Helling and Krivonak, 1978].

3.4 Conclusion

In this study, we investigated the incorporation processes leading to the formation of non-extractable NP isomer residues in soil derived organo-clay complexes after distinct incubation periods. The preferred release of residues after alkaline hydrolysis led to the assumption of a linkage via ester (amide) bonds, an sequestration into cavities of the humic material, or the intercalation into clay mineral interlayers. Due to the rather reversible character of hydrolysable bonds as compared to ether or *C-C*-linkages, biogenic degradation or the change of soil-pH could lead to a release of non-extractable (bound) residues into the environment and thus, they may still be considered as a potential risk. It could be shown that the applied parent NP isomer persisted in soil and particularly in organo-clay complexes as extractable and non-extractable residue over an incubation period of 180 days. The high discrepancies between the amount of the NP

isomer calculated from radioactivity measurements and the values obtained by GC-MS based quantitation suggested further complex incorporation processes (e.g. not identified transformation products, part of biomass or organic macromolecule, linked to oligomers) which were not traceable by the executed methods. With the comprehensive application of complementary analytical methods (e.g. humic matter fractionation, ^{13}C-CP/MAS-NMR, sequential chemical degradation) it was possible to obtain a comparatively detailed insight into the mode of incorporation of the applied NP isomer.

Table 3.3: Extractable ^{14}C-labeled NP isomer.

Incubation period	Amount ^{14}C-NP isomer[(a)]	Mass of organo-clay complexes	NP isomer per gram organo-clay complex[(b)]	Percentage of applied ^{14}C[(c)]	Calculated amount of ^{14}C-NP isomer[(d)]	($NP_{GC\text{-}MS}$ / NP_{MAX})-percentages[(d)]
(days)	(µg)	(g)	(µg NP/g clay)	(%)	(µg)	(%)
1	3.17	4.55	0.70	34.11	25.58	12.41
7	0.56	5.19	0.11	13.75	10.31	5.45
14	1.64	5.74	0.29	16.24	12.18	13.45
30	1.50	3.94	0.38	17.69	13.27	11.34
90	0.37	3.53	0.11	9.97	7.48	4.96
180	tr	3.44	-	3.23	2.42	-

(a) Actual amount of ^{14}C-NP in the solvent extract as determined by GC-MS (NP_{GC-MS}).
(b) Quotient of ^{14}C-NP amount and mass of organo-clay complexes.
(c) Percentages of applied ^{14}C in the solvent extract according to recently published results [Riefer et al., 2011].
(d) Maximum identifiable amount of ^{14}C-NP (NP_{MAX}) as calculated from the determined percentage of applied ^{14}C in the solvent extract. (e) Percentages of accordance between the actual amount of NP as determined by GC-MS and the calculated maximum identifiable amount. For the calculation, the latter was set to 100% each.
tr = trace amount (below the limit of quantitation)

Table 3.4: Extractable ^{13}C-labeled NP isomer.

Incubation period	Amount ^{13}C-NP isomer[(a)]	Mass of organo-clay complexes	NP isomer per gram organo-clay complex[(b)]	Percentage of applied ^{14}C[(c)]	Calculated amount of ^{13}C-NP isomer[(d)]	($NP_{GC\text{-}MS}$ / NP_{MAX})-percentages[(e)]
(days)	(mg)	(g)	(mg NP/g clay)	(%)	(mg)	(%)
1	2.47	2.89	0.85	34.11	17.06	14.47
7	4.89	5.48	0.89	13.75	6.88	71.16
14	2.25	3.03	0.74	16.24	8.12	27.74
30	2.22	3.64	0.61	17.69	8.85	25.10
90	2.09	3.47	0.60	9.97	4.99	41.99
180	0.26	3.70	0.07	3.23	1.62	16.26

(a) Actual amount of ^{13}C-NP in the solvent extract as determined by GC-MS (NP_{GC-MS}).
(b) Quotient of ^{13}C-NP amount and mass of organo-clay complexes.
(c) Percentages of applied ^{14}C in the solvent extract according to recently published results [Riefer et al., 2011].
(d) Assuming the same behavior of the ^{13}C and ^{14}C-NP isomer in soil, the maximum identifiable amount of ^{13}C-NP (NP_{MAX}) was calculated from the determined percentage of applied ^{14}C in the solvent extract.
(e) Percentages of accordance between the actual amount of NP as determined by GC-MS and the calculated maximum identifiable amount. For the calculation, the latter was set to 100% each.

Table 3.5: Alkaline hydrolysis of samples incubated with the ^{13}C-labeled NP isomer.

Incubation period (days)	Humic fraction	Amount ^{13}C-NP isomer[a] (mg)	Mass of organo-clay complexes (g)	NP isomer per gram organo-clay complex[b] (mg NP/g clay)	Percentage of applied ^{14}C[c] (%)	Calculated amount of ^{13}C-NP isomer[d] (mg)	(NP_{GC-MS}/ NP_{MAX})-percentages[e] (%)
1	HA	0.091	2.89	0.031	4.15	2.08	4.38
	HU	0.051		0.018	3.48	1.74	2.92
7	HA	0.140	5.48	0.025	13.35	6.68	2.09
	HU	0.102		0.019	10.20	5.10	2.00
14	HA	0.102	3.03	0.034	12.20	6.10	1.67
	HU	0.067		0.022	5.32	2.66	2.53
30	HA	0.061	3.64	0.017	13.12	6.56	0.93
	HU	0.084		0.023	5.65	2.83	2.96
90	HA	0.069	3.47	0.020	14.88	7.44	0.93
	HU	0.061		0.018	8.90	4.45	1.37
180	HA	0.073	3.70	0.020	14.71	7.36	0.99
	HU	0.046		0.012	8.68	4.34	1.06

(a) Actual amount of ^{13}C-NP in the extract of alkaline hydrolysis as determined by GC-MS (NP_{GC-MS}).
(b) Quotient of ^{13}C-NP amount and mass of organo-clay complexes.
(c) Percentages of applied ^{14}C in humic sub-fractions according to recently published results [Riefer et al., 2011].
(d) Assuming the same behavior of the ^{13}C and ^{14}C-NP isomer in soil, the maximum identifiable amount of ^{13}C-NP (NP_{MAX}) was calculated from the determined percentage of applied ^{14}C in the distinct humic fractions.
(e) Percentages of accordance between the actual amount of NP as determined by GC-MS and the calculated maximum identifiable amount. For the calculation, the latter was set to 100% each.

4 Mode of incorporation of the herbicide MCPA in soil derived organo-clay complexes examined by specific degradation of humic materials

ABSTRACT. The incorporation of xenobiotics into soil, especially via covalent bonds or sequestration has a major influence on their toxicity, mobility and bioavailability in the environment. Thus, the mode of incorporation of MCPA (4-chloro-2-methylphenoxyacetic acid) into organo-clay complexes has been investigated under a low level (8.5 mg MCPA/kg soil) and high level (1,000 mg MCPA/kg soil) concentration, during an incubation period of 120 days. Emphasis was laid on the elucidation of distinct covalent linkages between non-extractable MCPA residues and humic sub-fractions (humic acids, fulvic acids and humin). The release of compounds by a sequential chemical degradation procedure (1. alkaline hydrolysis, 2. BBr_3, 3. oxidation, 4. thermochemolysis) revealed ester (amide) bonds as the preferred mode of incorporation followed by ether linkages. A possible influence of the microbial activity of the soil on the mode of incorporation was observed. Structure elucidation identified MCPA as the only non-extractable substance, whereas the metabolite 4-chloro-2-methylphenol was additionally found as water extractable (bioavailable) and organic solvent extractable (bioaccessible) compound.

4.1 Introduction

Each year more than two million tons of pesticides are used worldwide predominantly in agriculture [Alavanja, 2009]. Among the different types, herbicides playing the major role followed by insecticides and fungicides [Kiely et al., 2004]. Phenoxy acid herbicides, such as 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-chloro-2-methylphenoxyacetic acid (MCPA), have been widely used for the post-emergent control of broad leaf weeds since the middle of the 20th century [Roberts et al., 1998, Thorstensen and Lode, 2001]. Usually, these compounds are formulated as esters and inorganic or amine salts which are rapidly converted in soil to their acidic form. Main degradation pathways of the active compound are microbially induced. Moreover, loss of the applied herbicide occur also

through volatilization, photolysis and leaching processes [Smith and Hayden, 1980].
MCPA as a selective, phytohormone-type herbicide inhibits the growth of plants in the same manner as natural auxins [Roberts et al., 1998]. Once released into the environment it is rapidly degraded showing half-life values between 7-41 days [Foster and McKercher, 1973, EU, 2008]. The rate of degradation depends on several factors, such as soil pH, moisture content, type of soil or the applied concentration [Sattar and Paasivirta, 1980]. Degradation pathways of MCPA in soil lead via 4-chloro-2-methylphenol as comparatively stable metabolite, to more reactive metabolites which are immediately transformed to harmless forms [Crespín et al., 2001]. In contrast to the parent compound which shows only minor toxic effects, 4-chloro-2-methylphenol was found to be highly toxic to aquatic organisms [OECD-SIDS, 1998].
To assess the risk of soil applied pesticides and their metabolites, detailed knowledge of the processes responsible for the transformation and the formation of both, extractable and non-extractable (bound) residues are required. As compared to extractable compounds, non-extractable residues (NER) are thought to exhibit a reduced toxicity and bioavailability and the potential to be (further) microbially degraded [Gevao et al., 2000]. NER are characterized by interactions with soil organic matter or mineral surfaces [Stevenson, 1994]. Depending on the mode of interaction, covering the range from weak adsorptive to reversible or irreversible covalent bonds [Senesi, 1992, Bollag et al., 1992, Dec and Bollag, 1997], NER can eventually be solubilized by natural processes (e.g. extracellular enzymes); thus, the parent compound or toxicologically relevant metabolites may become remobilized and bioavailable again. The formation of non-extractable residues can also result from entrapment (sequestration) in cavities of the macromolecular structure [Dec and Bollag, 1997].
Among the different soil components, organo-clay complexes are an important sink for anthropogenic substances, since they show unique physicochemical properties (e.g. surface functional groups, exchangeable interlayer cations, surface area) and are the preferential association site for soil organic matter [Wang et al., 2005, Cox et al., 1997]. Soil organic material itself contains numerous functional groups which are acting as reaction sites also for foreign compounds. Up to now, the fate and degradation of MCPA in soil were well investigated [Roberts et al., 1998, Foster and McKercher, 1973, Crespín et al., 2001, Jensen et al., 2004]. Nevertheless, information on the detailed mode of incorporation is lacking regarding the distinct (physico-)chemical linkages between (non-extractable) MCPA residues and organo-clay complexes. In order to obtain information on the incorporation processes of MCPA, a sequential chemical degradation of its non-extractable residues was executed in the present study. According to this procedure, the release of MCPA derived chemical structures from humic macromolecules was thought to occur either by degradation of the macromolecule resulting in unaltered residues incorporated by non-covalent bonds and sequestration, or by cleavage of true covalent bonds between

the organic matrix and the residues. As result of four consecutive degradation steps (i. ester (amide) cleavage, ii. ether (ester) cleavage, iii. oxidation, and iv. thermochemolysis) we hope to obtain sufficient information in order to draw conclusions regarding the nature of non-extractable MCPA residues, to predict the corresponding re-mobilization potential and hence, to evaluate the risk of these non-extractable residues for the environment.

Incubation experiments in soil were carried out with two different concentrations using ^{14}C-labeled and non-labeled MCPA. After incubation periods of up to 120 days, the organo-clay complexes were separated from other soil components. The complexes were first extracted with organic solvents; secondly humic sub-fractions (humic acids, fulvic acids, humin) were prepared, which were subjected to the degradation methods mentioned before. Released MCPA residues were extracted and analyzed by means of liquid scintillation counting (LSC), Radio-HPLC and GC-MS. The aim of the present study was to i) investigate the impact of the applied concentration on the incorporation ii) balance the releasable portion of the residues during each degradation step, iii) follow the dynamic behavior of specific bonds during the incubation period, and iv) elucidate the chemical nature of incorporated compounds.

4.2 Materials and Methods

4.2.1 Soil

Soil used for the experiments originated from an Ap horizon of a (Gleyic) Podzol, collected in Fuhrberg (located near Hannover, Germany). The sandy loam was air-dried, sieved to obtain particles ≤ 2 mm and stored at 4°C in the dark until use. Main soil characteristics are summarized in Table 4.1. X-ray diffraction analysis revealed a mineral composition of the clay fraction of 85.5% smectite, 8.4% kaolinite, 2.7% muscovite and 3.4% quartz.

Table 4.1: Properties of the soil utilized for the experiments.

Particle distribution			TOC[(a)]				WHC_{max}[(b)]	pH (soil)[(c)]
sand	silt	clay	sand	silt	clay	soil		
(%)	(%)	(%)	(%)	(%)	(%)	(%)	(g H_2O/100 g soil)	
57.2	30.7	12.1	2.1	13.1	14.8	5.7	58.3	5.4

(a) TOC: total organic carbon (b) WHC_{max}: soil maximum water holding capacity
(c) $CaCl_2$ method

4.2.2 Chemicals

[Ring-U-^{14}C] 4-chloro-2-methylphenoxyacetic acid (MCPA, 59.94 MBq/mmol) was provided by Prof. M. H. Gerzabek (University of Vienna) as a mixture of 92% MCPA methyl

ester and 8% of the free acid. Non-labeled MCPA, MCPA methyl ester, and 2-methyl-4-chlorophenol (MCP) were purchased from Sigma-Aldrich (Taufenkirchen, Germany). Other (non-labeled) chemicals were obtained from Sigma-Aldrich (Taufenkirchen, Germany), Merck (Darmstadt, Germany), and ABCR (Karlsruhe, Germany). Solvents were distilled, and their purity was controlled by gas chromatography before use.

4.2.3 Incubation experiments

Incubation experiments were carried out by applying separately two concentrations of MCPA to the air-dried soil. A detailed description of the application and incubation methods has been published recently [Klausmeyer, 2011]. Briefly, in case of the low level samples, 842 µg (0.25 MBq) ^{14}C-labeled MCPA dissolved in methanol was applied to 100 g of air-dried soil, leading to a concentration of 8.5 mg MCPA/kg soil. In case of the high level samples 50 mg (49.16 mg non-labeled and 0.84 mg ^{14}C-labeled) MCPA (0.25 MBq) dissolved in methanol was applied to 50 g air-dried soil, leading to a concentration of 1 g MCPA/kg soil. After application, the solvent was immediately evaporated and the flasks were shaken for 15 min by means of an overhead shaker. Deionized water was added to adjust the samples to 60% of the maximum water holding capacity (WHC_{max}). Incubation was executed in the dark at 20°C. All experiments were conducted in duplicate. Sample preparation was performed after 1, 7, 14, 30, 60 and 120 days of incubation. The microbial activity of the soil samples was determined immediately after sample drawing by means of the DMSO reduction assay according to Alef und Kleiner [Alef and Kleiner, 1989].

4.2.4 Degradation procedures

Sequential chemical degradation was performed with the separated humic fractions. To achieve fulvic acids (FA), humic acids (HA) and humin (HU), organo-clay complexes were separated from soil by a wet sieving and ultrasonification procedure. In order to release extractable portions of radioactivity, the clay fraction was extracted with methanol and dichloromethane by means of a Soxhlet apparatus. The humic materials contained in the extracted organo-clay complexes were subsequently fractionated according to the conventional alkaline separation procedure. A detailed description of the methods executed were described [Klausmeyer, 2011]. The workflow of sample preparation is displayed in Figure 4.1.

4.2.5 Alkaline hydrolysis

Humic sub-fractions were separately filled into glass vessels (100 ml) and suspended in 15 mL of a mixture of methanol, water and potassium hydroxide (1:1.2:250 v/v/w). After closing the vessel, the 2 M alkaline mixture was heated for 24 h at 105°C, and

then acidified to pH=2 with HCl. Solubilized MCPA residues were extracted with 15 mL diethyl ether by shaking the vessel for 10 min at 190 rpm on a horizontal shaker (GFL 3017, Burgwedel, Gemany). Subsequently, the sample was centrifuged for 3 min at 600 x g using a J20 XPI centrifuge (Beckman Coulter, CA, USA). The organic layer was removed. Remaining solid materials were re-suspended in 15 mL diethyl ether, and after shaking the supernatant was decanted. The procedure was then repeated twice using each 15 mL ethyl acetate. All organic layers were combined, the solution was dried using anhydrous sodium sulfate and filtered. The crude extract was concentrated to approx. 0.5 mL by means of a rotary evaporator. The resulting concentrate was separated by column chromatography on 2 g of 40 µm silica gel (Baker) using as eluents consecutively 5 mL n-pentane/dichloromethane (60:40 v/v) (fraction 1), 5 mL dichloromethane (fraction 2), and 5 mL methanol (fraction 3). All fractions were examined by liquid scintillation counting and GC-MS.

4.2.6 Boron tribromide treatment

Remainings of the humic sub-fractions derived from alkaline hydrolysis were neutralized with NaOH solution. Then, the sample was dried at 50°C and 15 mL of a 1 M boron tribromide in dichloromethane was added. The vessel was closed and treated for 15 min in an ultrasonic bath. After shaking for 10 min using a horizontal shaker (170 rpm) the entire procedure was repeated twice. Subsequently, the mixture was shaken for 24 h at room temperature. In order to terminate the reaction, the solution was cooled to 0°C and 10 mL of deionized water was added. The mixture was extracted using 10 mL diethyl ether and shaking for 10 min at 190 rpm. Then, the sample was centrifuged for 3 min at 600 x g. The organic layer was removed and the remaining aqueous suspension extracted another two times with 15 mL diethyl ether, each. After combining the organic layers, the solution was dried using anhydrous sodium sulfate and filtered. The crude extract was concentrated to approx. 0.5 mL and the resulting concentrate was separated by column chromatography using consecutively 5 mL n-pentane/dichloromethane (95:5 v/v) (fraction 1), 5 mL dichloromethane (fraction 2), and 5 mL methanol (fraction 3). All fractions were examined by liquid scintillation counting and GC-MS.

4.2.7 Ruthenium tetroxide oxidation

Remainings of the humic sub-fractions obtained after BBr_3-treatment were dried at 50°C. To each sample, 10 mg of RuO_4 and 500 mg of $NaIO_4$, 8 mL CCl_4, 8 mL acetonitrile and 1 mL deionized water were added. The vessel was closed and shaken for 4 h at 170 rpm using a horizontal shaker. Reaction was terminated by adding 50 µL MeOH and 2 drops of concentrated H_2SO_4. The sample was centrifuged at 600 x g for 3 min and the organic layer was removed. The precipitate was washed using 8 mL of

CCl_4, centrifuged, and the supernatant was removed. After combining the organic layers, 5 mL of deionized water was added and the mixture was immediately shaken for 3 min at 190 rpm. The aqueous layer was removed and immediately extracted three times with 5 mL diethyl ether. Both, the diethyl ether and CCl_4 extracts were combined and dried with anhydrous sodium sulfate. After filtration and concentration to a volume of approx. 0.5 mL a saturated $Na_2S_2O_3$ solution (0.5 mL) was added. The organic layer was removed, dried with anhydrous sodium sulfate, and filtered. The resulting crude extract was concentrated to approx. 0.5 mL and separated by means of column chromatography using 5 mL dichloromethane (fraction 1) and a mixture of 2 mL diethyl ether and 3 mL methanol (fraction 2). All fractions were examined by liquid scintillation counting and GC-MS.

4.2.8 Thermochemolysis (TMAH)

Samples derived from RuO_4-treatment were separately filled in a digestion bomb containing a 45 mL teflon tube. Then, 5 mL of a 25% methanolic tetramethylammonium hydroxide (TMAH) solution was added. After thorough suspension in an ultrasonic bath, methanol was removed by a gentle stream of nitrogen and the bomb closed. The pasty suspension was heated for 2 h at 270°C. After cooling the sample to -18°C, 10 mL of diethyl ether was added to extract compounds liberated from humic macromolecules. The sample was ultrasonically treated for 5 min and the organic layer was removed. The extraction procedure was repeated once using another 10 mL diethyl ether, twice using 10 mL dichloromethane, each, and twice using 10 mL n-hexane, each. After combining all organic extracts, the resulting solution was dried using anhydrous sodium sulfate and filtered. The crude extract was concentrated to approx. 0.5 mL and fractionated by column chromatography using as eluents consecutively 5 mL n-pentane/dichloromethane (95:5 v/v; fraction 1), 5 mL dichloromethane (fraction 2), and 5 mL methanol (fraction 3). All fractions were examined by liquid scintillation counting and GC-MS.

4.2.9 Analytical methods

Radioanalysis

Portions of radioactivity contained in all extracts were determined (in duplicate) by liquid scintillation counting (LSC) using a LS 6500 TD analyzer (BeckmanCoulter, Krefeld, Germany) and Lumasafe Plus scintillation cocktail (PerkinElmer, Rodgau, Germany). The ^{14}C content of solid samples (four replicates per sample) was determined by combustion in a biological oxidizer OX 500 (Harvey Instruments/Zinsser, Frankfurt, Germany).

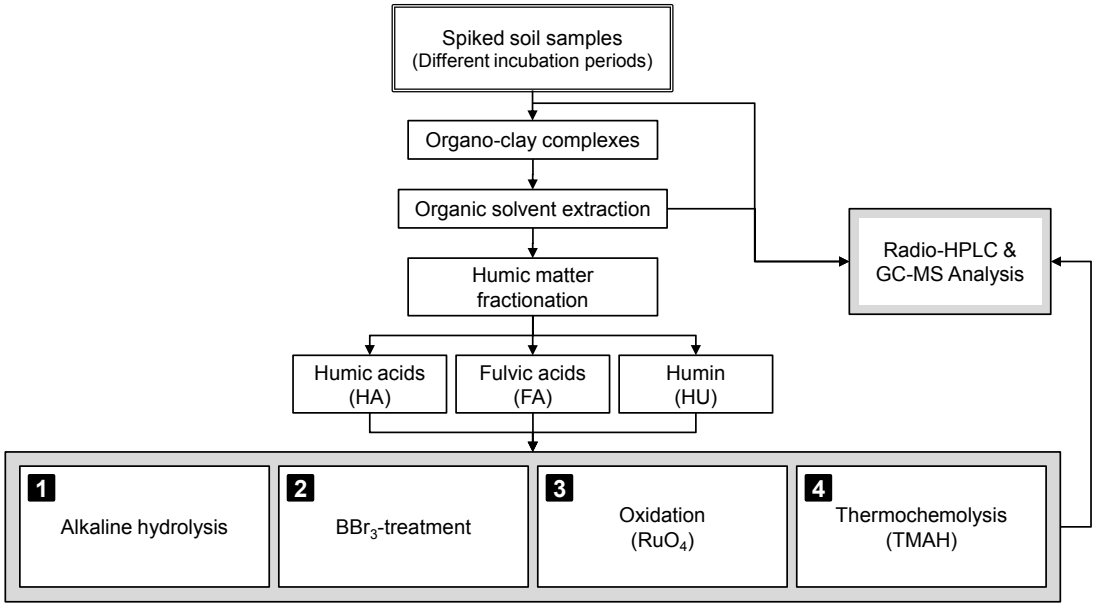

Figure 4.1: Workflow of sample preparation.

HPLC

Chromatographic characterization of extracted and released radioactivity was executed by HPLC using available reference compounds. HPLC analyses were performed at 35°C using a HP 1100 (Agilent, Waldbronn, Germany) and a Nucleodur 100-5 C18 ec column (250 x 4.0 mm, 5 µm; Macherey-Nagel, Düren, Germany). Gradient elution was programmed as follows: A (water + 0.2% acetic acid, v/v) and B (MeOH + 0.2% acetic acid; v/v): A/B 60:40 (v/v) for 5 min, linear 15 min gradient to 95% B, isocratic B (95%) for 5 min, and return to initial conditions within 5 min; flow rate 0.8 mL/min. The system was connected to a Ramona Star radiodetector equipped with a 1655 quartz cell (glass, 32-54 µm; internal diameter: 4.0 mm; volume: 0.4 mL; Raytest, Straubenhardt, Germany). Quicksafe Flow 2 (Zinsser Analytic, Frankfurt, Germany) was added as scintillation cocktail with a flow rate of 2 mL/min. UV-detection was performed at 228 and 280 nm. All samples were filtered (45 µm) prior to injection.

GC-MS

For unequivocal structure elucidation, GC-MS analyses were executed on a Magnum iontrap (FinniganMat, Bremen, Germany) linked to a gas chromatograph 3400 (Varian, Darmstadt, Germany) equipped with a Zebron ZB-1 (Phenomenex, Aschaffenhausen, Germany) capillary column (30 m x 0.25 mm x 0.25 µm). Oven temperature was programmed as follows: 3 min isothermal at 60°C, followed by heating with 6°C/min to 300°C, 15 min isothermal at 300°C, and return to initial conditions. Temperature of

the split/splitless injector was 270°C, splitless time was 60 s and helium carrier flow was 1.5 mL/min. Iontrap parameters were set to electron impact ionization with an emission current of 9 µA), full scan mode ranged from 35 to 650 amu, an interface temperature of 270°C and a manifold temperature of 220°C. Measurements were performed by manual injecting 1 µL of the sample. The integrals of two representative analyte parent or fragment ions were used for quantitation and compared with those of the internal standard (2,4-D methyl ester, 80 ng/µL). The limit of quantification (signal-to-noise ratio of 10:1) was in the range of 45 ng/g clay calculated from GC-MS analysis of reference compounds (MCPA, MCPA methyl ester, and 4-chloro-2-methylphenol). Regarding the influence of varying matrices, no attempts were made to quantify the compounds at concentrations less than 180 ng/g clay. Recoveries determined by spiking experiments on pure montmorillonite, were in the range of around 40%.

4.3 Results

In order to investigate the fate and incorporation of MCPA derived residues (in extractable and non-extractable form) into the individual particle size fractions, a preceding study using the same soil as that used for the present experiments was performed with ^{14}C-MCPA. The results of this study, which are summarized briefly, showed that residues of the herbicide were predominantly and in almost equal portions incorporated into the silt and clay fractions (low level concentration: average of 20% in the silt and 18% of applied ^{14}C in the clay fraction; high level concentration: 13% in the silt and 9% in the clay fraction). As compared to the silt fraction, the mass of the clay particles was lower and amounted to only one third of that of the silt fraction. These findings on the whole pointed to the major influence of the organo-clay complexes regarding the incorporation of MCPA residues. Mineralization to $^{14}CO_2$ (about 46% (low level) and 57% (high level) after 120 days of incubation) was supposed to occur mainly via transformation of extractable MCPA residues. As compared to the samples with the low concentration of MCPA (8.5 mg/kg soil), the high level concentration (1,000 mg MCPA/kg soil) had a strong negative impact on the microbial activity of the soil samples. The initial considerable reduction in microbial activity (average about 86%) however, recovered after about 60 days of incubation supposedly due to adaptation of the microorganisms to the high level conditions resulting in an increase of the microbial activity and mineralization after 30 days of incubation [Klausmeyer, 2011].
Emphasis of the present paper is on the mode of incorporation of MCPA derived residues into the organo-clay complexes. For that purpose, the non-extractable fraction of the complexes was examined in detail by a sequence of chemical degradation methods. The entire work-up of the samples including procedures leading to the data outlined before is shown in Figure 4.1.

4.3.1 Radioactivity balancing and incorporation of MCPA derived ^{14}C in individual sub-fractions of the organo-clay complexes

Sequential chemical degradation methods were executed with the extracted humic acid (HA), fulvic acid (FA) and humin (HU) fractions. Such techniques, including numerous chemical reactions, were performed frequently for structure elucidation of organic macromolecules (e.g. kerogen, humic matter) and to investigate the incorporation mode of xenobiotics in soils and sediments [Schaeffer-Reiss et al., 1998, Almendros and González-Vila, 1987, Richnow et al., 1993]. In case of the present experiments, the strength of the chemicals used to cleave and release incorporated MCPA residues increased with each degradation step. In the first selective step, alkaline hydrolysis was executed to cleave ester (amide) bonds followed by a treatment with boron tribromide (BBr_3) to release ether (ester) linked compounds (second step). Ruthenium tetroxide (RuO_4) - formerly used in catalytic oxidation of coals, lignite and humic acids - was applied as third degradation step to oxidize aromatic rings and functionalized carbon atoms under mild conditions (e.g. room temperature) [Richnow et al., 1992, Stock and Tse, 1983, Válková et al., 2009]. In a fourth step, TMAH thermochemolysis combined efficient pyrolytical cleavage with subsequent methylation of a number of functional groups. Oxidation and thermochemolysis are both abrasive methods leading to degradation of the humic matter and thus, release strongly incorporated residues.

Sequential chemical degradation of humic sub-fractions of samples with the low level MCPA concentration

In Figure 4.2 and Table 4.2 the portions of radioactivity released after each degradation step are displayed. Percentages refer to the initial amount of radioactivity in the individual humic sub-fractions. As consequence of alkaline hydrolysis, higher portions of radioactivity were releasable from FA and HA as compared to HU. Thus, after one day of incubation, 49% of the initial non-extractable ^{14}C was releasable from FA, 39% from HA and 13% from HU. Until 30 days of incubation, the radioactivity releasable decreased in case of the FA and remained constant up to day 120 (11%). In contrast, ^{14}C releasable from both, HA and HU fractions remained constant over the entire incubation period. Hydrolytic treatment of the HU fractions generally liberated the lowest portions of radioactivity among the humic sub-fractions, possibly indicating a different nature of incorporation of MCPA residues as compared to the FA and HA fractions. In general, the highest portion of hydrolytically releasable ^{14}C was found with the HA fraction (average of 38%). Subsequent BBr_3-treatment of the remaining samples, which was performed in order to cleave possible ether (ester) bonds [Bhatt and Kulkarni, 1983] released considerably lower amounts of radioactivity in case of all humic sub-fractions as compared to alkaline hydrolysis. The corresponding portion releasable from FA increased

until day 30 (8%) and remained constant during the following 90 days of incubation (day 120: 8%). These results contrast with those of alkaline hydrolysis - a finding which may point to a modification of the character of binding with time. However both, the ester and ether linkages appeared not to account completely for the entire radioactivity and thus, modes of incorporation occurring between MCPA derived residues and FA. Radioactivity releasable by BBr_3-treatment from HA remained constant during the entire incubation period (average: 5%), whereas in case of HU, the radioactivity which was liberated, decreased until day 120 (3%). The latter result was thought to point to ageing effects of either the residues themselves or the mode of incorporation. Oxidative as well as thermochemolytical degradation released only negligible portions of the incorporated radioactivity. No distinct trends were observed. In case of thermochemolysis, only three representative samples were measured to assess the potential of the method to cleave and release non-extractable MCPA residues.

Sequential chemical degradation of humic sub-fractions of samples with the high level MCPA concentration

The portions of radioactivity released after each degradation step are displayed in Figure 4.3 and corresponding data in Table 4.3. Percentages refer to initial amounts of radioactivity in the individual humic sub-fractions. Similar to the samples with a low level of MCPA, alkaline hydrolysis (first step) liberated the highest amounts of ^{14}C. Among the distinct organo-clay sub-fractions, highest portions of releasable radioactivity was found with the HA fraction (average: 60% of initial ^{14}C) followed by the FA fraction (average: 49%) and the HU fraction (average: 15%). These results again indicate a different mode of incorporation of MCPA derived residues into the HU fraction as compared to the FA and HA fractions. In case of BBr_3-treatment, the releasable radioactivity (possibly incorporated by ether (ester) bonds) was also considerably lower as compared to those released after alkaline hydrolysis. Portions liberated from the three sub-fractions were almost constant during the entire incubation period. In average, 13% of the initial radioactivity was released from FA, 10% from HA and 19% from the HU fraction. Oxidation as well as thermochemolytical cleavage showed a considerably low efficiency to release ^{14}C from the remaining non-extractable residues; a similar finding was already obtained with the low level samples. The data from all degradation steps performed showed no distinct trends with time which contrasts with the findings of the low level samples. Additionally, amounts of released radioactivity demonstrated a comparatively high variation. However, amounts of releasable portions decreased from incubation day 60 to 120, possibly indicating similar ageing effects as observed during the incubation of the samples with the low level concentration of MCPA.

With respect to the degradation methods used, recently published results showed that the non-extractable residues of low molecular weight compounds could be released and

analyzed either from soil or sediment constituents. So, Richnow *et al.* used alkaline and BCl_3-treatment to investigate the incorporation mode of metabolites derived from polyaromatic hydrocarbons (PAH) in humic substances [Richnow et al., 1993]. Kronimus *et al.* and Schwarzbauer *et al.* utilized the four degradation techniques applied in the present investigation in order to investigate non-extractable residues of xenobiotics and DDT metabolites in river sediments [Kronimus and Schwarzbauer, 2007, Schwarzbauer et al., 2003]. In all cases, boron trihalogenide-treatment, oxidation, and thermochemolytical cleavage led to a release/solubilization of reasonable amounts of the residues, which contrasts with our present results. Regarding the sediment samples, the contaminants were incorporated under anoxic conditions and incubated during longer periods of time. These findings indicate that environmental conditions, incubation time and the persistence (chemical structure) of the incorporated compounds are of major importance for the formation of residues releasable only under harsh conditions.
To verify whether the entire amount of incorporated ^{14}C or only portions were liberated by the degradation steps executed, total recoveries resulting from the chemical degradation procedure were determined. The recovery of solubilized ^{14}C was regarded crucial for the explanation of the incorporation processes of MCPA derived residues into organo-clay complexes in its entirety. Table 4.2 and 4.3 show the recovery rates obtained from the samples with low and high level concentrations of MCPA. In case of the low level samples, 15-57% (average: 32%) of the initial radioactivity in the individual humic sub-fractions could be released. Regarding the high level samples 11-99% (average: 58%) were released as soluble radioactivity. Thus, the degradation methods performed were obviously not capable to furnish information on the whole spectrum of modes of incorporation of MCPA residues into organo-clay complexes. Considerable amounts of residual ^{14}C still remained as non-extractable residues in the humic sub-fractions. Possible explanations for these findings might be, that either residues were incorporated in a way (e.g. *C-C* covalent bonds, entrapment) not cleavable by the applied degradation procedures, or the non-extractable radioactivity consisted of ^{14}C-labeled moieties of the completely degraded MCPA molecule, which were recycled and integrated into biomass or humic matter as natural building blocks [Roberts, 1984].

4.3.2 Structure elucidation of extractable and non-extractable MCPA residues derived from the soil and its organo-clay complexes

In terms of assessing the behavior and incorporation of xenobiotics in soil as well as their risk for the environment, a prerequisite is the knowledge of the chemical structure of extractable and non-extractable compounds derived from the xenobiotics. The main metabolite of MCPA in soil, 4-chloro-2-methylphenol, shows a considerably higher toxicity to aquatic organisms as the parent herbicide itself (MCPA: LC_{50} fish: $\rangle$ 90 mg/L; metabolite: LC_{50} fish: 2-6 mg/L) [EU, 2008, OECD-SIDS, 1998]. To elucidate

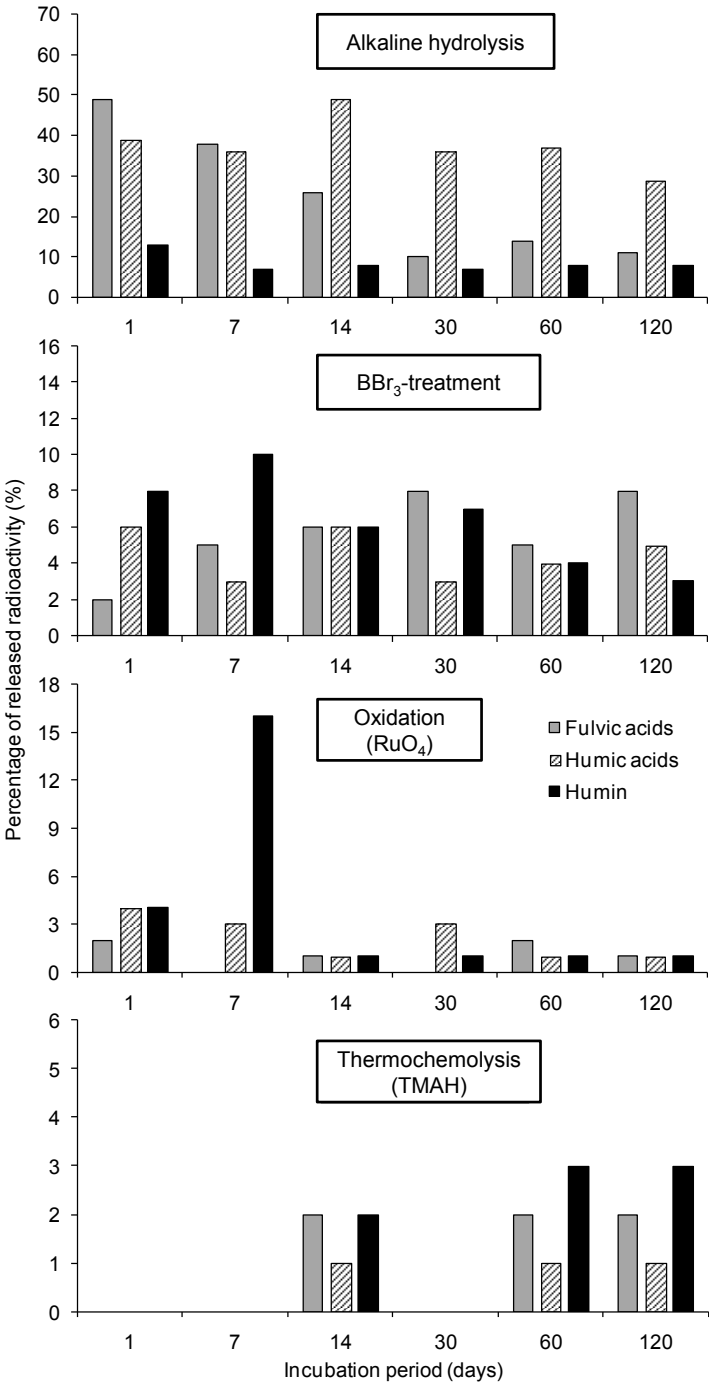

Figure 4.2: Low level concentration (8.5 mg MCPA/kg soil) - Percentage of ^{14}C released after sequential chemical degradation (% based on the total amount of radioactivity contained in the respective fraction). Grey bars: ^{14}C released from fulvic acids (FA); striped bars: ^{14}C released from humic acids (HA), black bars: ^{14}C released from humin (HU).

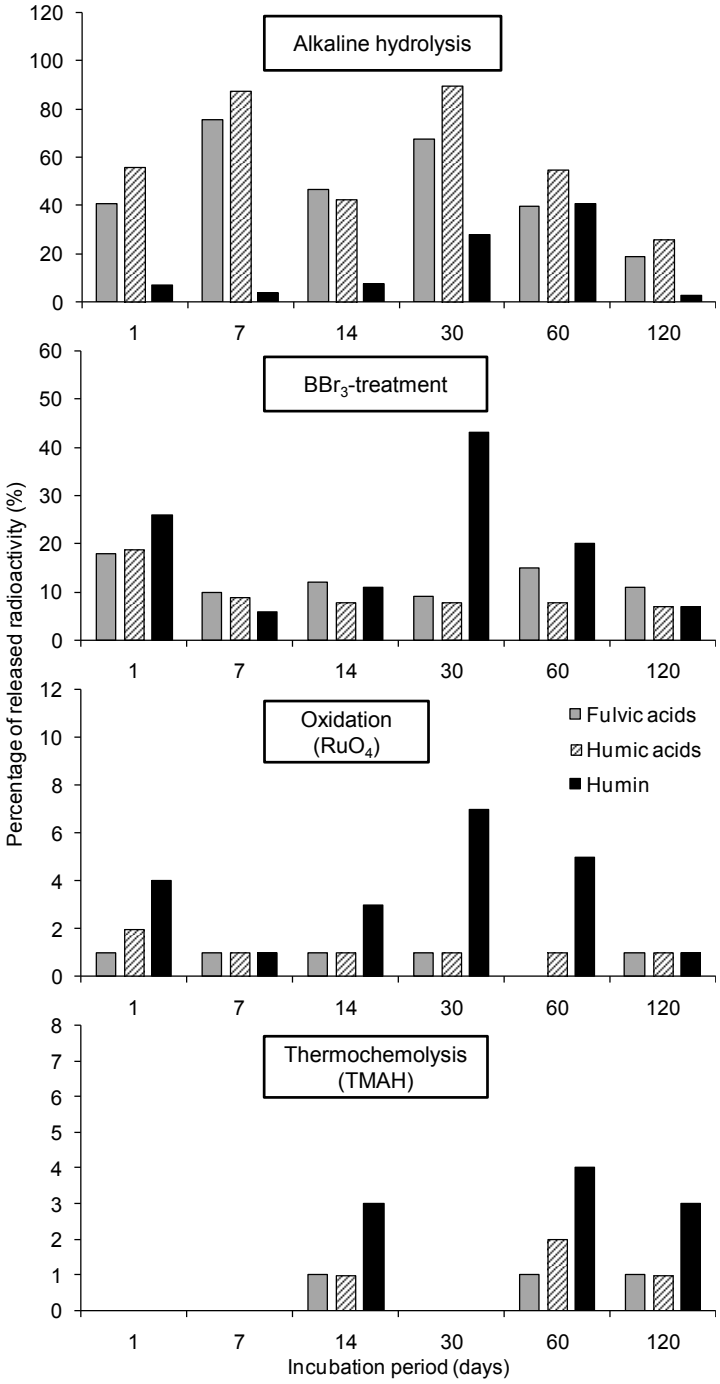

Figure 4.3: High level concentration (1,000 mg MCPA/kg soil) - Percentage of ^{14}C released after sequential chemical degradation (% based on the total amount of radioactivity contained in the respective fraction). Grey bars: ^{14}C released from fulvic acids (FA); striped bars: ^{14}C released from humic acids (HA), black bars: ^{14}C released from humin (HU).

the structure of extractable and non-extractable residues in the present investigation, the corresponding samples were examined by GC-MS and Radio-HPLC. Due to low amounts of MCPA derived residues in the extracts of the samples of the low level concentration and matrix interference especially in case of the alkaline hydrolysis extracts, GC-MS measurements were only performed with the high level samples. Representative chromatograms for the distinct extracts are displayed in Figure 4.5 – 4.8. With increasing incubation time, a rapid formation of the metabolite 4-chloro-2-methylphenol occurred. In the water used for particle size fractionation (= water extractable fraction of the entire soil) of the low and high level samples, the metabolite could be identified after one week of incubation (Figure 4.5 and 4.7B). In case of the high level samples, Radio-HPLC indicated an increase of this transformation product with increasing microbial activity up to 60 days of incubation (results not shown). In Table 4.4, the amounts quantified via GC-MS and Radio-HPLC as well as the concentrations of the identified residues in soil or its organo-clay complexes are shown. The metabolite 4-chloro-2-methylphenol was found to be below 1% (based on ^{14}C) of the initially applied amount of MCPA over the entire experimental period. Nevertheless, the metabolite was still identified after 120 days of incubation. The unexpected high concentrations of MCPA methyl ester occasionally measured by GC-MS in the water extract (day 7, 30 and 60) remained unclear (Figure 4.5). It is known, that esters of MCPA hydrolyze very rapidly to the corresponding acid in moistened soil [Smith and Hayden, 1980]. Therefore, we assumed that in our incubation experiments, the free acid was the predominant species shortly after application. The major water extractable compound from soil was thus, MCPA itself (Figure 4.5), which could be traced back to the polar character of the free acid. As in the case of the water extractable fraction, the organic extracts (methanol and dichloromethane) of the organo-clay complexes pointed to MCPA as the major extractable compound already after one day of incubation, besides 4-chloro-2-methylphenol. In contrast, no MCPA methyl ester was identified in the solvent extract of the clay fraction (Figure 4.6 and 4.7C). We assumed that the detection of the unexpected high concentrations of MCPA methyl ester in the water extract of the soil was due to artifacts during work-up of the samples, such as esterification of MCPA with the solvent methanol.

Regarding the non-extractable MCPA residues contained in the clay fraction, GC-MS chromatograms of the extracts derived from alkaline hydrolysis of the high level samples are shown in Figure 4.8. Corresponding data are summarized in Table 4.5. Both complementary methods, Radio-HPLC and GC-MS revealed that MCPA was the only detectable compound during the entire incubation. After 30 days of incubation, the portion of MCPA quantified decreased in all sub-fractions. However, radioactive balancing (Table 4.3) indicated a constant amount of ^{14}C remaining in the clay fraction until day 120. Together, these data on the whole may also illustrate - in addition to toxicologically not relevant, recycled moieties of MCPA due to complete degradation of the herbicide -

possible ageing processes, which effected the releasability of incorporated relevant MCPA residues.

4.4 Discussion

Mode of incorporation of MCPA residues into organo-clay sub-fractions

In this investigation, balances of radioactivity released from the non-extractable residues of MCPA associated with the organo-clay complexes obtained from a soil treated with ^{14}C-labeled MCPA were determined. To study both, the impact of the concentration of the herbicide and the influence of microorganisms on the mode of incorporation, two concentration levels of MCPA were separately applied to soil assays. Emphasis was laid on the soil derived organo-clay complexes due to their known high importance on the formation of non-extractable residues in general. Up to now to our knowledge, this is the first study dealing with a sequential chemical treatment of humic sub-fractions from organo-clay complexes for cleavage of defined chemical bonds in order to elucidate the mode of incorporation of MCPA into these fractions after incubation periods of up to 120 days.

The high concentration of MCPA applied (1,000 mg MCPA/kg soil) considerably influenced the microbial activity (DMSO assay) of the soil assays during the early phase of incubation. On the contrary, the low level assays (8.5 mg MCPA/kg soil) showed almost no reduction as compared to soil not treated with MCPA. According to recently published results, a nonylphenol isomer applied at a similar concentration rate of 1,000 mg NP/kg soil to the same soil revealed a noticeable reduction of the microbial activity, but the activity of the assays was still in the range of natural soils [Riefer et al., 2011]. As a conclusion of the present experiments, the chemical structure of the applied xenobiotics as well as its concentration have a significant impact on soil microbial activity. However, soils can be regarded as systems with the capacity to adapt to changing conditions. In case of the high level MCPA assays, the microbial activity recovered after 60 days of incubation reaching values comparable to those of natural soils [Sparling and Searle, 1993, Klausmeyer, 2011].

Both, the low and high level experiments gave clear evidence that ester or possibly amide bonds were involved to a considerable extent in the formation of the non-extractable residues of MCPA associated with the clay fraction. This conclusion was also confirmed by the fact, that only the acidic form of MCPA was released during alkaline hydrolysis, which may indicate a preferential cleavage of carboxylic acids esterified to hydroxyl groups at the humic material besides amide bonds. Moreover, the high portion of ^{14}C released during the first degradation step and the absence of 4-chloro-2-methylphenol in the corresponding extracts may point to an incorporation via hydrolysable bonds. Due to the complexity of the chemical structure of humic materials containing natural amide

and ester bonds, the sequestration of MCPA cannot be excluded from the present experiments. Ether linkages cleaved in step 2 appeared to show a lower but noticeable influence on the incorporation, whereas *C-C* bonds cleaved by oxidation (step 3) and thermochemolysis (step 4) seemed to play no role in the integration of MCPA residues in humic macromolecules. In contrast to the hydrolysable portion (step 1), ether bond cleavage released only minor amounts of radioactivity. The fact that no brominated compounds (the usual reaction products of ether cleavage using BBr_3) derived from MCPA were identified and ether bonds are frequent in humic substances indicated that the incorporation of residues released by BBr_3 were rather via sequestration processes.
The distribution of ^{14}C-labeled residues releasable from humic sub-fractions (mainly after alkaline hydrolysis) was in the order HA › FA › HU and similar with both concentration levels. This finding may indicate that the mode of incorporation was partially independent of the applied MCPA concentration and partially of microbial activity. It was supported by additional experiments executed with soil sterilized using γ-irradiation (30 kGy, dry soil). Thus, incorporation of MCPA residues into the clay fraction as non-extractable residues also occurred without any or at least with extremely reduced microbial activity. Within 60 days of incubation, approximately half of the radioactivity found in the clay fraction after soil particle separation could not be extracted and remained as non-extractable residues [results not shown]. However, extracellular enzymes, which were thought to be responsible for an incorporation [Bollag et al., 1992], may have remained still active even after γ-irradiation [McNamara et al., 2003] and may have led to the indirectly microbially assisted formation of non-extractable residues of MCPA residues under conditions of microbial inactivity.
However both, applied concentration of MCPA and present microbial activity distinctly influenced the dynamics of the incorporation and the amounts of releasable residues of MCPA´s non-extractable residues from the individual humic sub-fractions (clay fraction) regarding the high and low level concentration assays. In case of the high level assays, the increase of microbial activity from incubation day 30 until the termination of incubation correlated with the decrease of releasable residues after alkaline hydrolysis and BBr_3-treatment - thus, demonstrating that microbial activity led to a different mode of incorporation which was not cleavable by these methods. However, it should be noted that ageing effects may also have contributed to the decrease of releasable radioactivity. Humic sub-fractions differ in their chemical constitution, which might lead to divers interactions with incorporated xenobiotics. As discussed above, the incorporation of MCPA was thought to occur predominantly by reactions of the carboxyl function. Due to the higher amount of oxygen containing functional groups (e.g. hydroxyl, carboxyl) in fulvic acids as compared to other sub-fractions [Stevenson, 1994], incorporation of the phenoxyacetic acid herbicide via ester bonds may have preferentially occurred in this fraction. The highest (average) portion of radioactivity releasable after alkaline hydrol-

ysis however, was found with the HA fraction. This result may indicate an influence of further factors (apart from suitable functional groups) on the formation of ester bonds, such as access of microorganisms and products to reaction sites, stabilizing linkages, and repulsion effects as consequence of soil pH. The predominant clay mineral in the soil used was smectite (› 85%). Smectites are swellable minerals showing both, surface functional groups (mainly hydroxyl) and interlayers with exchangeable cations. The potential of organo-clay complexes to adsorb and incorporate xenobiotics was investigated by several authors [Hermosín and Cornejo, 1992, Liao et al., 2006, Iglesias et al., 2010]. As a consequence of the soil pH of 5.4 in the present investigation and MCPA's pKa (3.4) [EU, 2008], it was assumed that the applied herbicide was mainly de-protonated in soil exhibiting a polar, ionic character. Such compounds can interact with positively charged edges or cation bridges with certain multivalent metal ions at exchange sites of the clay minerals. Due to repulsions by the negatively charged mineral surface, these interaction sites however, are rather limited in case of the anionic MCPA [Cornejo et al., 2008]. Thus, the low releasable portions of non-extractable residues contained in the HU fraction (= humin associated with clay) can only partially be explained by clay-herbicide interactions, which leads to the assumption that further modes of incorporation either with the mineral or the organic matter are responsible for these portions of non-extractable radioactivity.

The recovery rates of the entire degradation procedure including the combusted aliquots of the humic sub-fractions revealed that radioactivity remained non-solubilized with the humic material after executing the four degradation steps. Chemical structures of these stably incorporated compounds could not be evaluated. Due to a relatively rapid degradation and mineralization of MCPA in the moistened soil, it is likely that late rather than initial transformation products of MCPA represented the remaining radioactivity. These products could have been residues complying with the definition of non-extractable residues (i.e. toxicologically relevant) or substances which were similar to natural occurring material (i.e. toxicologically not relevant). The latter can be analyzed only with difficulty. Furthermore, as result of ageing effects either of the xenobiotic compound itself or the natural humic material, complex incorporation processes could have occurred leading to structures which were not cleaved by the executed degradation methods. Several authors commented, that (^{14}C-labeled) compounds metabolized to a large extent can be incorporated into biomass or incorporated into humic matter during humification processes [Roberts, 1984, Kaestner et al., 1999, Barriuso et al., 2008]. As a consequence of the extraction and degradation procedures of the present investigation, such compounds derived from the ^{14}C-labeled MCPA could have been released resulting in measurable radioactivity. In addition to the latter substances, $^{14}CO_2$ produced in the course of mineralization may have been partly incorporated into soil organics and biomass; such phenomena were reported previously [Roberts, 1984, Kaestner et al., 1999, Barriuso et al.,

2008]. However, compounds not released by the techniques applied were supposed to be strongly incorporated in insoluble soil components or degraded and hence less toxic, bioavailable and mobile in soil [Senesi, 1992].

For the environment, xenobiotic compounds, which can be remobilized, are of great importance. In the present study, the water extractable portion (obtained during particle size fractionation) was thought to represent the bioavailable fraction due to comparatively weak incorporation forces. These compounds are easily available and can therefore be directly absorbed and assimilated by microorganisms or absorbed and partially metabolized by plants [Semple et al., 2004]. MCPA was found to be the main bioavailable compound in the present study followed by MCPA methyl ester (though its emergence remained inexplicable) and 4-chloro-2-methylphenol. Bioaccessible residues have the potential to be re-mobilized in the future and thus become available as a consequence of changing soil conditions (e.g. pH-value, temperature) [Semple et al., 2004]. We assumed that solvent extractable compounds (at least from the clay fraction) comply to such residues. Adsorption and weak incorporation forces prevented them to be extracted by water. Besides MCPA, the metabolite 4-chloro-2-methylphenol was identified as bioaccessible residue. Among the non-extractable residues it was possible to distinguish between reversible/irreversible and residues released easily, only with difficulty or not at all by applying the degradation sequence utilized (Figure 4.1). Ester (or amide) bond cleavage via hydrolysis is a naturally, e.g. enzymatically catalyzed occurring reversible process. Compounds incorporated in this manner may be easily released and hence become of concern for the environment. In our investigation, MCPA was proven to be a bioaccessible substance if present as non-extractable residue. In Figure 4.4, the proposed preferred modes of incorporation of MCPA and derived residues into humic materials are presented.

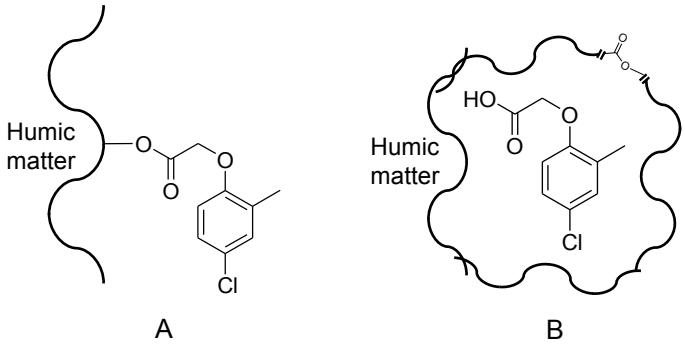

Figure 4.4: Model of the preferred incorporation mode of MCPA in organo-clay complexes. (A) Formation of covalent bonds (ester) between functional groups of humic substances and the carboxyl moiety of MCPA. (B) Entrapment (sequestration) of MCPA in cavities of the organic macromolecule.

4.5 Conclusion

The mode of incorporation of non-extractable MCPA residues in soil derived organo-clay complexes was investigated in detail by separating humic fractions (FA, HA, and HU) and executing a sequential chemical degradation procedure. Structure elucidation of the non-extractable residues incorporated via ester (amide) bonds, revealed the parent MCPA as the only traceable compound. In the extractable fractions of soil and soil derived organo-clay complexes, besides MCPA the metabolite 4-chloro-2-methylphenol was identified as bioavailable (extractable by water) and bioaccessible (extractable by organic solvents) compound. Independent from the applied MCPA concentration, hydrolysable covalent bonds were found to be the preferred incorporation mode of MCPA in all humic sub-fractions as indicated by alkaline hydrolysis. Additionally, ether bonds also contributed to the formation of non-extractable residues preferentially in the HU fraction but rather via sequestration processes than covalent bonds. As a consequence of the reversible binding character, such incorporated residues may be released easily (e.g. change of soil pH, enzymatically catalyzed hydrolysis), and thus they should still be seen as a potential environmental risk. However, in the course of incubation, the releasable portion deriving from humic sub-fractions decreased, especially in the assays with the high MCPA concentration, due to ageing effects of the incorporation mode, the non-extractable residues themselves or the organic matter. A correlation between an increasing soil microbial activity and a decreasing amount of compounds released, indicated a microbial influence on the mode of incorporation. Comprehensive sample preparation and analytical methods as used in this study (e.g. humic matter fractionation, sequential chemical degradation, Radio-HPLC, GC-MS), were found to be helpful in the investigation of the incorporation behavior of MCPA in soil and soil derived organo-clay complexes.

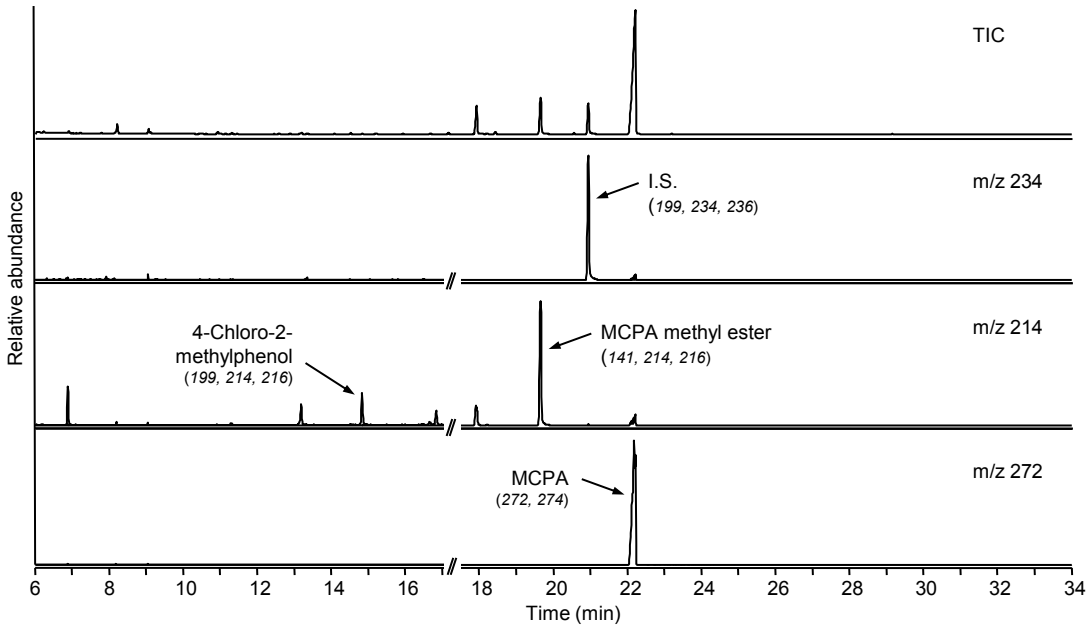

Figure 4.5: High level concentration (1,000 mg MCPA/kg soil) - GC-MS measurements of water extractable fraction (30 days of incubation). Top: total ion chromatogram (TIC); below: extracted ion chromatograms (EIC) at specific m/z values. I.S.: internal standard (2,4-D methyl ester). Left parts of the EICs were enlarged for a better illustration of the metabolite peak.

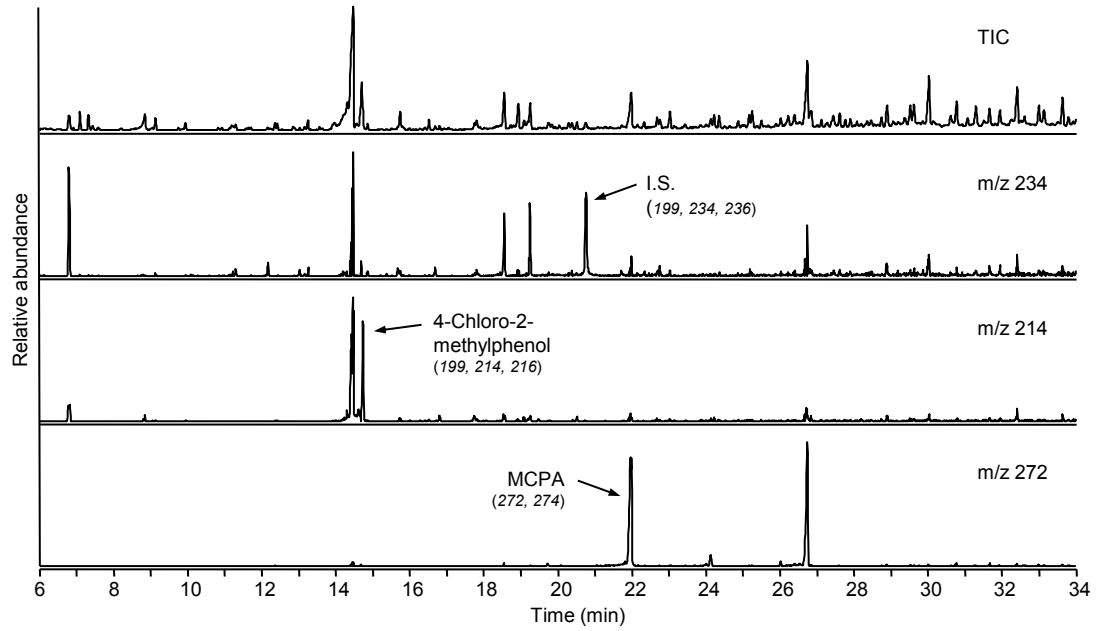

Figure 4.6: High level concentration (1,000 mg MCPA/kg soil) - GC-MS measurements of organic solvent extractable fraction (60 days of incubation). Top: total ion chromatogram (TIC); below: extracted ion chromatograms (EIC) at specific m/z values. I.S.: internal standard (2,4-D methyl ester).

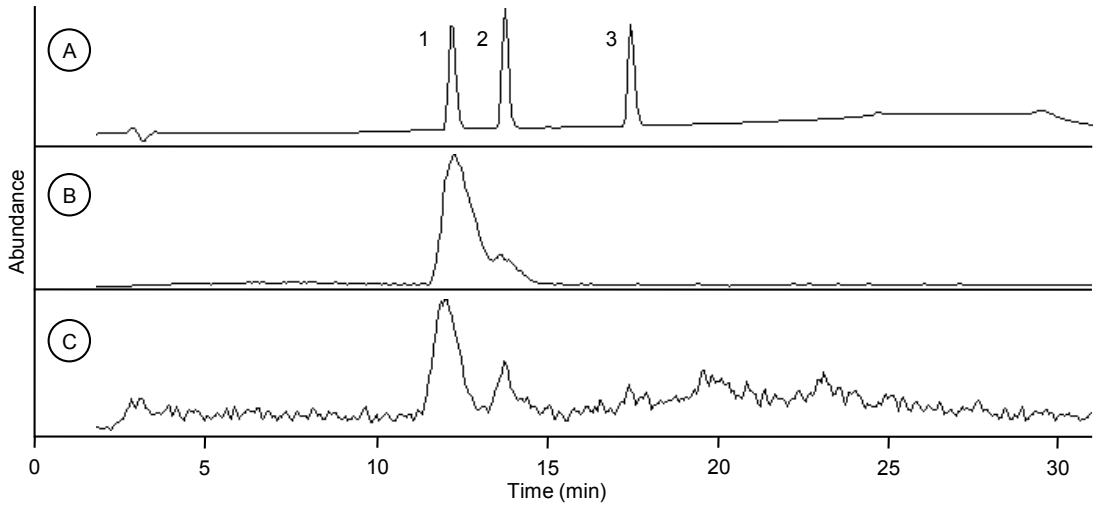

Figure 4.7: Low level concentration (8.5 mg MCPA/kg soil) - HPLC measurements of water (B) and organic solvent extractable fraction (C) after 7 days of incubation. Retention times of the reference substances are represented in chromatogram (A): MCPA (1), 4-chloro-2-methylphenol (2), MCPA methyl ester (3).

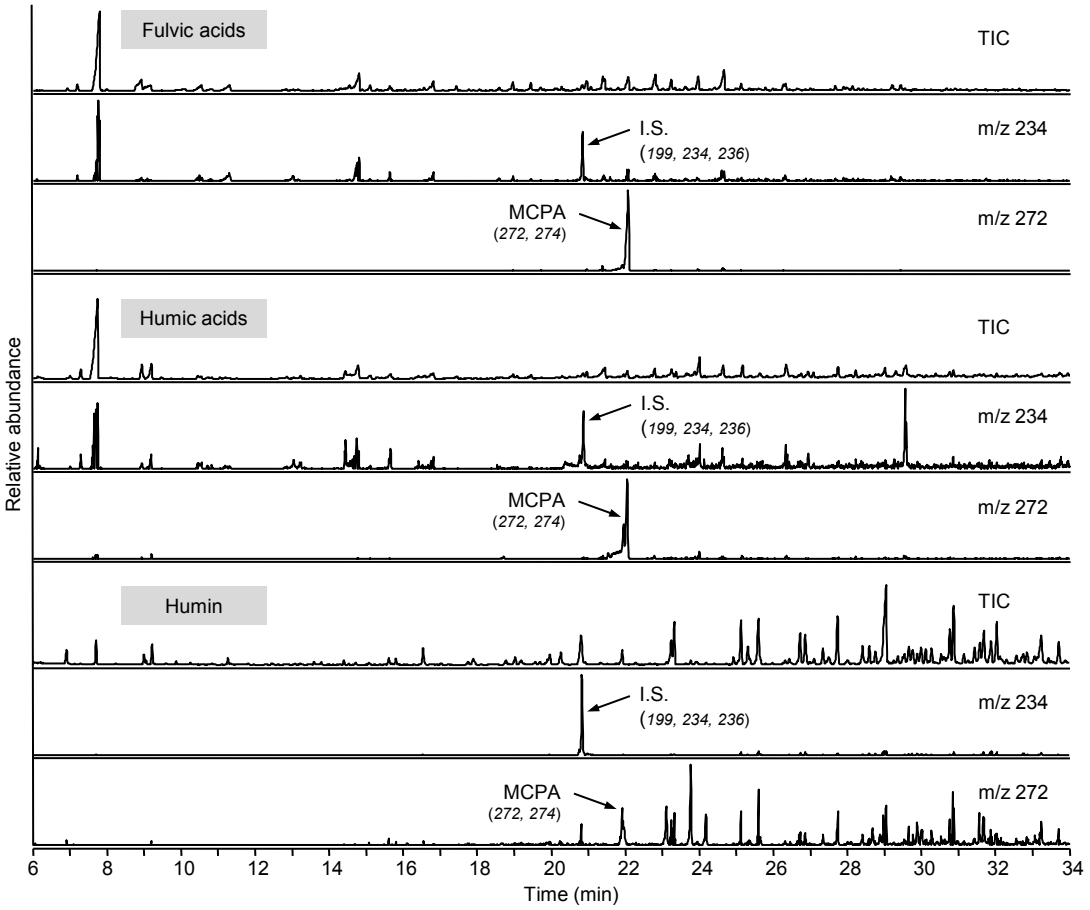

Figure 4.8: High level concentration (1,000 mg MCPA/kg soil) - GC-MS measurements of non-extractable fractions released after alkaline hydrolysis (30 days of incubation). Top: total ion chromatogram (TIC); below: extracted ion chromatograms (EIC) at specific m/z values. I.S.: internal standard (2,4-D methyl ester).

Table 4.2: Low level concentration (8.5 mg MCPA/kg soil) - Percentage of ^{14}C released after sequential chemical degradation (% based on the total amount of radioactivity contained in the respective fraction).

Humic fraction	Incubation period	STEP 1 Hydrolysis KOH/water/ methanol	STEP 2 BBr_3	STEP 3 Oxidation RuO_4	STEP 4 Pyrolysis TMAH	Recovery	Residual $^{14}C^{(a)}$
	(days)	(%)	(%)	(%)	(%)	(%)	(%)
FA	1	49	2	2		53	
	7	38	5	0		43	
	14	26	6	1	2	35	35
	30	10	8	0		18	
	60	14	5	2	2	23	
	120	11	8	1	2	22	27
HA	1	39	6	4		49	
	7	36	3	3		42	
	14	49	6	1	1	57	36
	30	36	3	3		42	
	60	37	4	1	1	43	
	120	29	5	1	1	36	40
HU	1	13	8	4		25	
	7	7	10	16		33	
	14	8	6	1	2	17	25
	30	7	7	1		15	
	60	8	4	1	3	16	
	120	8	3	1	3	15	38

(a) determined by combustion

Table 4.3: High level concentration (1,000 mg MCPA/kg soil) - Percentage of ^{14}C released after sequential chemical degradation (% based on the total amount of radioactivity contained in the respective fraction).

Humic fraction	Incubation period	STEP 1 Hydrolysis KOH/water/ methanol	STEP 2 BBr_3	STEP 3 Oxidation RuO_4	STEP 4 Pyrolysis TMAH	Recovery	Residual $^{14}C^{(a)}$
	(days)	(%)	(%)	(%)	(%)	(%)	(%)
FA	1	41	18	1		60	
	7	76	10	1		87	
	14	47	12	1	1	61	9
	30	68	9	1		78	
	60	40	15	0	1	56	
	120	19	11	1	1	32	20
HA	1	56	19	2		77	
	7	88	9	1		98	
	14	43	8	1	1	53	13
	30	90	8	1		99	
	60	55	8	1	2	66	
	120	26	7	1	1	35	21
HU	1	7	26	4		37	
	7	4	6	1		11	
	14	8	11	3	3	25	35
	30	28	43	7		78	
	60	41	20	5	4	70	
	120	3	7	1	3	14	35

(a) determined by combustion

Table 4.4: High level concentration (1,000 mg MCPA/kg soil) - Identification (GC-MS and Radio-HPLC) and quantitation (GC-MS) of extractable compounds.

Compound	Incubation period	Water extractable[a]			Solvent extractable[b]		
		Amount	Concentration	Percentage of applied ^{14}C	Amount	Concentration	Percentage of applied ^{14}C
	(days)	(µg)	(µg/g clay)	(%)	(µg)	(µg/g clay)	(%)
4-chloro-2-methyl-phenol	1				80	22	0.16
	7	327	77	0.65	1	0.4	0.01
	14	22	4	0.04	61	12	0.12
	30	302	70	0.60	13	3	0.03
	60	344	79	0.69	91	21	0.18
	120	76	15	0.15	14	3	0.03
MCPA	1				297	82	0.59
	7	27343	6466	54.69	n.a.	n.a.	n.a.
	14	1750	329	3.50	603	113	1.21
	30	22011	5103	44.02	n.a.	n.a.	n.a.
	60	29363	6766	58.73	556	128	1.11
	120	620	119	1.24	14	3	0.03
MCPA methyl ester	1				n.a.	n.a.	n.a.
	7	8151	1927	16.30	n.a.	n.a.	n.a.
	14	22	4	0.04	n.a.	n.a.	n.a.
	30	5778	1340	11.56	n.a.	n.a.	n.a.
	60	7625	1757	15.25	n.a.	n.a.	n.a.
	120	32	6	0.06	n.a.	n.a.	n.a.

n.a.: not analyzable (below the limit of detection) (a) water used for particle size fractionation
(b) organic solvent extract (methanol) of clay fraction grey area: samples not measured

Table 4.5: High level concentration (1,000 mg MCPA/kg soil) - Quantitation (GC-MS) of non-extractable MCPA residues contained in the clay fraction.

Humic fraction	Incubation period	Non-extractable (bound) MCPA		
		Amount	Concentration	Percentage of applied ^{14}C
	(days)	(µg)	(µg/g clay)	(%)
FA	1	1053	290	2.11
	7	1496	354	2.99
	14	1260	237	2.52
	30	1670	387	3.34
	90	708	163	1.42
	180	n.a.	n.a.	n.a.
HA	1	388	107	0.78
	7	1306	309	2.61
	14	1147	216	2.29
	30	2501	580	5.00
	90	454	105	0.91
	180	47	9	0.09
HU	30	26	6	0.05
	60	11	3	0.02
	120	9	2	0.02

n.a.: not analyzable (below the limit of detection)

5 First evidence for a stereoselective incorporation of nonylphenol diastereomers in soil derived organo-clay complexes

ABSTRACT. Environmental processes can affect the stereochemical properties of organic pollutants. In particular, biotic processes like microbial transformations or membrane penetration alter the ratios of enantiomers as well as diastereomers. These effects have been intensively used not only in environmental studies but also e.g. in medicine, toxicology, pharmacy and agricultural sciences. However, in order to identify unambiguously biotical initiated alteration of organic compounds, the knowledge on the stereoselective effect of all relevant processes is mandatory. Therefore, here we report on the first evidence for a stereospecific formation of non-extractable residues of a xeniobiotic in a highly relevant soil sub-fraction, the organo-clay complexes. In this study, soils were spiked with a labeled and non-labeled nonylphenol isomer and incubation experiments were performed to study its long term incorporation behavior in soil derived organo-clay complexes under abiotic and biotic conditions. Beside the extractable particle associated proportion, especially the humic fractions comprising bound residues have been analyzed by GC/MS. Our results from biotic experiments revealed alterations of the diastereomeric composition of the contaminant in the different soil humic sub-fractions. A depletion of the first eluting diastereomer as expressed by diastereomeric ratios around 0.6 has been observed for the extractable fraction, whereas the non-extractable proportion was enriched by the first diastereomer (diasteremoric ratio around 1.0). On the contrary, the diastereomeric ratios remained unaffected during the abiotic experiments (diasteremoric ratio around 0.8). These systematic observations gave clear evidence that the process of microbial assisted incorporation of nonylphenol into soil organo-clay complexes is a stereoselective process. To our knowledge, this is the first report on a stereoselective incorporation process of organic substances forming non-extractable residues. Consequently, the formation of non-extractable residues has to be considered in environmental studies dealing with stereoselective analysis of organic pollutants in soils.

5.1 Introduction

Environmental processes can affect the stereochemical properties of organic pollutants. In particular biotic processes like microbial transformations alter the ratios of individual enantiomers as well as diastereomers [Huehnerfuss and Shah, 2009]. For example, the enantioselective degradation of α-HCH by marine bacteria has been identified [Huehnerfuss et al., 1992]. Stereoselectivity has been reported also for adsorption of amino acids on clay minerals [Siffert and Naidja, 1992], for which implications for the origin of life has been discussed [Zaia, 2004]. Further on, it is also well known that physiological effects of chiral organic compounds can often be related to only one enantiomer. As a representative example, the enantioselective bioactivity of the pesticide malathion has been described by Hassan and Dauterman already in 1968 [Hassan and Dauterman, 1968].
Since stereoselective degradation, transfer and (eco)toxicology are well known phenomena, numerous studies dealt with the enantioselective analysis of chiral compounds under natural conditions to trace microbial assisted transformation or to evaluate the risk potential of individual enantiomers. A summary of knowledge on stereoselective trace analysis has been given in a book by Kallenborn and Huehnerfuss [Kallenborn and Huehnerfuss, 2001], in which the state-of-the-art and the future development as well as its implication in ecotoxicological studies are presented. More recent research work followed e.g. the stereochemical fate of polycyclic musk fragrances in wastewater, pharmaceuticals in sewage effluents, and organochlorine pesticides in soils [Berset et al., 2004, Kurt-Karakus et al., 2005, MacLeod and Wong, 2010]. Further on, recently a review regarding chiral chromatographic separation has been published [Huehnerfuss and Shah, 2009], in which not only principals but in particular actual trends and developments in stereoselective analysis have been described.
However, the formation of non-extractable residues (also called bound residues) has not been considered as a stereoselective process so far, although microbial assistance in the formation of bound residues is evident for decades. The process of incorporation or ageing of organic pollutants is an important environmental aspect in the evaluation of the state of pollution of particulate matter. For instance, bioavailability and environmental persistence is highly influenced by the formation of bound residues [Senesi, 1992].
To our knowledge no study has been published yet reporting on stereoselective formation of bound residues of organic pollutants in soils or sediments. Therefore, in this study the environmental fate of an individual nonylphenol isomer during its incorporation into soil derived organo-clay complexes has been investigated with a special focus on its stereoselective behavior.

5.2 Experimental

5.2.1 Chemicals

^{14}C-labeled 4-(3,5-dimethylhept-3-yl)phenol (353-NP, 304.14 MBq/mmol) was synthesized via Friedel Crafts alkylation using 3,5-dimethylheptan-3-ol and a mixture of non-labeled and [ring-U-^{14}C]phenol (60 mCi/mmol) according to Russ et al. [Russ et al., 2005]. The resulting radioactive material exhibited a specific activity of 8.22 mCi/mmol with a radiochemical purity of 94% and a chemical purity of 90%. ^{13}C-labeled 353-NP was synthesized accordingly.

5.2.2 Soil material and model organo-clay complexes

For spiking with nonylphenol, soil samples from Fuhrberg area (Lower Saxony, Germany) were used. This soil is characterized by a clay content around 5%, a maximum water holding capacity of approx. 42 g H_2O/100 g soil and total organic carbon (TOC) values in the clay fraction around 15%. More details are reported elsewhere [Riefer et al., 2011]. For the first two experiments soil samples have been used either without further treatment or after sterilization by γ-radiation. A third experiment was performed on model organo-clay complexes. These complexes have been prepared with montmorillonite as clay mineral phase and fulvic/humic acids extracted from peat also derived from Fuhrberg area. The resulting TOC content was approx. 5%. After spiking, the organo-clay complexes were mixed with pure sand (1:3 clay:sand, w/w) for a better experimental handling.

5.2.3 Incubation experiments

Incubation experiments were performed on approx. 50 to 100 g of natural or sterilized soil and approx. 5 g of model organo-clay complexes spiked with ^{13}C and ^{14}C-labeled nonylphenol, respectively. Resulting concentrations in the samples ranged from 1.25 to 1,000 µg/g dry weight. Corresponding amounts of 353-NP were applied to air-dried, homogenized, and sieved ($\leq$ 2 mm) soil samples from Fuhrberg, Germany, as well as air-dried and homogenized model organo-clay complexes. Directly after application, the solvent was evaporated and the flasks were shaken for 15 min in an overhead shaker. Thereafter, the water content of the samples was adjusted to 60% of maximum water holding capacity and the flasks were stored at 20°C in the dark. After different incubation periods of 1, 7, 14, 30, 90 and 180 days aliquots of approx. 20 g of the samples were suspended in 100 mL of water and subjected to ultrasonic assisted disaggregation (total energy input 22 kJ) as described by Morra et al. [Morra et al., 1991].

5.2.4 Particle size fractionation and extraction of organo-clay complexes

In case of soil samples the sand, silt and clay fractions were obtained by an initial manual wet sieving for separating the sand from silt and clay. Following, silt and clay were separated by centrifugation according to Stemmer et al. [Stemmer et al., 1998]. The clay fraction, consisting dominantly of organo-clay complexes, was subjected to a Soxhlet-extraction procedure using methanol and dichloromethane in order to obtain the extractable or free fraction of organo-clay related 353-NP. More detailed information on the sample procedure have been published recently [Riefer et al., 2011].

5.2.5 Separation of humic substances and alkaline hydrolysis

Further on, the organic material on the organo-clay complexes was separated according to the well known alkaline/acidic separation procedure for humic substances. Details are described elsewhere [Riefer et al., 2011]. Thus, three sub-fractions were obtained: alkaline soluble humic acids (HA), alkaline and acidic soluble fulvic acids (FA) as well as insoluble humin (HU). Following, these individual humic sub-fractions were subjected to alkaline hydrolysis to release ester (amide) bound NP residues. Details on this procedure are reported by Schwarzbauer et al. [Schwarzbauer et al., 2003]. After hydrolysis, the released contaminants had been extracted with diethyl ether and subsequently fractionated on a silica gel column by liquid chromatography according to Riefer et al. [Riefer et al., 2011].

5.2.6 Gas chromatographic-mass spectrometric analyses

GC-MS analyses were executed on a Magnum iontrap linked to a gas chromatograph Varian 3400 (Varian, Darmstadt, Germany) equipped with a ZB-1 capillary column (30 m x 0.25 mm x 0.25 µm). Oven temperature was programmed as follows: 3 min isothermal at 60°C, followed by heating with 6 K/min to 300°C, 15 min isothermal at 300°C. Temperature of the split/splitless injector was set to 270°C, splitless time was 60 s and helium carrier flow was 1.5 mL/min. Iontrap parameters were set to electron impact ionization with an emission current of 9 µA, full scan mode ranged from 35 to 650 amu, an interface temperature of 270°C and a manifold temperature of 220°C. Measurements were performed by injection of approx. 1 µL of the sample volume.
Diastereomeric ratios were calculated from ion chromatograms of 121 m/z (^{12}C-NP isomer) and 122 m/z (^{13}C-labeled NP isomer). Random repetitions (triplicates) of measurements revealed relative standard deviations between 1% and 35%. The absolute stereochemical conformation of the diastereomers 1 and 2 (see Figure 5.1) were not determined in this study.

5.3 Results and Discussion

Isomer mixtures of nonylphenol are well known environmental pollutants of high relevance, because they are stable in soils and sediments as well as are characterized as endocrine disruptors. Within these isomer mixtures 4-(3',5'-dimethyl-3'-heptyl)phenol (353-NP) is not only the most prominent isomer but exhibits the same relative estrogenic effect as compared to the technical mixtures [Preuss et al., 2006]. Beside its toxicological properties, the molecular structure of 353-NP contains two chiral carbon atoms (see Figure 5.1), that results in four possible stereoisomers comprising two pairs of enantiomers. Since diastereomers, that are not enantiomers, exhibit slightly different physico-chemical properties, a gas chromatographic separation of these stereoisomers is generally possible. Using these analytical approach, this study focused on the stereoselective behavior of 353-NP during its incorporation into a very relevant soil fraction, the organo-clay complexes.

5.3.1 Stereoselective incorporation of 353-NP diastereomeres into natural soil derived organo-clay complexes

A first set of incubation experiments followed the incorporation behavior of 353-NP into natural soil during a period of 180 days. Special focus was laid on organo-clay complexes since these are known to play an important role for the formation of bound residues [Wang and Xing, 2005]. After 1, 7, 14, 30, 90 and 180 days aliquots of soil material were taken and subjected to particle size fractionation in order to isolate these organo-clay complexes. During the total incubation time, the microbial activity was followed by the dimethyl sulphoxide (DMSO) reduction approach as reported in more detail elsewhere [Alef and Kleiner, 1989].

The organo-clay complexes were extracted obtaining an extractable, so-called free fraction of contaminants. Following, the residual organic matter was separated into the three major classes of humic substances: humic and fulvic acids as well as humin. These humic sub-fractions were subjected to alkaline hydrolysis to release the hydrolysable bound fraction of the nonylphenol contamination, because in a previous work evidence for a dominant ester linkage of bound nonylphenol has been revealed (unpublished results).

Radioanalysis applied on the extractable as well as the different bound fractions from the ^{14}C-experiments indicated a specific distribution of the spiked 353-NP as discussed in more detail elsewhere [Riefer et al., 2011]. Briefly, major proportions of 353-NP accumulated in the extractable as well as the hydrolysable fractions of humic acids and humin with absolute quota between 4 to 35% of the total applied radioactivity (see Table 5.1). Over the time course of 180 days, a decrease of extractable as well as an increase of bound 353-NP proportions was observed.

Therefore, fractions of the ^{13}C-experiment containing the dominant proportion of spiked

353-NP (as revealed by radioanalysis) were subjected to gas chromatographic-mass spectrometric analysis. Two separated peaks of 353-NP in the gas chromatogram were detected representing the two diastereomers as illustrated in Figure 5.1. Since a variation of the relative proportions of diastereomers was obvious, the ratio of the first and second eluting diastereomer was calculated (diastereomer 1/diastereomer 2 ratio, see Figure 5.1 and Table 5.1). The reference material exhibited a diasteromeric ratio of 0.85 with a standard deviation of 0.4. However, the ratios in the extractable and bound fractions were different. In the extractable fraction diastereomeric ratios between 0.47 and 0.82 were detected without any time trend. On the contrary, the diastereomeric ratios of 353-NP bound to humic acids ranged from 0.80 to 1.58 and the fraction associated to humin exhibited ratios from 0.80 to 1.17. Corresponding mean values were 0.56 for the free 353-NP fraction as well as 1.03 and 0.97 for the bound fractions, respectively. Hence, based on these data it became obvious, that the free and bound fractions of 353-NP exhibited different diastereomeric compositions, which is illustrated in Figure 5.2. A clear shift towards enrichment of the first eluting diastereomers during the incorporation process was evident.

5.3.2 Stereoselective behavior of nonylphenol during abiotic incorporation

To study the microbial influence on the observed stereoselective incorporation a similar experimental set-up was also applied to synthetic model organo-clay complexes and sterilized soil samples. For both systems, a very low microbial activity during the incubation experiments was confirmed by the DMSO reduction approach. As a major consequence of missing microbial activity the overall formation rate of non-extractable 353-NP residues in the artificial as well as in the sterilized natural organo-clay complexes was dramatically reduced. Only a very low proportion of total applied radioactivity was detected in the bound fraction of organo-clay complexes, but a major proportion of up to 71% remained as extractable components on the particles (see Table 5.1).
Accordingly, due to the low degree of incorporation the diastereomeric composition of 353-NP bound to organo-clay complexes was not detectable from the ^{14}C-experiments. On the contrary, in the extractable fraction diastereomeric ratios were measured and calculated to be between 0.68 and 1.0 as well as 0.72 and 0.92 for sterilized natural and artificial organo-clay complexes, respectively. These values fit very well the ratio of the original spiking material (0.85). Since the dominant 353-NP proportion applied to sterilized soil was accumulated in the extractable fraction of the sand particles, also for these sand associated 353-NP the diastereomeric ratio was calculated with values between 0.77 and 1.12.
Comparing the data from the different incubation experiments, the diastereomeric ratio of the extractable organo-clay fractions from abiotic experiments fell between the values of the free and bound 353-NP diastereomer composition. This can be demonstrated more

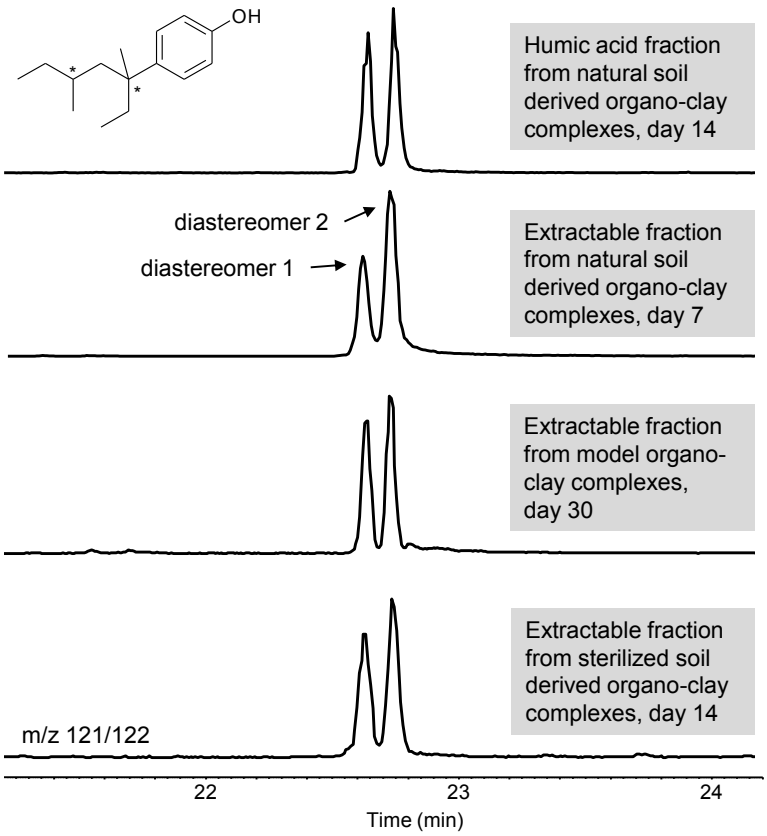

Figure 5.1: Ion chromatograms (m/z 121 or m/z 122) of 353-NP diastereomers detected in different fractions (humic acids and extractable fractions) derived from the incubation experiments with natural soil, sterilized soil and model organo-clay complexes. The molecular structure of 353-NP is given in the inset (*= chiral carbon atom). The chromatograms demonstrate the variations of diastereomeric composition of 353-NP (4-(3,5-dimethylhept-3-yl)phenol) according to incubation experiments and soil sub-fractions.

clearly by comparison of the mean values, for which their differences are illustrated as dotted lines in Figure 5.2. As mentioned above, mean diastereomeric ratios for 353-NP incubated in natural soil was 0.56 and around 1.0 for the free and bound fractions, respectively. The corresponding average values of extractable 353-NP in natural sterilized and artificial organo-clay complexes were 0.85 and 0.81, respectively, and fit very well the ratio of the original spiking material (0.85). Hence, a stereoselective alteration of 353-NP under abiotic conditions can be excluded. Since the average value of free and bound 353-NP in the natural soil experiment fit very well the ratios from abiotic experiments as well as the original spiking, also a selective microbial transformation under biotic conditions can be excluded as an alternative process affecting the stereochemical properties of the particle associated contamination.

In summary, a lack of microbial activity did not alter the diastereomeric composition as

compared to the original composition, whereas the diastereomeric composition becomes partitioned under biotic conditions. Hence, it can be concluded, that microbial assistance is not only essential for incorporation of 353-NP into organo-clay complexes but is also responsible for a stereoselective incorporation process.

5.4 Conclusion

Spiking experiments with labeled and non-labeled 353-NP followed the long term incorporation behavior of this contaminant into soil derived organo-clay complexes under abiotic and biotic conditions. Analysis of the diastereomeric composition of the contaminant in the extractable and non-extractable (or bound) fractions and comparison of these ratios from biotic and abiotic experiments revealed significant alterations. These systematic changes gave clear evidence that the process of microbial assisted incorporation of 353-NP into soil organo-clay complexes is a stereoselective process. To our knowledge, this is the first report on a stereoselective incorporation process of organic substances forming non-extractable residues. Beside physiological transfer processes (e.g. penetration through biological membranes) and biotic transformation, now also the microbial formation of bound residues has to be considered as an environmental process affecting the stereochemical composition of organic contaminants.

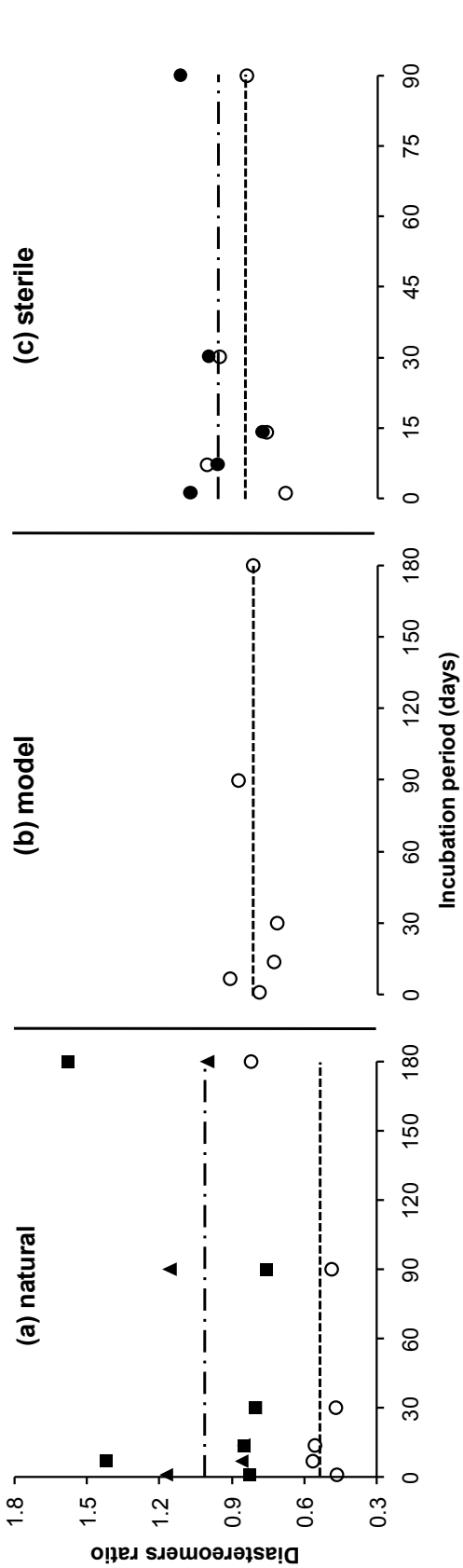

Figure 5.2: The diastereomeric ratios as detected in organo-clay complexes from different incubated materials: (a) natural soil, (b) synthetically prepared model organo-clay complexes and (c) sterilized soil. Differences in the diastereomeric composition in the extractable and non-extractable fractions from the biotic incubation experiment (a) are depicted by their mean values (dotted lines). Noteworthy, under abiotic conditions (b,c) the average values (dotted lines) are unalterated as compared to the initial diastereomeric ratio (0.85). ∘ = extractable fraction from organo-clay complexes, ▪ = hydrolysable fraction from humic acids, ▲ = hydrolysable fraction from humin, ● = extractable fraction from sand. Dotted lines represent average values (- - - = extractable fraction from organo-clay complexes; – · – · – = (a) non-extractable fractions / (c) extractable fraction from sand).

Table 5.1: Diastereomer ratios of 353-NP in the extractable and bound fraction as revealed from spiking experiments on natural soil, sterilized soil, and model organo-clay complexes: depletion of the first eluting diastereomer in the extractable fractions (diastereomeric ratios around 0.6), enrichment in the non-extractable proportion (ratio around 1.0), unaffected in the abiotic samples (ratio around 0.8). Diastereomeric ratio of the original spiking material: 0.85

NATURAL SOIL DERIVED ORGANO-CLAY COMPLEXES

	Extractable fraction			Non-extractable fraction			
				Humic acids		Humin	
Incubation period (days)	Diastereomeric ratio[a]	SD (n=3)[b]	Percentage ^{14}C[c] (%)	Diastereomeric ratio	Percentage ^{14}C	Diastereomeric ratio	Percentage ^{14}C (%)
1	0.47	0.07	35	0.82	4	1.17	3
7	0.56	0.07	15	1.42	13	0.86	10
14	0.56	0.17	17	0.85	12	0.85	5
30	0.47	0.07	18	0.80	13	0.80	6
90	0.49	0.06	9	0.76	12	1.16	9
180	0.82	0.15	9	1.58	15	1.00	9
Average	**0.56**			**1.03**		**0.97**	

STERILIZED SOIL

	Extractable fraction (organo-clay complexes)			Extractable fraction (sand)		
Incubation period (days)	Diastereomeric ratio	SD (n=3)	Percentage ^{14}C (%)	Diastereomeric ratio	SD (n=3)	Percentage ^{14}C (%)
1	0.68	0.05	14	1.07	0.48	50
7	1.00	0.15	10	0.96	0.15	55
14	0.76	0.04	11	0.77	0.27	58
30	0.95	0.34	15	1.00	0.29	44
90	0.84	0.17	9	1.12	0.38	52
180	n.a.	n.a.		n.a.	n.a.	
Average	**0.85**			**0.98**		

MODEL ORGANO-CLAY COMPLEXES

	Extractable fraction		
Incubation period (days)	Diastereomeric ratio	SD (n=3)	Percentage ^{14}C (%)
1	0.79	0.01	60
7	0.92	0.37	67
14	0.73	0.42	75
30	0.72	0.03	71
90	0.88	0.26	66
180	0.82	0.16	58
Average	**0.81**		

n.a.: not analyzable (below the LOD) (a) diasteremeric ratio=diastereomer 1/diastereomer 2 (see Figure 5.1). Data obtained from spiking experiments with ^{13}C-labeled and mixed $^{12}C/^{14}C$-labeled 353-NP. (b) SD = standard deviation (c) Percentage of total applied ^{14}C. Data obtained from spiking experiments using ^{14}C-labeled 353-NP.

6 Conclusion

In this study, the incorporation processes of a branched NP isomer and the herbicide MCPA were investigated in detail. Labeling (^{13}C and ^{14}C) of the applied compounds facilitated the balancing of incorporated and released compounds as well as structure elucidation via Radio-HPLC and GC-MS. Emphasis was laid on the incorporation processes of non-extractable residues during an incubation period of 180 or 120 days and the determination of chemical bonds between organo-clay complex derived humic sub-fractions and bound residues to assess the risk of a potential re-mobilization in the environment. Non-invasive ^{13}C-CP/MAS NMR and invasive sequential degradation methods were executed. The latter techniques have been used prior to investigate both, the structure of organic macromolecules (e.g. humic substances, lignin) and the incorporation mode of low molecular weight xenobiotics within soils and sediments.

Balancing of radioactivity incorporated in soil and soil derived organo-clay complexes revealed for both compounds a fast formation of non-extractable residues during 48 h of incubation. Organo-clay complexes were found to be the major sink for residues of the NP isomer and MCPA. For further investigations of non-extractable residues, pre-separated organo-clay complexes were fractionated into humic acids, fulvic acids and humin. Each fraction was sequentially degraded (1. alkaline hydrolysis, 2. BBr_3-treatment, 3. RuO_4 oxidation, 4. TMAH thermochemolysis) whereat the strength of the used chemicals to cleave and release incorporated residues increased. In both approaches using NP and MCPA, it was clearly demonstrated that non-extractable residues are predominantly formed via ester (amide) bonds as indicated by alkaline hydrolysis. However, among the humic sub-fractions the amount of releasable radioactivity differed and thus indicating diverse incorporation modes due to distinct chemical compositions (e.g. amount of oxygen containing functional groups, presence of clay minerals). During the incubation period, radioactivity liberated in course of chemical treatment decreased as a result of possible ageing processes leading to compounds which were on the one hand strongly incorporated (e.g. change of binding character) or on the other hand highly transformed (e.g. incorporated into biomass). Additionally, in case of the MCPA assays, a correlation between the microbial activity of the soil and the formation of non-releasable residues was found. Structure elucidation obtained only the NP isomer as solvent extractable (bioaccessible) and non-extractable substance. In case of the MCPA approach, within the water (bioavailable) and solvent extractable fraction, MCPA and the metabolite 4-chloro-2-methylphenol

could be identified, whereas MCPA was found as the only non-extractable compound. The four executed degradation steps could not trace the incorporation processes of the NP isomer and MCPA in its entirety as shown by radioactivity remaining in humic subfractions. Thus, other processes such as incorporation into humic matter (humification), formation of non-identified residues or the incorporation of CO_2 (mineralization) represent further possible modes of incorporation. Nevertheless, the use of comprehensive analytical methods (e.g. humic matter fractionation, sequential chemical degradation, ^{13}C-CP/MAS NMR, Radio-HPLC and GC-MS) led to a detailed insight into the incorporation processes of the low molecular weight compounds MCPA and NP into soil derived organo-clay complexes. With the introduced techniques, a stereoselective incorporation during the formation of the non-extractable NP isomer was determined for the first time and it was possible to assess the risk of extractable and non-extractable residues for the environment. Besides bioavailable and bioaccessible compounds, the formation of non-extractable (bound) residues via hydrolysable linkages gave evidence for a potential to be re-mobilized in soil and thus still important in terms of toxicity and bioavailability.

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