Effects of Soil Organic Matter Molecular Conformation and Substrate Additions on the Formation and Release of Xenobiotics Bound Residues

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# Table of Contents

List of Abbreviations ............................................................................................................. 9

Chapter I: Introduction ........................................................................................................ 11

  General introduction to the study .................................................................................... 12
  Background: The role of soil organic matter in the fate of xenobiotics bound residues in soil ................................................................................................................................... 14
  Objeceives and outline of this study................................................................................... 26

Chapter II ............................................................................................................................. 37

  Interacting effects of cation saturation and drying, freezing, or aging on the extractability of nonylphenol and phenanthrene from a sandy soil

  Shchegolikhina A., Schulz S., Marschner B.


Chapter III ............................................................................................................................ 65

  Cation treatment and drying-temperature effects on nonylphenol and phenanthrene sorption to a sandy soil

  Shchegolikhina A., Kunhi Mouvenchery Y., Woche S.K., Bachmann J., Schaumann G.E., Marschner B.


Chapter IV ........................................................................................................................... 87

  Effects of sterile storage, cation saturation and substrate additions on the degradability and extractability of nonylphenol and phenanthrene in soil

  Shchegolikhina A., Marschner B.

  Chemosphere: submitted
Chapter V ........................................................................................................................................................................................................................................................................ 107

Effects of γ irradiation, storage and incubation on enzyme activities in a sandy soil treated with different salts

Shchegolikhina A., Marschner B.

Soil Biology & Biochemistry: submitted

Chapter VI: Summary Discussion and Conclusions ................................................................................................................................. 117

The influence of the nonylphenol and phenanthrene properties on their behavior in soil .................................................................................................................................................................................................................................................................. 118

The effect of the SOM conformational structure alteration on the soil–xenobiotic interactions .................................................................................................................................................................................................................................................................... 120

The influence of the soil properties on the sorption and sequestration of nonylphenol and phenanthrene .................................................................................................................................................................................................................................................................... 123

The effects of the soil microbial activity on the formation of nonylphenol and phenanthrene bound residues .................................................................................................................................................................................................................................................................. 125

The aging of soils as a factor of soil alteration and the formation of xenobiotics bound residues .................................................................................................................................................................................................................................................................... 129

Summary and final conclusions .......................................................................................................................................................................................................................................................................................... 131

Acknowledgements ...................................................................................................................................................................................................................................................................................... 139

Curriculum Vitae ...................................................................................................................................................................................................................................................................................................................................................... 141

Appendix I .......................................................................................................................................................................................................................................................................................................................................................... 143

Effects of soil cation treatments and aging on nonylphenol and phenanthrene extractability: study of in situ pore-water extraction as an alternative method for evaluation of xenobiotics bioavailability in soils
Appendix II ......................................................................................................................................... 151

Supplementary data for the Chapter V
List of Abbreviations

1,2,4-TCB  
1,2,4-trichlorobenzene

³H NMR  
Proton nuclear magnetic resonance

Al-soil  
Soil treated with AlCl₃

ANOVA  
Statistical analysis of variance

CA  
Contact angle

CaB-WaMB  
Cation-bound water molecular bridges

Ca-soil  
Soil treated with CaCl₂

d/w  
Dry weight

DOC  
Dissolved organic carbon

DOM  
Dissolved organic matter

EEW  
Experiment with excess of water

H₂O-soil  
Soil treated with H₂O

K₊oc  
Carbon normalized partitioning coefficient

K₊ow  
Octanol-water partitioning coefficient

mo  
Month

Na-soil  
Soil treated with NaCl

NP  
Nonylphenol

PAHs  
Polyaromatic hydrocarbons

Phe  
Phenanthrene

PWE  
Pore-water extraction

SOC  
Soil organic carbon

SOM  
Soil organic matter
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>SUVA$_{254}$</td>
<td>Specific ultraviolet adsorption (at 254 nm) coefficient</td>
</tr>
<tr>
<td>T$_2$</td>
<td>$^1$H NMR spin-spin relaxation time</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>w/w</td>
<td>Wet weight</td>
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Chapter I

Introduction
Chapter I

1. General introduction to the study

The organic contaminant in soil can be degraded by biochemical transformation reactions, which lead to its complete mineralization and/or to the formation of xenobiotic metabolites (Semple et al., 2003). Bioavailability and bioaccessibility of hydrophobic organic pollutant and its degradation products are limited by processes of xenobiotic sorption to soil constituents, mainly to the soil organic matter (SOM) (Semple et al., 2004). It was shown that long-term xenobiotic–soil interaction may lead to slow sorption and entrapment of organic compound in the SOM structure. Therefore, aging of organic xenobiotic in soil can decrease its bioavailability and bioaccessibility in the course of time (Alexander, 2000). The aging also reduces a chemical extractability of the contaminant (Semple et al., 2003). Therefore, aging leads to the formation of xenobiotic non-extractable fraction or “bound residues” (Führ et al., 1998). The sorption of hydrophobic organic pollutant and the fate of xenobiotic in soil in general are preferentially governed by the properties of SOM, such as its aromaticity, condensity, structural conformation (Chefetz and Xing, 2009).

The SOM structure consists of supramolecular associations of small organic molecules (Piccolo, 2001). The degree of cross-linking of the SOM structure depends on many factors, such as, for example, C:N ratio of the SOM, cation composition of soil, and environmental conditions (e.g., soil temperature and moisture regimes, pH and salinity of soil water) (Gevao et al., 2000; Mordaunt et al., 2005; Northcott and Jones, 2000). The monovalent cations (e.g., K⁺ or Na⁺) can expand the SOM structure and make it more flexible, while polyvalent complexing cations (e.g., Ca²⁺ or Al³⁺) can increase the degree of cross-linking and make the SOM structure more condensed and rigid (Buurman et al., 2002; Lu and Pignatello, 2004; Yuan and Xing, 2001). The conformational properties of SOM also depend on the state of water molecules in the SOM structure, which in turn can be significantly altered by drying or temperature treatment of soil (Schaumann and Bertmer, 2008; Leite et al., 2012).

The fate of xenobiotic in soil also depends on its properties, such as hydrophobicity, polarity, toxicity for microorganisms (Haritash and Kaushik, 2009). Microbial activity can enhance the formation of bound residues, most likely due to the release of more reactive metabolites that covalently bind to SOM. On the other hand, the stimulation of microbial activity, for
example through organic substrates addition, can also remobilize bound residues and thus increase their biodegradation (Semple et al., 2006).

The objectives of this study were based on three main hypotheses: 1) Mono- and polyvalent cations can induce different cross-linking of the SOM and alter its conformational properties, which affects the process of sorption of organic xenobiotics in soil and formation of bound residues; 2) Bound residues formation can be influenced by changes in the physicochemical environment (pH, temperature, soil moisture), because they induce changes in the structural conformation of SOM; 3) The biodegradation of xenobiotics and their bound residues can be enhanced by increase of soil microbial activity due to addition of organic substrates, either through direct co-metabolic degradation of the xenobiotics or through enhanced degradation of the SOM matrix.

Referring to these hypotheses the main objectives of this study were: 1) Determination of sorption and desorption of xenobiotics model compounds in the soil saturated with different cations (Na\(^+\), Ca\(^{2+}\) or Al\(^{3+}\)), treated by different temperatures, dried and/or moistened; 2) Determination of the biodegradability of xenobiotics sorbed to SOM matrix with altered structural conformation; 3) Assessment of the role of long-term storage of xenobiotics in soil on the formation of bound residues; 4) Evaluation of the effect of organic substrate addition to soil on the biodegradation of xenobiotics and formation of bound residues; 5) Determination of xenobiotics extractability from soils at different stages of sorption and/or degradation process, using different solvents (water, cyclodextrin, ethanol).
2. Background: The role of soil organic matter in the fate of xenobiotics bound residues in soil

2.1. Soil organic matter structure

The SOM consists of a mixture of plant and animal residues at various stages of decomposition and of substances synthesized microbially and/or chemically from the breakdown products (Schnitzer, 1991). SOM plays a significant role in the functioning of soil in the environment. Quantity and quality (e.g., chemical and conformational properties) of SOM regulate the processes of sorption, sequestration and biodegradation of xenobiotics in soil.

SOM can be subdivided into non-humic and humic substances. First include substances with still recognizable chemical characteristics. These are, for example, carbohydrates, amino acids, proteins, peptides, fatty acids, resins and other low-molecular weight organic substances, which generally can be easily degraded in soil (Tan, 1998).

The main constituents of SOM are humic substances, the products produced through humification of plant residues and other organic materials. Humic substances have large chemical heterogeneity and geographical variability, and they range in molecular weight from a few hundred to several thousand daltons. These are amorphous, partly aromatic, dark-colored, chemically complex, polyelectrolyte-like materials (Schnitzer, 1991; Tan, 1998). Humic substances have largely remained uncharacterized at the molecular level and are still operationally defined in terms of the methods used to extract or isolate them (Kelleher and Simpson, 2006).

It was suggested (Piccolo, 2001) that humic substances are not single molecules, but can rather be interpreted by the concept of loosely bound humic supramolecular associations (Fig. I-1). In this concept humic substances are viewed as relatively small and heterogeneous molecules of various origins that self-organize in supramolecular conformations (Piccolo, 2001). Hydrophilic and hydrophobic domains of humic molecules can interact with each other and form large molecular size associations, which are stabilized only by weak forces such as dispersive hydrophobic interactions. These forces determine the conformational structure of humic substances, and the complexities of the multiple noncovalent interactions.
control their environmental reactivity (Kunhi Mouvenchery et al., 2012; Piccolo, 2001; Schaumann, 2006).

It was hypothesized that characterization of matrix structure of soil organic matter may be similar to characterization of interconvertible structural states of pure polymers: rubbery and glassy states (Graber and Borisover, 1998; Xing and Pignatello, 1997). Molecular chains in a rubbery structure have a relatively high flexibility. Reduction of molecular mobility and chain segment flexibility may be caused by the increase of humic substances molecular weight, the degree of unsaturation (due to multiple bonds, aromatic rings), and the degree of chain branching. Introduction of covalent cross-links between chains also leads to the reduction of molecular mobility in humic substances supramolecular structure, and to its transformation to glassy state (Yuan and Xing, 2001). It is important to note, that SOM may consist of a mixture of organic matter particles with different structures. Also individual particles of SOM may contain micro domains, which vary widely in rubbery-glassy character (Lu and Pignatello, 2004).

The rubbery state can reattain equilibrium quickly following an incremental change in temperature or other environmental condition. Unlike, the glassy state, is a nonequilibrium metastable state, which cannot quickly respond to environmental changes (Pignatello, 2012). In a rubbery polymer the diffusion of sorbate (organic compound) involves a
cooperative interchange of penetrant and polymer matrix. This process is more or less reversible because the rubbery polymer is relatively flexible. The diffusion through the glassy state (sorption or desorption) is more complex and may be considerably slower. Sorption of organic compounds on glassy parts of SOM is coupled to structural deformation of SOM, which happens in the course of time. This means that process of sorption itself can alter the soil organic matter structure over a time of soil-xenobiotic interaction (Pignatello, 2012). Hereby, the most important property of SOM – its ability to sorb and retain organic pollutants – can be altered during the long-term aging of soil. Short-term sorption and long-term aging studies are used for the evaluation of chemical and conformational properties of SOM, as well as for the investigation of effects of different agents on the features of SOM (Mavi et al., 2012; Paya-Perez et al., 1992; Zhang et al., 2009).

2.2. The effect of different agents on soil organic matter properties

2.2.1. Mono- and polyvalent cations in soil and soil solution

The cations in soil can be divided into three major categories: solid phase, exchangeable, and soluble ions. Cations in solid phase are presented by soil primary minerals and cations, which were initially released by weathering and/or organic decay and then reprecipitated. The exchangeable cations can be released to the solution, but they tend to associate with surfaces of the solid phase of soil, i.e. the minerals and SOM. The soluble cations are poor competitors of exchangeable cations for surface charge; they can be removed from soil by water alone, and therefore remain predominantly in the bulk soil solution (Bohn et al., 2001).

It was supposed that individual molecules of SOM are linked between each other and the mineral part of soil by bridges of water molecules and/or by cations in so-called exchange sites (Aquino et al., 2011; Piccolo, 2001). At these sites one cation can be replaced by another cation. When ions have the same valency, the cation with smallest hydrated size is preferably adsorbed by soil organo-mineral matrix according to the lyotropic series, e.g., $\text{Al}^{3+} > \text{Mg}^{2+} > \text{Ca}^{2+} > \text{K}^+ > \text{Na}^+$ (Tan, 1998).
Polyvalent cations are strong complexing agents, which can form up to four linkages with functional groups of one macromolecule and/or few relatively small molecules of SOM (Fig. 1-1b). These linkages may be characterized by high conformational stability (Nuzzo et al., in press; Schulten and Leinweber, 2000). The extent of such bond formation, and thus the alteration of SOM conformation, depends on the molecular size, composition and initial configuration of SOM, as well as on the chemical properties of cation, and cation/SOM ratio (Schaumann and Thiele-Bruhn, 2011). A number of studies with dissolved organic matter (DOM) and humic acids in aqueous solution showed that addition of polyvalent cations to the solution increased condensation, matrix rigidity, thermal stability and stability against biological degradation, as well as hydrophobicity and water repellency of these components of SOM (Buurman et al., 2002; Lu and Pignatello, 2004; Nebbioso and Piccolo, 2009; Polubesova et al., 2007; Saison et al., 2004; Scheel et al., 2007; Scheel et al., 2008). It is also important to note that in the process of formation of cation-mediated organic complexes the acidity and ionic strength of the soil solution are also very important factors (Kunhi Mouvenchery et al., 2012). Generally it is possible to suppose that the polyvalent cations may lead to the formation of more condensed SOM structure than the monovalent cations, and the effect on the SOM properties amplified with the increase of valency of applied cation (Kaiser, 1998; Luo et al., 2008; Yuan and Xing, 2001).

The knowledge about the effects of different cations on the SOM molecular structure is still insufficient and further research of the cation-SOM interactions is needed. Also there is a lack of systematic studies addressing the effects of various cations on the soil properties in changing environmental conditions (Kunhi Mouvenchery et al., 2012).

**2.2.2. Water in soil and formation of water-cation bridges in SOM structure**

Supramolecular structure of SOM is a multi-component system, in which molecules of organic matter and ions are held together by non-covalent interactions like van der Waals forces, \( \pi-\pi \) interactions, hydrogen bonds, or electrostatic effects. The stability of supramolecular assembly is lower compared to the stability of its constituents. Therefore, slow transformation of SOM caused by changes in environmental conditions, such as moisture, temperature, physicochemical characteristics of soil and soil solution, may result from alterations in supramolecular structure of SOM (Schaumann et al., 2013).
The cross-links between individual molecule sections through intermediary water molecules are significantly important for the matrix rigidity of SOM supramolecular structure (Aquino et al., 2011; Schaumann and Bertmer, 2008). The formation of cation bridges between protonated functional groups at the ends of molecular chains of SOM can occur via hydration of cation. The relevance of water molecules for cation bridging process depends strongly on the protonation/deprotonation status of the soil solution, in the other words on the pH level (Kunhi Mouvenchery et al., 2012).

Depending on the presence or absence of the hydration shell of the cation, the outer sphere or inner sphere complexes in the SOM matrix can be formed, respectively (Kalinichev and Kirkpatrick, 2007). Preference for either inner sphere or outer sphere complexes depends on the ion charge, ion radius and on such steric factors, as the distance between functional groups and the gain in free energy. In this context, the water molecule bridges play a central role in stabilizing SOM supramolecular structure (Aquino et al., 2011; Kalinichev and Kirkpatrick, 2007; Kunhi Mouvenchery et al., 2012; Schaumann and Bertmer, 2008). However, it is important to note that the stabilizing effect of water molecular bridges can appear only at intermediate water content, because with the increase of water content in soil the swelling and plasticizing processes predominate (Schaumann and LeBoeuf, 2005).

The obvious effect of water content on the SOM conformational structure was detected by contact-mode atomic force microscopy. Studies of the conformational structure of humic acids adsorbed on mica surface showed specific ring-shaped networks with holes inside (Fig. I-2a, c) (Leite et al., 2012). Samples of humic acids that were rinsed with water and then dried did not show the ring-shaped structures (Fig. I-2b). The authors suggested that the formation of more condensed structures of humic acids could be due to two processes: a) either hydrophobic humic acids self-associated into condensed supramolecular structures and separated from water medium while rinsing, or b) subsequent slow air-drying at room temperature allowed lateral movement and stretching of the humic acid structures along the mica surface (Leite et al., 2012). In both proposed mechanisms, water plays the dominant role in configuration of the SOM molecular structure.

It was recently hypothesized that the hydrophobicity of the SOM surface may be controlled by the formation of water and water-cation bridges in the interior of the SOM matrix.
The conducted study revealed that the soil treated with cations of different valency showed significant variability of the surface hydrophobicity. It was suggested that possibly the interior formation of water and water-cation bridges determined the thickness or continuity of the water film on the surface of SOM and thus affected its hydrophobicity (Schaumann et al., 2013). Based on the results of $^1$H-NMR-relaxometry, $^1$H wide-line NMR spectroscopy and contact angle studies, the conceptual model of the relation between structural features of the SOM and wetting properties of SOM surface was proposed (Fig. I-3). It was suggested that the SOM structure and wetting situation on the SOM surface can be manipulated depending on the configuration of hydration shell of cation and on the bond force between the cation and water molecules.

The studies that describe the effect of water molecules distribution along the SOM structure on the properties of SOM are very limited. However, researchers often conduct temperature treatments (e.g., heating, freezing) of SOM or soil samples to evaluate their influence on SOM, i.e. possible changes of its wetting and conformational properties (Kleber et al., 2010). Previous studies showed that in comparison with air-dried humic acids, the freeze-dried substances exhibit a significantly higher initial surface area, which however is not stable upon contact with water (Hung et al., 2012). Drying of soils and sediments led to the increase of their hydrophobicity and significantly altered the sorption of polycyclic aromatic hydrocarbons (Liu and Lee, 2006; Tang and Weber, 2006). Repeated wetting and drying of
soils decreased extractability of sorbed organic xenobiotics (Kottler et al., 2001). However, Eschenbach et al. (1998) reported earlier that repeated freezing-thawing cycles conducted with contaminated soils did not affect extractability of polycyclic aromatic hydrocarbons. In contrast, it was shown that drying of soil significantly increased release of dissolved organic carbon (DOC) from it, while freezing gave similar, but less evident effect on SOM (Kaiser et al., 2001). Moreover, drying of soil was found to also decrease the SOM mineralization rates (Lamparter et al., 2009; White et al., 1998). Thus, the available knowledge about the effects of dehydration and temperature treatments on SOM structure is limited and reports from different studies are often controversial.

To summarize, SOM is a very complex organo-mineral matrix, and processes of its alteration in soil is still not-well understood. Nevertheless, it is known that different environmental factors affecting the soil, such as temperature regime, drying cycles, changes in acidity of soil solution, etc., may in turn significantly alter features of SOM.
2.2.3. Changes of SOM as an ecosystem property

Soil is a complex system in which solid, liquid, gas and biology all interact in the course of time. The functioning of this system is difficult to incorporate into one model. In frame of this conceptual problem the understanding of processes of SOM formation and transformation in soil is very challenging for scientific community (Kleber et al., 2010).

For a long time it was considered that humic substances are the most persistent natural organic constituents, which are present in soil in a quasi-stable state. Recent studies however provide the evidence that SOM can be characterized as both “dynamic” and “refractory” at the same time (Kleber et al., 2010). This dual behavior of SOM may be caused, on the one hand, by its chemical and physical properties, such as complexity, condensity, aromaticity, elemental composition and other features of organic carbon, which govern stability of its molecular structure. On the other hand, SOM is constantly affected by the physicochemical and biological agents of the surrounding environment that can increase (or decrease) the probability, and therefore rate, of SOM decomposition (Schmidt et al., 2011). In other words, the persistence and alteration of SOM, and of humic substances in particular, in soil is not only a function of its molecular properties, but also a property of ecosystem.

It is important to note that the prevalence of humic substances in soil compared to other organic matter in soil, which has been assumed for decades, needs reconsideration. Laboratory studies, based on the extraction methods, define humic substances as large, complex macromolecules. On the contrary, the direct, in situ studies rather than confirming the existence of such molecules, in fact find smaller, simpler organized molecular structures (Kelleher and Simpson, 2006; Schmidt et al., 2011). In the light of new knowledge the importance of studying soil as a whole complex system, rather than analyzing its isolated SOM, becomes more evident.

The results of novel high-resolution in situ studies of non-destructed soil samples are still rare (Kögel-Knabner, 2000; Rennert et al., 2012; Sparks, 2006). The analyses of interaction of soil with different organic chemicals are the most common methodological approach for investigation of functioning of SOM in soil (Kleber et al., 2010). A great number of long-term
sorption, degradation, mineralization, and extraction studies with $^{14}$C-labelled compounds were conducted for indirect analyses of SOM hydrophobicity, density, structural organization, microporosity, bioavailability, affinity for mineral particles, etc. Nevertheless, the understanding of SOM molecular structure, its chemical properties and functioning in the environment is still not comprehensive.

2.3. The fate of xenobiotics in soil and processes of bound residues formation

When organic contaminants reach soils or sediments, they become involved in a number of processes, which can lead to their gradual disappearance or immobilization (Dec and Bollag, 1997). The interaction between soil and xenobiotics is governed by biotic and abiotic factors, which together affect the fate of pollutants in soil.

The most important abiotic influential factors are: chemical properties of xenobiotic and its concentration in soil; chemical and conformational properties of soil constituents interacting with pollutant (e.g., properties of SOM and minerals present in soil bulk and in soil solution); extracellular soil enzyme activity; soil water properties (e.g., pH level, salinity); soil temperature, aeration and moisture regimes (Gevao et al., 2000; Mordaunt et al., 2005; Northcott and Jones, 2000). The biotic factors significantly depend on the above listed soil properties and in general can be specified by the type of soil. The major biological influential parameters, which affect the fate of xenobiotics in soil, are: microbial biomass; homogenization of microbial community in a bulk of soil; presence of microorganisms or enzymes able to degrade the applied xenobiotic; biological diversity; plant cover (Chaplain et al., 2011; Haritash and Kaushik, 2009). The kinetics of all transformations occurring with xenobiotic in the soil cannot be evaluated without taking into account the duration of soil–xenobiotic interactions. This means that the aging is a crucial factor affecting the fate of pollutants in soil (Reid et al., 2000).

Obviously, the fate and persistence of xenobiotics in soil are the subjects of comprehensive, intensive and continuous research (Gevao et al., 2000). However, systematic and detailed studies of xenobiotics transformation in soils are limited. Nevertheless, the accumulated
knowledge about the fate of various xenobiotics in different soils and sediments allows to predict the most likely scenario of pollutant behavior in the environment (Reid et al., 2000).

The organic pollutants are subjected to several processes in soil: losses due to volatilization and leaching of xenobiotics and their metabolites; losses by chemical reactions, such as oxidation, photolysis, hydrolysis, etc.; losses due to biological accumulation, transformation and mineralization; and binding to the solid phase of soil. As a result, the amount of organic pollutants in soil declines, but significant portion of compounds and/or their metabolites remains in soil. The persistent fraction of xenobiotics is presented in soil by labile and/or bound residues. The amount of labile residues of xenobiotics tends to decrease with time, while bound residues increase (Eschenbach et al., 1998; Northcott and Jones, 2000; Semple et al., 2003).

Conceptually, the bound residues in soils, sediments, plants, or animals represent the parent xenobiotic component or its metabolite(s), which are unextracted by methods that do not significantly alter the chemical nature of compounds and/or the structure of the preserving matrix (Führ et al., 1998). Consequently, the amount estimated as bound residues is operationally defined by the applied extraction procedure. At the moment there is no standard internationally approved method for evaluation of bound residues fraction in different soils, and comparison of results of different studies should be made with great care (Chaplain et al., 2011). The quantification of bound residues in soils is performed using radiotracer techniques, but the qualification of the bonds and forces occurring between xenobiotics and soil can only be made using indirect analyses based on a large number of various techniques and methods (Northcott and Jones, 2000).

The most important mode of soil–xenobiotic interaction is adsorption, which can vary from complete reversibility to total irreversibility, and depends on soil features and the compound properties, such as its size, shape, molecular structure, configuration, solubility, chemical functions, polarity, polarizability and charge distribution, as well as the acid-base nature of the xenobiotic molecule (Gevao et al., 2000). The sorption phenomenon includes both the physical and chemical interactions between soil and pollutants or/and their metabolites. The introduction to some possible binding forces, which govern the sorption and entrapment of xenobiotics on SOM, is presented below. The fundamental studies and review works of Wais
(1998), Senesi (1992), Gevao (2000), Dec and Bollag (1997) were used for preparation of this condensed description.

**Ionic bonds** occur between SOM and xenobiotics, which exist in cationic form or can act as proton acceptors. Ionic bonding involves ionized, or easily ionizable, carboxylic and phenolic hydroxyl groups of humic substances, and thus can be pH dependent. Ionic bonding of xenobiotics on the structure of SOM is highly stable.

**Hydrogen bonding** is suggested to play an important role in the adsorption of non-ionic polar pesticides on humic substances. Hydrogen bonding can be formed between oxygen- and hydroxyl-containing functional groups of humic substances. Xenobiotic molecules compete with already existing ligands, e.g. water molecules, for these binding sites.

Compared to chemical bonds mentioned above, **van der Waals forces** are relatively weak short-range intermolecular forces, which have different origin. Often, in humic substances chemistry, the term “van der Waals forces” is a synonym for dipole-dipole forces, which occur between nonionic or non-polar xenobiotics and SOM. Van der Waals forces exist in addition to stronger bonds, and decay rapidly with distance increase between interacting molecules.

**Ligand exchange** is a process that occurs in SOM complexes with polyvalent metal ions, which are generally also associated with water molecules. These relatively weak ligands can be replaced by appropriate functional groups of organic pollutants.

**Charge-transfer complexes** can be formed via electron donor-acceptor mechanisms, when molecules with a high electron density react with molecules with electron deficiency. The amount of complexes formed in the SOM structure is determined, for example, by the aromatic structure of the xenobiotic and quinone structures of the humic substances. Charge-transfer complexes are characterized by relatively high resistance and stability.

**Hydrophobic partitioning** is interaction mechanism between hydrophobic groups of humic substances and non-polar xenobiotics. SOM functional groups are pH-dependent, which in turn may alter the internal and external hydrophobic surfaces of SOM matrix, and affect hydrophobic partitioning of xenobiotics.
Covalent bonding between pollutants and/or their metabolites and SOM are very strong forces leading to stable, irreversible retention of xenobiotic in the humic substances matrix. Processes of covalent bonding of xenobiotic onto SOM are often mediated by chemical, photochemical or enzymatic catalysts and may occur in soil due to oxidative coupling mechanism. Covalently bound pollutants become integral components of the SOM and cannot be extracted without changing the SOM properties. This means that covalent bonding may be one of the most important processes, which govern formation of bound residues in soil.

Sequestration (aging) is a physicochemical process of slow integration and diffusion of non-polar and hydrophobic xenobiotics into the structure of SOM. Sequestration is closely related with sorption phenomena, which are generally ascribed to binding mechanisms, occurring between xenobiotics and SOM surfaces instantaneously upon the first contact.

It was suggested that rubbery regions of SOM, discussed above, are domains for partitioning during the fast sorption processes. Glass-like parts of SOM are believed to be responsible for both partitioning and filling of holes (diffusion) in the structure of humic substances during long-term aging of xenobiotics in soil (Pignatello, 2012; Subramaniam et al., 2004). Thus, it is possible to summarize that organic pollutants generally exhibit biphasic behavior within the soil: (i) a portion of xenobiotic can be sorbed quickly within minutes to a few hours; (ii) the remaining part is sorbed much slower over weeks and months (Xing and Pignatello, 1997). The amount and forces of bonds between soil and xenobiotics and/or their metabolites determine the content of different fractions of pollutants in soil.

Xenobiotic fractions in soil can be evaluated using a great number of biological (e.g., incubation) and chemical (e.g., extraction) methods. Various methods allow to distinguish labile, sequestered and bound forms of the organic contaminants in soil and sediments (Northcott and Jones, 2000).

The presence of the following fractions of organic pollutants in soils can be determined: (i) degradable, or removable fraction, which can be referred to easily extractable and
degradable compounds; (ii) readily available fraction, which can also be referred to bioavailable compounds, although with lower extractability and bioaccessibility; (iii) recalcitrant fraction, which mainly refers to sorbed and sequestered compounds; and (iv) non-extractable fraction, which is equal to xenobiotics bound residues fraction (Fig. I-4).

The speciation of different fractions of xenobiotics in soil is the subject of current research. There is no standard approach for investigation of the nature of binding and sequestration of xenobiotics in soil, but the importance of such studies is obvious: processes of physical and chemical sequestration of organic contaminants in soil determine the processes of their biodegradability and bioaccumulation, as well as rates of xenobiotics dissolution and release with natural waters (Bollag et al., 1992; Nam and Alexander, 2001; Wilcke, 2000).

3. Objectives and outline of this study

It is well known that when hydrophobic organic pollutant reaches the soil it can be strongly sorbed to SOM and the fate of the xenobiotic is defined by the properties of the soil (mainly of SOM) and by environmental conditions. Many studies, mentioned in the previous chapter, showed that alteration of the SOM structural properties may significantly affect the

![Figure I-4](image.png)
processes of adsorption, degradation and mineralization of organic chemicals in soil, which in turn govern the formation of xenobiotics bound residues. Our study was based on three main hypotheses:

1) The sequestration of xenobiotics in soil and formation of bound residues are affected by the structural conformation of SOM, which in turn depends on the cation composition of the soil matrix. In particular, introduction of polyvalent cations into the SOM structure increases the degree of its cross-linking and rigidity (glassiness), which results in the reduction of fast partitioning of xenobiotic in favor of slow sorption process leading to a higher proportion of bound residues during aging.

2) Changes in the physicochemical environment (pH, temperature, soil moisture) affect the formation of bound residues, because they induce changes in the structural conformation of SOM.

3) The biodegradation of xenobiotics and their bound residues can be enhanced by increase of soil microbial activity due to addition of organic substrates, either through direct co-metabolic degradation of the xenobiotics or through enhanced degradation of the SOM matrix.

Referring to these hypotheses, the main objectives of this study were:

1) Determination of sorption and desorption of xenobiotics model compounds in the soil depending on the different SOM structural conformation induced by varying
   - complexing agents (cations),
   - environmental conditions (temperature, soil moisture),
   - duration of soil storage (aging).

2) Determination of the biodegradability of xenobiotics after sorption to SOM matrix with altered structural conformation.

3) Assessment of the role of long-term storage of xenobiotics in soil (sterile and biologically active) on the formation of bound residues and alteration of other soil properties (e.g., enzyme activity).
4) Evaluation of the effect of the addition of organic substrate to soil on the biodegradation of xenobiotics and formation of bound residues.

5) Determination of xenobiotics extractability from soils at different stages of sorption and/or degradation process, using different solvents (water, cyclodextrin, ethanol).

Two organic chemicals were chosen as model compounds: phenanthrene (Phe) and p353-nonylphenol (NP). The NP and Phe are widely presented in the environment. The NP is a primary breakdown product of nonylphenol ethoxylates. These chemicals are non-ionic surfactants, which are used in agricultural and industrial applications (Guenther et al., 2002). The Phe is a representative compound of the large group of polycyclic aromatic hydrocarbons, which can be formed in the environment during incomplete burning of oil, gas, coal or other organic substances; and due to decay and decomposition of some dyes, plastics and pesticides (Van der Perk, 2006). NP and Phe are potentially hazardous to microorganisms, plants and animals due to their toxicity and carcinogenicity (Frutos et al., 2010; Soares et al., 2008). Depending on the concentration the effect of xenobiotic on the soil microbiology can be substantial and in some cases drastic (Imfeld and Vuilleumier, 2012). Our preliminary (unpublished) studies showed that neither NP nor Phe at concentration of 10 $\mu$g g$^{-1}$ of soil significantly affected the soil respiration, which most probably indicates the negligible effect of this concentration of xenobiotic on the soil microbiology. In this study the NP and Phe were used at concentration of 10 $\mu$g g$^{-1}$ of soil.

The NP and Phe were chosen for our study as model compounds because of their wide spread occurrence in the environment and of their different chemical properties (Fig. I-5). Both compounds are known as hydrophobic chemicals. The octanol/water partitioning coefficient $K_{ow}$ of NP and Phe varies in a similar range: log $K_{ow}$ is 4.7 and 4.5, respectively for NP and Phe (Montgomery and Welkom, 1990; Porter and Hayden, 2002). This coefficient is commonly used for prediction of the sorption rates of hydrophobic xenobiotics in soil (Ying, 2006). However, we speculated that these two compounds may interact with soil differently, because one of them, the NP, has the phenolic group, which determines the lipophilic properties of this xenobiotic. Therefore, in contrast to the Phe, the NP is ionizable and pH-dependent chemical (Murillo-Torres et al., 2012).
Alteration of SOM structure was conducted via treatment of sandy soil with different cations: H⁺, Na⁺, Ca²⁺ and Al³⁺ using deionized H₂O or 0.1 M NaCl, CaCl₂ and AlCl₃ aqueous solutions, respectively. Obtained soil samples were sterilized by γ-radiation, and then one part of soil was spiked with ¹⁴C-labelled Phe or NP at 10 μg g⁻¹ of soil, while another part remained uncontaminated. The series of sorption, extraction and degradation experiments was carried out. The additional alteration of soil properties was achieved by drying and freezing of samples, long-term sterile aging, and incubation of re-inoculated soils with substrate and water additions. The scheme of experimental approach is presented in the Fig. I-6.

The studies presented in this thesis can be divided into four main topics:

1) Extraction study. The main objective of this study was to investigate the effect of long-term soil aging and incubation on the extractability and degradability of NP and Phe in cation-treated soils. In addition it was tested if drying or freezing of soils can accelerate the relevant aging processes. Samples were then used in four parallel experimental setups: (i) 9 months of storage under sterile conditions (aging), (ii) inoculation by native original soil with further 7 months of incubation (bioaging), (iii) drying and wetting or (iv) freezing and thawing of soils. After different time intervals, the extractability of xenobiotics with water, cyclodextrin, and ethanol was investigated.
Chapter I

The research was published in: “Interacting effects of cation saturation and drying, freezing, or aging on the extractability of nonylphenol and phenanthrene from a sandy soil” Shchegolikhina A., Schulz S., Marschner B., 2012, Journal of Soils and Sediments, 12, pp. 1280-1291. In this thesis it is presented in the Chapter II.

The unpublished results of the additional study of xenobiotics extractability, which was carried out using alternative in situ pore-water extraction method, are presented in the Appendix I.

II) Sorption study. The objective of this study was to investigate the effects of incorporating mono- and polyvalent metal cations into the structure of SOM on alterations of the soil's sorption properties. The samples were then sterilized and either stored moist, or dried at room temperature or at 20°C, 60°C or 105°C in a vented oven. Treated soils were used for batch sorption experiments with NP and Phe. The research was accepted for publication: “Cation treatment and drying-temperature effects on nonylphenol and phenanthrene

III) Degradation study. The main objective of this study was to determine the effects of long-term abiotic processes during aging of NP and Phe in soil on their microbial degradability and formation of bound residues. The specific aims of study were to investigate how the fate of NP and Phe in soils might be affected by: (i) saturation of soil by cations, (ii) addition of organic substrate (wood flour) during incubation period, (iii) different soil moisture levels. The research was submitted for publication: “Effects of sterile storage, cation saturation and substrate additions on the degradability and extractability of nonylphenol and phenanthrene in soil” Shchegolikhina A., Marschner B., Chemosphere. In this thesis it is presented in the Chapter IV.

The unpublished results of additional study of xenobiotics mineralization by specific microorganisms are reported in the Appendix I.

IV) Enzyme study. The study of enzymatic activity of soils aged for 1 and 10 months was submitted for publication: “Effects of $\gamma$ irradiation, storage and incubation on enzyme activities in a sandy soil treated with different salts” Shchegolikhina A., Marschner B., Soil Biology & Biochemistry. Here it is presented in the Chapter V.
References


Chapter II

Interacting effects of cation saturation and drying, freezing, or aging on the extractability of nonylphenol and phenanthrene from a sandy soil

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Abstract

Purpose: The structure and properties of the soil organic matter (SOM) and its interactions with solutes may be altered by changes in soil chemistry and by the aging of soil. The main objective of this study was to investigate the effect of long-term aging and cation saturation of soil on the extractability and degradability of two hydrophobic xenobiotics in soil. In addition it was tested if drying or freezing of soils can accelerate the relevant aging processes.

Materials and methods: The sandy topsoil was treated by either 0.1 M NaCl, CaCl₂, AlCl₃ solutions or water and samples were sterilized by γ-radiation and spiked with ¹⁴C-labelled nonylphenol (NP) or phenanthrene (Phe) at 10 μg g⁻¹ of soil. Samples were then used in four parallel experimental setups: (i) 9 months of aging under sterile conditions, (ii) inoculation by native original soil with further 7 months of aging (bioaging), (iii) drying and wetting or (iv) freezing and thawing of soils. After different time intervals, the extractability of xenobiotics with water, cyclodextrin, and ethanol was investigated.

Results and discussion: During 9 months of aging under sterile conditions a continuous decrease of NP and Phe extractability and an increase of the non-extractable fraction occurred. During the 7 months of biologically active aging the mineralization of NP was lower than of Phe while more NP remained extractable than Phe. In comparison to the sterile aging, the bioaging led to a less formation of non-extractable residues of NP and Phe. The long-term sterile aging effects on NP extractability were also achieved by short-term freezing and thawing of the soils, while aging of Phe was better mimicked by drying-wetting cycles. The effects of cation saturation of soils on xenobiotics extractability were less pronounced.

Conclusions: Sterile aging, bioaging, freezing and thawing facilitate the formation of the non-extractable fraction of NP and Phe in the soils. Different cation treatments alter soil properties, but the effects on aging of NP and Phe in soils were negligible.
Interacting effects of cation saturation and drying, freezing, or aging on the extractability of nonylphenol and phenanthrene from a sandy soil

1. Introduction

Many organic pollutants reaching soils are strongly sorbed to organic and mineral soil constituents which reduces their biodegradability, mobility and volatilization (Northcott and Jones, 2000; Semple et al., 2003). Initial sorption of xenobiotics to soil is a rapid and reversible process, which is often followed by a period of slow sorption, occurring over months and even years of aging (Bollag et al., 1992; Northcott and Jones, 2000). Generally aging leads to the progressive formation of a fraction resistant to a desorption, biodegradation, and mineralization (Puglisi et al., 2007; Scow et al., 1995; Tang et al., 1998). In this context, the extractable and bioavailable fractions decrease with time while the irreversibly bound or firmly bound fractions of contaminants increase (Northcott and Jones, 2000; Senesi, 1992).

Many studies that report on aging of xenobiotics in soils, describe the effects without differentiation between biotic and abiotic transformation processes (Fava et al., 1998; Jin et al., 2008; Puglisi et al., 2007; Telscher et al., 2005; Topp and Starratt, 2000). In such cases, it remains unclear if the formation of non-extractable residues is due to either physicochemical processes, such as slow diffusion into micropores (Pignatello and Xing, 1996), entrapment into hydrophobic cavities or the glassy phase of the soil organic matter (SOM) (Xing and Pignatello, 1997), or due to biochemical reactions, such as biological incorporation of metabolites (Berns et al., 2008; Nowak et al., 2011) or partial oxidation by exoenzymes and subsequent covalent bonding to SOM (Bollag et al., 1992).

Biological activity can be almost completely excluded from the processes of aging or soil development via sterilization (Powlson and Jenkinson, 1976). In sterile soils only the physicochemical processes may govern sorption and sequestration of xenobiotics during aging. Formation of extractable and non-extractable fractions will be strongly influenced by the soil moisture, since most of chemical reactions and many physical processes in soils depend on interactions and transport of compounds in the aqueous phase. Furthermore, the amount of water molecules and its aggregation state define the expansion rate of the SOM structure and occurrence of sorption domains (Aquino et al., 2011; Schaumann and Bertmer, 2008; Schneckenburger et al., 2012). Aging of xenobiotics in soils with constant moisture has
been widely investigated, while studies on the effects of drying and wetting, freezing and thawing are limited. It was reported that drying-wetting or freezing-thawing cycles conducted with contaminated soils can enhance, decrease or have no significant effect on the sequestration of organic compounds in soils (Chung and Alexander, 2002; Eschenbach et al., 1998; Kottler et al., 2001; Northcott and Jones, 2003; White et al., 1998; Zhao et al., 2009). Some of these studies indicate that drying and freezing has a comparable sequestering effect like aging of soils under sterile conditions (White et al., 1998; Zhao et al., 2009).

Another factor which can affect the SOM structure conformation and thus influence its sorptive properties is the presence of different cations. It is known that monovalent cations, like $K^+$ or $Na^+$, can expand the SOM structure and make it more flexible. In contrast, polyvalent complexing cations, like $Al^{3+}$, make the SOM structure more condensed and rigid (Buurman et al., 2002; Lu and Pignatello, 2004; Yuan and Xing, 2001). Previously, different SOM fractions with defined characteristics, for example humic acids, were generally used in order to study the effect of SOM structural conformation on the sorption of xenobiotics (Lu and Pignatello, 2004; Polubesova et al., 2007). Much less efforts were dedicated to investigate cation effects on the behavior of pollutants in natural soils (Kunhi Mouvenchery et al., 2012).

The main objective of this study was to investigate the interactive effect of aging and cation saturation on the extractability of two hydrophobic xenobiotics in soil. In addition it was tested if drying or freezing of soils can accelerate the relevant aging processes. Two compounds, with different chemical structure and polarity were used in our study: the hydrophobic phenanthrene (Phe) comprised of three aromatic rings ($\log K_{ow}$ 4.5, $S_w$ 1.3 mg L$^{-1}$) (Montgomery and Welkom, 1990) and the branched nonylphenol isomer 4-(3,5-dimethyl-3-heptyl)-phenol with both hydrophobic (aliphatic) and polar (phenolic -OH) subunits ($\log K_{ow}$ 4.7, $S_w$ 6 mg L$^{-1}$) (Porter and Hayden, 2002). The specific aims of our study were: (i) to evaluate the NP and Phe extractability from sterile soils during a 9 months aging experiment, (ii) to characterize xenobiotics degradability as well as extractability from microbially active soils incubated for 7 months, (iii) to analyze the influence of drying-wetting and freezing-thawing cycles on the formation of non-extractable residues in soils.
2. Materials and methods

2.1. Soil preparation

The sandy soil was collected from 0-20 cm of an agricultural field near Hannover, Germany. It was dried at 20°C and sieved ≤ 2 mm. For the alteration of the cation saturation of the exchange sites, 100 g of soil was filled into steel cylinders of 5 cm height and 4.5 cm diameter and placed into percolation units. The samples were then leached with 0.1 M aqueous solutions of NaCl, CaCl$_2$, or AlCl$_3$ at a ratio of 1:50 (w/w) over 18 h, then these three samples were leached with deionized water at a ratio 1:15 (w/w) until electric conductivity of the leachate reached constant values. An additional reference sample was obtained by leaching with water instead of salt solutions. After the percolation treatment, the soils were dried at room temperature. The percolation was performed at low rates (approx. 45 mm h$^{-1}$), thereby minimizing physical disturbances.

The soil-water potential of all five samples (original soil, H$_2$O-, Na-, Ca- and Al-soil) was determined in a pressure chamber. Samples were placed on the sand or clay layer and the defined pressure was applied. Water content at each pressure level was measured by weighing the sample.

In accordance with the determined soil-water potential values the soil samples were moistened to pH 2.8 (-630 hPa). The moist soils were then sterilized three times by $\gamma$-radiation with 24 hour intervals for a retained microbial activation and growth. In total, the soils were exposed to a dose of 75 kGy. After sterilization all further treatments and experiments were performed under sterile conditions. In cases when acidity of soils changed due to salt and water percolation, the pH was adjusted to the same level for all samples by adding of certain amounts of 0.05 M HCl to soils. Final water content of all soils corresponded to pF 2 (-100 hPa).

A portion of the sterilized unspiked soil was used for determining general soil properties. Amounts of the total soil organic carbon and nitrogen, dissolved organic carbon (DOC), water soluble, exchangeable and total cations were determined. Acidity of soil solution and particle-size distribution were analyzed (see below).

For spiking of soils, two compounds were used. $^{14}$C-ring-labeled and unlabeled nonylphenol
(NP) (98% purity, RWTH Aachen, Aachen, Germany) was dissolved in methanol. $^{9-14}$C-labeled phenanthrene (Phe) (99.7% purity, Campro Scientific GmbH, Berlin, Germany) and unlabeled Phe (99% purity, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) were separately dissolved in ethanol. The chemicals were applied to the dry sea sand (washed, untreated by acid, Sigma-Aldrich Chemie GmbH, Steinheim, Germany), solvents were evaporated and then the spiked sand was mixed into the moist soils (1%, d/w) to achieve the concentration of $10^3$ g $^{-1}$ soil for both compounds. Radioactivity of the spiked soils was 1800 Bq $g^{-1}$. The total concentration of NP or Phe in the samples was controlled by combustion (see below).

Sterilized original, $H_2O$-, Na-, Ca- and Al-soils contaminated by NP or Phe were divided into four parts for different sets of the experiments:

1. Sterile aging. Sterilized soils were divided into four parts. One part was analyzed after 24 h from contamination. Another three were investigated after 2, 5 and 9 months of storage in closed jars at constant 20°C in the dark. The loss of water from samples was monitored by weighing of the jars with soil at the beginning and at the end of aging. The weight change and water loss were negligible. Aged soils were used for water-ethanol, cyclodextrin and ethanol extraction experiments (Table II-1). Additionally, a sterility test was performed in soils aged for 9 months (see below).

2. Drying. Spiked unaged soils were dried at 20°C until constant weight was reached. Then dry soils were divided into two parts. One part was stored in closed jars. Another part was rewetted with the amount of water evaporated from samples. Rewetted soils were dried again. In total, four drying-wetting cycles (48 h each) were performed, and as soon as this procedure was completed the dry and wet-dry treated soils were subjected to the water extractions.

3. Freezing. Spiked unaged soils were divided into two parts, placed into jars and closed. One part was stored at $-30°C$. Another part was frozen at $-30°C$ for 24 h and then thawed at 20°C, after which the samples were frozen again. In total, four freezing-thawing cycles were performed, and as soon as this procedure was completed samples treated with one and four freeze-thaw cycles were subjected to the water extractions.
Interacting effects of cation saturation and drying, freezing, or aging on the extractability of nonylphenol and phenanthrene from a sandy soil

4. Bioaging. Sterile soils were mixed with non-sterile original soil preincubated moist for 14 days (5%, w/w), placed in jars with screw caps, and then incubated for 7 months in the dark at 20°C. Once a week jars were opened for 30 sec and then closed. The loss of water from samples was monitored by weighing of jars with soil at the beginning and at the end of the incubation. The weight loss was negligible. Loss of volatilized NP or Phe was also assumed to be negligible. After incubation soils were used for water-ethanol extraction experiments. Furthermore, the total content of $^{14}$C in the samples at the end of the bioaging experiment was analyzed by combustion.

All extraction experiments carried out after aging, drying, freezing and bioaging are summarized in Table II-1.

**Table II-1** Extraction experiments carried out with sterile, dried, frozen, and incubated soils.

<table>
<thead>
<tr>
<th>Extraction</th>
<th>Water-ethanol</th>
<th>Cyclodextrin</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent</td>
<td>Deionized water</td>
<td>Ethanol absolute 99.9%</td>
<td>1 mM cyclodextrin aqueous solution</td>
</tr>
<tr>
<td>Sequential cycle</td>
<td>I</td>
<td>II</td>
<td>III</td>
</tr>
<tr>
<td>Duration of shaking, [hours]</td>
<td>3</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Experiment and treatment mode</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sterile aging</td>
<td>24 hours</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>2 months</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>5 months</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>9 months</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Drying-wetting</td>
<td>1 time dried</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>4 times dried</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Freezing-thawing</td>
<td>1 time frozen</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>4 times frozen</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Bioaging</td>
<td>7 months</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

2.2. Analytical methods

Contents of the organic carbon and nitrogen were determined with an elemental analyzer (Vario EL, Elementar Analysensysteme GmbH, Hanau, Germany) in dry ground soil samples. The total cation content was analyzed after microwave pressure combustion with HNO$_3$ (ISO 15587-2-2002-07). The exchangeable cations were determined in NH$_4$Cl extracts (Lüer and
Böhmer, 2000). The water soluble xenobiotic fraction was determined by shaking the moist samples with water at a ratio of 1:10 (d/w) for 20 h. In the solutions the pH was determined with the glass electrode (InoLab 730, WTW GmbH, Weilheim, Germany). The water suspensions were filtered through a 0.45 µm membrane. In the filtrates the water soluble cations and anions were analyzed using the ICP-AES (SPECTRO CIROS\textsuperscript{CCD}; SPECTRO Analytical Instruments GmbH, Kleve, Germany) and ion chromatography (ProfIC, Metrohm AG, Herisau, Switzerland). The content of dissolved organic carbon was determined with a TOC-analyzer (Dimatoc 2000, DIMATEC Analysentechnik GmbH, Essen, Germany). The particle size distribution was analyzed with a laser particle analyzer (ANALYSETTE 22, Fritsch GmbH, Idar-Oberstein, Germany).

The concentrations of $^{14}$C-labelled NP and Phe in soils were analyzed by dry combustion. Dried homogenized soil was placed in the sample boat and burned for 4 min in an oxygen stream at 900°C (OX-300, Zinsser Analytic GmbH, Frankfurt, Germany). Released $^{14}$CO$_2$ was trapped with the scintillation cocktail and measured by liquid scintillation counting with background correction.

In order to determine the extractability of NP and Phe from the soils the following experiments were carried out: (i) extraction by water and ethanol; (ii) extraction by cyclodextrin aqueous solution; (iii) extraction by pure ethanol. Xenobiotics water extractability is an indicator for their bioavailability and for a potential risk of leaching to ground waters (Eschenbach et al., 1998; Kilbane, 1998). An extractant that mimics microbial interactions with contaminants and thus enables to assess the microbial bioavailability of xenobiotics in soils, is $\beta$-cyclodextrin (Doick et al., 2005; Hartnik et al., 2008; Papadopoulos et al., 2007). Cyclodextrin is a cyclic oligosaccharide with polar exterior and nonpolar interior cavities. Molecules of cyclodextrin may interact with hydrophobic compounds as versatile complexing agents, which encapsulate hydrophobic xenobiotics in the cavity, thereby increasing their water solubility (Reid et al., 2000) and thus promoting the release and microbial accessibility of contaminants in soil and solution (Fava et al., 2003; Fava et al., 1998). Many studies report that the cyclodextrin extracted fraction strongly correlates with biodegradation and mineralization of mono- and polycyclic aromatic hydrocarbons (Doick et al., 2005; Hartnik et al., 2008; Papadopoulos et al., 2007).
Deionized water, 1 mM (2-Hydroxypropyl)-β-cyclodextrin (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) aqueous solution, and ethanol absolute 99.9% (J.T. Baker, Avantor Performance Materials, Center Valley, USA) were used as solvents in analyses of xenobiotics extractability.

Contaminated soils were used for serial and sequential extractions. All extraction experiments were carried out in 10 mL glass centrifuge vials (Novodirect GmbH, Kehl, Germany) closed with Teflon-lined screw caps. Soil in amount of 0.8 g (d/w) and 8 ml of solvent were placed into the vials, mixed using an overhead shaker with 15 rpm at 20°C for certain periods of time (Table II-1). Subsequently, vials were centrifuged for 15 min at 2700 g, and 80% of supernatant was replaced by a fresh solvent. An aliquot of the removed supernatant was mixed with the scintillation cocktail Ultima Gold (PerkinElmer, Waltham, USA) to determine the radioactivity by the liquid scintillation counting using a Tri-Carb 2800TR analyzer (PerkinElmer, Waltham, USA). This extraction cycle was repeated several times depending on the extraction experiment (Table II-1). Remaining solution from every previous extraction cycle and the amount of solute therein was taken into account in calculations of NP or Phe extractability.

The sterility of the irradiated soils was tested after 7 months of storage under sterile conditions. The test was carried out by incubation of aliquots in liquid cultures using 0.1 x Luria-Bertani medium after Miller (Atlas, 1993). Cultures were incubated under aerobic and anaerobic conditions at 25°C for 72 h. In addition, soil extracts obtained with 0.9% NaCl were spread onto 0.1 x Luria-Bertani agar plates and incubated aerobically at 25°C for two weeks. Since no microbial growth was observed in any sample, irradiated soils can be regarded as sterile.

All experiments were carried out with a minimum of three replicates.

2.3. Calculations

The process of the compound extraction is defined as a particular case of desorption. The amount of unextracted xenobiotic was calculated from the relationship
where \( q_e \) (\( \mu g \) \( g^{-1} \) of organic carbon) is the mass of xenobiotic sorbed to the soil organic carbon, \( C_0 \) is the initial concentration of compound in the system (\( \mu g \) \( mL^{-1} \)), \( C_e \) is the concentration of dissolved xenobiotic at the end of one extraction cycle (\( \mu g \) \( mL^{-1} \)), \( V \) is the volume of the solvent (\( mL \)), \( W \) is the mass of soil (\( g \)), and \( f_{oc} \) is the weight fraction of organic carbon in soil.

The carbon normalized distribution coefficient \((K_{oc})\) was calculated using the equation

\[
K_{oc} = \frac{q_o}{C_e} = \frac{V}{W \cdot f_{oc} \cdot C_e} \left( C_0 - C_e \right).
\]  

For convenience, \( \log K_{oc} \) was used. All presented \( K_{oc} \) values were calculated only for the first extraction cycle, which characterizes the degree of dissolution of xenobiotics from soil at the beginning of desorption process.

For each of the extraction cycles the percentage of extracted xenobiotic was calculated from the relationship:

\[
E_n = \frac{C_{en}}{C_{0n}} \cdot 100\%,
\]  

where \( E_n \) (\%) is the percentage of extracted compound by the \( n \)-th extraction cycle.

The total extractability of xenobiotic at the end of experiment was calculated as a sum

\[
\sum_{k=1}^{n} E_k = E_1 + E_2 + \ldots + E_n.
\]  

The difference between the xenobiotics concentration in the soil at the beginning of the bioaging experiment and the \(^{14}\text{C}\) concentrations determined by combustion at the end of the incubation was assumed to be lost by mineralization. Therefore, the sequestered organic compounds fraction (non-extractable residues) in soil after bioaging was calculated by
subtraction of the mineralized, the water soluble, and ethanol extracted fractions from the initial content of xenobiotic applied to the soil.

For each parameter the mean, maximum and minimum values were calculated. Statistical significant differences between means were established by subjecting data to the ANOVA and Tukey’s test ($P < 0.05$).

3. Results

3.1. General properties of the soils

The soil texture was not affected by the treatments and consisted of 68.6% sand, 29.0% silt and 2.4% clay. However, the percolation treatments slightly reduced the amounts of organic C and N from 1.88% and 0.24% in the control soil to 1.72-1.83% and 0.11-0.18% in all treated soils (Table II-2). The contents of organic C showed no significant differences between soils, whereas all treatments significantly reduced the N-content compared to the control, resulting in increased C:N ratios. The highest and the lowest amounts of organic N were detected in original soil and Al-soil, respectively. Treatment of the soil by water or salt solutions also affected the soil-water potential and final moisture level of the samples (Table II-2). The pH of the water extracts changed slightly, but differences between soils were not statistically significant, except in the Al-treated soil where pH dropped to 4.6. The extractable DOC concentrations were highest in the Na-soil (352 mg kg$^{-1}$) and significantly lower in Ca- and Al-soils (273 and 272 mg kg$^{-1}$ respectively). Original non-percolated soil showed also high values of the DOC in solution (355 mg kg$^{-1}$).

The total amount of water soluble cations as well as anions was highest in the Na$^+$ treated soil (Table II-2), indicating that salt removal during washing was incomplete. The original soil, H$_2$O-, and Ca-soil released similar amounts of ions into water. The lowest total amount of water-soluble ions was detected in soil treated by Al$^{3+}$.

The percolation treatments changed the cation composition in all extracted fractions. Water treatment led to a slight elution of all cations, whereas percolation by Na-, Ca- or Al-salt solutions increased the amount of these cations and reduced the contents of other ions in
Table II-2 General properties of soils. Values followed by the same letter in each row are not significantly different (ANOVA, Tukey’s test, P < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>Original soil</th>
<th>H₂O-soil</th>
<th>Na-soil</th>
<th>Ca-soil</th>
<th>Al-soil</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Water soluble</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>cations</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na⁺</td>
<td>0.22C</td>
<td>0.09BC</td>
<td>12.33D</td>
<td>0.08AB</td>
<td>0.07AB</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>6.73D</td>
<td>6.62CD</td>
<td>0.94A</td>
<td>9.69E</td>
<td>1.89B</td>
</tr>
<tr>
<td>Al³⁺</td>
<td>0.57B</td>
<td>0.41A</td>
<td>1.27C</td>
<td>0.49AB</td>
<td>1.59D</td>
</tr>
<tr>
<td>K⁺</td>
<td>0.27C</td>
<td>0.12B</td>
<td>0.13B</td>
<td>0.07A</td>
<td>0.07A</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>1.27D</td>
<td>1.07C</td>
<td>0.12A</td>
<td>0.20B</td>
<td>0.23B</td>
</tr>
<tr>
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<td>8.32B</td>
<td>14.79E</td>
<td>10.52D</td>
<td>3.85A</td>
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<td>F⁻</td>
<td>0.15C</td>
<td>0.05B</td>
<td>0.13C</td>
<td>0.06B</td>
<td>0.02A</td>
</tr>
<tr>
<td>Cl⁻</td>
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<td>6.48B</td>
<td>17.89D</td>
<td>8.67C</td>
<td>2.92A</td>
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<tr>
<td>NO₃⁻</td>
<td>0.69D</td>
<td>0.23C</td>
<td>0.15B</td>
<td>0.18BC</td>
<td>0.11AB</td>
</tr>
<tr>
<td>PO₄³⁻</td>
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<td>0.62B</td>
<td>1.98E</td>
<td>1.01C</td>
<td>0.00A</td>
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<td>0.12A</td>
<td>0.14A</td>
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<td>7.52B</td>
<td>20.36E</td>
<td>10.05D</td>
<td>3.20A</td>
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<tr>
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<td>16.01B</td>
<td>0.11A</td>
<td>0.15A</td>
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<td>18.60B</td>
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<td>4.14A</td>
</tr>
<tr>
<td>Al³⁺</td>
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<td>5.58B</td>
<td>1.16A</td>
<td>21.90C</td>
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<td>K⁺</td>
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<td>0.29B</td>
<td>0.35B</td>
<td>0.17A</td>
<td>0.15A</td>
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<tr>
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<td>2.81C</td>
<td>0.79B</td>
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<td>47.35C</td>
<td>41.34B</td>
<td>48.64D</td>
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<td><strong>cations</strong></td>
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<td></td>
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<tr>
<td>Na⁺</td>
<td>1.98A</td>
<td>1.89A</td>
<td>18.96B</td>
<td>1.36A</td>
<td>1.89A</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>52.00D</td>
<td>47.08C</td>
<td>22.63B</td>
<td>55.48D</td>
<td>6.71A</td>
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<tr>
<td>Al³⁺</td>
<td>204.67D</td>
<td>188.18C</td>
<td>181.36BC</td>
<td>171.27AB</td>
<td>233.72E</td>
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<td>Fe³⁺</td>
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<td>43.95B</td>
<td>40.18A</td>
<td>38.97A</td>
<td>40.79A</td>
</tr>
<tr>
<td>K⁺</td>
<td>5.98C</td>
<td>5.37B</td>
<td>4.94AB</td>
<td>4.43A</td>
<td>4.69A</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>10.16C</td>
<td>9.21B</td>
<td>6.79A</td>
<td>6.07A</td>
<td>6.14A</td>
</tr>
<tr>
<td>Sum</td>
<td>318.59C</td>
<td>295.68B</td>
<td>274.86A</td>
<td>277.59AB</td>
<td>293.93B</td>
</tr>
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<td><strong>SOM</strong></td>
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<tr>
<td>C [%]</td>
<td>1.88A</td>
<td>1.83A</td>
<td>1.79A</td>
<td>1.76A</td>
<td>1.72A</td>
</tr>
<tr>
<td>N [%]</td>
<td>0.24C</td>
<td>0.18B</td>
<td>0.14AB</td>
<td>0.12AB</td>
<td>0.11A</td>
</tr>
<tr>
<td><strong>C: N</strong></td>
<td>7.76A</td>
<td>10.28B</td>
<td>13.00C</td>
<td>14.39D</td>
<td>15.29D</td>
</tr>
<tr>
<td><strong>DOC [mg kg⁻¹]</strong></td>
<td>355 B</td>
<td>303 AB</td>
<td>352 B</td>
<td>273 A</td>
<td>272 A</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td>5.0 B</td>
<td>5.2 B</td>
<td>5.3 B</td>
<td>5.0 AB</td>
<td>4.6 A</td>
</tr>
<tr>
<td><strong>Gravimetric moisture content at pF 2 [%]</strong></td>
<td>10.5 B</td>
<td>9.0 A</td>
<td>11.8 B</td>
<td>11.6 B</td>
<td>11.3 B</td>
</tr>
</tbody>
</table>
the treatment significantly reduced the concentrations of Na\(^+\) in the water soluble and of Al\(^{3+}\) in the HNO\(_3\)-digestible fractions (Table II-2).

Cation exchange capacity was altered in all percolated soils. Nevertheless, differences between sums of exchangeable cations in original and Ca-soils were statistically not significant (Table II-2). In comparison to the original soil the cation exchange capacity was substantially reduced after water and NaCl treatment, but the strongest decrease occurred in the Al-soil. Significantly higher concentrations of exchangeable Na\(^+\), or Ca\(^{2+}\), or Al\(^{3+}\) were detected in soils treated with the respective cations compared to the control soil. It is interesting to note the relatively high amount of Al\(^{3+}\) in the Na-soil.

3.2. Sterile aging experiment

At each of the different aging intervals, the extractability of the two compounds was not significantly affected by the soil treatments, while the extractability of NP was always significantly higher than that of Phe. Therefore, no statistically significant cation treatment effects were detected regarding the formation of a non-extractable fraction (data not shown). Based on this, the data of control and treated soils was pooled for each time point of the aging experiment (24 hours or 2, 5 or 9 months) (Table II-3). Since the amount of xenobiotics extracted by the sequential water-ethanol extraction (Table II-1) was not significantly different from the sequential ethanol extraction (data not shown) only the yield of the first 3 sequential water cycles from the water-ethanol extractions are presented in Table II-3.

During the 9 months of sterile aging the extractability of NP and Phe decreased continuously with all extractants, which is reflected in the increase of the carbon normalized partitioning coefficients. Cyclodextrin was more efficient than water for extracting both NP and Phe, especially during the first months of the aging experiment. The log\(K_{oc}\) values of the first water extraction increased during the 9 months aging from 4.21 to 4.37 for NP and from 4.43 to 4.71 for Phe, whereas the same parameter of the first extraction with cyclodextrin solution increased from 2.85 to 3.29 for NP and from 3.74 to 4.31 for Phe. The log \(K_{oc}\) values
for the water and cyclodextrin extractions of NP became almost constant after 5 months of aging.

After the 9 months of aging under sterile conditions the total water extractability of NP and Phe (obtained by three sequential extraction cycles) was reduced by 34 and 51%, respectively. Cyclodextrin extractability declined from 67.5 to 40.6% for NP and from 23.0 to 7.7% for Phe, which is equivalent to relative reductions by 40 and 66% for NP and Phe, respectively. Cyclodextrin extractability of both xenobiotics decreased until 5 months of aging, and then remained stable.

Table II-3 Extractability of nonylphenol and phenanthrene from moist soils aged under sterile conditions. Data of all samples (original, H₂O-, Na-, Ca- and Al-soils) were used to calculate the presented mean values and standard deviations. Values followed by the same letter in each row are not significantly different (ANOVA, Tukey’s test, P < 0.05).

<table>
<thead>
<tr>
<th>Time of aging</th>
<th>24 hours</th>
<th>2 months</th>
<th>5 months</th>
<th>9 months</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>log Kₒc of the first extraction cycle</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extraction of nonylphenol by:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>4.21 ± 0.03 A</td>
<td>4.28 ± 0.04 B</td>
<td>4.33 ± 0.06 C</td>
<td>4.37 ± 0.06 C</td>
</tr>
<tr>
<td>Cyclodextrin aqueous solution</td>
<td>2.85 ± 0.03 A</td>
<td>3.03 ± 0.03 B</td>
<td>3.26 ± 0.04 C</td>
<td>3.29 ± 0.04 C</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1.09 ± 1.00 A</td>
<td>--</td>
<td>2.01 ± 0.12 A</td>
<td>2.13 ± 0.05 A</td>
</tr>
<tr>
<td>Extraction of phenanthrene by:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>4.43 ± 0.05 A</td>
<td>4.57 ± 0.03 B</td>
<td>4.64 ± 0.00 C</td>
<td>4.71 ± 0.02 D</td>
</tr>
<tr>
<td>Cyclodextrin aqueous solution</td>
<td>3.74 ± 0.06 A</td>
<td>3.92 ± 0.02 B</td>
<td>4.22 ± 0.02 C</td>
<td>4.31 ± 0.03 D</td>
</tr>
<tr>
<td>Ethanol</td>
<td>2.29 ± 0.13 A</td>
<td>--</td>
<td>2.51 ± 0.06 B</td>
<td>2.63 ± 0.04 B</td>
</tr>
</tbody>
</table>

| Total extractability [%] |           |          |          |          |
| Extraction of nonylphenol by: |           |          |          |          |
| Water         | 7.12 ± 0.26 D | 6.39 ± 0.43 C | 5.80 ± 0.72 B | 4.77 ± 0.40 A |
| Cyclodextrin aqueous solution | 67.49 ± 2.61 C | 55.72 ± 1.84 B | 43.21 ± 2.01 A | 40.60 ± 2.05 A |
| Ethanol       | 94.43 ± 5.02 B | --        | 87.35 ± 3.19 A | 82.20 ± 1.62 A |

| Extraction of phenanthrene by: |           |          |          |          |
| Water         | 4.51 ± 0.52 C | 3.40 ± 0.24 B | 2.94 ± 0.11 B | 2.20 ± 0.10 A |
| Cyclodextrin aqueous solution | 23.00 ± 3.06 C | 17.67 ± 0.96 B | 9.38 ± 0.46 A | 7.65 ± 0.75 A |
| Ethanol       | 77.04 ± 5.29 B | --        | 67.66 ± 3.53 A | 59.51 ± 2.83 A |

Ethanol was the most efficient extractant for both NP and Phe. At the beginning of the aging experiment, 94.4% of NP and 77.0% of Phe were extracted by the three sequential ethanol
Interacting effects of cation saturation and drying, freezing, or aging on the extractability of nonylphenol and phenanthrene from a sandy soil

extraction cycles. After 9 months of storage, ethanol extractabilities were significantly reduced to 82.2 and 59.5% for NP and Phe respectively.

3.3. Drying-wetting and freezing-thawing experiments

In comparison to the untreated soils, the water extractability of both NP and Phe (obtained by three sequential extraction cycles) decreased after drying as well as after freezing of soils. The repeated drying or freezing cycles additionally reduced the contaminants’ extractability in most of the studied soils. Nevertheless, the differences between singly treated and four times treated samples were not significant in any soils ($P > 0.05$), and therefore the data of all dried or frozen treatments was pooled. For comparison, the water extractabilities of xenobiotics from untreated by drying or freezing, but stored for 24 hours or for 9 months, soils are also shown (Fig. II-1).

![Fig. II-1 Water extractability of nonylphenol and phenanthrene from moist, dried and frozen soils. Data of singly treated and four times treated samples were pooled for calculation of mean, maximum and minimum values for dried and frozen soils. Extractability values of treated samples (moist not aged, moist aged for 9 months, dried or frozen soils) characterized by the same capital letter are not significantly different; for each soil (original, H$_2$O-, Na-, Ca- or Al-soil) extractability values characterized by the same lowercase letter are not significantly different (ANOVA, Tukey’s test, $P < 0.05$).](image)

Water extractability of NP from frozen soils varied between 4.4 and 5.1% which was similar to NP extractability from 9 months aged samples. Differences between cation-treated soils were negligible except for the Al-soil, which showed lower values. The soils dried at room
temperature showed intermediate NP extractability values between 24 h aged samples and
frozen soils. A slight cation treatment effect on the water extractability of NP from dried soils
was observed. Thus, Na- and Al-soils released around 5.3%, which was significantly lower
than values of original soil, H₂O- and Ca-soil (around 6.1, 6.5 and 6.0% respectively).
Nonylphenol water extractability from dried samples was 11.5–28.9% lower than from the
24 h aged soils but still 4.7–24.5% higher than from the frozen or 9 months aged soils
(Fig. II-1).

In contrast to NP, the extractability of Phe was more strongly reduced after drying than after
freezing of the samples. Water extractability of Phe from dried soils showed an effect of the
cation treatments and varied between 1.9 and 2.3% (Fig. II-1). Interestingly, the effect of
drying on Phe extractability was as strong as 9 months of aging of soils. The Phe water
extractability from frozen samples was 29–44% lower than from the 24 h aged soils, and 20–
35% higher than from the dried or 9 months aged soils. Frozen salt-treated samples showed
a slight, but statistically significant trend of increasing Phe extractability in accordance with
valence of cation used for soil treatment: Na-soil < Ca-soil < Al-soil.

3.4. Bioaging experiment

During the long-term bioaging experiment a clear effect of the cation treatments appeared
after 7 months of incubation (Fig. II-2). For NP, the mineralization was lowest in the original
soil, higher in Na- and Al-soils and highest in the H₂O- and Ca- samples (24, 37, 43, 46 and
52% respectively). In comparison to NP, the mineralization of Phe was much higher in all
studied soils, reaching up to 71%. Mineralization of Phe in H₂O-treated soil was not
significantly different from the original soil. In comparison to the other treated samples the
Na- and Al-soils showed lower mineralization of both NP and Phe. Phenanthrene
mineralization in the Na⁺ treated soil was 60% and 66% in the Al³⁺ treated soil.

At the end of the bioaging experiment the total water extractability of NP amounted to 6.3–
7.7% of the remaining compound in all samples (Table II-1). For Phe, water extractability was
very low in the Na- and Al-soils (1.2 and 1.7%, respectively), higher in the original and
H₂O-soil (3%) and highest in the Ca-soil (3.9%) (Fig. II-2). For Phe a significant exponential
relation ($R^2 = 0.9$) was found between mineralized and water extractable fractions in the bioaged soils (data not shown).

Sequential ethanol extraction exhaustively removed the remaining NP from all samples, except the original soil. Here, a non-extractable NP fraction of 15% remained. For Phe, the ethanol extractable fraction was almost equal in the original, H$_2$O- and Ca-soils (3.0, 3.2 and 3.6% respectively), and much lower than in Na- and Al-soils (20.2 and 20.9% respectively). This was significantly and negatively correlated to the mineralization of Phe ($R^2 = 0.9$).

![Fig. II-2 Fractions of nonylphenol and phenanthrene determined by sequential extraction with water and ethanol in soils after 7 months of bioaging. Values followed by the same letter in each row are not significantly different (ANOVA, Tukey's test, $P < 0.05$). Orig. – original soil, H$_2$O – soil treated by water, Na – soil treated by NaCl, Ca – soil treated by CaCl$_2$, Al – soil treated by AlCl$_3$.](image)

Bioaging for 7 months led to the formation of similar amounts of Phe in the nonextractable fraction in the original and H$_2$O-soils (23%), in the Na- and Ca-soils (18.7 and 19.9%, respectively), whereas in Al-treated sample it was only 11.5%.

When comparing NP and Phe mineralization in the bioaged soils with water or ethanol extractability from sterile aged soils, no correlations were found. Similarly, no correlations exist between the extraction data sets from bioaged and sterile samples (data not shown).
When comparing the non-extractable fractions of the two xenobiotics in the long-term sterile aged and bioaged incubation assays (Fig. II-2 and Fig. II-3), it is evident that this fraction was reduced in the biologically active samples. After 7 months of bioaging, the mean non-extractable NP fraction amounted to 3.0%, while it reached 13.0% after 9 months of storage under sterile conditions. For Phe the values are 19.3 and 38.3% for bioaging and sterile aging, respectively.

![Fig. II-3 Fractions of nonylphenol and phenanthrene determined by sequential extraction with water and ethanol in soils after 9 months of aging under sterile conditions. Values followed by the same letter within each row are not significantly different (ANOVA, Tukey's test, $P < 0.05$). Orig. – original soil, H$_2$O – soil treated by water, Na – soil treated by NaCl, Ca – soil treated by CaCl$_2$, Al – soil treated by AlCl$_3$.](image)

### 4. Discussion

Although the cation treatments altered total and exchangeable cation composition as well as DOC concentrations in the soil samples, the effects on the extractability of NP and Phe were low. This stands in contrast to other studies (Lu and Pignatello, 2004; Polubesova et al., 2007) in which strong cation-dependent effects on the sorption of non-polar compounds were reported. However, these and other similar studies were generally carried out with isolated and purified humic acids, which are generally more aromatic and less polar than other organic matter fractions or the overall SOM. Similarly, the observed decrease in...
N-content and increase in C:N ratio in the washed and cation-treated soils had no effect on sorptive properties of the soils, although Hur et al. (2009) identified this to be a controlling factor for the sorption of phenanthrene to isolated humic acids. By using natural SOM in a sandy soil we had expected to detect cation-induced effects on the polarity and sorptivity of the organic matter more easily than in a soil with higher amounts of reactive clay or iron oxide minerals, which are considered to be essential for the stabilization of SOM (Kögel-Knabner et al. 2008, Marschner et al. 2008). However, since these stabilizing interactions are also affected by cation-induced conformational changes of organic matter structure (Bohn et al., 2001), they may also control the sorptive properties of SOM.

Sterile aging of the soils led to a decrease of NP and Phe extractability with all extractants over time. Independent of aging time the extractability of xenobiotics by cyclodextrin was considerably higher than by water, which can be explained by higher ability of this cyclic oligosaccharide to solubilize hydrophobic compounds (Reid et al., 2000). During the first five months, the cyclodextrin extractable fraction decreased with higher dynamics than the other investigated fractions, indicating that this fraction is most sensitive to the so-called slow sorption processes associated with diffusion into micropores and other less accessible sorption sites (Northcott and Jones, 2000, 2001; Richnow et al., 1999; Zhao et al., 2009). However, ethanol extractability of the compounds became also stable after 5 months, indicating that the slow sorption processes had decelerated even more or reached equilibrium (Northcott and Jones, 2001). Generally, extractability of NP was greater than of Phe, independent of solvent and soil. This most probably can be explained by higher water solubility of NP (Brix et al., 2001; Tang and Weber, 2006).

The stabilizing effects of drying-wetting or freezing-thawing on the extractability of NP and Phe from freshly contaminated soils showed no significant difference between one and four cycles of the treatments. This indicates that once a fraction is stabilized by such a treatment, it cannot be further stabilized or destabilized by subsequent drying or freezing events (Eschenbach et al., 1998). Drying and freezing of soils had different effects on the extractabilities of the two organic contaminants which partly explains the conflicting reports from other studies, where enhanced or decreased, or no effects on the formation of non-extractable bound residues in SOM were determined (Kalbitz et al., 1997; Kottler et al.,
In our study, soil freezing reduced the water extractability of NP most strongly, while Phe extractability was most reduced in dried soils. This can be explained by the fact that during drying, water films disappear from soil surfaces, making them more hydrophobic (Lamparter et al., 2009) which enhances the sorption of the purely hydrophobic Phe. In contrast, freezing of moist soils will lead to a partial destruction of organic macromolecules or organo mineral structures through crystallized water which will expose additional functional groups (Yu et al., 2010), which appears to affect the sorption of NP (having a polar hydroxyl group) more strongly. Interestingly, in the dried soils, NP extractability with water was significantly correlated to the content of water-extractable and exchangeable Ca\(^{2+}\) which was lowest in the Na and Al-treated soils. Although the differences in NP-extractabilities are small (5.1 to 5.3 vs. 5.8 to 6.2\%) this indicates that NP sorption sites are stabilized through cross-linking of organic molecules by Ca\(^{2+}\) only after dehydration.

In spite of the higher water, cyclodextrin and ethanol extractability of NP in comparison to the Phe extractability from the sterile aged samples, the mean mineralization rate of NP in the re-inoculated soil samples was lower than of Phe (0.19 and 0.32\% d\(^{-1}\) respectively). These rates are at the low end of mineralization rates reported in previous studies which vary between 0.62 to 20\% d\(^{-1}\) for NP (Barber et al., 2009; Chang et al., 2007b; Hesselsoe et al., 2001; Telscher et al., 2005; Topp and Starratt, 2000) and from 0.06 to 62.1\% d\(^{-1}\) for Phe (Hatzinger and Alexander, 1995; Nam and Alexander, 2001; Watanabe et al., 2005), which, however, also indicate a greater persistence of NP in the environment. Interestingly, contrasting mineralization rates would be expected from the position of the \(^{14}\)C-label in the chemical structures of the compounds. In Phe, the \(^{14}\)CO\(_2\) used for the determination of the mineralization rates originates from the least degradable central benzene ring of the Phe molecule, while NP has only one uniformly labeled ring, which should be more easily accessible for microbial degradation (Mirsal, 2004). Mineralization of both compounds was lower in the Na- and Al-soils which most likely is primarily due to the negative effects of these cations on the microbial activity and therefore degradation of xenobiotics in soils reported in earlier studies (Chang et al., 2007a; Yuan et al., 2004) and not due to cation effects on the compound's accessibility, since this should have had opposite effects from the monovalent Na\(^+\) and the trivalent Al\(^{3+}\). Interestingly, NP mineralization was lowest in the
original soil compared to the water or salt-treated samples. Apparently, percolation and washing of soils either removed certain components or changed soil properties that reduce NP mineralization. One soil property that was altered in all treated samples is the N-content which decreased and was accompanied by an increase in the C:N ratio, indicating that mineral N compounds or N-rich organic compounds were selectively leached from the soils. Since high N-availability reduces the production of oxidizing enzymes involved in the degradation of aromatic structures (Sinsabaugh, 2010) a treatment-induced N-limitation quite likely has stimulated the production of oxidizing enzymes.

Generally, in most bioaged samples the water soluble xenobiotic fraction was higher than in the sterile aged soils. During non-sterile incubation, both the ethanol extractable and the non-extractable fractions strongly decreased and in case of NP the non-extractable fraction was not even detectable at the end of 7 months of bioaging. Apparently, the long-term incubation led to a partial transformation of non-extractable and ethanol extractable fractions to water soluble and mineralized fractions. This transformation is apparently a microbially mediated process, since the compounds’ ethanol extractabilities were negatively correlated with their mineralization rates, which agrees with previous research, where the interdependency between bioavailable and sequestered fractions of xenobiotic in soils was observed (Nam and Alexander, 2001; Puglisi et al., 2007; Watanabe et al., 2005).

For the other extractants, no relationships were found between cyclodextrin extractability of NP and Phe and their mineralization although this extractant has been described to solubilize the bioaccessible or bioavailable fraction of hydrophobic compounds (Doick et al., 2005; Hartnik et al., 2008; Papadopoulos et al., 2007). The significant exponential relationship between Phe mineralization and water extractable fractions in the incubated soils shows that apart from mineralization to CO₂, the microbial degradation of Phe releases water soluble metabolites (Parikh et al., 2004).

In contrast to other studies (Benoit and Barriuso, 1997; Nowak et al., 2011), the formation of non-extractable residues was not enhanced in the bioaged soils, but even reduced compared to the sterile aged soils. This may partly be due to the relatively polar solute ethanol used for obtaining the extractable fraction, which will thus selectively remove more polar metabolites formed during microbial degradation. When using less polar extractants such as butanol,
hexane, or dichloromethane (Northcott and Jones, 2001; Zhao et al., 2009) a higher recovery of the sorbed or entrapped original hydrophobic compounds can be expected. As pointed out by Northcott and Jones (2000), the term “non extractable fraction” depends of the extraction method used. Another reason for the different results than in other similar studies may be that we used the relatively "mild" sterilization method of γ-radiation because despite its high efficiency it has the least effects on SOM structure (Berns et al., 2008). In contrast, the commonly in other studies used method of autoclaving disrupts chemical bonds in SOM as seen in the strong release or water-soluble compounds and may thus greatly increase the accessibility of sorption sites for the extractants.

5. Conclusions

Our study showed that under both sterile and non-sterile conditions the extractability of the two test compounds decreased with time and this depends on both solute and solvent properties. Freezing and drying treatments of the spiked soils, decreased the extractabilities of the xenobiotics similarly as by long-term ageing. Freezing was most efficient in decreasing NP extractability, while drying was more effectively reduced for Phe extractability. Aging of the organic pollutants in soils under sterile conditions leads to a higher formation of non-extractable residues than in biologically active soils. Since no differentiation between original compounds and metabolites was possible in the extracts, this may well be due to a higher proportion of more polar metabolites in the biologically active soils. On the other hand, the sorptive properties and accessibility of sorption sites may also have been altered by microbial growth. An influence of cation composition in soil organo-mineral structure on the aging of xenobiotics in soils was less apparent.

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Interacting effects of cation saturation and drying, freezing, or aging on the extractability of nonylphenol and phenanthrene from a sandy soil

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Chapter II


Interacting effects of cation saturation and drying, freezing, or aging on the extractability of nonylphenol and phenanthrene from a sandy soil


Chapter III

Cation treatment and drying-temperature effects on nonylphenol and phenanthrene sorption to a sandy soil

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Abstract

The objective of this study was to investigate the effects of incorporating mono- and polyvalent metal cations into the structure of soil organic matter (SOM) on sorption properties of the soil, with special reference to hydrophobic organic compounds. To this end, a sandy soil was leached with 0.1 M NaCl, CaCl$_2$, AlCl$_3$ aqueous solutions and then washed with deionized water to remove excess salts. The samples were then sterilized and either stored moist, dried at room temperature, or at 20°C, 60°C, or 105°C in a vented oven.

Saturation with Na$^+$ led to an increase of dissolved organic carbon (DOC) content in the soil water extracts, whereas the polyvalent cations Ca$^{2+}$ and Al$^{3+}$ decreased it. Proton nuclear magnetic resonance ($^1$H NMR) relaxometry studies showed that Al$^{3+}$ restricted the mobility of water molecules that are confined in nano- and micropores within the SOM structure to a higher extent than Ca$^{2+}$ or Na$^+$. According to contact angle (CA) analyses, cation treatment did not change the wetting properties of the samples significantly. The CA of the soils increased significantly after drying at elevated temperatures.

Batch sorption and desorption experiments carried out with the two hydrophobic compounds nonylphenol (NP) and phenanthrene (Phe) showed no clear salt-treatment effects on sorption and desorption kinetics or on isotherms. Nevertheless, the sorption of the two xenobiotics increased for dry soil, and successive desorption was reduced. Sorption log $K_{oc}$ values of moist and room temperature dried samples were 3.7–3.8 and 3.8–4.0 for NP and 4.1–4.2 and 4.2–4.3 for Phe, respectively. Sorption of both xenobiotics increased significantly with the drying temperature. The log $K_{oc}$ values of NP and Phe sorption to 105°C treated soils were around 4.0 and 4.4 respectively. In general, drying and heating increased the sorptive capacity and decreased the reversibility of sorption. We conclude that structural modifications of SOM due to incorporation of polyvalent cations into the interphase structure do not modify the sorption characteristics of the soil for hydrophobic compounds. Instead, increasing hydrophobization of soil constituents due to heat treatment significantly increased the accessible sorption sites for non-polar organic compounds in this soil.
1. Introduction

SOM plays a dominant role in the sorption of non-polar, uncharged organic xenobiotics in soils. SOM is composed of polydisperse organic compounds with highly variable chemical structures. According to some model concepts, humic substances contain expanded and more condensed molecular domains with a higher or lower flexibility (Xing, 1997). This flexibility may be controlled by bridges of water molecules (Schaumann et al., 2013). Similarly, polyvalent metal ions, such as Al$^{3+}$, Fe$^{3+}$, Ca$^{2+}$, and Mg$^{2+}$ can be coordinated to multiple functional groups of organic matter (Schnitzer and Scinner, 1965) and form cation bridges or water-cation complexes (Kunhi Mouvencehery et al., 2012). This may decrease the flexibility of the SOM matrix and hence raise the diffusional resistance of partitioned molecules (Lu and Pignatello, 2004; Yang et al., 2001). Alteration of SOM by formation or breaking of these cross-linking bridges may cover or open sorption sites in soil aggregates, affect the surface charge of macromolecules and organo-mineral complexes, and thus have an influence on the fate of xenobiotics in soil, namely on their sorption (Varadachari et al., 1997).

Previous studies on the effect of mono- and polyvalent cations (like Na$^+$, Ca$^{2+}$, and Al$^{3+}$) on SOM sorptive properties may be divided into three types: (a) investigations with dissolved or solid purified organic substances (e.g., humic acids), (b) studies with soil organic colloids, and (c) studies with the non-fractionated entire soil matrix. However, the reported findings are quite diverse and do not show any consistent pattern, which can partly be explained by the variety of chemical properties of the sorbates and sorbents and by the differences in cation concentrations and applied cations that were added before, during, or even after addition of the sorbates. Charles et al. (2006) saturated peat soils with different exchangeable mono- and divalent cations. The cation type was found to minimally affect sorption of p-nitrocyanobenzene, 1,2,4-trichlorobenzene and 1,4-dinitrobenzene. Lu et al. (2002) reported that the capacity of anionic polyacrylamide sorption increased significantly as the total amount of dissolved salts in the solution increased. The divalent cations like Ca$^{2+}$ and Mg$^{2+}$ were about 28 times more effective with respect to polyacrylamide sorption than monovalent cations like Na$^+$ and K$^+$ (Lu et al., 2002). As the authors suggested, this was due to the stronger flocculation power and, consequently, to a higher charge screening ability of...
polyvalent cations. Laegdsmand et al. (2004) reported that addition of K\(^{+}\) to the soil solution enhanced pyrene sorption on soil colloids, whereas Ca\(^{2+}\) led to its decrease. Experiments with humic acids saturated with Ca\(^{2+}\) or Al\(^{3+}\) revealed that the type of cation can significantly influence the sorption of naphthalene, α-naphthol, and phenanthrene (Phe) (Yuan and Xing, 2001). Other data showed that in comparison to untreated humic acids the treatment with Al\(^{3+}\) enhanced hysteresis and nonlinearity of naphthalene and 1,2,3-trichlorobenzene sorption (Lu and Pignatello, 2004). Luo et al. (2008) investigated the effect of Na\(^{+}\), Ca\(^{2+}\), and Al\(^{3+}\) on sorption of Phe to soils. Cations were dissolved in the sorption solution at different concentrations. Polyvalent calcium and aluminum ions at a concentration of 0.01 M significantly increased Phe sorption compared to the monovalent sodium. Summarizing, information about the effect of mono- and polyvalent cations effect on SOM sorption properties is very diverse and not consistent.

Drying and heating of soil may lead to a breakdown of water molecule bridges in the SOM structure (Schaumann and Thiele-Bruhn, 2011). This in turn may significantly alter the sorptive properties of soil materials. For example, drying of salt marsh sediments significantly increased sorption of hydrophobic compounds such as tyrosine, aniline, and naphthalene, while sorption of lysine was reduced (Liu and Lee, 2006). These results suggested that drying affected sorption sites through increased hydrophobicity and reduced polarity of the organic matter. Drying can also greatly affect the ability of soil to release DOC to the solution and alter properties of dissolved organic matter (Zsolnay et al., 1999), which can notably alter the sorption-desorption balance. Batch experiments with soils showed that the release of DOC from air-dried samples exceeded that of field-fresh samples by up to a factor of four (Kaiser et al., 2001).

Since hydrophobic interactions play a key role in the sorption of hydrophobic chemicals to soils, the solid surface free energy or wettability may be a good indicator for the sorption potential. The wettability of soil, as assessed by contact angle (CA) measurements, varies within a wide range and affects major physical processes like infiltration and important soil properties like aggregate stability (Bachmann and van der Ploeg, 2002). Despite their fundamental importance, investigations that link wetting properties and surface chemistry with sorption phenomena are rare. Ferguson and Whiteside (1992) differentiated between
the bulk of the solid and the interfacial region ("interphase"). Diffusion of functional groups within the interphase will modify not only the structure of the interphase but also the wettability at the outermost layer.

One means to study water confined in organic matter or small pores is \(^1\)H NMR relaxometry. The proton relaxation time provides information on the molecular mobility of water molecules in soil matrixes. Water molecules in nano- and micropores of SOM structures and water molecules bound to the functional groups are restricted in mobility. As the mobility of water molecules can also be affected by the rigidity of the SOM matrix, the transversal relaxation time (\(T_2\)) is expected to decrease when cross-linking agents like multivalent cations stabilize the organic matter structure (Bayer et al., 2010). Therefore, \(T_2\) can serve as additional means to characterize the matrix rigidity, which in turn may be response for sorptive properties of SOM (Bonin and Simpson, 2007).

The objectives of this study were therefore (i) to determine the effects of various cations and temperature pretreatments on the sorption and sorption kinetics of nonylphenol (NP) (4-(3,5-dimethyl-3-heptyl)phenol) and phenanthrene (Phe) to soil and to (ii) relate this to specific physical properties of the samples. For our study, a sandy soil was selected to exclude effects of salts on clay dispersion and aggregation interacting with sorptive processes.

2. Materials and methods

2.1. Soil preparation

The sandy soil was collected from the upper 0-20 cm of an agricultural field near Hannover, Germany. It was dried at 20°C and sieved (≤ 2 mm). The content of sand, silt and clay in the original soil was 68.6, 29.0, and 2.4% respectively, the total organic carbon was 1.88%, the water-extractable organic carbon (DOC) content was 355 mg kg\(^{-1}\), pH (determined in water) was 5.0.

To obtain soil material with a dominance of one kind of cation at the exchange sites, 100 g of the original soil was filled into steel cylinders of 5 cm height and 4.5 cm inner diameter and
placed into percolation units. The samples were leached under saturated conditions with aqueous solutions of 0.1 M NaCl, CaCl₂, or AlCl₃ at a ratio of 1:50 (w/w) during 18 h and then leached with deionized water at a ratio of 1:15 (w/w) until the electric conductivity of the leachate reached constant values. An additional reference sample was obtained by leaching the original soil with deionized water instead of salt solutions. After the percolation treatment, the soils were dried at room temperature and a relative air humidity of around 40%. These treatments lead to a significant increase of the concentrations of exchangeable Na⁺, Ca²⁺, or Al³⁺ in the soil after treatment with the respective cation. In contrast, the organic carbon content and the particle size distribution were similar in control and percolated samples (Shchegolikhina et al., 2012).

In accordance with the determined soil-water potential values, the soil samples were moistened to pF 2.8 (~630 hPa) and then sterilized three times by γ-radiation with a total radiation dose of 75 kGy. After sterilization, all further treatments and experiments were performed under sterile conditions. Since the pH of soils changed due to salt and water percolation, the pH of the original soil and of the H₂O-, Na-, and Ca-treated samples was adjusted with additions of 0.05 M HCl to the pH of the Al-treated sample (4.6), based on titrations performed at a soil:solution ratio of 2:5. As seen in the equilibrium solutions of the sorption experiments, this adjustment generally was not fully successful. The final matric potential of all samples corresponded to pF 2 (~100 hPa). The sample preparation is described in more detail by Shchegolikhina et al. (2012).

Soil samples were divided into three parts and prepared for three different sets of experiments:

Moist soils were packed into jars with screw caps and stored at room temperature. These soils were used for the analysis of the water soluble organic matter fraction and studies of sorption and desorption isotherms and kinetics.

Moist soils were dried at room temperature until constant weight (almost 1 week) and stored as described above. The same studies as with the moist soils were performed. Dry soils were used also for ¹H NMR relaxation time studies.
Moist soils were placed into open jars and dried at 20°C, 60°C or 105°C until constant weight was reached (at 20° and 60° for 24 h; at 105°C only for 1 h to avoid a sustained heating effect on the SOM). Samples were cooled to room temperature in a desiccator and then directly used for investigations of sorption and wetting properties.

2.2. Analytical methods

To investigate the water-soluble organic matter fraction released from soils during the sorption experiment, moist and dry samples were agitated in water at a ratio of 1:10 (d/w) for 20 h. In the solutions, pH was determined with a glass electrode (InoLab 730, WTW GmbH, Germany). The water suspensions were filtered through a 0.45 µm membrane. The content of DOC was determined with a TOC-analyzer (Dimatoc 2000, Imatec Analysentechnik, Germany). The same solutions were analyzed by an ultraviolet (UV) and visible absorption spectrophotometer (Lambda 2, PerkinElmer Inc., USA).

The 1H NMR spin-spin relaxation times (T2) were determined in cation-treated soils to monitor changes in matrix mobility and in water binding, resulting from the presence of polyvalent metal cations. Dry control and cation-treated samples were moistened with deionized, distilled water. Water content in rewetted soils was 20% of soil dry weight. T2 measurements were performed using a NMR relaxometer (Minispec 7.5, Bruker, Germany) at a magnetic field intensity of 0.176 T. Decay of transverse magnetization was recorded using the CPMG (Carr-Purcell-Meiboom-Gill) pulse sequence with the following acquisition parameters: recycle delay = 1 s, echo-time = 1 ms, number of echoes = 400, number of scans = 128. The decay functions were parameterized on the basis of two parallel first order exponential decay functions with their respective characteristic time constants indicating the relaxation times T2-1 and T2-2. Model fits were obtained using the Origin 7.5 software.

To characterize the wettability of the soil samples, CAs were determined with the sessile drop method (Bachmann et al., 2003). The placement of a drop of deionized water on the soil surface and its subsequent behavior was recorded with a CCD equipped contact angle microscope (OCA 15, DataPhysics, Filderstadt, Germany). Then CA was evaluated after 30 ms (initial CA), 1000 ms, and 5000 ms, using the software SCA 20 (DataPhysics, Filderstadt,
Germany). For the measurement a dry soil sample was sprinkled and gently pressed on double sided adhesive tape fixed on a glass slide. Excess soil material was removed in order to achieve a one-grain-layer. The drop volume was 3 µL, and the recording frequency was 30 Hz. Per slide 6 drops were placed and the mean CA was determined.

All mentioned analyses were done with a minimum of three replicates.

2.3. Sorption and desorption studies

Two xenobiotic compounds with distinct chemical structures and essentially different wetting properties were used in the sorption-desorption studies. NP has a phenolic group and a hydrophobic aliphatic group with a water solubility of about 5 mg L\(^{-1}\) at pH 7 (Brix et al., 2001). Phe is a hydrophobic polycyclic aromatic hydrocarbon with a water solubility below 1 mg L\(^{-1}\) (Tang and Weber, 2006). Both compounds have similar octanol-water partition coefficients \((\log K_{ow})\) of about 4.5 (Montgomery and Welkom, 1990; Porter and Hayden, 2002).

\(^{14}\)C-ring-labeled and unlabeled (98% purity, RWTH Aachen, Germany) 353-NP (4-(3,5-dimethyl-3-heptyl)phenol) was dissolved in methanol. \(^{14}\)C-uniformly-labeled (99.7% purity, Campo Scientific GmbH, Germany) and unlabeled (99% purity, Sigma-Aldrich Chemie GmbH, Germany) Phe was dissolved in ethanol.

All sorption and desorption studies were carried out with 0.8 g soil and 8 mL of the aqueous xenobiotic solution in 10 mL glass centrifuge vials (Novodirect GmbH, Germany) closed with Teflon-lined screw caps. The components were mixed in an overhead shaker with 15 rpm at 20°C. Based on sorption kinetics, the final equilibration time was set to 20 h. Then the samples were centrifuged for 15 min at 2700 \(\times\) g, and an aliquot of the supernatant was removed and mixed with a scintillation cocktail (Ultima Gold, PerkinElmer, USA) to determine the radioactivity by liquid scintillation counting using a Tri-Carb 2800TR analyzer (PerkinElmer, USA). Control assays without soil were handled in the same way.

Because of very low water solubility of NP and Phe, the concentrations of the chemicals used in this study varied for NP from 1 to 37 \(\mu\)g g\(^{-1}\) and for Phe from 1 to 13 \(\mu\)g g\(^{-1}\) of soil. In all
sorption-desorption studies, deionized water was used as a solvent. The addition of salt to the sorption working solution, e.g. CaCl$_2$, used in many studies with cation-saturated materials (Guanshu and Xing, 2001; Lu and Pignatello, 2004), was avoided to make the preliminary effect of the ions adsorbed during the percolation and its impact on the surface properties of the SOM more clear. To minimize cosolvent effects, the concentration of methanol or ethanol in the sorption solution was always below 0.1% (v/v). After centrifugation, the acidity of the supernatant was monitored. Additional studies revealed that pH was independent of the xenobiotic concentration in the solution and of the time of mixing (data not shown).

After the sorption experiments, the same assays were used for a short-term desorption study, where 80% of the supernatant in the vial was replaced by deionized water and then samples were mixed for 30 min in the overhead shaker with 15 rpm. The soil solution was separated and radioactivity was measured as described above. All sorption-desorption experiments were carried out with four replicates.

Sorption equilibrium studies were carried out with moist and dried (at room temperature) soils using five different xenobiotic concentrations. The minimum concentration was defined by the limit of analytical detection and the maximum concentration was dependent on the xenobiotic’s water solubility (Brix et al., 2001; Tang and Weber, 2006).

2.4. Calculations

The specific UV absorbance coefficient (SUVA$_{254}$) was calculated by dividing the absorbance at 254 nm (in cm$^{-1}$) by the DOC content (in mg L$^{-1}$) and then multiplying by 100 cm M$^{-1}$ (EPA, 2009).

Within the tested concentration range, all sorption isotherms were found to be linear, so that the amount of the xenobiotic associated with the solid phase was calculated from the relationship

$$ q_e = \frac{V}{W \cdot f_{oc}} (C_0 - C_e) $$  \hspace{1cm} (III-1)
where $q_e$ (µg g$^{-1}$ of organic carbon) is the mass of xenobiotic sorbed to soil organic carbon, $C_0$ is the initial concentration of the compound (µg mL$^{-1}$), $C_e$ is the aqueous phase concentration of the xenobiotic at the end of the sorption/desorption experiment (µg mL$^{-1}$), $V$ is the volume of the aqueous phase (mL), $W$ is the mass of soil (g), and $f_{oc}$ is the weight fraction of organic carbon in the soil.

The carbon normalized distribution coefficient ($K_{oc}$) was calculated using the equation

$$K_{oc} = \frac{q_e}{C_e} = \frac{V}{W \cdot f_{oc} \cdot C_e} (C_0 - C_e)$$ (III-2)

For convenience, log $K_{oc}$ was used.

For each parameter the mean, maximum, and minimum values were calculated. Statistically significant differences ($P < 0.05$) were established by subjecting the data to t-test and ANOVA analysis.

3. Results and discussion

3.1. Effects of cation saturation on general soil properties

Cation and water treatment altered the acidity of water extracts from the cation-treated soils (Fig. III-1a). For moist soils, the differences between pH values of original soil and any of the treated samples were not significant. Nevertheless, the difference between pH of Na-soil (5.3) and Al-soil (4.6) was significant. All soils, except the Ca-soil, showed a significantly lower pH after drying compared to moist soils. Original, H$_2$O-, Na-, and Ca-soils dried at room temperature showed similar pH values (4.7–5.0), whereas Al$^{3+}$ treatment significantly decreased soil pH (4.1) in comparison to the other samples (Fig. III-1a).

The amount of water-extractable DOC was also affected by the treatments (Fig. III-1b). It was considerably higher in the original and Na-soil compared to the other samples. Surprisingly, drying of the soils reduced the release of DOC in all samples except for the Na-soil by 18–28%, which is contrary to the general observation that air-dried soils release more DOC than
Cation treatment and drying-temperature effects on nonylphenol and phenanthrene sorption to a sandy soil

field-moist soils (Kalbitz et al., 2000). Most probably the increase of DOC concentration for Na-soil was due to dispersion effects of Na$^+$ on SOM (Chorom et al., 1994).

\[ \text{SUVA}_{254} \text{ coefficients were smallest for Al-soil with 0.36 and 0.66 L mg C}^{-1} m^{-1} \text{ for the moist and the dry samples respectively (Fig. III-1c). The strongly complexing properties of the Al}^{3+} \text{ apparently leads to a preferential precipitation or sorption of aromatic components from soil solution, which is reflected in the decline of UV absorbance (Kaiser, 1998; Scheel et al., 2008). The retention of this aromatic DOM fraction can increase the amount of aromatic fraction of organic matter remaining in the soil. In contrast, the monovalent Na$^+$ enhanced dissolution of organic matter (Fig. III-1b) and also increased DOC aromaticity as reflected by highest SUVA$_{254}$ values (Fig. III-1c). Notably, the SUVA$_{254}$ coefficients for dried samples in comparison to moist soils were higher by factors of 1.3, 1.8, and 2.3 for Na-, Ca-, and Al-soils, respectively. Together with the generally lower DOC concentrations after drying, this indicates that the less aromatic DOM fraction was most likely preferentially depleted due to microbial degradation during the one week air drying period. Overall, the differences in DOC} \]

**Fig. III-1** Properties of water extracts from moist and dried at room temperature soils. Mean, maximum and minimum values are presented on graphs. The pH and dissolved organic carbon values of moist soils were taken from Shchegolikhina et al. (2012).
aromaticity in treated and control soils, may strongly influence sorption and desorption processes by providing mobile sorbents (Chefetz and Xing, 2009).

Cation induced changes of SOM properties are also reflected in the results from the $^1$H NMR analyses. The best fits of the relaxation curves were obtained with two component functions ($R^2 > 0.999$). These revealed two types of water proton populations characterized by distinguishably different $T_2$ values. $T_2$ of the fast relaxing fraction ($T_{2,1}$) was comparable in all samples (4.4–4.7 ms), indicating that the immobile and strongly bound water experiences comparable binding states in all samples.

$T_2$ values of the more slowly relaxing population ($T_{2,2}$) did not differ between original and water-treated samples (13.5 and 13.6 ms, respectively). But the cation-treated samples showed a clear trend with increasing $T_{2,2}$ in the order: Al-soil < Ca-soil < Na-soil, although the differences were very small (11.7, 13.5 and 14.4 ms, respectively). This trend is in accordance with the increase of cation valency. However, such small $T_{2,2}$ values do still not correspond to mobile water, but can be due to water molecules which are less confined than the fast relaxing population. That is, confinement of water was highest for Al-soil, least for Na-soil, and intermediate for Ca-soil.

As expected from their valency, trivalent Al$^{3+}$ confined water to a higher extent than divalent Ca$^{2+}$ or monovalent Na$^+$ do. Moreover, the higher $T_{2,2}$ values for Na treated samples than for the control samples suggested that hydrated Na$^+$ is less involved in cross-linking between organic matter segments due to a higher mobility of the water molecules in their hydration shell. A similar trend in proton relaxation times at 20 MHz was found for a cation-treated peat sample (Schaumann et al., 2013), which suggests that the effects of cation treatment on the mobility of water molecules are similar for different soils. Alternatively, water molecules can be entrapped in organic matter network cross-linked by polyvalent cations as suggested by Schaumann and Thiele-Bruhn (2011).

However, the cation effects on water mobility may also be influenced by pH which was altered by the treatments. The $T_{2,2}$ values of the more mobile water population increased with increasing pH of soil solution (Pearson correlation coefficient $> 0.96$), possibly due to an increasing destabilization of the matrix with increasing soil pH.
3.2. Effects of soil moisture on sorption and desorption of xenobiotics

Sorption of Phe was higher than sorption of NP in all studied samples (Tab. III-1). This agrees with other studies, which generally report lower log $K_{oc}$ for NP (3.8–5.9) than for Phe (3.7–6.8) (Bonin and Simpson, 2007; During et al., 2002; Karapanagioti et al., 2001; Telscher et al., 2005). NP sorption coefficients were equal in all moist soils, independent of sample pretreatment, with log $K_{oc}$ values of 3.7–3.8 ($P < 0.05$). The same was observed for Phe sorption, with log $K_{oc}$ of 4.1–4.2. Desorption NP and Phe $K_{oc}$ values were higher than sorption $K_{oc}$ for all samples. And again no significant effect of the cation treatment on the desorption $K_{oc}$ values of either NP or Phe was observed. Hysteresis between sorption and desorption of NP and Phe was also not influenced by the cation treatment (data not shown).

**Tab. III-1** Sorption and desorption of nonylphenol and phenanthrene to and from moist and room temperature dried soils*. Nonylphenol and phenanthrene log $K_{oc}$ values followed by the same letter in each column are not significantly different (ANOVA, Tukey’s test, $P < 0.05$).

<table>
<thead>
<tr>
<th>Xenobiotic</th>
<th>Soil</th>
<th>Original soil</th>
<th>H$_2$O-soil</th>
<th>Na-soil</th>
<th>Ca-soil</th>
<th>Al-soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonylphenol</td>
<td>Moist</td>
<td>3.73A</td>
<td>3.70A</td>
<td>3.71A</td>
<td>3.72A</td>
<td>3.80A</td>
</tr>
<tr>
<td></td>
<td>Desorption</td>
<td>4.01B</td>
<td>3.98B</td>
<td>4.01B</td>
<td>4.02BC</td>
<td>4.06C</td>
</tr>
<tr>
<td></td>
<td>Dry</td>
<td>3.96B</td>
<td>3.96B</td>
<td>3.78A</td>
<td>3.93B</td>
<td>3.96B</td>
</tr>
<tr>
<td></td>
<td>Desorption</td>
<td>4.14C</td>
<td>4.17C</td>
<td>4.12C</td>
<td>4.10C</td>
<td>4.13C</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>Moist</td>
<td>4.12A</td>
<td>4.12A</td>
<td>4.13A</td>
<td>4.13A</td>
<td>4.18A</td>
</tr>
<tr>
<td></td>
<td>Desorption</td>
<td>4.35B</td>
<td>4.34B</td>
<td>4.36B</td>
<td>4.36B</td>
<td>4.39B</td>
</tr>
<tr>
<td></td>
<td>Dry</td>
<td>4.28B</td>
<td>4.29AB</td>
<td>4.31AB</td>
<td>4.26AB</td>
<td>4.19A</td>
</tr>
<tr>
<td></td>
<td>Desorption</td>
<td>4.86C</td>
<td>4.87C</td>
<td>4.97C</td>
<td>4.82C</td>
<td>4.75C</td>
</tr>
</tbody>
</table>

* Common logarithms of carbon normalized partitioning coefficient ($\log K_{oc}$) were calculated from sorption and desorption isotherms. Equilibration time for sorption was 20 h, for desorption it was 30 min. Nonylphenol concentrations: for experiments with moist soils were 1, 10, 19, 28 and 37 $\mu$g g$^{-1}$ of soil and 2, 7, 14 and 21 $\mu$g g$^{-1}$ of soil for studies with dried soils. Phenanthrene concentrations: for experiments with moist soils were 1, 4, 7, 10 and 13 $\mu$g g$^{-1}$ of soil and 0.5, 1.75, 3 and 6 $\mu$g g$^{-1}$ of soil for studies with dried soils.

After drying, the sorption and desorption properties of the soils changed. Sorption of NP and Phe generally increased in all samples with two exceptions: sorption of NP to Na-soil and of Phe to Al-soil was not notably changed. NP sorption increased in some dried samples more than two-fold, while Phe sorption increased less drastically (Tab. 1). An increase of sorption of different contaminants on dry rather than moist organic material has been shown repeatedly in previous studies (Berkowitz et al., 2008; Kottler et al., 2001; Shelton and
Parkin, 1991). It appears to be due to a better accessibility of hydrophobic sorption sites, either through removal of the protective water layer or due to re-orientation of flexible organic molecules by cross-linking of the polar groups, leading to a more condensed structure with the non-polar moieties being oriented to the outside. This effect was explained in detail by Ferguson and Whiteside (1992) for the temporal diffusion of polar functional groups into functionalized polyethylene carboxylic acid as a function of the ambient temperature. Apparently, this modification of the SOM surface due to drying is very stable, even after 20 hours of contact with bulk water and additional mechanical shear forces imposed through the overhead shaking procedure.

Despite the fact that differences between treatments were not significant, it is interesting to note that sorption $K_{oc}$ values of NP on cation-treated dry soils increased in the order: Na-soil < Ca-soil < Al-soil. In contrast, Phe sorption showed an opposite trend: Al-soil < Ca-soil < Na-soil. Different concentrations of dissolved organic matter in the soil solution (Fig. 1b) probably influenced the direct xenobiotic sorption on soil. High amounts of released DOC from the Na-soil may have interacted with NP in solution and thus prevented it from further sorption to the soil. It is unclear, why Phe was not affected similarly. Possibly, this is due to the steric differences between the condensed Phe and the branched aliphatic NP. In any case, the generally higher $K_{oc}$ values of Phe (Tab. 1) showed that it was more strongly attracted to solid SOM through hydrophobic interactions, which made it less prone to interact with the water soluble organic matter fraction. But the underlying mechanisms are not yet understood. Other authors also report conflicting results of the effects of cation-induced DOC release on the sorption of xenobiotics (Graber and Borisover, 1998; Luo et al., 2011).

After soil drying, desorption of the xenobiotics was generally more affected than sorption, as is reflected in higher $K_{oc}$ values. This was most pronounced for Phe where the ratios between desorption and sorption $K_{oc}$ values increased two-fold compared to the moist soils (Tab. 1). This can be explained by the fact that the hydrophobicity of soils generally increased during drying, which may have enhanced the sorption of non-polar compounds (Berkowitz et al., 2008). Phe desorption $K_{oc}$ values followed the order: Na-soil > Ca-soil > Al-soil (Tab. 1), which showed that desorption was most efficient in Al-, and less efficient in Ca- and in Na-soil dried
at room temperature. In general, drying increased the sorption of the xenobiotics and decreased the reversibility of this process.

3.3. Effects of drying temperature on soil wetting and sorptive properties

Soils dried at 20, 60, and 105°C and prepared for kinetic sorption experiments were used also for CA determination (Fig. III-2). The 20°C treated soils were very hydrophilic, 105°C heated samples were exceedingly hydrophobic, whereas 60°C treated soils achieved intermediate values. In general, the CA of the soils increased with treatment temperature, which agrees with previous studies (Doerr et al., 2005). The technique used in this study allowed detecting more distinct differences between salt-treated soils only in case of the 60°C-treatment of the samples. Specifically, this experiment revealed that the Al-soil showed significantly higher CA than the other samples. In general, the CA of 60°C heated soils after a contact time of 5000 ms decreased in the order: Al-soil > H₂O-soil ≥ Ca-soil ≥ Na-soil ≥ original soil. The detected wetting properties of the samples had an effect on the ability of the water to penetrate the soil surface, which in turn are reflected in the xenobiotics sorption.

Fig. III-2 Wetting properties of soils dried at different temperatures. Mean values of contact angles are presented; error bars represent maximum and minimum values.
The log $K_{oc}$ values, presented in Fig. III-3, characterize the sorption of xenobiotics after 20 h of equilibration time. In comparison to the sorption of NP and Phe on moist soils, the sorption to all heated soils was significantly higher. Differences between 20 and 60°C treated samples were statistically significant. Heating to 105°C resulted in a further increase of xenobiotic sorption $K_{oc}$ values, but the difference to 60°C treated soils were not significant, except for $\mathrm{H_2O}$- and Na-soils for NP, and Na-soil for Phe. The effect of heat treatment on the sorption of xenobiotics and on wetting properties of soils are closely related: the lower contact angles occurring at lower drying temperatures correspond to lower sorption coefficients of both compounds, while higher sorption coefficients are found in soil samples with higher contact angles. This relationship (Fig. III-4) is more pronounced for Phe than for NP, regarding the change in log $K_{oc}$ values (slope) and the goodness of the fit of the regression equation. Generally, the increase of treatment temperature leads to water evaporation and to the reduction of water films on the soil particle surfaces (Bachmann and van der Ploeg, 2002; Doerr et al., 2005). The strong dehydration effect of the interphase leads consequently to an increase in the sorption $K_{oc}$ values (Fig. III-3). Apparently, this effect was still significant after 20 h of contact with water, $K_{oc}$ values were highest for Al-soil in all experiments with heat treated samples. The kind of salt treatment did not have a distinct effect on sorption properties of heat treated soils.

Sorption kinetics of NP and Phe were not strongly affected by the heat treatments (Fig. III-5). Sorption of Phe was higher than that of NP even after only one hour of interaction of the xenobiotic with the soil. It is interesting to note that the sorption kinetics on moist, 20, and 60°C treated samples were very similar. The sorption of NP or Phe at equilibrium time was similar for the mentioned samples, but sorption rates were different already at the beginning of the experiment: sorption $K_{oc}$ values increased from 20°C to 60°C treatment, but sorption to 105°C treated soil was retarded. Most probably during the first hours of interaction of the 105°C heated soil with the aqueous solution the penetration of water and dissolved xenobiotic molecules to the soil surfaces was severely hindered due to the distinct wetting resistance ($\mathrm{CA} > 90^\circ$) (Fig. III-2). Increasing soil wetting led to closer interaction with the xenobiotics dissolved in the liquid phase and to an increase of sorption due to a larger total amount of wetted interfaces.
Cation treatment and drying-temperature effects on nonylphenol and phenanthrene sorption to a sandy soil

Fig. III-3 Sorption of nonylphenol (a) and phenanthrene (b) to soils dried at 20, 60, and 105°C. Experiments were done with a xenobiotic concentration of 10 µg g⁻¹ of soil, equilibration time was 20 h. Mean, maximum, and minimum values are presented.

Fig. III-4 Relationship between wetting and sorption properties of soils dried at different temperatures. Contact angles refer to a contact time 33 ms.
Chapter III

From the beginning of the experiment, the sorption of xenobiotics increased with the time of contact of contaminants with soils. Nevertheless, after 7 h, no further increase of sorption was observed. The most hydrophobic 105°C treated soil with the highest contact angle showed the maximum of NP and Phe sorption at the equilibrium time of 20 h (log $K_{oc}$ 3.99 and 4.41 respectively).

4. Conclusions

This study demonstrated the combined effects of temperature and cation treatment on the sorption of NP and Phe in soil. Contrary to expectation, no clear cation effects on the sorption and desorption of xenobiotics was detected for moist soils. After drying NP and Phe sorption $K_{oc}$ values were increased and only drying led to a certain differentiation between the cation-treated soil samples. The difference was reflected in the $K_{oc}$ values, the contact angle, and the proton relaxation time, which suggests that cations force re-orientation of hydrophilic functional groups towards multivalent cations in the matrix with the
consequence of cross-link formation. These cross-links increase matrix rigidity and impair re-orientation to the outer surface.

Heating of the soils had the most pronounced effect on sorption and desorption of NP and Phe. With increasing temperature the contact angle of the soil increased substantially and sorption of both xenobiotics was enhanced. Apparently, heating of the samples leads to a physicochemical modification of the interphase changing its free energy of interacting with water and its sorptive properties for organic solutes. In our study it has been become evident, that the contact angle sensitive layer of the interphase is also sensitive for the specific binding processes for NP and Phe. It remains a subject for further investigations how persistent these reorientation effects are in contact with water and, especially, how the dynamics of reorganization will be affected by the binding state of the contacting water. The present study clearly shows that the sorptive properties of soils are affected by heat treatment. This implies that the pre-treatment and storage conditions of soil samples are very important factors when investigating sorption properties. On the other hand, environmental factors like the soil thermal regime might also affect sorption characteristics to a higher extent than commonly assumed. At the field scale, other effects of soil water repellency such as the creation of preferential flow paths may interact with sorption processes and thus determine the long-term transport behavior of hydrophobic organic solutes similar to NP and Phe. Since this study was carried out with only one soil, further investigations with other soils with different properties (e.g., grain size distribution, organic carbon content, wetting properties) are needed for evaluating the general validity of the findings.

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References


Chapter IV

Effects of sterile storage, cation saturation and substrate additions on the degradability and extractability of nonylphenol and phenanthrene in soil

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Abstract

The main objective of this study was to determine the effects of long-term abiotic processes during aging of organic pollutants in soil on their microbial degradability and formation of non-extractable residues. The specific aims of our study were to investigate how the fate of p353-nonylphenol (NP) and phenanthrene (Phe) in soils might be affected by: (i) saturation of soil by cations with different valency (Na$^+$, Ca$^{2+}$ or Al$^{3+}$), (ii) addition of organic substrate (wood flour) during incubation period, (iii) different soil moisture levels.

This study showed positive effect of long-term aging of sterilized samples on respiration of re-inoculated samples. However, the lack of aging effects on the mineralization of NP and Phe indicates that slow sorption processes by diffusion into less bioaccessible domains were not relevant in studied soils. Similarly, the lower respiration and xenobiotic mineralization rates in the Na$^+$ and Al$^{3+}$ treated soils indicate that this is due to toxic effects on microbial activity and not due to xenobiotic accessibility. Instead, the formation of non-extractable residues was strongly promoted by biological activity, most likely through formation of more reactive metabolites. The addition of wood flour greatly stimulated microbial respiration and enhanced NP mineralization while inhibiting that of Phe. Along with negligible effect of water addition after 4 weeks of incubation on kinetics of soil respiration, the soil moisture effect on xenobiotics mineralization indicates that most probably the bioavailability of NP and Phe increased due to bridging role of water films in soil.
1. Introduction

Hydrophobic organic pollutants reaching the soil are generally strongly sorbed by soil constituents. Furthermore, xenobiotics can be transformed by microbial degradation and mineralization, which in turn may contribute to the formation of bound residues in soil. The intensity of these processes depends on bioaccessibility and bioavailability of pollutant, which are governed by both sorbent and sorbate properties (Gevao et al., 2000; Semple et al., 2003).

One important factor which limits the bioaccessibility of pollutants is the strength of their sorption. Sorbing properties of soils are essentially conditioned by the properties of soil organic matter (SOM) (Alexander, 2000). Depending on the aromaticity and degree of condensation of SOM, sorption and desorption rates of pollutants may vary (Pan et al., 2007). One of the factors which can alter the SOM is the presence of strong complexing polyvalent cations. For example, in the presence of Al$^{3+}$ and Ca$^{2+}$ in solution the sorption of phenanthrene (Phe) was significantly increased in comparison to experiments with Na$^+$ (Lu and Pignatello, 2004). Another study showed that metals Cd, Cu, Pb, and Zn significantly increased Phe sorption to an agricultural soil (Saison et al., 2004). In contrast, cation saturation of soil with Na$^+$, Ca$^{2+}$ or Al$^{3+}$ before mixing with xenobiotics did not show significant effect of cations on short-term sorption of nonylphenol (NP) or Phe (Shchegolikhina et al., 2013).

It is well-known that long-term interaction of xenobiotics with soil or so-called aging, causes its strong sorption and gradual increase of non-degradable and non-extractable fractions (Semple et al., 2003). One of the processes responsible for this has been proposed to be the slow diffusion of the compounds into poorly accessible micropores of soil organic macromolecules which by some authors is also attributed to sorption or entrapment within less flexible (“glassy”) structures (Lu and Pignatello, 2004; Pignatello, 2012). Our studies with aging of xenobiotics in a sterilized sandy soil for 9 months showed a decrease of the ethanol extractability of NP and Phe from 94.4 to 82.2% and from 77.0 to 59.5% respectively (Shchegolikhina et al., 2012). Investigation of soils with different properties showed decrease of Phe mineralization and extractability up to 9.6–35.0% and 14.1–54.3%
Chapter IV

respectively after 200 days of non-sterile aging (Chung and Alexander, 2002). Though the role of biotic and abiotic factors in the formation of bound residues was investigated using different materials and under various conditions (Kristensen et al., 2001; Chung and Alexander, 2002; Li et al., 2007; Ncibi et al., 2007), the processes occurred in soil during aging are still not well understood.

Besides the abiotic factors the fate of xenobiotics in soil depends on biotic factors, such as the activity of soil microorganisms. Microbial activity can enhance the formation of xenobiotics bound residues, most likely due to release of more reactive metabolites that covalently bind to SOM (Gevao et al., 2000; Li et al., 2007). On the other hand, the stimulation of microbial activity, through nutrient addition or organic substrates can also remobilize bound residues and thus increase their biodegradation (Wicke and Reemtsma, 2010). Along with availability of nutrients, the soil moisture is one of the essentially important soil properties for microbiological activity (Brockett et al., 2012). Water content in soil can limit the living space for microorganisms. The water films formed between soil particles play important transporting role for microbiology (Paradelo and Barral, 2009), which in turn can stimulate the bioaccessibility and bioavailability of water-solved chemicals in soil, including xenobiotics and their bound residues (Kottler et al., 2001).

The main objective of this study was to determine the effects of long-term abiotic processes during aging of organic pollutants in soil on their microbial degradability and formation of non-extractable residues. Two model compounds, with different chemical structure and properties were used in our study: the hydrophobic phenanthrene comprised of three aromatic rings ($\log K_{ow} 4.5$, $S_w 1.3 \text{ mg L}^{-1}$) (Montgomery and Welkom, 1990) and the branched nonylphenol isomer 4-(3,5-dimethyl-3-heptyl)-phenol with both hydrophobic (aliphatic) and polar (phenolic -OH) subunits ($\log K_{ow} 4.7$, $S_w 6 \text{ mg L}^{-1}$) (Porter and Hayden, 2002). The Phe is a representative compound of the large group of polycyclic aromatic hydrocarbons, which can be formed in the environment during incomplete burning of oil, gas, coal or other organic substances; and due to decay and decomposition of some dyes, plastics and pesticides (Van der Perk, 2006). The NP is a primary breakdown product of nonylphenol ethoxylates. These chemicals are non-ionic surfactants, which are used in agricultural and industrial applications (Guenther et al., 2002). The specific aims of our study
were to investigate how the fate of NP and Phe in soils might be affected by: (i) saturation of soil by cations with different valency, (ii) addition of organic substrate (wood flour) during incubation period, (iii) different soil moisture levels.

2. Materials and methods

2.1. Soil preparation

The sandy soil was collected from the upper 0–20 cm layer of a gleyic podzol from the Fuhrberger Feld (Lakwiese), Fuhrberg, Lower Saxony, Germany. It was dried at 20°C and sieved (≤ 2 mm). The content of sand, silt and clay in the original soil was 68.6, 29.0 and 2.4% respectively, pH (determined in water solution) was 5.0 (Shchegolikhina et al., 2012).

To obtain soil material with a dominance of one cation at the exchange sites, the original soil was placed into percolation cylinders and leached with aqueous solutions of 0.1 M NaCl, CaCl$_2$, or AlCl$_3$ at a ratio of 1:50 (w/w) during 18 h. Then samples were leached with deionized water at a ratio 1:15 (w/w) until electric conductivity of the leachate reached constant values. An additional reference sample was obtained by leaching the original soil with water instead of salt solutions. After the percolation treatment, the soils were dried at room temperature and humidity around 40%.

In accordance with the determined soil-water potential values (Shchegolikhina et al., 2012), the soil samples were moistened to pF 2.8 (~630 hPa) and then sterilized 3 times by γ-radiation with a total radiation dose of 75 kGy. After sterilization, all further treatments and experiments were performed under sterile conditions. The pH of the samples was altered by the salt solution and water percolation. To adjust the pH of the samples to the same value (4.6), the soils were mixed with certain amounts of 0.05 M HCl. The final matric potential of all samples corresponded to pF 2 (~100 hPa), which is equal to water content 10.5% in original soil, 9.0% in H$_2$O-treated soil, 11.8% in Na-treated soil, 11.6% in Ca-treated soil, 11.3% in Al-treated soil. The preliminary incubation experiments with original soil moistened to different levels (5–15%) showed that the soil moisture corresponded to pF 2 is optimal for microbial activity (data not shown).
The sterilized soils were spiked with $^{14}$C-ring-labeled and unlabeled NP or Phe. $^{14}$C-U-ring-labeled and unlabeled p353-nonylphenol (NP) (98% purity, RWTH Aachen, Aachen, Germany) was dissolved in methanol. 9-$^{14}$C-labeled phenanthrene (Phe) (99.7% purity, Campro Scientific GmbH, Berlin, Germany) and unlabeled Phe (99% purity, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) were separately dissolved in ethanol. The chemicals were applied to the dry sea sand (washed, untreated by acid, Sigma-Aldrich Chemie GmbH, Steinheim, Germany), solvents were evaporated and then the spiked sand was mixed into the moist soils (1%, d/w) to achieve the concentration of 10 $\mu$g g$^{-1}$ soil for both compounds. Radioactivity of the spiked soils was 1800 Bq g$^{-1}$.

The concentrations of $^{14}$C-labelled NP and Phe in soils were determined by dry combustion. Dried homogenized soil was placed in a crucible and burned for 4 min in an oxygen stream at 900ºC (OX-300, Zinsser Analytic GmbH, Frankfurt, Germany). Released $^{14}$CO$_2$ was trapped in an alkaline scintillation cocktail and measured by liquid scintillation counting with background correction. The determined recovery rate was 88–94%.

The sterility of the irradiated samples was tested after 7 months of storage of soils under sterile conditions. The test was carried out by incubation of aliquots in liquid cultures using 0.1x Luria-Bertani medium after Miller (Atlas, 1993). Cultures were incubated under aerobic and anaerobic conditions at 25ºC for 72 h. In addition, soil extracts obtained with 0.9% NaCl were spread onto 0.1x Luria-Bertani agar plates and incubated aerobically at 25ºC for two weeks. Since no microbial growth was observed in any sample, it was assumed that the microbial activity in the irradiated samples was negligible.

Sterilized original, H$_2$O-, Na-, Ca- and Al-soils spiked with NP or Phe were prepared for different sets of experiments. Some of them were carried out directly after spiking with xenobiotics (non-aged samples), others were performed after 2, 5, 9 months of dark storage of soils at room temperature in closed jars under sterile conditions (aged samples).
2.2. Mineralization experiments

Aging effect

The original non-sterilized soil was preincubated moistly for 14 days and then added to sterilized samples as an inoculum at 5% (w/w). The following incubation was carried out in a Respicond-apparatus (A. Nordgren Innovations AB, Bygdeå, Sweden) that hourly monitors CO$_2$ production and collects respired CO$_2$ in 0.6 M KOH solution. Every 4 days the KOH solution was sampled for analysis of the $^{14}$C-activity and then refreshed. To determine the radioactivity of the KOH solution (99% purity, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) the aliquot was mixed with a scintillation cocktail Ultima Gold (PerkinElmer, Waltham, USA) and analyzed by liquid scintillation counting (Tri-Carb 2800TR analyzer, PerkinElmer, Waltham, USA). The concentrations of $^{14}$C-labelled NP or Phe and their undegraded residues in soils after incubation were also determined in selected samples by dry combustion as described above. The differences between the values calculated from the mineralization data and obtained from the combustion analysis were statistically insignificant, which showed that losses of the CO$_2$ during the study in the Respicond-apparatus were negligible.

Wood flour addition effect

At the beginning of the incubation, subsamples of the inoculated soils were mixed with dry wood flour (1%, d/w), which was produced by grinding of beech saw dust (“Chipsi” made by JRS J. Rettenmaier & Söhne GmbH + Co. KG, Rosenberg, Germany). Soil/wood flour mixtures were additionally moistened with deionized water (2.5 mL g$^{-1}$ of wood flour) to reach the same pH value, as in soils without substrate addition (pH 2). Soil/wood flour mixtures incubated as described above.

Water addition effect

After 4 weeks of incubation a part of soils was additionally moistened by mixing with deionized water in amount 50% from initial moisture. Therefore the water content was increased to 15.8% in original soil, 13.5% in H$_2$O-treated soil, 17.7% in Na-treated soil, 17.4% in Ca-treated soil, 17.0% in Al-treated soil. Moistened soils were further incubated as described above.
2.3. Extraction experiments

Six sequential cycles of water-ethanol extractions were performed with the soils before (Shchegolikhina et al., 2012) and after each of the incubation experiments. Soil in amount of 0.8 g (d/w) and 8 ml of solvent were placed into the 10 mL glass centrifuge vials (Novodirect GmbH, Kehl, Germany), closed with Teflon-lined screw caps and mixed using an overhead shaker with 15 rpm at 20°C for certain periods of time. Subsequently, vials were centrifuged for 15 min at 2700 g, and 80% of supernatant was replaced by a fresh solvent. An aliquot of the removed supernatant was mixed with the scintillation cocktail Ultima Gold (PerkinElmer, Waltham, USA) to determine the radioactivity by the liquid scintillation counting using a Tri-Carb 2800TR analyzer (PerkinElmer, Waltham, USA). This extraction cycle was repeated six times. The first three extraction cycles were performed with deionized water (duration of shaking 3, 3 and 7 h, respectively for the I, II and III cycle). The next three cycles were carried out with ethanol absolute 99.9% (duration of shaking 1 h for each cycle). The rest of the supernatant from every previous extraction cycle was taken into account in calculations of NP or Phe extractability.

2.4. Calculations

For each 4 days of the degradation experiment when the respired CO₂ was collected in the KOH solution the percentage of mineralized xenobiotic $M_n [%]$ was calculated from the relationship:

$$M_n = \frac{C_n}{C_0} \cdot 100\%,$$

where $C_n$ [Bq] is the total radioactivity detected in the KOH solution at time $n$, $C_0$ [Bq] is the initial radioactivity of the soil sample spiked with NP or Phe. Mineralized fraction or the total mineralization was calculated as a sum of mineralization rates at each of sampling time periods.

For each of the extraction cycles $n$ the percentage of extracted xenobiotic $E_n [%]$ was calculated from the relationship:
\[ E_n = \frac{S_n}{C_0} \cdot 100\% , \quad (IV-2) \]

where \( S_n \) [Bq] is the total radioactivity detected in the solution at the end of one extraction cycle.

The total extractability or extracted fraction of xenobiotic at the end of experiment was calculated as a sum of extracted fractions determined at each extraction cycle. It is assumed that the extracted fraction of xenobiotic in incubated soil may include the initial chemicals as well as the products of their microbial degradation. The change in xenobiotic extractability, caused by incubation, was calculated by subtraction of the total NP or Phe extractability detected in incubated soils from the total xenobiotic extractability determined during the study of non-incubated soils presented by Shchegolikhina et al. (2012).

In the experimental set-up the three parallel groups of soil samples were treated. The relevant soil samples then were thoroughly mixed before taking samples for the analytical studies mentioned above. All analyses were done with a minimum of three replicates. The data were analyzed using the STATISTICA-10 program (StatSoft, Inc., Tulsa, USA). For each parameter the mean value and the standard deviation were calculated. Statistical significant differences between means were established by subjecting data to an ANOVA followed by Tukey’s test \((P < 0.05)\).

### 3. Results and discussion

#### 3.1. General properties of soils

Soil texture and organic carbon content, the properties which can greatly influence the sorption of organic pollutants (Chung and Alexander, 2002), were not significantly changed by percolation of soil with water, NaCl, CaCl\(_2\) or AlCl\(_3\) aqueous solutions (Shchegolikhina et al., 2012). However, all treatments significantly reduced the organic nitrogen content compared to the control, resulting in increased C:N ratios (Tab. II-2). The percolation changed the cation composition in all soils. Significantly higher concentrations of exchangeable Na\(^+\), or Ca\(^{2+}\), or Al\(^{3+}\) were detected in soils treated with the respective cations.
compared to the control soil. $^{1}H$ NMR studies of original and percolated soils determined that the water molecules had various confinements in the structure of soils treated with different cations, which probably indicated alteration of SOM with cations (Shchegolikhina et al., 2013).

### 3.2. Soil respiration

Uncontaminated soils and samples spiked with NP or Phe released similar amounts of CO$_2$, which suggests that the xenobiotics at concentrations of 10 $\mu$g g$^{-1}$ did not affect the soil respiration.

All incubation experiments, except the one with 9 months aged soils, showed the highest respiration in the original soil (Fig. IV-1). Respiration of percolated soils was generally lower. Incubation experiments carried out with non-aged and aged soils showed an unclear effect of water and salt treatments on soil respiration. Thus, the non-aged samples inoculated with original soil showed a slight trend in the total soil respiration, which followed the order: original soil $>$ H$_2$O-soil $\geq$ Ca-soil $\geq$ Na-soil $\geq$ Al-soil, but this order was not repeated in the incubation experiments with aged soils.

After 5 months of sterile aging, the subsequent soil incubation showed that soil respiration was significantly increased in comparison to the non-aged soils ($P < 0.05$). Most probably the observed aging effect indicates that undetermined abiotic processes, taken place in the sterilized soils, altered those properties of soil, which then affected the activity of microbial community introduced to soil after aging.

The aging effect on soil respiration was intensified by substrate addition. Generally, wood flour addition increased the total soil respiration by a factor of 4.6 in original soil and by a factor of 2.9 in percolated soils. Notably, the effect of substrate addition was constant and independent of any other studied factors, like aging period, soil moisture or duration of incubation. The additional factor – the organic substrate – revealed that microbial community may adapt and grow easier and faster in soil, which was not percolated and thus was apparently not depleted of essential nutrients or substrates. One of them could be for
example nitrogen content (Jassal et al., 2011), which was significantly decreased by percolation of soils (Tab. II-2).

3.3. Mineralization of xenobiotics in soil

In all investigated soils the mineralization of the Phe was considerably higher than that of NP (Fig. IV-1), which agrees with previous studies of xenobiotics mineralization during the long-term incubation of soils (Shchegolikhina et al., 2012). Contrasting mineralization rates would be expected from the results of sorption experiments, which showed that in comparison to NP the Phe sorption in soils was significantly higher (Shchegolikhina et al., 2013). This indicates that most probably the bioavailability of the NP and Phe in the studied sandy soils was less depended on the sorption processes taken place in the contaminated soils during sterile aging, than on the properties and activity of the certain microbial community formed in the samples after inoculation of sterilized soils.

All performed studies, except 9 months aged soil incubation, showed that Na\(^+\) and Al\(^{3+}\) treatments significantly retarded mineralization of both xenobiotics \((P < 0.05)\). Most probably this occurred due to negative effects of Na\(^+\) and Al\(^{3+}\) on the soil microbiology (Yuan et al., 2004; Chang et al., 2007) and therefore on the degradation of xenobiotics in soils. In comparison to the control the retarding effect of Na\(^+\) and Al\(^{3+}\) was much stronger in case of Phe mineralization, than of NP mineralization. This indicates that mineralization of xenobiotics was conducted a) by different microorganisms with different ability to degrade NP and Phe; or/and b) by the same microbial community via different biochemical degradation pathways.

The addition of water to non-aged soils after 4 weeks of incubation significantly increased xenobiotics mineralization during the next 4 weeks (data not shown). For example, in non-aged Na- and Al-soils without wood flour, the addition of water and following incubation increased the Phe mineralization by a factor of 33 and 18, respectively. However, after 5 months of aging this effect became less pronounced in all soils; and even in some samples, particularly in the original and Ca\(^{2+}\) treated soils without wood flour, water addition
reduced NP and Phe mineralization. Thus, positive effect of water addition on xenobiotics mineralization decreased with aging of contaminated soils.

**Fig. IV-1** Total soil respiration and xenobiotic mineralization for 4 weeks of incubation performed after different periods of sterile aging of soils.
Along with negligible effect of water addition after 4 weeks of incubation on kinetics of soil respiration (data not shown), the soil moisture effect on xenobiotics mineralization indicates that most probably the soil microbiology was not altered by water addition, but the bioavailability of NP and Phe increased due to bridging role of water films in soil (Paradelo and Barral, 2009). Addition of water and prolongation of incubation period up to 8 weeks allowed determining the significant effect of the sterile aging on the NP and Phe mineralization. Specifically, the longer incubation (8 weeks) allowed to detect the negative effect of 5 months aging on NP and Phe mineralization ($P < 0.05$) in all samples without wood flour, except Na-soil (Fig. IV-2), whereas the shorter incubation study (4 weeks) did not reveal a clear effect of aging (Fig. IV-1). This indicates that evaluation of non-mineralized fraction of xenobiotics requires longer incubation period or/and optimal soil moisture, which could ensure entire mineralization of bioavailable and biodegradable fraction of pollutants in soil.

Except for a few samples, the wood flour application enhanced the NP mineralization and retarded the Phe mineralization in most of the performed studies independently of the aging period or incubation time. For example, incubation of 5 months aged soils showed that in the average for all soils the wood flour changed the NP and Phe mineralization by a factor of 1.4 and 0.8 respectively. Taking into account that wood flour application gave also the positive effect on the respiration of soils with both xenobiotics, there could be two possible reasons for such substrate effect on the xenobiotics mineralization: a) the addition of wood flour changed the sorption properties of soils and thus may alter the bioaccessibility of xenobiotics (Semple et al., 2003) or/and b) substrate addition induced a shift in the composition of the microbiological community with different ability to mineralize xenobiotics (Lashermes et al., 2010).

Wood flour addition also made the aging effect on the mineralization of NP more pronounced: at the end of the incubation experiment NP mineralization was significantly lower ($P < 0.05$) in 5 months aged soils than in non-aged soils (Fig. IV-2). Mineralization rates of NP in aged and non-aged soils incubated without substrate addition were less different from each other. The negative aging effect on the Phe mineralization was detected only for original, $H_2O$ and $CaCl_2$ treated soils.
The mineralization of NP was closely related to soil respiration if non-amended and wood flour treated samples are considered separately (Fig. IV-3). For the wood flour treated soils, the relationship is even closer if the original soil with very high respiration rates is excluded. These results show that the mineralization of NP is dependent on the overall metabolic activity in the samples, thus indicating a mainly co-metabolic mineralization (Mueller and Shann, 2007). For the Phe statistically significant relationships were also determined, but considering the high vertical scatter of the data points and the disjunctive grouping within the treatments, the mineralization of Phe is only poorly related to the respiratory activity in the samples.

**Fig. IV-2** Relationship between xenobiotics mineralization and their water-ethanol extractability determined after 8 weeks incubation of soil. Or – original soil, H$_2$O – water treated soil, Na – NaCl-treated soil, Ca – CaCl$_2$-treated soil, Al – AlCl$_3$-treated soil.
Effects of sterile storage, cation saturation and substrate additions on the degradability and extractability of nonylphenol and phenanthrene in soil

**Fig. IV-3** Relationship between soil respiration and xenobiotics mineralization in samples aged for 5 mo and then inoculated. Or - original soil, H₂O - water treated soil, Na - NaCl-treated soil, Ca - CaCl₂-treated soil, Al - AlCl₃-treated soil. Linear regression lines are included for the untreated and wood flour amended samples separately, the dotted lines show linear regression models calculated without the original soils (Or).
3.4. Extractability of xenobiotics from aged and incubated soils

Water-ethanol extraction carried out at the end of the 8 week of incubation experiments revealed that NP extracted fraction (51.7–72.7%) was substantially higher than Phe extracted fraction (5.4–39.8%) (Fig. IV-2). Most probably that was defined by abiotic processes of sorption and entrapment of xenobiotics in soil during aging (Shchegolikhina et al., 2013), and intensified by processes of microbiological transformation of compounds during incubation.

Addition of wood flour significantly enhanced NP mineralization, which most probably increased amount of NP residues in soil, and those might strongly bound to SOM (Li et al., 2007). This in turn led to formation of NP non-extractable fraction, which in average increased by a factor of 1.5. Wood flour addition increased distinction of the water and cations treatment effect, as well as of the aging effect, on NP mineralization and formation of non-extractable residues. However, in comparison with other studies of the p353-NP (Li et al., 2007; Riefer et al., 2011; Shan et al., 2011), its mineralization in the studied soils was extremely low, even when organic substrate was added. This most probably was the main reason for the lack of clear correlations between mineralized and extracted fractions of NP. In contrast to the NP, the relationship between Phe extractability and mineralization fitted well to a power function ($R^2=0.78$).

It was observed that microbial degradation of NP and Phe in soils significantly reduced xenobiotics extractability. Thus, incubation reduced extracted fractions of NP and Phe by 9.2–33.4% and 28.3–72.9% respectively (Fig. IV-4). However, the change in extractability of compounds and their metabolites during incubation exceeded the xenobiotics mineralization rates. For Phe, a substantial change of the amount of extracted fraction occurs even in samples with low mineralization, which underlines the importance of biological activity for the formation of bound residues. For NP, no clear trend in relationship between change of the amount of extracted fraction, caused by incubation, and mineralization was observed. For Phe, this relationship was well described by an exponential function ($R^2=0.84$) and showed that the higher the Phe mineralization was during the incubation period, the more intensive was the formation of the non-extractable fraction of Phe in soils.
4. Conclusions

This study showed that some still undetermined chemical or biochemical processes continue to alter soil properties even during sterile aging of soils spiked with NP or Phe. Increased soil respiration in re-inoculated aged soils indicates higher substrate availability from microbial residues or hydrolysis products. On the other hand, the lack of aging effects on the mineralization of the two xenobiotics shows that slow sorption processes by diffusion into less bioaccessible domains were not relevant in this soil. Similarly, the lower respiration and xenobiotic mineralization rates in the Na- and Al-treated soils indicate that this is due to toxic effects on microbial activity and not due to xenobiotic accessibility. Instead, the formation of non-extractable residues was strongly promoted by biological activity, most
likely through formation of more reactive metabolites. The addition of wood flour greatly stimulated microbial respiration and enhanced NP mineralization while inhibiting that of Phe. It remains unclear if this differential response is due to sorptive interactions or due to stimulating and inhibiting effects on certain members of the microbial community.

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References


Chapter V

Effects of $\gamma$ irradiation, storage and incubation on enzyme activities in a sandy soil treated with different salts

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Abstract

This study was performed to test the effects of (i) γ irradiation, (ii) long-term storage and (iii) salt treatments of soil on the activities of different enzymes. Re-inoculation of the γ-irradiated soil samples greatly increased enzyme activities within 1 month. After 10 months of moist storage, total enzyme activity decreased in all soils but more strongly in the non-sterile soils than in sterile. Among the enzymes most affected by sterilization were chitinase, acid phosphatase and leucine-aminopeptidase, while activities of β-glucoronidase, β-cellobiosidase and β-glucosidase were around 30–60% of the re-inoculated controls. Among the cation treatments, percolation of soil with NaCl, CaCl$_2$ and AlCl$_3$ solutions most strongly reduced total enzyme activities in sterile soils. Enzyme activity analyses of re-inoculated samples showed a significant negative treatment effect only in the H$_2$O-treated soil.
Various studies have shown a strong effect of time and storage conditions on the soil enzyme activities (Lee et al., 2007; Turner and Romero, 2010; Wallenius et al., 2010). Other investigations showed that soil enzyme activity changes during incubation of soil under influence of different factors (Burns et al., 2013; DeForest, 2009; Stumpe et al., 2012). None was done to investigate the effects of sterilization by γ irradiation and subsequent sterile aging on soil enzyme activities although it is well known that free or soil-bound extracellular enzymes may take contribute to substrate turnover in sterile soils or when microbial biomass is low (Dick and Burns, 2011; Stursova and Sinsabaugh, 2008). Only few studies have been carried out to investigate effect of different cations on enzyme activities, while the effect of heavy metals on soil enzyme activity in well known (Kuperman and Carreiro, 1997; Shen et al., 2005; Wang et al., 2007). This study was performed to test the effects of (i) γ irradiation, (ii) long-term aging and (iii) water and various salt treatments of soil on the activities of different enzymes in a sandy soil.

The sandy soil was collected from an agricultural field, dried at 20°C and sieved (≤ 2 mm). The original soil was placed into percolation cylinders and leached with aqueous solutions of 0.1 M NaCl, CaCl₂, or AlCl₃ at a ratio of 1:50 (w/w) during 18 h (Na-, Ca- and Al-soils). Then samples were leached with deionized water at a ratio 1:15 (w/w) until electrical conductivity of the leachate reached constant values. An additional reference sample was obtained by leaching the original soil with water instead of salt solutions (H₂O-soil). After the percolation treatment, the soils were dried at room temperature until constant weight was reached. Dry soil samples were moistened to pF 2.8 (~630 hPa) and sterilized 3 times by γ radiation with a total radiation dose of 75 kGy. After sterilization, all further treatments and experiments were performed under sterile conditions. pH of the samples was adjusted to the same level (4.6) using 0.05 M HCl. The final matric potential of all samples corresponded to pF 2 (~100 hPa). Detailed description of soil sampling and percolation, as well as general properties of samples were presented by Shchegolikhina et al. (2012) (Chapter II).

Half of the sterile soil was divided into two parts, which were placed in jars with screw caps, and stored at constant 20°C in the dark. Another half of sterile soil was re-inoculated with non-sterile original soil preincubated moist for 14 days (5%, w/w). These samples were also divided into two parts, which were placed in jars with screw caps, and then incubated at
20°C in the dark. Once a week jars were opened for 30 sec and then closed. The loss of water from samples was monitored; it was negligible.

After 1 and 10 mo of sterile aging or non-sterile incubation one set of soil samples was used for enzyme activity analyses. 4-Methylumbelliferone-linked substrates were used to quantify the activities of acid phosphatase, β-D-xylosidase, N-acetyl-β-D-glucosaminidase, β-D-glucoronidase, β-D-celllobiosidase, and β-D-glucosidase. The leucine, tyrosine and arginine aminopeptidases activating substrates were linked with 7-amino-4-methyl coumarin as fluorescence dye, following the method of Marx et al. (2001) (see the method description in the Appendix II: Supplementary data). Fluorescence was measured using a microplate reader (Infinite 200, Tecan, Germany) with 365 nm excitation wavelength and emission at 450 nm. After correcting for negative controls and quenching, activities were expressed in [nmol h⁻¹ g⁻¹]. The total enzyme activity of the sample was calculated as a sum of activities of all analyzed enzymes. Statistical significant differences between values were established by subjecting data to the ANOVA and Tukey's test ($P < 0.05$).

Comparison of enzyme activities in sterile and bioactive soils showed that after 1 mo of incubation the total enzyme activities in the re-inoculated soils were greatly increased over the sterile soils (Fig. V-1). In spite of the fact that enzyme activity decreased with time of storage in most samples, it was still significantly higher than in sterile soils after 10 mo.

In comparison with 1 mo data, a long-term sterile aging or incubation generally decreased soil enzyme activities (Fig. V-1). They remained stable only in the sterile original soil (147–170 nmol g h⁻¹). In the sterile H₂O-, Na-, Ca- and Al-treated soils it decreased by 48, 29, 64 and 59%, respectively. In the non-sterile original, H₂O-, Na-, Ca- and Al-treated soils total enzyme activity decreased by 55, 77, 49, 63 and 63%, respectively.

In soils stored sterile for 1 mo the total enzyme activity was significantly lower in the Na- and Al- treatments compared to the original soil (Fig. V-1). 10 mo sterile aging increased this negative effect and it became significant also for the Ca-soil. In the non-sterile soils incubated for 10 mo, only the H₂O-treatment showed a significantly lower total enzyme activity than the other treatments. After 1 mo the non-sterile soils showed that the percolation with NaCl increased activity of all enzymes, except acid phosphatase, leucine and
Effects of γ-irradiation, storage and incubation on enzyme activities in a sandy soil treated with different salts

arginine aminopeptidases (Tab. V-1). In comparison to the original soil, the Al$^{3+}$ treatment led to significant increase of activity of β-D-xylosidase, N-acetyl-β-D-glucosaminidase and β-D-glucoronidase after 1 mo of non-sterile incubation. After 10 mo of incubation this positive effect of AlCl$_3$ treatment was observed only for β-D-xylosidase activity. Other studies with the salt affected soils generally show that enzyme activity mainly depends on anion type, pH and electrical conductivity of the soil solution (El-Shinnawi and El-Shimi, 1981; Frankenberger and Bingham, 1982; Yuan et al., 2007). But variation of these factors was excluded in our study. Acid phosphatase activity has been shown to be significantly inhibited by Al$^{3+}$ and Ca$^{2+}$ (Onthong et al., 2007).

Water and cation treatments did not alter total organic carbon content of the samples, but total amounts of exchangeable cations were significantly changed and organic nitrogen was depleted via percolation (Shchegolikhina et al., 2012) (Chapter II). Therefore, these amendments can have an indirect effect on soil enzymes. For example, positive correlations between N content and N-acetyl-β-D-glucosaminidase, β-D-glucosidase and tyrosine aminopeptidase activities were found in the sterile soils. Interestingly, while for 1 mo aged soils Pearson r was 0.68, 0.49 and 0.60 for N-acetyl-β-D-glucosaminidase, β-D-glucosidase and tyrosine aminopeptidase activities, respectively, the 10 mo aged soils showed much lower activity.
stronger correlations for these enzymes— in all cases, Pearson $r$ was higher than 0.94. This indicates that soil N played an important role in processes of aging of these enzymes.

**Tab. V-1** Enzyme activity of stored under sterile conditions or incubated for 1 or 10 mo soils and coefficient of Pearson’s correlation between enzyme activity and total organic nitrogen content in soils (Shchegolikhina et al., 2012). $\beta$-Xyl – $\beta$-D-xylosidase, N-acet – N-acetyl-$\beta$-D-glucosaminidase, Pho – acid phosphatase, $\beta$-glucoro – $\beta$-D-glucoronidase, $\beta$-cello – $\beta$-D-cellobiosidase, $\beta$-glu – $\beta$-D-glucosidase, Leu – leucine aminopeptidase, Tyr – tyrosine aminopeptidase, Arg – arginine aminopeptidase, n/d – not detected.

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<th>Ca-soil</th>
<th>Al-soil</th>
<th>Enzyme activity [nmol g h$^{-1}$]</th>
<th>P-value</th>
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Incubation affected individual enzymes activity differently (Tab. V-1). In comparison with enzyme activity in sterile soils, the highest effect of 1 mo incubation was observed for N-acetyl-$\beta$-D-glucosaminidase, acid phosphatase, leucine and arginine aminopeptidases activity as the mean for all 5 soils (Fig. V-2). Fig. V-2 shows the combined effects of biogeochemical processes, underwent the long-term sterile aging and incubation, on soil enzyme activity. For example, increase of difference between enzyme activity of sterile and incubated soils, detected for $\beta$-D-xylosidase and leucine aminopeptidase, allows suggesting that in soils incubated for 10 mo enzyme inactivation through sorption or degradation were more intense than formation of new enzymes from microbial activity. Hereby, our study
Effects of γ-irradiation, storage and incubation on enzyme activities in a sandy soil treated with different salts

shows the importance of abiotic enzyme transformation and degradation processes, which are still not well understood, on the prediction of the fate of enzymes in soil.

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References


Chapter VI

Summary Discussion and Conclusions
1. The influence of the nonylphenol and phenanthrene properties on their behavior in soil

The experiments with moist and dry soils, as well as with samples heated at different temperatures, showed significant differences in the sorption of NP and Phe in soils. The NP sorption was lower than sorption of Phe in all studied samples: the log $K_{oc}$ values of NP sorption on the moist, dried and heated soils were 3.70–3.80, 3.78–3.96 and 3.84–4.07, respectively; for the Phe these parameters were 4.12–4.18, 4.19–4.31 and 4.26–4.43 (Chapter III). The determined log $K_{oc}$ values of xenobiotics were very close to the previously observed ranges of the log $K_{oc}$: for NP and Phe it was 3.8–5.9 and 3.7–6.8, respectively (Bonin and Simpson, 2007; During et al., 2002; Karapanagioti et al., 2001; Telscher et al., 2005).

The sorption of NP on the salt-treated dried soils followed the order: Na-soil < Ca-soil < Al-soil, while the Phe sorption had an opposite trend: Al-soil < Ca-soil < Na-soil (Chapter III, Tab. III-1). Possibly, the sorption of xenobiotics was affected by cation-induced DOC in the soil solution (Graber and Borisover, 1998; Luo et al., 2011), the amount of which was maximal in the Na-soil, minimal in Al-soil and intermediate in Ca-soil (Fig. III-1). The DOM in solution most likely interacted with the branched aliphatic compound NP, which was reflected, for example, in the decrease of its sorption on the Na-soil (Murillo-Torres et al., 2012). The data of pore-water extraction analyses (Appendix II) confirmed that the apolar compound Phe was essentially sorbed on the solid soil constituents, which did not release to the soil solution, on the contrary to DOM.

The sorption experiments with the heated soils showed a significant enhancement of the sorption of both compounds with the increase of the temperature of soil heating. This dependency was more evident for Phe, than for NP. With rise of the temperature of the soil heating the hydrophobicity also increased, which was reflected in the CA values (Fig. III-2). The drying and high temperature treatments of soil can significantly affect the conformational structure of the SOM and aromaticity of some of its fractions (Schneckenburger et al., 2012; Vergnoux et al., 2011), which in turn can significantly influence the Phe sorption rates (Pan et al., 2007).
Depending on the different ability of xenobiotics to be sorbed in the soil, their extractability also varied accordingly (Scow and Johnson, 1997). Hence, our batch experiments showed that in general the NP extractability was higher than extractability of Phe. For example, water extractability of NP and Phe was 4.77–7.12 and 2.20–4.51%, respectively (Chapter II, Tab. II-3). The rates of pollutants desorption and leaching from soils can give information about the nature of soil–xenobiotic interactions (Richnow et al., 1998). The water extraction experiments showed that freezing of contaminated soil most strongly reduced the NP extractability. On the contrary, the Phe extractability was substantially reduced after drying of contaminated soils. Drying as well as heating of soils leads to hydrophobization of the soil surfaces (Wang et al., 2010b), which probably induced the stronger sorption forces between soil and purely hydrophobic Phe, and thus decreased its extractability. The effect of drying on the extractability of xenobiotics in soils was reported earlier (Belkessam et al., 2005). Multiple freezing and thawing cycles could lead to a partial destruction of the molecular structure of soil organo-mineral complexes and to the formation of the additional domains for NP sorption (Yu et al., 2010), which can explain considerable decrease of NP extractability.

The xenobiotics extractability from soils shows the rate of their sequestration in the soil matrix, and can also indirectly characterize the bioavailability and bioaccessibility of pollutants for soil microorganisms (Riding et al., 2013). From the results of the sorption and extraction experiments carried out with original and cation-treated soils the higher mineralization of NP than of Phe can be expected. However, the incubation studies (bioaging and a series of 2 months degradation experiments), which were carried out with the samples after inoculation with the preincubated original soil, showed that the mineralization of Phe was considerably higher than of NP (Fig. II-2, IV-1). After incubation the water-ethanol extractability of NP from soils (51.7–72.7%) was substantially higher than the Phe extractability (5.4–39.8%) (Fig. IV-2). The significant exponential relationship between the Phe mineralization and its water extractability from incubated soils suggests that during the mineralization the water-soluble metabolites of the Phe were formed. Such relationship was not observed for the NP mineralization and extractability. This confirmed that the two xenobiotics behave essentially different in the soil during incubation, which leads to the
different fate of NP and Phe in soils. The additional evidence for this suggestion was obtained in the incubation experiments with substrate addition. The wood flour application generally increased the NP mineralization and decreased the Phe mineralization. There could be several reasons for the observed effect of substrate addition on the xenobiotics mineralization: the wood flour particles might play the role of the additional sorption domains for the Phe and its metabolites; and/or wood flour activated specific microorganisms in the soil, with higher ability to degrade the NP; and/or NP and its metabolites were degraded through co-metabolism induced by the wood flour addition.

The performed studies revealed the significant differences in the behavior of the NP and Phe in soils. The special features of these compounds became evident during their sorption, sequestration, and microbial transformation leading to xenobiotics mineralization. However, the formation of xenobiotic metabolites and their fate in soils were not particularly studied in the frame of the present work, and these issues require further comprehensive investigation.

2. The effect of the SOM conformational structure alteration on the soil–xenobiotic interactions

The performed saturation of the original soil with cations of different valences and various complexing features led to the significant alteration of the soil properties, such as the pH, the ability to release DOC to the soil solution, the cation composition and the cation exchange capacity (Chapter II). In frame of the presented study, the performed $^1$H NMR analysis allowed to conclude that the conformational structure of the SOM was also affected by the percolation of the soil with different salt solutions (Shchegolikhina et al., in press). The NMR studies indicated that in the control and cation-saturated soils the binding states of the immobile and strongly bound water molecules in the soil matrix were similar (Chapter III). However, the analysis also revealed that the less confined water molecules in the molecular structure of SOM had different mobility depending on the valency of the cation used for soil treatment. The confinement of the water molecules increased in the following order: Na-soil < Ca-soil < Al-soil. One possible explanation of the observed salt-
treatment effect could be the complexing and stabilizing influence of polyvalent cations, like Al$^{3+}$, on SOM, which leads to stronger binding and entrapment of the water molecules in the SOM structure (Aquino et al., 2011; Schaumann et al., 2013).

The strong correlation between the $T_2$ values of the more mobile water population, determined by the NMR studies, and the pH of soil, analyzed in the water extracts of soil, indicate the possible cross effect of these chemical and/or conformational properties of the soil matrix. Other characteristics of soil solution were also significantly altered by the percolation treatment of the soil: the content of DOC and the SUVA$_{254}$ coefficient, which were decreased with the increase of the valency of the cation used for soil treatment (Fig. III-1). The SUVA$_{254}$ Coefficient values, which reflect the aromaticity of the DOC (Kaiser, 1998; Scheel et al., 2008), showed that, for example, strong complexing Al$^{3+}$ led to the preferential precipitation or sorption of aromatic components from the soil solution, while Na$^+$ in soil matrix increased dissolution of the SOM and also enhanced the aromaticity of the DOC. Interestingly, as indicated by SUVA$_{254}$ coefficients, the aromaticity of the DOC significantly increased after drying of soil, and the increase of this parameter was greater in soils treated with stronger complexing cations. Importantly, recent studies (Rodriguez-Liebana et al., 2013) indicate that the properties of the soil solution, such as electrical conductivity and specific UV absorbance, indirectly influence the sorption of organic pollutants in soil, and therefore have to be considered in studies of xenobiotics fate in soils and sediments.

The alteration of sorptive properties of the isolated fractions of SOM, such as humic substances, or of the soil as a whole after its treatment with different cations could be the indirect confirmation of the induced changes of the SOM structure (Lu and Pignatello, 2004; Polubesova et al., 2007). However, our sorption-desorption studies, carried out with moist soils did not reveal a clear effect of cation saturation on the sorption or desorption of NP and Phe (Chapter III). Similarly, the effects on the extractability of NP and Phe from sterile soils, caused by cation or water treatments, were low. However, other studies showed a significant role of cations in the fate of xenobiotics interaction with soil. The addition of Ca$^{2+}$ to the soil solution decreased the pyrene sorption on soil colloids, while addition of K$^+$ showed the opposite effect (Laegdsmand et al., 2004). The presence of the Na$^+$, Ca$^{2+}$, and
Al\(^{3+}\) in the solution significantly affected the Phe sorption in soil: the polyvalent cations at a concentration of 0.01 M substantially increased sorption in comparison to the little effect of Na\(^+\) (Luo et al., 2008).

There could be several possible reasons for the observed low and unclear effects of cation saturation on the sorption and sequestration of NP and Phe. Although SOC plays the dominant role in the sorption of hydrophobic organic compounds in soils, the xenobiotics can also interact with other soil constituents (Luthy et al., 1997; Wilcke, 2000). Perhaps, the sorption of NP and Phe on the soil constituents, which were not significantly affected by cation treatments, for example mineral particles, made the cation effect unclear and less distinct. Nevertheless, based on the correlation analyses of the obtained data, we suggest that some cations had a direct influence on the processes of xenobiotics sorption in soil. For instance, the NP water extractability from dried soils showed a significant correlation with the content of the water soluble and exchangeable Ca\(^{2+}\) in soil (Chapter II). This is indicative of the stabilizing role of Ca-ions in the processes of the NP sorption in studied samples, but only after dehydration of the soil.

In summary, the alteration of the interactions of soil with different agents in the environment (including xenobiotics) that occurs after the cations treatment of soil is indicative of the changes in the conformational structure of SOM, as well as of the alteration of other soil constituents affected by cations saturation. However, the question about the effect of mono- and polyvalent cations on the SOM structure can be answered only in the case of the detailed characterization of the chemical and conformational properties of the SOM using the methods, which allow (i) to determine the binding partners (e.g., water molecules) of cations and their coordination geometry, (ii) to assess the rigidity of the SOM matrix, and (iii) to distinguish inter- and intramolecular features of the SOM (Kunhi Mouvenchery et al., 2012).
3. The influence of the soil properties on the sorption and sequestration of nonylphenol and phenanthrene

In the previous section the alteration of the SOM, as a particular part of soil, and the influence of these changes on the sorption of NP and Phe were discussed. In this section the soil is considered as an entire natural sorbent for the xenobiotics. The treatments of soil, which can alter its properties in general, and the effects of these changes on the sorption of xenobiotics are discussed.

As it was mentioned above, the treatment of the original soil by water and different salt solutions led to the significant changes of some general properties of soil. For example, the SOC amount was not altered by percolation, but the N-content significantly decreased in the water- and cation-treated samples, in comparison with the original soil (Tab. II-2). The alteration of C:N ratio can influence the sorptive properties of soil, as it was shown for the sorption of the Phe in the isolated humic acids, where Phe sorbed stronger on the humic acids with higher C:N ratio and aromaticity (Hur et al., 2009). The C:N ratio also plays an important role for the microbial activity (Xia et al., 2008), and thus can affect the mineralization rates of xenobiotics in soils (Yang et al., 2011). The influence of the soil properties on the mineralization and formation of the bound residues of xenobiotics will be discussed in the next section.

The performed salt solution treatment altered the wetting properties of the soil, such as soil-water potential, which reflects the ability of soil to retain water. However, the soil texture, which controls the soil water capacity (Tan, 1998), was not significantly affected by the percolation of soil (Chapter II). Therefore, the alteration of the wetting properties of soil occurred preferentially due to the changes of its chemical features, such as cation composition in soil matrix, pH, and ability of soil to release organic matter to the water solution (DOC content). These changes were detected in the percolated soil (Tab. II-2). For example, the Na-treated soil, which showed the highest volumetric water content (Appendix I), released the maximal amount of the DOC to the soil solution. It is well-known that the Na$^+$ in the soil matrix enhances the expansion of the SOM, which increases the
water capacity of the sample; and leads to the growth of the SOM dispersion in the soil solution (Chorom et al., 1994), which increases the DOC content.

The changes of the general properties of soil induced by the water- and salt-treatments of soil, described above, affected the sorption and sequestration of the NP and Phe insignificantly. The sorption-desorption experiments carried out with the moist soils, where the additional factors of soil treatment (such as drying, freezing, aging, microbial transformation) were not applied, did not show clear effects of the water- and cation-treatments (Tab. III-1).

Drying and heating of soil led to the increase of the NP and Phe sorption in all studied samples. The effect of the soil moisture content on the sequestration of organic compounds in soils is well-known (Berkowitz et al., 2008; Kottler et al., 2001; Shelton and Parkin, 1991). Our sorption-desorption experiments with the moist and dried soils revealed that drying decreased the reversibility of the NP and Phe sorption (Tab. III-1), which was also confirmed by the extraction studies (Fig. II-1). The sorption kinetics of the NP and Phe on heated soils (20, 60 and 105°C) showed that the heating effect was still significant even after 20 hours of shaking of soil with water (Fig. III-5). This means that changes, which happen in the soil matrix under stressful environmental conditions like drying and/or heating, are substantial. The soil matrix altered in such a way interacts with organic xenobiotics differently, which needs to be taken into account when assessing the pollutants fate in the soils and sediments.

To investigate the influence of the stress conditions on the fate of xenobiotics, which are already sorbed in soil, the soil samples spiked with the NP and Phe were subjected to one and four drying-wetting or freezing-thawing cycles (Chapter II). Previously, several groups reported different and in some cases controversial findings about the influence of drying-wetting or freezing-thawing on the extractability of xenobiotics from soils (Chung and Alexander, 2002; Eschenbach et al., 1998; Kottler et al., 2001). Our results showed that drying-wetting and freezing-thawing treatments led to the substantial decrease of the xenobiotics extractability (Fig. II-1). This means that the xenobiotics present in the soil become involved in all transformations of the soil matrix that occur due to the influence of abiotic factors. In some cases, like in our study, these changes lead to the sequestration of
xenobiotics in soils. The differences between the effect of one compared to four cycles of
drying-wetting or freezing-thawing on the NP and Phe extractability from soils were not
significant (Chapter III). This suggests that the alterations, which once occurred in the
studied soils because of their drying or freezing, cannot be reversed or intensified by the
multiple repetition of the treatment.

4. The effects of the soil microbial activity on the formation of
nonylphenol and phenanthrene bound residues

The sequestration of xenobiotics and formation of their bound residues in biologically active
soils are governed by the bioavailability of compounds to soil microorganisms (Haritash and
Kaushik, 2009; Semple et al., 2004). On the one hand, the bioavailability and bioaccessibility
are determined by the physicochemical properties of soil, which also influence the sorption
and entrapment of pollutants in soil (Scow et al., 1995). On the other hand, the ability of
microorganisms to degrade the xenobiotics adsorbed in soil depends on the different
parameters of microbial community inhabiting a particular soil. The most important
microbiological characteristics of soil for the xenobiotics fate are the quantity and diversity
of soil microorganisms, as well as the distribution homogeneity of the microorganisms
among the soil bulk (Semple et al., 2003).

The enzymes present in soil are mainly produced by the soil microorganisms and play an
important role in the chemical transformation and fate of xenobiotics in the environment
(Haritash and Kaushik, 2009; Wicke and Reemtsma, 2010). Not surprisingly, the analyses of
the sterile and incubated soils showed that the total enzyme activity was substantially higher
in the bioactive soils. In the soils incubated for 1 month the total enzyme activity significantly
varied in different samples (Fig. V-1). But the total respiration values of the 1 month
incubated soils were very similar in all percolated samples (Fig. IV-1). These observations
suggest that the composition of the microbial community may have varied in different soils,
which led to the distinct variations of the total enzyme activity values in water- and
salt-treated samples. In addition, previous studies of different soil enzymes showed that
they are mainly influenced by the pH and the electrical conductivity of the soil solution (El-
In addition to substantial differences of the total enzyme activity mentioned above, the mineralization of NP and Phe in the incubated for 1 month soils also varied significantly among the samples (Fig. IV-1). The mineralization of both NP and Phe was lower in the Na- and Al-treated soils compared to the other samples. The negative effects of these cations on the microbiology in some soils was reported earlier (Chang et al., 2007a; Yuan et al., 2004). In comparison with the NP mineralization, the negative effect of Na$^+$ and Al$^{3+}$ on the Phe mineralization was much stronger. This indicates that the mineralization of these two compounds was most probably conducted by different microorganisms present in soils, with different ability to degrade NP and Phe.

The incubation of soils inoculated with the microbial community, which is capable to mineralize the 1,2,4-TCB (Wang et al., 2010a; Wang et al., 2007), showed that this soil microbiology was significantly more effective in case of the Phe mineralization, compared to the NP mineralization (Appendix I). During 2 months of incubation the Phe and NP mineralization was 23.9–32.5% and 0.8–0.9%, respectively (data not shown). In comparison with the effect of the original soil inoculum, the inoculation of the sterile soils with the 1,2,4-TCB mineralizing microbial community significantly increased the Phe mineralization.

The detected effect of the soil microbiology on the xenobiotics mineralization confirms the important role of microorganisms in the fate of xenobiotics in soils (Haritash and Kaushik, 2009). However, the mechanisms that underlie the microbial degradation and mineralization of organic pollutants, as well as the formation of bioavailable, bioaccessible or bound residue fractions of xenobiotics, are still poorly understood (Gevao et al., 2000; Haritash and Kaushik, 2009; Wilcke, 2000). This often makes the remediation and recultivation techniques, applied to the polluted soils, unique depending on the type of soil, climate, environmental conditions and features of pollutants (Wilson and Jones, 1993).

Another factor, which significantly affected the xenobiotics mineralization, but not the soil respiration, was the water addition (50% from initial moisture) to soils after one month of incubation. The enhancement of the NP mineralization after the increase of the soil moisture was more substantial compared to the growth of the Phe mineralization. One possible
explanation of the observed water addition effect on the pollutants mineralization could be the increase of NP and Phe bioavailability due to the bridging role of the water films between soil constituents (Paradelo and Barral, 2009). The correlation between the NP mineralization and its pore-water extractability from the non-incubated soils (Appendix I) also indicates the importance of the soil water for the bioavailability of the NP in the studied soils.

It is common to apply different organic substrates and nutrients to investigate their effects on microbial activity and formation of extractable and non-extractable residues of xenobiotics in soils (Semple et al., 2006). The addition of the wood flour, produced by grinding of beech sawdust, to the soils significantly increased the soil respiration by a factor of 4.6 in original soil and by a factor of 2.9 in percolated soils (Chapter IV). In general, the wood flour addition led to the increase of the NP mineralization and to the reduction of the Phe mineralization. Such distinct effects on the xenobiotics mineralization are indicative of substantially different processes of the microbial transformation of NP and Phe in the studied soil samples. Besides the most likely effect on soil microorganisms (Lashermes et al., 2010), the wood flour could also play a role of additional sorbent in soil matrix, which in turn may alter the bioaccessibility of xenobiotics (Semple et al., 2003). Both of these factors can affect the formation of xenobiotics bound residues. For instance, together with stimulation of the soil respiration and NP mineralization, the wood flour addition led to the increase of the non-extractable fraction of NP in soils.

The series of extraction experiments carried out with soils, which were incubated without substrate additions or with wood flour addition, showed a significant relationship between the Phe water-ethanol extractability and mineralization, fitted to a power function (Chapter IV). Meaning that the higher was the mineralization of the Phe during the incubation period, the lower was its extractability after the degradation. For the NP no regular dependency of the extractability on the mineralization was detected. However, the Na- and Al-treated soils with the lowest mineralization rates showed the highest NP extractability after the incubation period. The correlation between the xenobiotics extractability and mineralization is the commonly observed effect of the microbial degradation of pollutants on their sequestration in soils (Northcott and Jones, 2000; Semple
et al., 2006). Nevertheless, the mechanisms of the formation of xenobiotics extractable and non-extractable fractions in soils are still not well-understood.

As it was shown previously, the incubation time of organic pollutants in soils can also significantly influence their fate in the environment (Hofman et al., 2008; Ling et al., 2010; Regitano et al., 2006; Scelza et al., 2010; Vessigaud et al., 2007). The degradation of the NP and Phe in the control and cation-treated soils during their 2 months incubation led to the formation of the significant amount of the non-extractable fraction of xenobiotics (Chapter IV). However, the subsequent incubation up to 9 months caused the diminution of the non-extractable fraction of xenobiotics (Fig. II-2). For instance, the long-term bioaging of the NP in the percolated soils led to the decrease of the bound residues fraction down to the undetectable amounts. The analysis of the mineralization and extraction data suggests that the NP and Phe bound residues formed in all soils, but incubation resulted in the partial transformation of the non-extractable and ethanol-extractable fractions into water-soluble and mineralized fractions. Interestingly, however, the observed decrease of the NP and Phe bound residues fraction does not correlate with other studies, which on the contrary reported the growth of the non-extractable fraction of organic pollutants in soils with time of incubation (Benoit and Barriuso, 1997; Nowak et al., 2011).

The mean mineralization rates of the NP and Phe (0.19 and 0.32% d\(^{-1}\), respectively), determined in our study, are in the lowest range compared to mineralization rates reported in the previous works: 0.62–20% d\(^{-1}\) for NP (Barber et al., 2009; Chang et al., 2007b; Hesselsoe et al., 2001; Telscher et al., 2005; Topp and Starratt, 2000) and 0.06–62.1% d\(^{-1}\) for Phe (Hatzinger and Alexander, 1995; Nam and Alexander, 2001; Watanabe et al., 2005). The prediction of organic pollutants behavior in soils is generally based on the knowledge accumulated through a multitude of sorption, degradation, extraction and other studies of different chemicals in various soils. Currently, however, there is no uniform methodological approach available that would allow to make strong prognosis regarding the fate of a particular compound in a given soil (Riding et al., 2013).
5. The aging of soils as a factor of soil alteration and the formation of xenobiotics bound residues

It is believed that soil is a system, which stays in a quasi-equilibrium and slowly reacts to the changes in the environment (Targulian and Krasilnikov, 2007). The kinetics of soil response to the influence of some external agent depends not only on the soil properties, but also on the duration of this stimulus (Subramaniam et al., 2004).

Our long-term study of soil, which was stored under sterile conditions in the dark at constant temperature, and which was not disturbed, mixed or affected by any external agent, showed significant changes of the soil properties after 5–10 months of aging. The pore-water extraction analyses of the non-aged and aged for 5 months soils revealed an interesting effect of the aging on the ability of soil to release the pore-water: unexpectedly, the amount of extracted pore-water significantly decreased after the 5 months aging of the original, H$_2$O-, Ca- and Al-soils, while in the Na-soil it did not changed (Fig. AI-1). The observed effect most probably indicates that the physicochemical properties of soils can change in the course of time also under influence of some still unknown internal agents present in the soil. Interestingly, it was shown recently that the molecular structure of the SOM of peat changes with time, namely, the SOM rigidity increases. The authors assumed that these structural conformations can occur due to the formation of the water molecular bridges in the SOM matrix (Schneckenburger et al., 2012). The extractions of pore-water performed in our study showed that the behavior of water in the soil bulk during the long-term aging is determined by the initial properties of soil, namely, by the presence of mono- or polyvalent cations in the soil matrix.

The enzyme studies of the sterile soils revealed that the 9 months aging decreased the soil enzyme activity in all percolated soils, while in the original untreated soil it reduced insignificantly (Fig. V-1). The negative effect of the time of soil storage on the enzyme activity is well-known and was shown for different soils previously. The reduction of the enzyme activity can be caused by the chemical processes of enzyme degradation and modification due to reactions with other soil constituents (SOM, minerals, chemicals in soil solution) (DeForest, 2009; Lee et al., 2007; Turner and Romero, 2010; Wallenius et al., 2010).
Thereby, the enzyme activity could be one of the possible internal factors, which induce the alteration of the soil properties during aging.

The changes in the soil functioning (e.g., interaction of soil with xenobiotics) detected after its aging can indirectly indicate the alterations of soil properties, which occurred during the aging. The comparison of the respiration rates of the non-aged and aged for 5 months sterile soils, which were then inoculated and incubated for more than 4 weeks, revealed that the long-term sterile aging of soil can influence the activity of the microbiology, which was introduced to the soil with inoculation: aging led to a significant increase of the soil respiration (Fig. IV-1). This might indicate that during the sterile aging of the samples some processes of soil changing (e.g., activity of soil enzymes) create more favorable conditions for soil microorganisms, which result in growth advantages and subsequently higher release of CO$_2$, detected in our studies. However, the 2 months of incubation studies carried out with non-aged and 5 months aged soils showed that the aging considerably reduced the NP and Phe mineralization in all soils, except Na-treated sample (Fig IV-2). The decrease of the bioavailability and mineralization of the NP and Phe became evident from the extractability data: the water, cyclodextrin and ethanol extractability of xenobiotics from sterile samples reduced with the time of aging of contaminated soils (Chapter II). A number of previous studies of the fate of different pollutants in various soils and soil materials during the sterile aging showed the same trends in the xenobiotics extractability and mineralization (Hatzinger and Alexander, 1995; Ncibi et al., 2007; Northcott and Jones, 2001; Sharer et al., 2003; Slizovskiy and Kelsey, 2010; Tang et al., 1998; Zhao et al., 2009).

In summary, our findings, on the one hand, indicate that the long-term storage of soil can significantly change its properties, and therefore the analyses of soil have to be carried out as soon as possible after sampling. On the other hand, the aging experiments showed that pollutants sequestration in soils depends on the duration of xenobiotic–soil interaction, and that changes taking place in the soil without microbial influence may significantly affect the formation of xenobiotics bound residues.
6. Summary and final conclusions

Organic chemicals that are introduced into soils by human activities (xenobiotics) and their metabolites are strongly sorbed and sequestered in soil, mainly through interactions with the soil organic matter (SOM). The fate of organic pollutants and formation of their non-degradable and non-extractable residues (bound residues) in soil are preferentially defined by the properties of SOM, environmental conditions and duration of soil–xenobiotic interaction. However, the mechanisms underlying the bound residues formation and factors influencing the sorption, mineralization and sequestration of xenobiotics in soil are still not well-understood.

The main goal of this study was to determine the effects of SOM properties, specifically of its structural conformation, on the sorption, mineralization and formation of extractable and non-extractable (bound) residues of the two model compounds nonylphenol (NP) and phenanthrene (Phe) under influence of different environmental conditions: long-term sterile storage (aging), incubation, drying, freezing or heating of soils. The alteration of the SOM properties was performed by the saturation of soil with mono- or polyvalent cations, which was suggested previously to affect its structural conformation.

The obtained results revealed that, in spite of the significant changes of the cation composition of the soil matrix, there was no clear effect of the cation-treatment of soil on the sorption of the NP and Phe. However, the SOM structure was indeed altered by the treatment of soil with NaCl, CaCl₂, AlCl₃ solutions: the ¹H NMR studies showed that the confinement of the water molecules in the SOM structure increased in the following order: Na-treated soil < Ca-treated soil < Al-treated soil, as was anticipated.

The subsequent studies of the influence of different environmental conditions on the fate of xenobiotics in cation-treated soils showed that:

1) The soil hydrophobicity and the sorption of the NP and Phe on dried samples increased significantly with elevated temperature of drying, while the reversibility of sorption process decreased.

2) The long-term sterile aging of xenobiotics in soils substantially increased their bound residues fraction. Moreover, the aging effect on NP extractability could also be achieved by
short-term freezing and thawing of the soils, while aging of Phe was better mimicked by drying-wetting cycles.

3) The inoculation of sterile soils with the native microorganisms and subsequent incubation resulted in the substantially lower mineralization of the NP compared to the Phe. The relationship between the Phe extractability and mineralization fitted well to a power function, while it was not the case for NP. The mineralization of xenobiotics increased significantly after growth of the soil moisture content, while the soil respiration did not change. The addition of wood flour to soils increased soil respiration and mineralization of NP, while Phe mineralization decreased.

4) The incubation of contaminated soils with native microbiology for 2 months increased the amount of xenobiotics bound residues fraction. However, the long-term incubation for 9 months caused the diminution of this fraction. In comparison to the sterile aging, the long-term incubation resulted in the lower amounts of non-extractable residues of NP and Phe in soils.

5) The effects of the SOM structure alteration by cation saturation of soils on the sorption and long-term aging of NP and Phe were negligible. However, treatments with Na\(^+\) and Al\(^{3+}\) significantly reduced the mineralization of both xenobiotics, which resulted in the increase of their extractability.

In conclusion, the sequestration of NP and Phe in sterile soils and formation of bound residues were insignificantly affected by the structural conformation of SOM, performed by saturation of soil with mono- or polyvalent cations. Though the polyvalent cations most probably induced the increase of cross-linking in the SOM structure, the effect of SOM alteration on the NP and Phe sorption and formation of bound residues in studied soils was not evident. Our findings indicate that the environmental factors, like heating, freezing or drying of soil, had a stronger influence on the fate of the NP and Phe in comparison with effects of the SOM alteration due to the saturation of soil with different cations. The increase of microbial activity due to addition of organic substrate (wood flour) led to opposite effects on the mineralization of two different model compounds.
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Appendix I

Effects of soil cation treatments and aging on nonylphenol and phenanthrene extractability: study of *in situ* pore-water extraction as an alternative method for evaluation of xenobiotics bioavailability in soils
**1. Introduction**

The most frequently applied techniques for estimation of leaching potentials of organic pollutants from soil are extraction batch experiments with excess of water (EEW) or other solvents (de Wilde et al., 2008; OECD, 2000). However, it was suggested that the results of these experiments cannot reflect the behavior of xenobiotics in natural soils, as well as the interactions between soil and soil solution (Folberth et al., 2009b). The authors of the *in situ* pore-water extraction (PWE) method reported that the amount of dissolved isoproturon determined by batch extraction method had a significant correlation with the content of the total organic carbon in soil; while results of the PWE analyses had a clear correlation with the total isoproturon mineralization (Folberth et al., 2009a; Folberth et al., 2009b). It was suggested that extraction of the compound using the batch method leads to the disaggregation of soil, and therefore to the increase of availability of the sorption sites for a solvent. This cannot happen in the undisturbed soil samples, which are used for the pore-water extraction.

The main objective of our study was the comparison of nonylphenol (NP) and phenanthrene (Phe) extractability determined by two methods - PWE and EEW analyses. The obtained results were then correlated with the mineralized fraction of contaminants, in order to evaluate if PWE method can be used for the assessment of xenobiotic mineralization. For extraction studies, the sterile moist original soil and H\(_2\)O, NaCl, CaCl\(_2\) and AlCl\(_3\) treated soils freshly spiked with the NP and Phe or aged for 5 months were used. To evaluate the mineralization rates of NP and Phe, all soils were inoculated either with original soil or with microbial community capable of 1,2,4-trichlorobenzene (1,2,4-TCB) mineralization.

**2. Materials and methods**

**Soil preparation**

The detailed description of the soil treatment with water and different salts, of moistening, sterilization and spiking with NP and Phe, as well as the determination of the volumetric water content in soil samples is presented in the Chapter II.
Effects of soil cation treatments and aging on nonylphenol and phenanthrene extractability: study of in situ pore-water extraction as an alternative method for evaluation of xenobiotics bioavailability in soils

Sterile moist contaminated soils were used for extraction and incubation studies directly after spiking with xenobiotics and after 5 months of storage (aging) under sterile conditions in the dark at the constant temperature. During aging the change of moisture of soil samples was monitored and was found to be negligible.

**Extraction methods**

All extraction studies were carried out with the sterile soils.

*Batch extraction with excess of water (EEW)*

Contaminated soils were used for water sequential extraction. The soil in amount of 0.8 g (d/w) and 8 ml of solvent were placed into the vials, mixed using an overhead shaker with 15 rpm at 20°C for certain periods of time (3, 3 and 7 h for the I, II and III extraction cycles, respectively). Subsequently, vials were centrifuged for 15 min at 2700 g, and 80% of supernatant was replaced by a fresh solvent. The radioactivity was determined by the liquid scintillation counting. This extraction cycle was repeated 3 times. The remaining solution from every previous extraction cycle and the amount of solute therein was taken into account in calculations of NP or Phe dissolved fraction.

*Pore-water extraction (PWE)*

Contaminated soils were placed to custom-build centrifuge containers and centrifuged at 21°C for 90 min at 9170 g. The extracted pore-water was filtered with 0.45 μm polycarbonate filters. The aliquot of filtrate was measured by liquid scintillation counting. The amount of xenobiotic dissolved in soil water was calculated by multiplication of the determined concentration with the total water content in the sample.

**Mineralization studies**

The original non-sterile soil was preincubated moist for 14 days and then added (5%, d/w) to the sterile samples as an inoculum. Another part of soils was inoculated with a different 14 days preincubated soil with native capacity of 1,2,4-TCB mineralization (Wang et al., 2010; Wang et al., 2007). The following incubation was carried out in a Respicond-apparatus that hourly monitors CO₂ production and collects respired CO₂ in 0.6 M KOH solution. Every 4 days the KOH solution was sampled for analysis of the ^14C-activity and then refreshed. To
determine the radioactivity of KOH solution the aliquot was analyzed by liquid scintillation counting. Incubation was carried out for 4 weeks. The concentrations of $^{14}\text{C}$-labelled NP or Phe in soils after incubation were also determined in certain samples by dry combustion. The differences between the values calculated from the mineralization data and obtained from the combustion analysis were statistically negligible.

The analyses of xenobiotics mineralization by microbial community of original soil were carried out with non-aged and 5 months aged soils (original and four percolated samples). Incubation experiments with microbial community of soil with native capacity of 1,2,4-TCB mineralization were conducted only with 5 months aged soils (original, NaCl and AlCl$_3$ treated samples).

3. Results

**General properties of soils**

Volumetric water content in soils at pF 1 – pF 0 was 43–49, 41–42, 43–47, 40–43 and 39–43% in original soil, H$^+$, Na$^+$, Ca$^{2+}$, and Al$^{3+}$ treated samples, respectively. The maximal content of the volumetric water was detected in the Na-soil.

The amount of extracted water, determined after centrifugation of soil samples (pore-water), was different in all samples (Fig. AI-1) and did not correlate with initial water content in soils, which was 10.5, 9.0, 11.8, 11.6 and 11.3% in the original soil, H$^+$, Na$^+$, Ca$^{2+}$, and Al$^{3+}$ treated samples, respectively. Notably, water extractability significantly decreased in all soils after 5 months of aging, but not in Na-treated sample, where it did not change.

**Extractability of NP and Phe by PWE compared to EEW**

Maximal pore-water NP extractability of xenobiotics was detected from Na-soil (Fig. AI-2). In the salt-treated soils the pore-water extractability of NP reduced with increase of the cation valency. For the Phe this tendency was not observed, all salt-treated soils showed similar values, which were higher than in the original and H$_2$O-treated soils. The H$_2$O- and Al-treated soils revealed a tendency for the minimal extractability of both xenobiotics. Generally, the pore-water extracted fraction of NP was 0.11–0.15 and 0.04–0.08% in the non-aged and
Effects of soil cation treatments and aging on nonylphenol and phenanthrene extractability: study of in situ pore-water extraction as an alternative method for evaluation of xenobiotics bioavailability in soils

5 months aged soils, respectively. For the Phe, these parameters were 0.11–0.13 and 0.03–0.05%.

The 5 months aging of the contaminants in soil greatly decreased their pore-water extractability. The reduction of NP extractability caused by 5 months aging amounted to 51 and 19% of xenobiotic extractability from non-aged soils as determined by the PWE and EEW

Fig. AI-1 The amount of extracted water (percentage of dry weight of soil sample) determined by pore-water extraction method. The mean values and standard deviations are presented.

Fig. AI-2 Extractability of (a) nonylphenol and (b) phenanthrene determined by pore-water extraction method. The mean values and standard deviations are presented.

The 5 months aging of the contaminants in soil greatly decreased their pore-water extractability. The reduction of NP extractability caused by 5 months aging amounted to 51 and 19% of xenobiotic extractability from non-aged soils as determined by the PWE and EEW
(Chapter II), respectively. The corresponding parameters for Phe were 69 and 35%. In general, the extractability of both compounds determined by EEW was significantly higher compared to the PWE results.

The correlation between the extractability and mineralization of xenobiotics in soils

No clear correlation was found between the mineralization of compounds and their extractability as analyzed by EEW (Chapter II). The only possible exception could be the behavior of the Phe in 5 months aged soils. The Pearson coefficient characterizing the correlation of the Phe extractability and mineralization was −0.84.

The comparison of the pore-water dissolved fraction and mineralization of Phe did not reveal any correlation (Fig. AI-3b). However, NP pore-water extractability and mineralization had a relation, particularly if excluding Na-soil results from analyzed data (Fig. AI-3a). Here again the Na-treated soil showed outlying results, as well as in studies of the volumetric water content and of the amount of total extracted pore-water, described above.

**Fig. AI-3** Correlation between the pore-water dissolved fraction (determined in sterile soils) and mineralized fraction of (a) nonylphenol and (b) phenanthrene (determined after 4 weeks of incubation). The white and black dots represent non-aged and 5 months aged soils inoculated with original soil, respectively. Gray dots represent 5 months aged soils inoculated with 1,2,4-trichlorobenzene-degrading microbial community. Linear regression lines are included for the non-aged and aged soils, except Na-sample. The dotted line shows linear regression only for Na⁺-treated samples.
4. Summary

The outlying results obtained for the Na-soil – the highest volumetric water content at the pF 0, the lack of the aging effect on the extractability of the total pore-water from soil, significantly different behavior of the pore-water extractable fraction of NP and Phe, in comparison with other soils – all these indicate that the status of the water molecules in the soil treated with Na\(^+\) played a very important role in the soil functioning. Probably, the behavior of water in the original soil and samples treated with other cations also influenced the formation of non-extractable residues of xenobiotics, but these effects were not evident in our study.

The behavior of polar compound Phe in studied soils was likely governed by its hydrophobic properties. Thus, the pore-water extractability of the Phe did not correlate with the amount of extracted water, which suggests that this compound was strongly sorbed by soil and did not interact with compounds dissolved in the soil solution.

The unexpected effect of aging reflected in the decrease of soil ability to release the pore-water was observed in all samples, except for Na-treated soil. This phenomenon revealed that during sterile aging the unknown processes of possible soil matrix reorganization, leading to stronger binding or entrapment of water molecules, take place in soils.

On the contrary to the results presented by the authors of the in situ pore-water extraction method, our results do not demonstrate clear correlation between pore-water extractability and mineralization of two xenobiotics in different soils. This indicates that the PWE analysis can not be uniformly applied to all soils, as well as to the various chemicals, as an alternative method for evaluation of xenobiotics bioavailability, without preliminary studies.
References

Folberth, C., Scherb, H., Suhadolc, M., Munch, J.C., Schroll, R., 2009a. In situ mass distribution quotient (iMDQ) - A new factor to compare bioavailability of chemicals in soils? Chemosphere 75, 707-713.


Appendix II

Supplementary data for the Chapter V
Method for enzyme activity measurement in soils

1 g of soil was mixed in 100 ml sterile deionizer water and sonicated for 120 s at 50 J. Afterwards, the suspensions were continuously stirred while aliquots were dispensed into each of a black polystyrene 96-well microplate. All enzyme assays were carried out under buffered conditions. Depending on the final substrate concentration in the wells, the appropriate amount of sterile MES buffer (2-(N-morpholino)-ethanesulfonic acid) or TRISMA base (tris-(hydroxymethyl)-aminomethane) was also added into each well. Finally, 50 µl of soil suspension, 50 µl of buffer and 100 µl of 4-Methylumbelliferone or 7-amino-4-methyl coumarin linked substrate solution were added to each microplate well (Tab. AII-1). Each microplate also included three replicates of a quench standard and a substrate control. The plates were covered and incubated in the dark at 30°C for 3 h. Fluorescence was measured after 30, 60, 120, and 180 min using a microplate reader (Infinite 200, Tecan, Germany) with 365 nm excitation wavelength and emission at 450 nm. After correcting for negative controls and quenching, activities were expressed in [nmol h⁻¹ g⁻¹].

Tab. AII-1 Enzymes with corresponding substrates and buffers.

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<th>Enzyme</th>
<th>Substrate</th>
<th>Sigma no.</th>
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<td>N-acetyl-β-D-glucosaminidase</td>
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<td>M2133</td>
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<tr>
<td>acid phosphatase</td>
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<td>M8168</td>
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<td>β-D-glucosidase</td>
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<td>M3633</td>
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<tr>
<td>β-D-xylisidase</td>
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<td>M6018</td>
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<td>L-Arginine-7-amino-4-methylcoumarin</td>
<td>A2027</td>
</tr>
</tbody>
</table>
Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass ich meine Dissertationsschrift mit dem Titel

“Effects of Soil Organic Matter Molecular Conformation and Substrate Additions on the
Formation and Release of Xenobiotics Bound Residues”

selbstständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe. Dies gilt sowohl für Textteile, als auch für Abbildungen und Grafiken.

Bochum, 19.04.2013

________________________________________________________________________

(Anastasia Shchegolikhina)