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NONEXTRACTABLE RESIDUES OF SULFONAMIDE ANTIMICROBIALS IN SOIL - FORMATION MECHANISMS WITH ORGANIC MATTER AND STABILITY

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Summary

The input of veterinary antimicrobials into the environment is of concern because of their potential to invoke bacterial resistance or to exert adverse effects on microbial communities. Veterinary sulfonamide antimicrobials enter soils via excretions of grazing livestock or manure application. A few weeks after application a major part of sulfonamides cannot be recovered from soil by conventional extraction methods, indicating a strong tendency of these antimicrobials to form nonextractable residues (NER). The mechanisms driving NER formation are either physical entrapment in organic matter or covalent bond formation. The latter seems more likely due to (i) the very rapid initial NER formation in soils and (ii) the possibility of sulfonamide covalent bonding to quinone moieties of natural organic matter via their aromatic amino group. To judge the long-term behavior and a possible remobilization as well as effects of sulfonamides in soils, it is essential to identify the binding pathway relevant for sulfonamides. The overall objectives of this PhD thesis were (i) to identify the main reaction pathways of sulfonamides with model compounds and organic matter isolates as well as the stability of the formed bonds, and (ii) to assess the factors and pathways governing NER formation of sulfonamides in soils.

The binding mechanisms and stability for the model sulfonamide sulfathiazole (STZ) in reactions with model humic constituents (quinone-forming and other carbonyl compounds) in the absence and presence of laccase was elucidated in Chapter 2 of this thesis. As revealed by high resolution mass spectrometry, the initial bonding of STZ occurred by 1,2- and 1,4-nucleophilic additions of aromatic amines to quinones, resulting in imine and anilinoquinone formation, respectively. The stability of covalent bonds against desorption and pressurized liquid extraction (PLE) was assessed. The results showed a release of covalently bound STZ by PLE, but no systematic differences between the two bonding types. This suggests that the strength of bonding is not controlled by the initial type of bond, but by the extent of subsequent incorporation of the reaction product into the observed polymerization products. This incorporation could be confirmed for ^{15}N aniline by ^{1}H-^{15}N HMBC NMR spectroscopy showing that initial 1,2- and 1,4-addition bonds were replaced by heterocyclic bonding with increasing incubation time.

In Chapter 3 of this thesis the covalent bonding of ^{14}C-labelled sulfamethazine (SMZ) as model sulfonamide to dissolved humic acid was studied. This was achieved in well-constrained single- and binary-solute systems using Leonardite humic acid (LHA) and four synthesized model humic

acids (SHAs) with different properties in the absence and presence of laccase. Only a small amount of added SMZ was covalently bound in the absence of laccase. The pool of reactive quinones increased considerably after oxidation of the organic matter, resulting in extensive covalent bonding. The competition with the stronger nucleophile *p*-ethoxyaniline was not of importance when many reactive sites were available. No or only negligible portions of SMZ were released from covalent interactions with natural humic acid using PLE. The overall covalent bond formation was considerably higher in incubations with SHAs than with LHA. This higher extent of covalent binding of SMZ to all SHAs confirmed the relevance of the oxidation state of organic matter.

Chapter 4 of this thesis contributed to the identification of limiting factors in NER formation of ^{14}C-labelled SMZ by manipulation of soil samples. The amount of quinones in soil available for nucleophilic addition was a limiting factor as indicated by (i) an (initial) increase of NER formation by adding quinone precursors or enhancing their formation by manganese oxide addition and (ii) a decrease of NER formation by limiting the formation of quinones under anaerobic conditions. These observations coincided well with the results obtained from the studies in Chapter 2 and 3 that covalent bonding by nucleophilic addition is initially the dominant pathway of NER formation and that NER formation is governed mainly by the availability of reactive quinones. For the slow formation of NER with increasing incubation time the limiting factor is not the formation of quinones by oxidants, but probably the availability of sulfonamides, which need to desorb from the solid phase prior to the reaction. This hypothesis is supported by the fact that no slow NER formation phase was observed during the anaerobic incubation of soil.

Zusammenfassung

Der Eintrag antimikrobieller Wirkstoffe in die Umwelt ist aufgrund negativer Auswirkungen auf mikrobielle Gemeinschaften und der möglichen Verbreitung von Resistenzgenen bedenklich. Antimikrobielle Sulfonamide aus der Tierhaltung werden durch Ausscheidungen weidender Tiere oder durch Applikation von Gülle in Böden eingetragen. Sulfonamide zeigen in Böden eine starke Tendenz zur Bildung gebundener Rückstände und sind mittels konventioneller Methoden nach einigen Wochen nicht mehr extrahierbar. Als Mechanismen für die Bildung gebundener Rückstände kommen physikalischer Einschluss in organische Substanz oder Bodenpartikel sowie die Bildung kovalenter Bindungen in Frage. Für eine kovalente Bindung sprechen die Befunde, dass die initiale Bildung gebundener Rückstände sehr schnell verläuft und eine Reaktion der aromatischen Aminogruppe der Sulfonamide mit Chinonen der organischen Substanz möglich ist. Um das Langzeitverhalten und eine mögliche Remobilisierung und damit verbundene Effekte in Böden zu verstehen, ist es notwendig, die Reaktionswege der Sulfonamide in Böden zu verstehen. Die Ziele dieser Arbeit waren es daher (i) die wichtigsten Reaktionswege von Sulfonamiden mit Modellverbindungen und Huminsäuren aufzuklären und die Stabilität dieser Bindungen zu bestimmen, sowie (ii) die Einflussfaktoren und Mechanismen der Bildung gebundener Rückstände in Böden aufzuklären.

Im Kapitel 2 dieser Dissertation wurden die Bindungsmechanismen und die Bindungsstabilität des Sulfonamides Sulfathiazol (STZ) im Vergleich zum stärkeren Nucleophil *para*-Ethoxyanilin für die Reaktion mit Modellverbindungen (Chinon-bildende und andere Carbonylverbindungen) in An- und Abwesenheit von Laccase untersucht. Mit Hilfe hochauflösender Massenspektrometrie konnte gezeigt werden, dass die initiale Bindung von STZ durch 1,2- und 1,4-nucleophile Additionen der aromatischen Aminogruppe an Chinone erfolgt, was zur Bildung von Iminen bzw. Anilinochinonen führt. Die Stabilität der kovalenten Bindungen wurde gegenüber Desorption und beschleunigter Lösemittelextraktion (PLE) bestimmt. Diese Versuche zeigten, dass kovalent gebundenes STZ durch PLE freigesetzt wird, jedoch wurden keine systematischen Unterschiede zwischen Iminen und Anilinochinonen festgestellt. Dieses Ergebnis deutet darauf hin, dass die Bindungsstärke nicht durch die Art der initialen Bindung, sondern vielmehr durch die darauffolgende Inkorporation der Moleküle in die beobachteten Polymerisationsprodukte gesteuert wird. Diese Inkorporation konnte für ^{15}N-markiertes Anilin mittels ^{1}H-^{15}N HMBC NMR-

Spektroskopie bestätigt werden. Dies zeigte, dass die 1,2- and 1,4-Addukte mit zunehmender Inkubationszeit durch N-Heterozyklen ersetzt werden.

Im Kapitel 3 dieser Dissertation wurde die kovalente Bindung von ^{14}C-markiertem Sulfamethazin (SMZ) an gelöste Huminsäuren untersucht. Dazu wurden Inkubationsexperimente mit Leonardite-Huminsäure (LHA) sowie mit vier synthetischen Huminsäuren (SHAs) mit unterschiedlichen Eigenschaften durchgeführt. In Abwesenheit von Laccase war die Anzahl der reaktiven Gruppen in LHA begrenzt, so dass nur ein kleiner Anteil des zugegebenen SMZ kovalent gebunden wurde. Die Anzahl der reaktiven Chinone wurde durch die Zugabe von Laccase erheblich erhöht, was zu einer verstärkten kovalenten Bindung des SMZs führte. In diesem Fall hatte auch die Konkurrenz des stärkeren Nucleophils *para*-Ethoxyanilin keinen Einfluss auf die gebundene Menge an SMZ. Nur ein geringer Teil des an die Huminsäure gebundenen SMZ wurde durch PLE freigesetzt. Für die SHAs zeigte sich ein deutlich höherer Anteil kovalent gebundenen SMZs im Vergleich zu LHA. Dies bestätigt, dass der Oxidationsgrad und damit die Zahl der Chinongruppen in der organischen Substanz für die Bindung relevant ist.

Kapitel 4 dieser Dissertation trug dazu bei aufzuklären, welche Einflussfakoren die Bildung gebundener Rückstände von ^{14}C-SMZ im Boden bestimmen, indem dieser selektiv manipuliert wurde. Ein limitierender Faktor war die Anzahl der vorhandenen Chinongruppen, weil (i) bei Zugabe von Hydrochinonen oder Manganoxid vor der Inkubation eine erhöhte Bildung gebundener Rückstände erfolgte, und (ii) eine erniedrigte Rückstandsbildung unter anaeroben Bedingungen beobachtet wurde, was die oxidative Chinonbildung verhinderte. Diese Ergebnisse stehen mit denjenigen der Kapitel 2 und 3 im Einklang, dass die kovalente Bindung durch nucleophile Addition als wichtigster initialer Schritt anzusehen ist und durch die Verfügbarkeit reaktiver Chinone bestimmt wird. Für die langsame Bildung gebundener Rückstände mit zunehmender Inkubationszeit ist dagegen die Chinonbildung nicht mehr limitierend, jedoch vermutlich die Verfügbarkeit der sorbierten Sulfonamide, die zunächst von der Festphase desorbieren müssen. Diese Hypothese wird durch die Beobachtung gestützt, dass unter anaeroben Bedingungen keine langsame Bildung gebundener Rückstände zu beobachten war.

Chapter 1

General introduction

Environmental relevance of veterinary sulfonamide antimicrobials

Since the discovery of the growth-inhibiting effects of penicillin on bacteria and the development of a broad range of antimicrobials, the medical profession has been able to successfully control a wide range of infectious diseases in humans and animals. However, owing to the use of antimicrobials over 70 years, researchers noted the ongoing emergence of antibiotic-resistant microorganisms, that continue to plague global health care.[1] Meanwhile, an equally alarming decline has occurred in the research and development of new antimicrobials to deal with the thread.[2] Development of antimicrobial resistance in microbes is attracting increasing attention because of their impeding influence on the effectiveness of clinically applied antimicrobials nowadays. Thus, the World Health Organization (WHO) cited the formation of resistance to antimicrobials as one of the key global problems facing future health policy.[3]

The development of resistance in bacteria has been attributed to the use of antimicrobials in human medicine. Recently, the contributions of veterinary medicine and agriculture to antimicrobial resistance have been widely discussed.[4] Roughly 50% of the more than 1 million tons of antimicrobials released into the biosphere during the last 60 years[5] were used in veterinary medicine.[6] The spread of antibiotics from agriculture via, for example, manure into the water and soil of the surrounding environment may select for resistant bacteria.[4]

Among different classes of antimicrobials, sulfonamides (SAs) are widely used in animal husbandry[7] to cure or prevent infectious diseases and to promote growth. A large part of the administered sulfonamides are excreted from animals unaltered or as acetyl conjugate, which reverts back to the sulfonamide by microbial cleavage.[8]

Special concern is given to SAs also due to their persistence in the environment. SA residues were detected in surface waters,[9] ground waters,[10,11] surface runoff,[12,13] and soils.[14,15] Inevitably, soil is a hot spot for SAs as it receives a large portion of excreted antibiotics through grazing livestock or the application of manure. Also, the high density of microbes in the soil environment encourages genetic changes, which could enhance the development of antimicrobial resistance.[16] Due to the high frequency of SA detection, the possible risk to adverse effects on microbial communities,[17] and the development of resistance in human pathogens SAs were grouped into a “high priority” category of veterinary medicines.[18]

SAs are quite water-soluble and show a pH-dependent speciation suggesting a high mobility in the environment. Indeed, studies investigating losses from grasslands and arable soils revealed very small apparent sorption coefficients, K_d, indicating significant potential for SAs losses to water

bodies.[19] However, the overall losses of SAs to the surrounding surface water due to leaching and surface runoff amounted to only 2.5%,[13,20,21] which was comparable to less hydrophilic pesticides,[22] a large fraction was retained in soil[12,14,23] suggesting chemical reaction and not just physical sorption interactions.

Interactions of sulfonamides with the soil solid phase

Knowledge about SA sorption is still limited and mainly based on short term studies focused on whole soils. Due to the fact that SA molecules ionize depending on matrix pH makes the sorption prediction even more difficult. Short-term sorption of SAs to soil is rather weak and increases with decreasing pH due to higher affinity of the SA cation than the uncharged and anionic species.[24-29] The complexity of the soil matrix restrained from making unanimous conclusions on key factors governing the strong sorption of SAs in soil.

The approach to study individual soil constituents helps to acquire a better understanding of the specific interactions that affect SA sorption. The contribution of clay minerals and pedogenic oxides to sorption were shown to be likely, yet it seems to be of minor importance in topsoils.[26,30-32] Consequently, the studies indicate that it is mainly the content of soil organic matter (SOM), and its chemical speciation that determine the extent of SA sorption.[31,33] Nevertheless, the plot[20,23] and laboratory[34] studies contradict picture on SA interactions depending on sorbent and experimental conditions.

Moreover, many of these studies did not take into account that sorption of SAs, after achieving an apparent equilibrium state after several hours or days, may increase with contact time. The sorbed amount of sulfathiazole increases regardless of the type of organic sorbent used from 1 to 14 d sorption time in sterile batch systems.[35] In soils Stoob *et* al. (2006)[36] showed a pronounced decrease in extractability for aged laboratory samples with time and Kreuzig and Höltge (2005)[23] provided first evidence that, apart from sorption, nonextractable residue (NER) formation contributes to a large extent to the dissipation of SAs in soil.

Nonextractable residues in soils

The International Union of Pure and Applied Chemistry (IUPAC) defines NERs as a parent compound or metabolites that are not released from the soil matrix by methods that leave

the compounds and/or the structure of organic matter unaltered.[37,38] NERs are hence operationally defined and depend on the method of extraction. In most cases, radiocarbon-labeled compounds are used in studies investigating NERs, thus a complete mass balance can be attained. Under these circumstances, all radioactivity not extractable in soils regardless of its chemical nature is considered as NER. Recent studies revealed, however, that in some cases also microbial metabolites and biomolecules not related to the original contaminant molecule structure contribute to this NER fraction.[39]

There are two fundamental pathways leading to the formation of nonextractable residues of organic contaminants of target compounds:

(i) The compounds diffuse to sites in the solid soil matrix, in which they resist extraction or desorption. One suggested mechanism is pore diffusion in the fixed intraparticle pore system, as it is present in lattice discontinuities of minerals, grain boundaries, or in the interlayer of expandable clay minerals.[40,41] The second mechanism is slow intraorganic matter diffusion in rigid organic matter phases, in which diffusing molecules are retained by adsorption to high-affinity microvoids or pores in the organic matter matrix.[41] Another suggested mechanism causing entrapment of contaminants in dissolved organic matter is micelle-like aggregation.[42] While more hydrophilic regions of the aggregates are directed to the surrounding water, its hydrophobic interior has the ability to entrap hydrophobic contaminants.[43,44]

(ii) The compounds undergo chemical reactions with the soil organic matter, which results in the formation of covalent bonds. Covalent bond formation depends on the reactivity of functional groups of both the contaminant and the organic matter and can be enhanced by the presence of enzymes or mineral catalysts.[38] If possible at all, a cleavage of these bonds occurs under harsh conditions (e.g., acid or alkaline hydrolysis)[45] or by enzymatic attack. Although there are not many studies on this topic, it is reasonable to assume that the parent compound is less likely to be released from covalently bound than from physically entrapped xenobiotics.[46]

To identify the binding sites of organic contaminants in soil, various sequential extraction and fractionation methods were used. Typically, the portion that is not removed by shaking, ultrasonic, soxhlet, or pressurized liquid extraction (PLE) is considered as a NER. For sulfonamides, several studies showed that the extractable fraction depends strongly on the method used. In laboratory studies, Kreuzig and Höltge (2005)[23] found a rapid decrease of extractable ^{14}C-sulfadiazine down to 40% of the applied amount after 3 d, and to less than 10% after 28 d in both sterile and nonsterile samples. While the NER fraction of ^{14}C-sulfadiazine amounted to 54-69% at day 0 if an ethanol:water shaking extraction was used, it was only 10-20%

for the same soil samples if a microwave-assisted extraction with acetonitrile:water at 140°C was applied.[47,48] Similarly, Stoob *et* al. (2006)[36] showed that the extractable fraction of different sulfonamides increased up to a factor of six if the extraction temperature in PLE was increased from 100°C to 200°C.

Hence, findings for SAs are in line with those for many other organic contaminants that show (i) prolonged sorption kinetics in soil, and (ii) increasing resistance to desorption or extraction from soil with contact time.[49] The formation of NERs leads to a decrease in the toxicity and bioavailability of contaminants.[50] However, the major question arising is the environmental significance of NERs, even when the magnitude of NERs formed is known. To this end, it is necessary to know whether these NERs are "harmless" biomolecules or largely unchanged "harmful" contaminant molecules. In the latter case, a portion could be released due to drastic changes in environmental conditions or by microbial degradation of the occluding organic matter.[51,52] Thus, there is a great need to understand the fundamental processes underlying NER formation in soils and their possible remobilization.

Nonextractable residue formation of sulfonamides

The rapid loss of extractability of sulfonamides from soil within several days[23] implies that covalent bonding rather than slow intraparticular diffusion is initially the dominant process of NER formation. This covalent bonding probably occurs via the sulfonamides' aromatic amino group, as many other aromatic amines show a significant covalent bonding to organic matter.[53-56] The proposed reaction pathways are summarized in Chapter 2 (Figure 1).[56-62]

Covalent bonding of SAs to model humic constituents and humic acid was demonstrated by Bialk *et* al. (2005, 2007, and 2008).[60-62] Accordingly, the nucleophilic aniline nitrogen of sulfonamide undergoes addition reactions at electron-poor sites of organic matter resulting in imine and aniliniquinone formation.[60] The reactive sites in OM are usually related to the presence of carbonyl groups in the form of ketones, aldehydes, carboxylic acid esters, or as *ortho*- and *para*-quinones. Furthermore, radical reactions involving aniline radical cations and semiquinone radicals are possible as these govern one-electron oxidation initiated polymerization reactions of aromatic amines and of quinones.[54,55] The studies of Bialk *et* al. (2005, 2007 and 2008)[60-62] laid a foundation for understanding the mechanisms of NER formation of SAs. However, the reversibility of these reactions and stability of the formed covalent bonds has not been studied, although these

are important factors when assessing a potential remobilization of SAs under appropriate conditions. Hsu and Bartha (1976)[63] suggested that the initial binding of aromatic amines might occur via an easily hydrolysable imine bond and that a stronger bonding occurred via 1,4-additions or a subsequent incorporation into heterocyclic forms. Thus, it is plausible that during extraction procedures using solvent-water mixtures at elevated temperatures a dissociation of covalent bonds occurs. The strong temperature dependency of sulfonamide extractability from soil[36] suggests that covalently bound SAs are partially released at high temperatures, as it seems not likely to occur for SAs retained by intraparticle or intra-OM diffusion.

In the first part of this PhD thesis (Chapter 2) the reaction mechanisms of SAs with a wide range of model humic constituents was systematically investigated in order to identify the pathways of covalent bond formation of sulfonamides with non-quinone carbonyls and quinone-type model humic constituents in the presence and absence of laccase. Furthermore, we evaluated the stability of the different types of covalent bonds formed against desorption and pressurized liquid extraction.

The mechanisms by which residues become bound to soil depend on the characteristics of both, the target molecule and the soil matrix.[18] Despite the importance of SOM, details on its structure and reactivity are only partially covered. Quinone carbonyls, i.e. *ortho*- or *para*-quinones probably belong to the most reactive functional groups that are involved in electron transfer reactions in soil organic matter (SOM).[64] They are formed from the corresponding hydroquinone or substituted phenol moieties through oxidation. Due to the electron-withdrawing effect of the sulfonamide group, SAs are relatively weak nucleophiles and require reactive sites for addition.[60-62] Quinones readily reacting with SAs are likely not abundant in humic acid as no evidence was found for covalent bonding of SAs to humic acid in the absence of oxidants and the binding was completely reversible.[34] Thus, it seems likely that quinone formation is the limiting factors for the SA covalent bonding, but could be influenced by competing organic molecules such as other amines or phenols present in the soil solution.

In the second part of this PhD thesis (Chapter 3) operationally defined extraction procedures were linked to the mechanisms of SAs covalent bonding with humic acids to obtain a better understanding of the limiting factors governing NER formation of SAs with organic matter.

With regard to soils, the humic acid fraction of SOM represents only the more easily extractable and probably less macromolecular part of the whole SOM. Thus, it is not representative for whole soils as NERs formation might also occur by reaction with soil minerals or by microbial attack. In fact, soil has been described as the most complex biomaterial on Earth[65] and extracting key molecular information such as binding mechanisms of organic contaminants currently challenges the limits of modern science.[66] Thus, there is only circumstantial evidence on mechanisms of NERs formation in soil which is derived from macroscopic observations made during sequential extraction procedures. It is, hence, not surprising that research directed towards understanding the nature of NERs of SAs in soils is limited to studies of Schmidt *et* al. (2008)[47] and Förster *et* al. (2009).[48] These authors applied sequential extraction and fractionation techniques to monitor the extractability of ^{14}C-labelled SAs in long-term soil incubation experiments. The authors observed two-phase dissipation kinetics with an initial rapid, followed by a slower NER formation rate. The initially fast formation suggests covalent bond formation and that the unstable electrophilic carbonyl moieties, which are sufficiently reactive to undergo nucleophilic attack by SAs are supposed to have a high turnover rate. Thus, oxidative enzymes or manganese and iron oxides might continuously generate quinones from phenolic moieties in SOM, but their actual contribution is not known. Studies on other aromatic amines have demonstrated the role of Mn oxides in NER formation,[67] although the reduction of Mn(III)/Mn(IV) was not sufficient to account for all NERs formed.

In the third part of this PhD thesis (Chapter 4) soil incubation experiments using ^{14}C-labelled sulfamethazine were carried out to assess *the factors governing its NER formation in whole soils. To this end, circumstantial evidence on mechanisms of NER formation was derived from a selective manipulation of soil samples based on the knowledge gained in Chapters 2 and 3.*

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Chapter 2

Reactions of sulfonamide with humic acid constituents

based on
Reactions of a sulfonamide antimicrobial with model humic constituents: Assessing pathways and stability of covalent bonding
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Introduction

Sulfonamides are among the most widely used veterinary antimicrobials worldwide[1] and reach soils by grazing livestock or the application of manure. A large fraction is retained in soil despite their substantial polarity.[2-4] Several studies have demonstrated that the long-term fate in soils is governed by an extensive formation of nonextractable residues (NERs), while only a limited transformation and a very low mineralization rate have been reported.[3,5,6]

NERs are defined as parent compounds or metabolite(s) which are not released from the soil matrix by extraction methods that do not change the compounds themselves and/or the structure of soil matrix.[7] Thus, the extent of NER formation is operationally defined by the extraction procedure used. For sulfonamides, several studies showed that the extractable fraction depends strongly on the method used. While the NER fraction of ^{14}C-sulfadiazine amounted to 54-69% at day 0 if an ethanol:water shaking extraction was used, it was 10-20% for the same soil samples if a microwave-assisted extraction with acetonitrile:water at 140°C was applied.[5,6] Similarly, Stoob *et* al. (2006)[8] showed that the extractable fraction of different sulfonamides increased up to a factor of six if the extraction temperature in PLE was increased from 100°C to 200°C. A second common finding of these studies was a rapid loss of extractability of sulfonamides from soil within several days, which implies that covalent bonding rather than slow intraparticular diffusion is initially the dominant process of NER formation. This covalent bonding probably occurs via sulfonamides' aromatic amino group, as many other aromatic amines show an extensive covalent bonding to organic matter.[9-12] Despite the fact that sulfonamides are relatively weak nucleophiles due to the electron-withdrawing effect of the sulfonamide group, a covalent bonding to model humic constituents and humic acid was demonstrated by Bialk *et* al. (2005, 2007, 2008).[13-15]

Several studies have so far addressed reaction mechanisms of aromatic amines with natural organic matter or simple model constituents. The possible reaction pathways are summarized in Figure 1.[12-18] The nucleophilic aniline nitrogen undergoes addition reactions at electron-poor sites of organic matter, which are usually related to the presence of a carbonyl group in the form of ketones, aldehydes, carboxylic acid esters, and as *ortho*- or *para*-quinones. On β-unsaturated carbonyls, a 1,4-nucleophilic conjugate addition might occur (Figure 1A, reaction I). A direct attack of the nitrogen at the carbonyl C results in the formation of a 1,2-addition product (reaction II), which is usually unstable. An elimination of water results in the formation of an imine group (Schiff base, reaction III). Both pathways have been confirmed for the binding of aromatic amines to monomers,[13,14,19-21] and humic acids.[15,22] Besides imine formation, the 1,2-addition of an aromatic

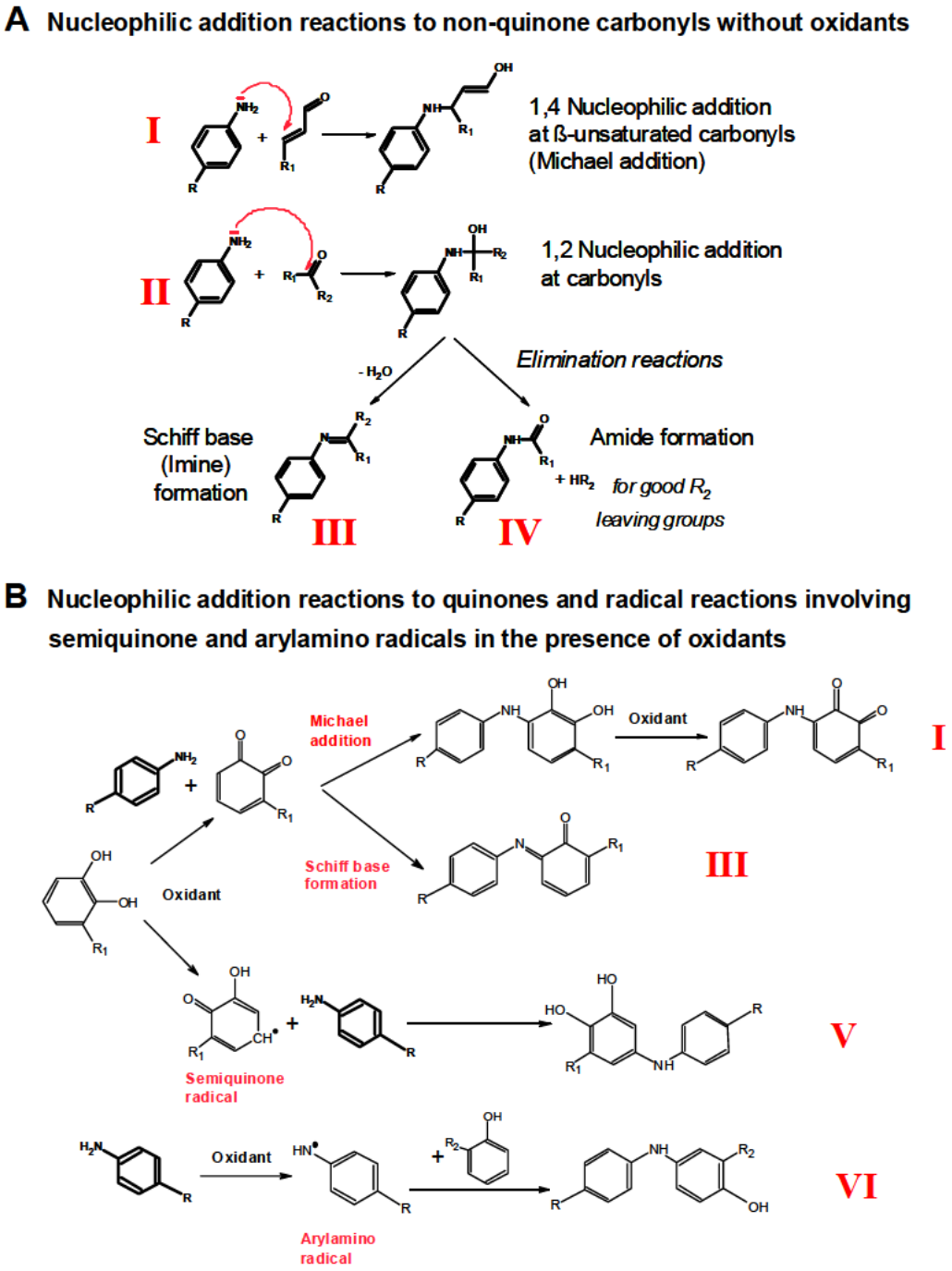

Figure 1. Schematic overview of possible reaction mechanisms for covalent bonding of aromatic amines to organic matter (A) in the absence and (B) in the presence of oxidants (i.e. MnO_2 and phenol oxidase enzymes; "oxidative coupling"). Note that no complete stoichiometry of the reactions is given. Adapted from refs. [12-18]

amine might also result in the formation of an amide bond (reaction IV), if a good leaving group is present. Typically, good leaving groups like those in carboxylic acid halogenides or anhydrides do not occur naturally. Whether these elimination reactions from naturally occurring functionalities are possible has so far not been elucidated. However, ^{15}N NMR spectroscopy suggested that also amide bonds might be formed between aromatic amines and organic matter.[10,23]

In principle, the same reactions occur at quinone carbonyls, i.e. *ortho*- or *para*-quinones (Figure 1B). These probably belong to the most reactive functional groups in organic matter,[24] and are formed from the corresponding hydroquinone or substituted phenol moieties through oxidation by phenol oxidase enzymes or sesquioxides. They might undergo either 1,4- or 1,2-nucleophilic addition reactions (reactions I and III). Benzoquinones having high standard reduction potentials and unprotected β-unsaturated carbons are the most sensitive to addition reactions with nucleophiles as well as polymerization, resulting in a fast removal of these unstable moieties from the quinone pool.[24] Quinones having lower reduction potentials such as naphthoquinones and anthraquinones are more stable[25] and will likely predominate in the soil quinone pool over time. The overall reactivity and prevalence of the 1,2- or the 1,4-pathway depends on the presence of electron-withdrawing or electron-donating substituents.[19,21]

Furthermore radical reactions involving aniline radicals and semiquinone radicals (reactions V and VI) are possible, as these govern one-electron oxidation initiated polymerization reactions of aromatic amines and of quinones.[10,11]

The reversibility of above mentioned reactions and stability of covalent bonds, resulting in a potential remobilization of sulfonamides under appropriate conditions has so far rarely been studied. Hsu and Bartha (1976)[26] suggested that the initial binding of aromatic amines might occur via an easily hydrolysable imine bond and that a stronger bonding occurred via 1,4-additions or a subsequent incorporation into heterocyclic forms. Thus, it seems possible that during extraction procedures using solvent-water mixtures at elevated temperatures a dissociation of covalent bonds could occur. The strong temperature dependency of sulfonamide extractability from soil[8] suggests that at high temperatures at least a partial release of covalently bound sulfonamides is possible.

The objectives of this study were (i) to identify the pathways of covalent bond formation of sulfonamides with non-quinone carbonyls and quinone-type model humic constituents in the presence and absence of laccase, and (ii) to evaluate the stability of the different types of covalent bonds against desorption and pressurized liquid extraction. For comparison experiments with other model aromatic amines exhibiting higher nucleophilicity were performed. Quinone-

forming compounds selected for investigation span a considerable range of standard reduction potentials and contain side groups relevant to those present in organic matter. These include both hydroquinones and substituted (hydroxy)benzenes and benzoic acids, for which we use the term "phenols" in the following.

Materials and methods

Chemicals

We used the sulfonamide sulfathiazole (STZ), aniline (ANL), and *p*-ethoxyaniline (EXA) as model aromatic amines (Table S1, SI). Nine substituted (hydroxy)phenols forming instable quinones upon oxidation, hydroquinone, the stable under ambient conditions compound *p*-benzoquinone and five non-quinone carbonyls were used as model humic constituents. Their structures and abbreviations are given in Table S2. Extracellular fungal laccase from *Trametes versicolor* (oxygen oxidoreductase E.C. 1.10.3.2.) with a reported activity of 22.4 U/mg was obtained from Sigma-Aldrich (Buchs, Switzerland). A fresh enzyme stock solution of 1 mg/mL in 0.1 mM ammonium acetate buffer (pH 5.6) was prepared before each set of experiments and diluted as required.

Short-term kinetic assays with laccase generated quinones

Kinetics were determined over 30 min for the reaction of STZ with phenols (CAT, 4mCAT, PRO, GEN, VAN, SYR, AcSYR, FER, and ESC). A total volume of 2 mL of reaction solution with an individual phenol concentration of 50 µg/mL was prepared in 0.1 M ammonium acetate buffer (pH 5.6) in amber glass vials. Unless specified otherwise, in all kinetic experiments the aromatic amine concentration was adjusted to achieve an initial molar ratio to that of the phenol of 1:3. A laccase solution with an activity of 0.22 U/mL was subsequently added. The reaction mixture was immediately stirred for 1-2 s, and an aliquot of 500 µL was withdrawn using a glass microsyringe. The solution was infused at a rate of 15 µL/min using a syringe pump and combined with 100 µL/min of 70:30 methanol/water (v/v) via a T-piece prior to the electrospray ion source of a TSQ Quantum MS (Thermo Finningan, San Jose, CA). Full scan mass spectra (*m/z* 80-800) were recorded at unit mass resolution at 1 s scan times. Data were continuously acquired for 30 min to monitor the time course of adducts and products. Control samples containing only phenols or STZ were used for calibration of the concentrations. The transformation of phenols and STZ alone by laccase was assessed in separate control experiments. In the reaction vials with the remaining reaction mixtures, the laccase activity was quenched with methanol and the aliquots were kept frozen at -30°C until identification of reaction products by high-resolution mass spectrometry (HRMS) as described below.

For selected phenols experiments with the stronger nucleophiles EXA and ANL were conducted (see Table S2). To evaluate the importance of radical reactions, additional experiments

were performed in the presence of the radical scavenger *tert*-butanol,[27] which was added in a 10-fold molar excess to the phenol prior to laccase.

Long-term kinetic assays

To test whether nucleophilic addition-elimination reactions at carbonyls other than quinones are likely to occur under natural conditions, we studied reactions of STZ and EXA with different carboxylic acid esters and ketones, namely 1-penten-3-one, acetylsalicylic acid, acetic acid *p*-tolyl ester, methylbenzoylformate, and 1-phenyl-1,2-propanedione. Additionally, the slowly reacting compounds hydroquinone (HQ) and its oxidation product benzoquinone (BQ) were incubated with STZ and EXA. Batch experiments were carried out in 10 mL of 0.1 M ammonium acetate buffer at pH 5.6 and additionally at pH 4.0 for BQ in 20 mL amber glass vials for 76 days at room temperature in the dark. Initial concentrations of STZ and EXA were 0.016 mM and 0.029 mM, respectively. The carbonyl compounds were added in a 3-fold molar excess. Reaction progress was monitored by withdrawing 50 µL aliquots from the reaction solution at 1, 2, 6, 13, 19, 25, 42, 49, 58, 66, and 76 days. Aliquots were transferred to 2 ml amber glass vials containing 950 µL of water/methanol 90:10 (v/v) and analyzed by LC-MS/MS immediately after collection (see SI for details). Reaction rate constants for the second-order reactions between humic acid constituents and aromatic amines accounting for their pH-dependent speciation were estimated using the chemical kinetics software KINTECUS.[28] Standard deviations of the rate constants were estimated from boot-strapping using 100 re-sampling runs.

Structure elucidation of reaction products

For structure elucidation of reaction products, full scan and MS/MS spectra were acquired using a high resolution LTQ Orbitrap XL instrument (Thermo Scientific, San Jose, CA). The spectra were acquired in positive and negative ion mode using electrospray ionization (ESI) and a resolving power of 60,000 and processed by averaging 10 microscans per scan in centroid mode. Samples were directly infused into the ion source using a syringe pump at a flow rate of 15 µL/min. MS/MS spectra were recorded after collision-induced dissociation and higher energy C-trap dissociation (HCD) at different collision energies. The mass accuracy of the measurements was < 5 ppm for all compounds.

Assessment of the stability of covalent bonds

To assess the stability of reaction products from different phenols STZ was incubated with CAT, 4mCAT, PRO, GEN, VAN, SYR, AcSYR in the presence and absence of laccase for 24 hours as described for the short-term kinetic assays. The free and sorbed fraction of STZ was determined by a direct injection of the reaction solutions into an LC-MS system, assuming a complete desorption of STZ from polymerized phenols in a methanol-water gradient.[29] The total extractable fraction was assessed by pressurized liquid extraction (PLE) based on a method for soil samples.[8] From these two approaches, the fraction of labile covalently bound STZ was defined as difference between the STZ determined by PLE and that determined by direct LC-MS injection. The stable covalently bound STZ was defined as difference between the total added and the PLE-extractable STZ fraction.

For direct LC-MS analysis 50 µL of reaction mixture was withdrawn and transferred to an LC vial with 100 µL of methanol and 850 µL of water. STZ was analyzed by LC-MS/MS as described in the SI.

An ASE 350 (Dionex, Sunnyvale, CA, USA) was used for pressurized liquid extraction. Aliquots of 1 mL of reaction mixture were dripped into an ASE cell prefilled with diatomaceous earth (Restek Corporation, Bellefonte, PA). The extraction was done with water/acetonitrile 85:15 (v/v) adjusted with ammonium acetate buffer to pH 5.6 at 140 ºC and two static extraction cycles of 5 min. After extraction, the cells were flushed with an additional cell volume of the extraction solvent and purged with N_2. To assess the effect of pH, extraction solutions were prepared additionally at pH 4.0 (0.1 M ammonium acetate buffer), 7.0 (0.1 M ammonium phosphate buffer), and 9.0 (0.1 M tris buffer). To assess the effect of temperature, the extractions were carried out additionally at 60 and 200 ºC. All samples were extracted and analyzed in triplicate. The pH of the PLE extract was adjusted to 5.0 using acetic acid. An aliquot of 900 µL was combined with 100 µL of methanol and filtered into a 2-mL vial using a 0.2 µm PTFE syringe filter. Compounds were analyzed by LC-MS/MS (see SI).

Incorporation of ^{15}N labeled aniline into CAT and SYR polymers

Since ^{15}N-labeled sulfathiazole was not available ^{15}N-labeled aniline was used to study the temporal changes in its laccase-mediated reaction with CAT and SYR. 250 mg of ^{15}N aniline reacted with each monomer (added in a 3-fold molar excess) in the presence of 22 U/mL of laccase

in 10 mL of acetate buffer (pH 5.6). Reaction progress was monitored in 2 mL aliquots of the reaction solution taken at 10 min, 2 h, 1, 5, and 20 d using ^{1}H-^{15}N heteronuclear multiple bond correlation (HMBC) NMR spectroscopy after freeze-drying and dissolution in acetone-d_6 (see SI for details).

Results and Discussion

Reactions with quinones formed by oxidation of phenols in short-term kinetic assays

For all tested phenols a rapid oxidation catalyzed by laccase was observed. A fraction of the phenols was not oxidized, as the closed system did not allow for a reoxidation of the laccase after depletion of the initially dissolved O_2 (Figure 2). Sulfathiazole was transformed in the presence of laccase and a threefold higher molar concentration of the tested phenols within 30 min, ranging from 30% (VAN), 80% (AcSYR, 4mCAT), 95% (PRO), and >98% (CAT, GEN, SYR). No reaction occurred with FER and ESC in the presence of laccase, as indicated by constant STZ concentrations and the absence of reaction products.

Direct infusion into the MS allowed for a highly selective monitoring of the reaction progress and an identification of reactants and products. However, it did not allow for an accurate quantification of the products due to the varying ionization efficiency of the ESI source. The reaction systems proved to be complex, as a range of consecutive and parallel reactions occurred simultaneously. Due to over-parameterization and uncertainty about the individual reaction steps occurring, an estimation of rate constants for individual reactions was not possible.

An overview of the reaction pathways of STZ with the different phenols based on the identification of products by HRMS is given in Table S2. For the reaction of STZ with CAT, 4mCAT, PRO, and GEN in the presence of laccase 1,4-reaction products were identified, as exemplified for 4mCAT in Figure S2 (SI). With increasing incubation time, the initial reaction products with CAT and PRO tended to disappear (Figure 2A). Several low-intensity ions in the higher *m/z* range emerged with increasing incubation time suggesting the formation of oligomers or polymers from STZ and the respective quinones. For the reaction of STZ with SYR, AcSYR and VAN in the presence of laccase (Figure 2B) 1,2-nucleophilic addition products were identified, as exemplified for SYR in Figure S3 (SI). The disappearance kinetics of both, VAN and STZ (Figure 2C) show that both, the formation of the quinone and the subsequent reaction of STZ were slower as for the reactions with other constituents. HRMS spectra showed that predominantly a 1,2-addition product was formed between a dimer of VAN and STZ, and with lower intensity a 1,4-addition and a 1,2-addition product between STZ and one VAN molecule. These results are similar to those of Tatsumi *et* al. (1994),[21] who reported a reaction product between chloroanilines and a VAN dimer, while no addition to a VAN monomer was observed.

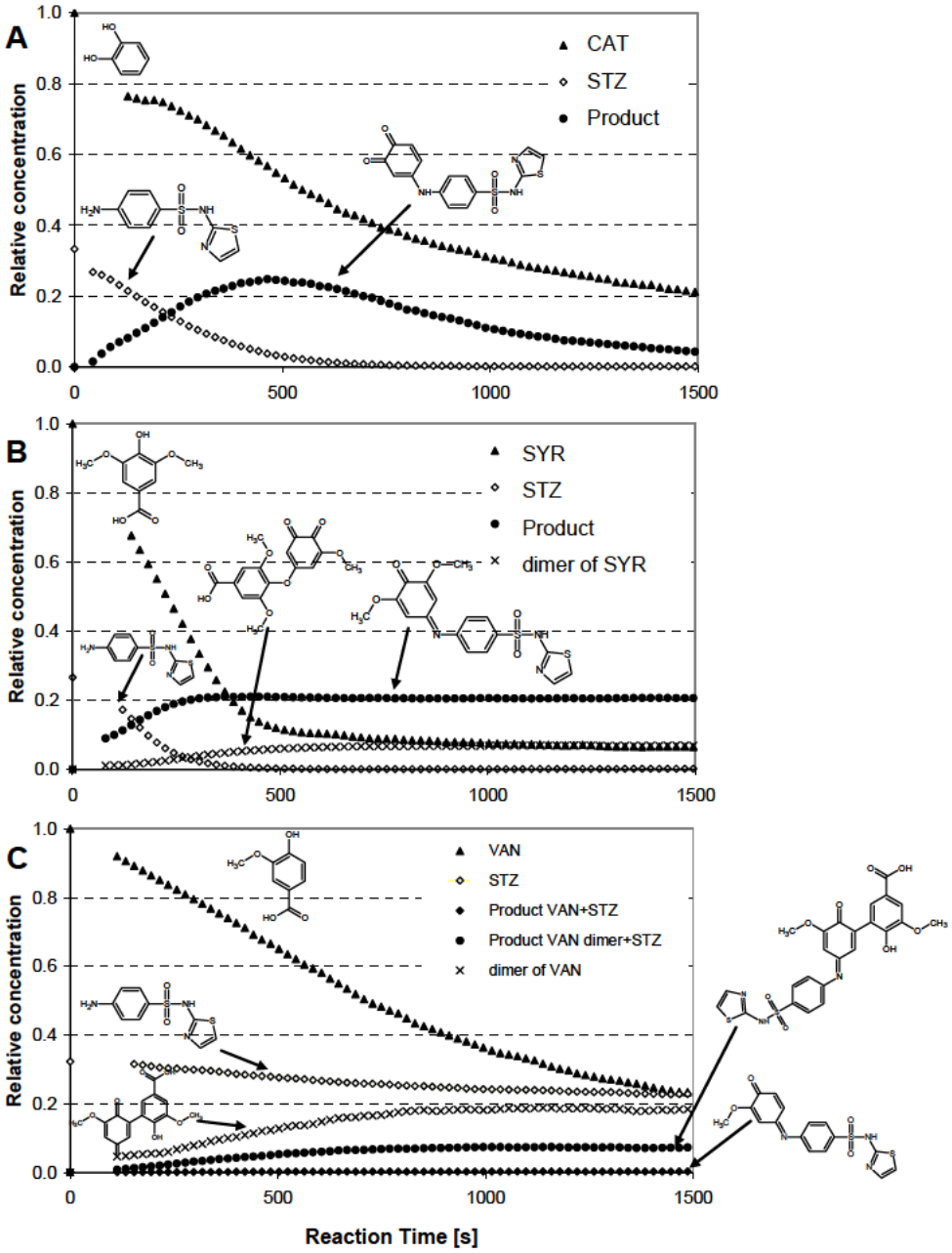

Figure 2. Short-term kinetic assays of the reaction of sulfathiazole with model monomers (A) catechol, (B) syringic acid, and (C) vanillic acid in the presence of laccase. Molar concentrations were normalized to those of the respective monomer for comparison. The relative concentrations of reaction products were estimated assuming the same MS ionization efficiency as sulfathiazole for all monitored compounds.

In contrast to the other phenols tested, AcSYR and FER form more stable semiquinone radicals upon laccase oxidation[30,31] and could, thus, give hints whether semiquinone radicals were involved in the reactions. No reaction between FER and STZ in the presence of laccase occurred, although FER was rapidly transformed to a range of condensation products observed by HRMS, which are reported elsewhere.[32,33] For AcSYR, the oxidation by laccase yielded the same quinone as for SYR, and the same reaction products and a similar kinetics was observed for both compounds (data not shown). Thus, semiquinone radicals were most likely not involved in the reaction of STZ and the phenols in the presence of laccase.

The reactivity towards nucleophilic attack and the observed reaction products of the different quinones can be largely explained with substituent effects. For *ortho*-quinones, the 1,4-addition was clearly preferred in case the electrophilic sites bear a hydrogen atom (CAT, 4mCAT, PRO). For ESC both positions for 1,4-addition are linked to carbon atoms, and consequently no reaction with STZ occurred. For *para*-quinones, the substituents significantly influence the type of product. The presence of an electron-withdrawing group in GEN facilitated 1,4-addition. Electron-donating methoxy groups in VAN and SYR resulted in the predominance of 1,2-addition reactions, due to a higher electron density at the carbon atoms of the double bond, although 1,4-addition was still possible for VAN.[34]

In contrast to the incubations with laccase, control experiments without the enzyme showed no reaction within 30 minutes. However, at storage times beyond 3 days a loss of CAT, 4mCAT, GEN, PRO and ESC was noted, probably due to autoxidation by O_2, which is common for (substituted) hydroquinones and catechols.[24] In most cases the same products were formed as in the presence of laccase. However, the initial dimerization of quinones was not observed. Consequently, incubations with VAN showed only a very limited STZ transformation in the absence of laccase.

Ethoxyaniline (EXA) was transformed to 80% with VAN and >98% with CAT, 4mCAT, PRO, GEN, SYR, and AcSYR. The disappearance of EXA and ANL was in general slightly faster than that of STZ (Figure 3), which is consistent with the nucleophilic reactivity of the amino group (Table S1). The reaction pathways were essentially the same for all three aromatic amines. While in the absence of phenols EXA showed dimerization during infusion with laccase, indicating its one-electron oxidation to form a radical cation, no dimerization was observed for STZ and ANL due to their higher oxidation potential.[13] In reaction mixtures with phenols in the presence of laccase, no EXA dimers were observed, indicating that the nucleophilic addition to the electrophilic quinone was favored above dimerization of the amine via radical reactions.

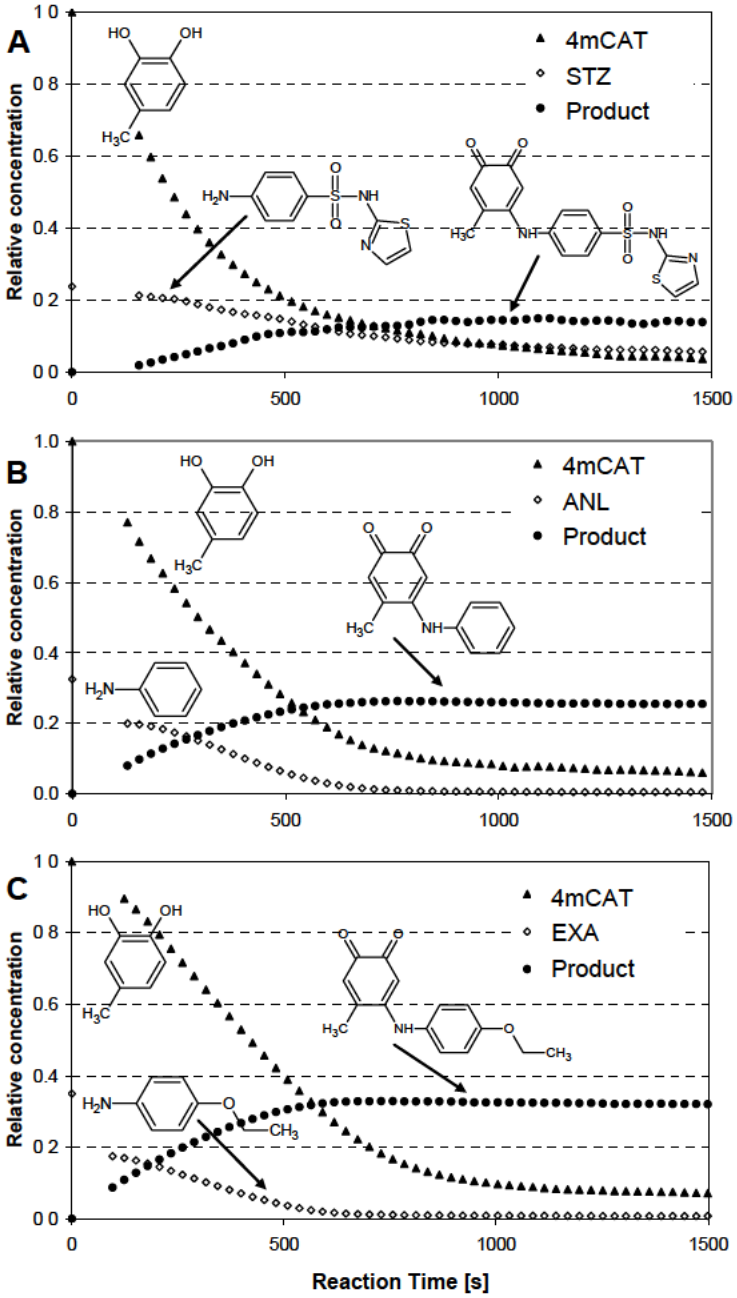

Figure 3. Short-term kinetic assays of the reaction of 4-methylcatechol with aromatic amines (A) sulfathiazole, (B) aniline, and (C) *p*-ethoxyaniline in the presence of laccase and 10-fold molar excess of *tert*-butanol. Molar concentrations were normalized to that of 4-methylcatechol for comparison. The relative concentrations of reaction products were estimated assuming the same MS ionization efficiency as sulfathiazole for all monitored compounds.

To further elucidate the possible involvement of radicals, assays with laccase were conducted in the presence of the radical scavenger *tert*-butanol for the reactions of STZ, EXA, and ANL with 4mCAT, CAT and SYR (Figure 3). As compared to the assays without scavenger, no differences in the disappearance kinetics of the aromatic amines and the initial formation kinetics of the reaction products were observed. However, the presence of *tert*-butanol during incubation limited further polymerization reactions of the primary aromatic amine-quinone 1:1 reaction products. The dimerization of EXA alone was also slower.

From many possible pathways outlined in Figure 1[12-18] our observations from the experiments indicate that nucleophilic 1,2- and 1,4 additions to quinones formed by laccase oxidation of phenols are the predominant pathways of covalent bonding for aromatic amines. The rate-limiting step is the formation of the quinone, while the subsequent nucleophilic addition to quinones is a rather fast process. Anilino radical cations are probably not important for this binding, while semiquinone radicals do not directly react with the aromatic amine, but are involved in a further incorporation into the polymer.

Reactions with non-quinone carbonyls, benzoquinone, and hydroquinone in long-term kinetic assays

The reaction of non-quinone type carbonyl compounds, *p*-benzoquinone (BQ) and hydroquinone (HQ) with STZ and EXA was tested in long-term kinetic assays over 76 d (Figure 4). Among non-quinone type carbonyls only the β-unsaturated ketone 1-penten-3-one underwent slow 1,4-addition (Figure 4A). The reaction rate for EXA was larger than that of STZ. For all other non-quinone carbonyls no reaction was observed within 76 days, suggesting that nucleophilic acyl substitution resulting in the formation of covalent bonds (i.e. amide bonds, Schiff bases) is probably of minor importance.

STZ and EXA reacted with BQ or BQ formed by autoxidation of HQ by 1,4-nucleophilic addition; no other reaction products were observed. The reactions of EXA and STZ with BQ were initially fast and slowed down considerably after 5-10 d (Figure 4C). While EXA almost completely reacted after 60 d, about 50% of the initial STZ concentration remained. The reason for this difference is probably the oligomerization reaction of BQ, which is faster than the addition of STZ, but not of EXA. The reaction of EXA with BQ was again faster than that of the weaker nucleophile STZ and the reactions of both aromatic amines were apparently slower at pH 4 than at pH 5.6. However, when accounting for the protonation of the aromatic amino group at lower pH, the second-order rate

constants for the reaction of the neutral species with BQ were higher at lower pH (EXA: $k_{pH\ 4}$ = 2.08 ± 0.11 $M^{-1}s^{-1}$; $k_{pH\ 5.6}$ = 0.43 ± 0.05 $M^{-1}s^{-1}$; STZ: $k_{pH\ 4}$ = 0.06 ± 0.01 $M^{-1}s^{-1}$; $k_{pH\ 5.6}$ = 0.032 ± 0.005 $M^{-1}s^{-1}$). The apparent lower rate resulted therefore from the lower concentration of the reacting neutral species. This is in line with the acid-catalyzed mechanism of the 1,4-addition reaction. Both aromatic amines were transformed to the same extent in reactions with HQ as by BQ (Figure 4B). Initial reaction rates, however, were slower due to the autoxidation of HQ to BQ by O_2 preceding nucleophilic addition.

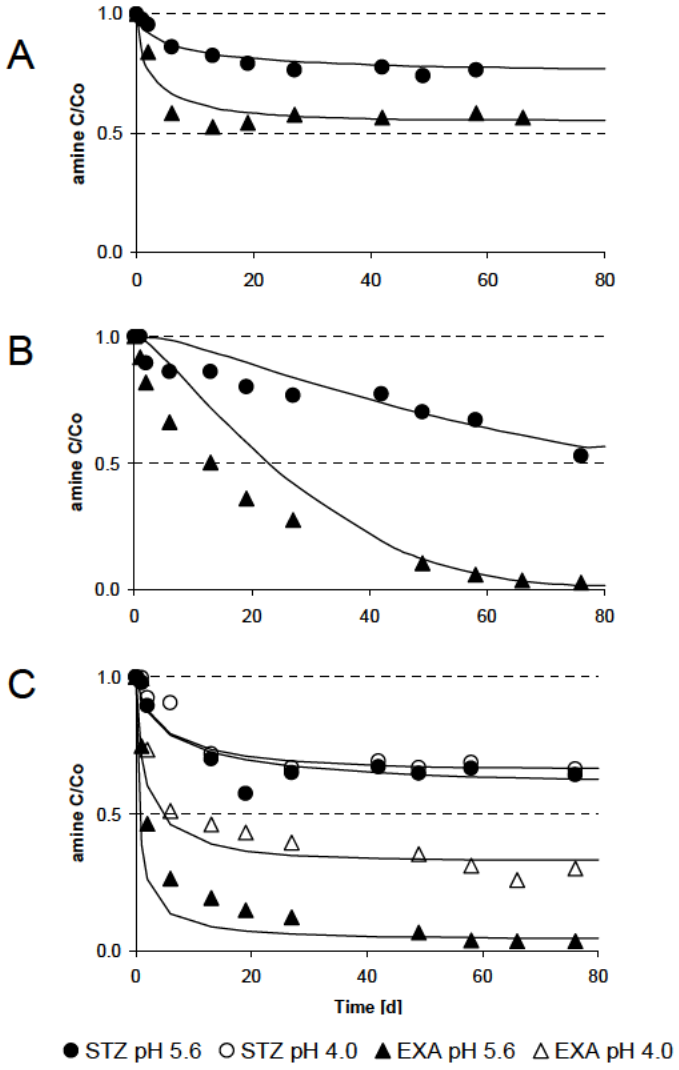

Figure 4. Kinetics of the reactions of STZ and EXA with (A) 1-penten-3-one, (B) hydroquinone (HQ), and (C) *p*-benzoquinone (BQ). Initial reactant concentrations are: (i) for the reaction with STZ (0.016 mM): 1-penten-3-one (0.016 mM), HQ (0.021 mM), BQ (0.020 mM); for the reaction with EXA (0.029 mM): 1-penten-3-one (0.068 mM), HQ (0.070 mM), BQ (0.069 mM). The fitted lines show the dissipation of aromatic amines assuming second-order kinetics for the reaction with the humic monomer.

Stability of covalent bonds

The stability of STZ covalently bound to phenols forming either anilinoquinones (CAT, 4mCAT, PRO, GEN) or imines (VAN, SYR, AcSYR) against desorption (direct LC-MS analysis) and PLE at 140°C and pH 5.6 is shown in Figure 5. In the absence of laccase (Figure 5A and 5B) the added STZ was fully recovered in all cases by direct LC-MS analysis, while PLE extraction recovered between 85% (VAN) and 30% (CAT). STZ without the presence of phenols was fully recovered. The extent of STZ binding to phenols followed the same order as for the short-term kinetic assays. These findings suggest that the phenols likely undergo oxidation during the extraction at elevated temperature and nucleophilic addition of STZ to the formed quinones is plausible, nevertheless, the mechanism behind the reaction under PLE conditions remains unknown. If CAT was incubated in the presence of laccase for 48 h before adding STZ, all STZ was recovered by PLE, confirming this finding. Reactions governed by autoxidation of phenols during the subsequent sample handling and LC-MS analysis did not result in a significant loss of STZ, as revealed by control samples. Purging of the extraction solvent with N_2 to remove O_2 prior to PLE or an addition of cysteine in 20-fold molar excess as a reducing agent did not result in higher STZ extractability, suggesting that the oxidation is not related to the presence of O_2.

Between 65 and 2% of STZ were covalently bound to the phenols in incubations with laccase as indicated by the low recoveries by LC-MS injection (Figure 5C and 5D). No or only a small fraction of this covalently bound STZ was released by PLE for CAT, 4mCAT, PRO, SYR and AcSYR, but about 50% for GEN. The extractability did not differ at different pH values (4.0, 7.0, 9.0) and increasing the extraction temperature from 60 to 200°C released only a slightly higher fraction of STZ for all phenols (data not shown). The assumed higher STZ release from imines as compared to anilinoquinones[26] by PLE was not observed. It is likely that not the initial type of bonding, but the further incorporation of STZ into the polymer controls its extractability, which depends on the structure of the phenols and their tendency to polymerize. Thus, the significantly higher recoveries in PLE extraction of GEN and VAN as compared to other phenols might be related to the lower polymerization observed in the short-term assay. Furthermore, the simultaneous formation of covalent bonds during PLE does not allow for a reliable determination of the releasable fraction in all cases.

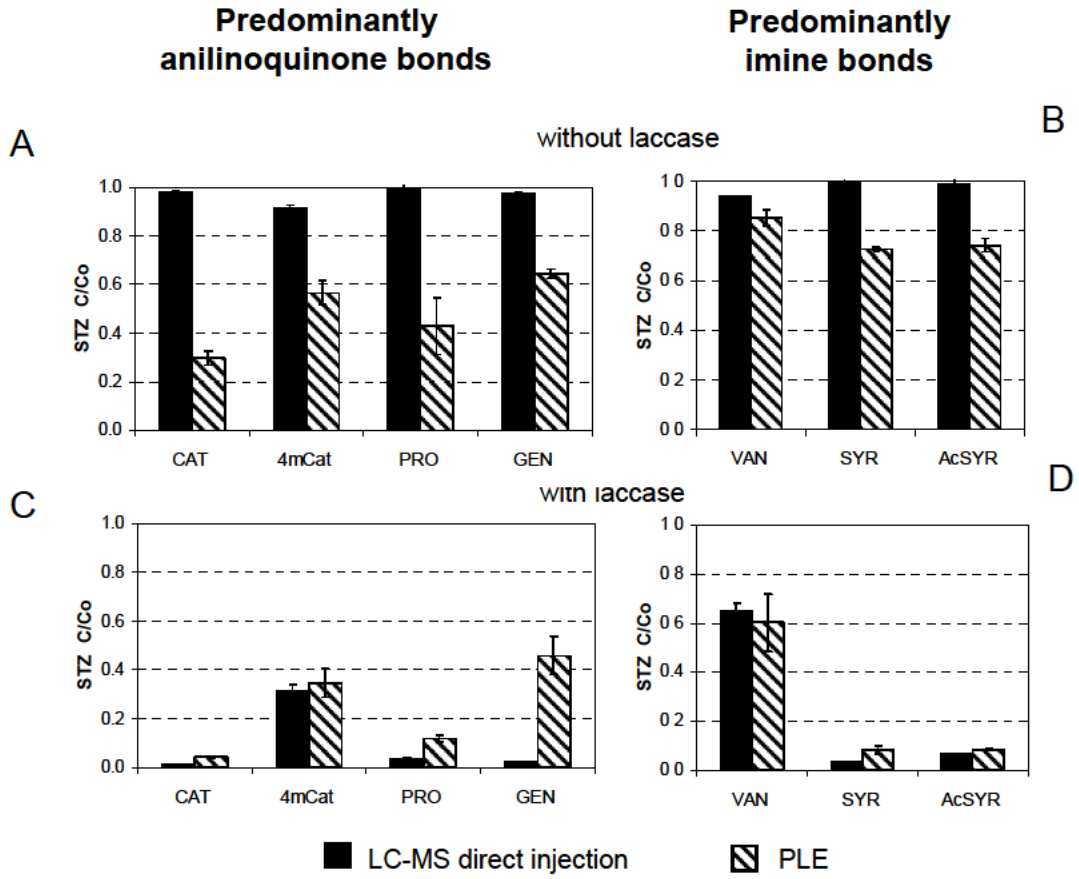

Figure 5. Extractability of STZ from anilinoquinones (A and C) and imines (B and D) in the absence (A and B) and presence (C and D) of laccase as determined by direct LC injection and pressurized liquid extraction (PLE) at 140°C and pH 5.6. Concentrations are given relative to the initial STZ concentration C_0. Error bars show standard deviation of three replicates.

Incorporation of ^{15}N labeled aniline into CAT and SYR polymers

To further elucidate the incorporation of aromatic amines into polymers generated by laccase-catalyzed oxidation of phenols, ^{15}N NMR spectroscopy was applied, as the wide range of different reaction products impeded an identification of individual compounds by HRMS. The temporal changes in laccase mediated reaction systems of ^{15}N aniline-CAT and ^{15}N aniline-SYR were monitored over 20 days by ^{1}H-^{15}N HMBC NMR. The detailed information on the assignment of NMR shifts and the obtained 2D spectra can be found in the SI.

A large numbers of resonances in both reaction systems changing within time indicate a multitude of reaction products and a very dynamic system (Figures S4 and S5). ANL reacted initially

with CAT mainly by a 1,4-addition generating anilinoquinones and anilinohydroquinones with $\delta^{15}N$ at 101/109 ppm and 70 ppm, respectively. A strong resonance at $\delta^{15}N$ 196 ppm appearing with longer incubation time could correspond to heterocyclic nitrogen,[35] suggesting that the initial aniline(hydro)quinones react further by an addition-tautomerization-oxidation sequence forming heterocyles. The incorporation of the nitrogen atom of aromatic amines as heterocyclic ring systems into soil organic matter was already proposed by Hsu and Bartha (1976), but not proven by spectroscopy.[12,26]

The initial reaction product of SYR and ANL was mainly quinone imine with the nitrogen atom at $\delta^{15}N$ 338 ppm. After two days, the resonance of anilinoquinone emerged, and after 20 days the resonance of an iminoquinone disappeared, while two major resonances corresponding to free aniline at $\delta^{15}N$ 58 ppm and to anilide or acetanilide nitrogens at 125 ppm were observed as predominant signals along with some less intense signals assigned to heterocyclic nitrogens. This indicates that the initial imine bonds are not stable and either (i) are tautomerized to anilinoquinones, or (ii) hydrolyze with time to release free aniline, which might partially undergo a 1,4-addition reactions forming anilinoquinone bonds *de novo*, implying that sites susceptible to these reactions are available. An anilide bonds formation in ANL-SYR system with laccase would require ring fission of initial product and nucleophilic acyl substitution (see Figure 1).

When extrapolating model compound studies to the soil environment, the large multiplicity of functional groups and the variation in the structural configurations of these functional groups in soil organic matter need to be considered. It is possible that heterocyclic condensation products form through reactions of aromatic amine with combination of different classes of functional groups in soil organic matter.

Implications for NER formation of sulfonamides in soil

Both, MS and NMR studies revealed that the initial bonding of studied aromatic amines occurs by 1,2- and 1,4- additions to quinones and that their products (imines and anilinoquinones, respectively) are further incorporated into polymers by subsequent reactions resulting in heterocylic nitrogens, which are likely more stable against pressurized liquid extraction.

In principle these processes could also hold true for soils, and a slow NER formation with time could be related to a slow increase of the amount of covalently bound sulfonamides and the strength of covalent bonding. However, extrapolations from an abiotic homogeneous model system to a spatially compartmented, heterogeneous system have to be done with caution, as also soil minerals or microorganisms might form reactive intermediates involved in NER formation. Our study also confirmed that sulfonamides are relatively weak nucleophiles and require reactive quinones for addition.[13] The rapid NER formation within the first days in soil[5,6] suggests a large available quinone pool. It remains an open question whether a relatively weak nucleophile STZ can undergo nucleophilic addition to quinones in natural soils without actions of phenoloxidase enzymes or oxidants such as manganese oxides. The whole soil NMR experiments have shown that aniline readily condenses with quinone groups in soil organic matter under nonenzymatic conditions.[36] The observed covalent bond formation of STZ with phenols during PLE offers as alternative explanation an analytical artifact, this is NERs are formed by the extraction method. However, mild solvent extractions such as shaking extractions at room temperature not suspected to cause artificial NER formation released even less sulfonamide from soil.[3] This implies that this artifact might be less relevant for real soil samples with organic matter in a rather oxidized state as compared to completely reduced quinone precursors. Nevertheless, our findings suggest that this possibility should be addressed when characterizing NER formation in soil based on harsh extraction procedures.

Acknowledgements

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Supporting Information Available

Chemical purity and supplier information, LC-HRMS analysis of aromatic amines, instrumental parameters for ^{1}H and ^{15}N NMR analysis, structure elucidation of reaction products by HRMS, an overview of the reaction pathway of STZ with different phenols, ^{1}H-^{15}N HMBC NMR spectra monitoring temporal changes in incorporation of ^{15}N aniline into CAT and SYR polymers. This information is available.

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Supporting information for Chapter 2

Materials and Methods

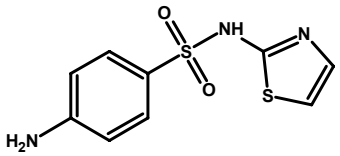
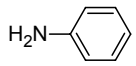
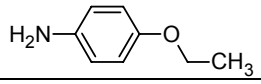
Chemicals

Sulfathiazole (STZ) (4-amino-*N*-(2-thiazolyl)-benzenesulfonamide, Sigma-Aldrich, St. Louis, MO), sulfamethazine (SMZ) (4-amino-*N*-(4,6-dimethyl-2-pyrimidynyl)-benzenesulfonamide), aniline (ANL) (Sigma-Aldrich), and *p*-ethoxyaniline (EXA) (for synthesis, Merck, Darmstadt, Germany) were used as received. For NMR studies ^{15}N aniline (98 atom % ^{15}N) was bought from Isotec (Miamisburg, OH).

The model phenols catechol (CAT) (1,2-dihydroxybenzene, purity > 99%), 4-methylcatechol (4-mCAT) (3,4-dihydroxytoluene, 95%), protocatechuic acid (PRO) (3,4-dihydroxybenzoic acid), gentisic acid (GEN) (2,5-dihydroxybenzoic acid), acetosyringone (AcSYR) (4-hydroxy-3,5-dimethoxyacetophenone, 97%), *trans*-ferulic acid (FER) (*trans*-4-hydroxy-3-methoxycinnamic acid, 99%), esculetin (ESC) (6,7-dihydroxycoumarin, 98%), syringic acid (SYR) (4-hydroxy-3,5-dimethoxybenzoic acid, 98%), hydroquinone (HQ), and *p*-benzoquinone (BQ) were purchased from Sigma-Aldrich, and vanillic acid (VAN) (4-hydroxy-3-methoxybenzoic acid) was obtained from Alfa Aesar (Karlsruhe, Germany). The model non-quinone carbonyl compounds 1-penten-3-one, acetylsalicylic acid, acetic acid *p*-tolyl ester, methyl benzoylformate, and 1-phenyl-1,2-propanedione with purities ranging between 98 and 99.5 % were purchased from Sigma-Aldrich (Buchs, Switzerland). Individual stock solutions for all compounds were prepared in methanol with concentrations of 1 mg/mL.

HPLC grade methanol, acetonitrile and water (Acros Organics, Geel, Belgium) were used as solvents for extraction and as liquid chromatography eluents. Ammonium acetate, formic acid, acetic acid, and tris(hydroxymethyl)aminomethane (Tris) of “pro analysi” grade were obtained from Merck.

Table S1. Chemical structures of sulfathiazole and other model aromatic amines used. The anticipated nucleophilic reactivity is given relative to each other (+ = lowest, +++ = highest).

Compound	Structure	Nucleophilic reactivity of amino group [a]
4-Amino-N-(2-thiazolyl)-benzenesulfonamide (Sulfathiazole, STZ)		+ pK_a = 2.3
Aminobenzene (Aniline, ANL)		++ pK_a = 4.60
1-Amino-4-ethoxybenzene (Ethoxyaniline, EXA)		+++ pK_a = 5.36

[a] The assumed nucleophilic reactivity is based on pK_a values taken from Richter *et* al. (2009)[1] for STZ and EXA and from Mackay *et* al. (2006)[2] for ANL, as the pK_a of the conjugate acid of the nucleophile and the rate of the reaction can described by a Brønsted linear free energy relationship[3] if the donor atom of all nucleophiles is similar and the same solvent is used.

LC-MS/MS analysis of aromatic amines

The LC-MS/MS system consisted of an autosampler (HTC PAL, CTC Analytics, Zwingen, Switzerland), a Rheos 2000 LC pumps (Flux Instruments, Basel, Switzerland), a column oven (Jones, Omnilab, Mettmenstetten, Switzerland), and a triple quadrupole mass spectrometer with an electrospray probe (TSQ Quantum, Thermo Finningan, San Jose, CA). 20 µL of samples were injected and separated at a flow rate of 200 µL/min using a Nucleodur C18 Gravity column (125 mm × 2 mm, 5 µm particle size; Marcherey-Nagel, Oensingen, Switzerland) equipped with a guard column by gradient elution. The mobile phases consisted of water (A) and methanol (B), both containing 0.1% of formic acid. The composition of the mobile phase was changed linearly from 5% B at the start to 95% B at 14 min before re-equilibration to starting conditions.

The mass spectrometer was operated in full scan mode with a range of m/z 80-800 and selected reaction monitoring (SRM) mode with the following electrospray ionization (ESI) conditions: positive mode, spray voltage 3500 V, sheath gas pressure 4 MPa, auxiliary gas pressure 0.5 MPa, ion transfer capillary temperature 350ºC.

The error of the analytical procedure was below 10%. External calibration curve of ten concentrations (2-1500 ng/mL) was constructed for the quantification of the analytes in the sample.

Quality control standards were measured after every 10 injections to check for instrumental drift. The analysis was stopped and a new calibration curve was constructed if the quality control standard was not within ± 10% of its theoretical value. Details on detection limits and recovery rates were published elsewhere.[1] Instrumental blank samples consisting of water/methanol 90:10 (v/v) were run to check for analyte carryover.

^{1}H and ^{15}N NMR analysis of ^{15}N aniline reactions products with CAT and SYR

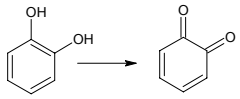
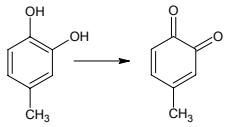
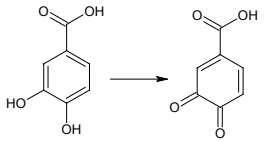
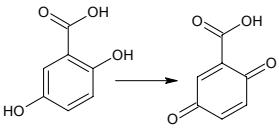
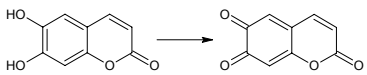
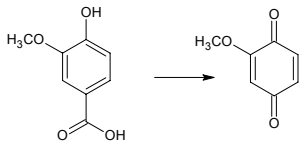
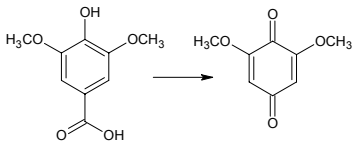
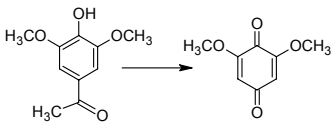
The ^{1}H and ^{15}N NMR spectra were recorded at 400.13 (40.56) MHz on a Bruker Avance-400 NMR spectrometer (Bruker Biospin AG, Fällanden, Switzerland). The 1D 1H and 2D ^{1}H-^{15}N HMBC correlation experiments were performed at 298 K using a 5 mm broadband inverse probe with z-gradient (100% gradient strength of 53.5 Gcm^{-1}) and 90° pulse lengths of 6.8 µs (^{1}H) and 20.7 µs (^{15}N). All NMR spectra were recorded with the Bruker standard pulse programs and parameter sets with selection of coupling constants of 4 Hz (gs-HMBC). The ^{1}H chemical shifts in water were referenced to an external sample of 3-trimethylsilyl tetradeutero sodium propionate in D_2O at 0.0 ppm and the ^{15}N chemical shifts were internally referenced to the signal of aniline at 58.0 ppm with respect to the NH_3 chemical shift scale with δ (^{15}N) = 0.0 ppm.[4]

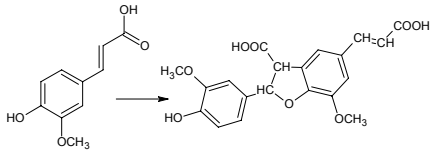
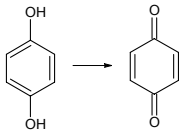
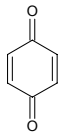
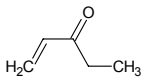
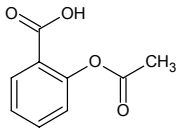
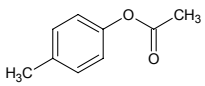
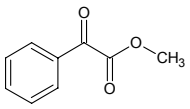
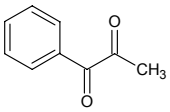
Results and Discussion

Structure elucidation of reaction products by HRMS. The reaction of STZ with 4mCAT in the presence of laccase (Figure S2A) resulted in the formation of one predominant reaction product, for which a monoisotopic mass of 375.0347 Da could be determined based on positive and negative ion HRMS ($[M-H)]^-$ at m/z 374.0273, Figure S3A; $[M+H]^+$ at m/z 376.0419). The predicted molecular formula $C_{16}H_{13}O_4N_3S_2$ corresponded to the 1,4-addition product of STZ and 4mCAT, which was reoxidized to an anilinoquinone structure. The MS/MS fragmentation yielded characteristic product ions as shown in Figure S2B. The ions at m/z 123.0454 and m/z 254.0060 in the full scan mass spectra corresponded to unreacted 4mCAT and STZ, respectively. Less intense ions were observed in full scan mass spectra corresponding to reaction products containing two and three 4mCAT units and one STZ unit. In the short-term kinetic assay, these products emerged at later reaction times as compared to the STZ:4mCAT 1:1 reaction product, for which the intensity slightly decreased from 1000 s reaction time onwards (Figure S1B). Due to their low intensity, no MS/MS spectra could be recorded. For the STZ:4mCAT 1:2 product, two principal structures are possible, either a linkage of the STZ aniline nitrogen to both 4mCAT molecules or a linkage of the STZ aniline nitrogen to a 4mCAT dimer.

For the reaction of STZ with SYR in the presence of laccase, HRMS spectra revealed the formation of 1,2-nucleophilic addition products, as exemplified for SYR in Figure S3A ($M-H^-$ at m/z 404.0369). The less intense ion at m/z 333.0604 corresponded to the SYR dimer, for which a structure was suggested by Tatsumi et al. (1994)[5] and confirmed by an MS/MS data. The reaction pathway involves the oxidation of SYR by laccase through decarboxylation to 2,6-dimethoxy benzoquinone and the carbonyl C is attacked in a 1,2-addition reaction, followed by the elimination of water (imine formation).

Table S2. Model humic constituents used to study NER formation mechanisms and reaction pathways proposed in this study. For hydroxyphenols, the oxidation reaction to the corresponding reactive quinone is shown; non-quinone carbonyl compounds are directly susceptible to nucleophilic addition reactions.

Phenols/Quinones	**Names, Abbreviations, Standard reduction potentials (pH 7) E_h^0, Mechanism**
	**Catechol (CAT)**, 0.390 V[Ref. 6] Rapid[1] Michael addition of STZ, EXA, and ANL
	**4-Methylcatechol (4mCAT)**, E_h^0- no reference found Rapid Michael addition of STZ, EXA, and ANL
	**Protocatechuic acid (PRO)**, 0.470 V[Ref. 6] Rapid Michael addition of STZ and EXA
	**Gentisic acid (GEN)**, 0.356 V[Ref. 7] Rapid Michael addition of STZ and EXA
	**6,7-Dihydroxycoumarin (esculetin, ESC)**, E_h^0- no reference found No[3] reaction of STZ and EXA with the formed quinone
	**Vanillic acid (VAN)**, 0.181 V[Ref. 8] Rapid Schiff base formation to VAN dimers and small fraction of Michael addition to monomers of STZ and EXA
	**Syringic acid (SYR)**, 0.101 V[Ref. 6] Rapid Schiff base formation of STZ, EXA, and ANL
	**Acetosyringone (AcSYR)**, 0.101 V[Ref. 6] Rapid Schiff base formation of STZ and EXA

	**Ferulic acid (FER)**, E_h^0- no reference found No reaction with STZ and EXA, oligomerization products of FER observed[Ref. 9]
	**Hydroquinone (HQ)**, 0.286 V[Ref. 6] Slow[2] Michael addition of STZ and EXA after oxidation to p-benzoquinone, addition reaction is rate limiting; rate for EXA > STZ
	***p*- Benzoquinone (BQ)**, 0.286 V[Ref. 6] Slow Michael addition; reaction rate of EXA > STZ
Non-quinone carbonyl compounds	
	**1-Penten-3-one** Slow Michael addition; reaction rate of EXA > STZ
	**Acetylsalicylic acid** No reaction after 76 days
	**Acetic acid *p*-tolyl ester** No reaction after 76 days
	**Methylbenzoylformate** No reaction after 76 days
	**1-Phenyl-1,2-propanedione** No reaction after 76 days

[1]Rapid reaction: conversion of aromatic amines > 60% within 30 minutes

[2]Slow reaction: conversion of aromatic amines > 20% within 2-76 days

[3]No reaction: = conversion of aromatic amines < 10% within 76 days, no reaction products detected by MS

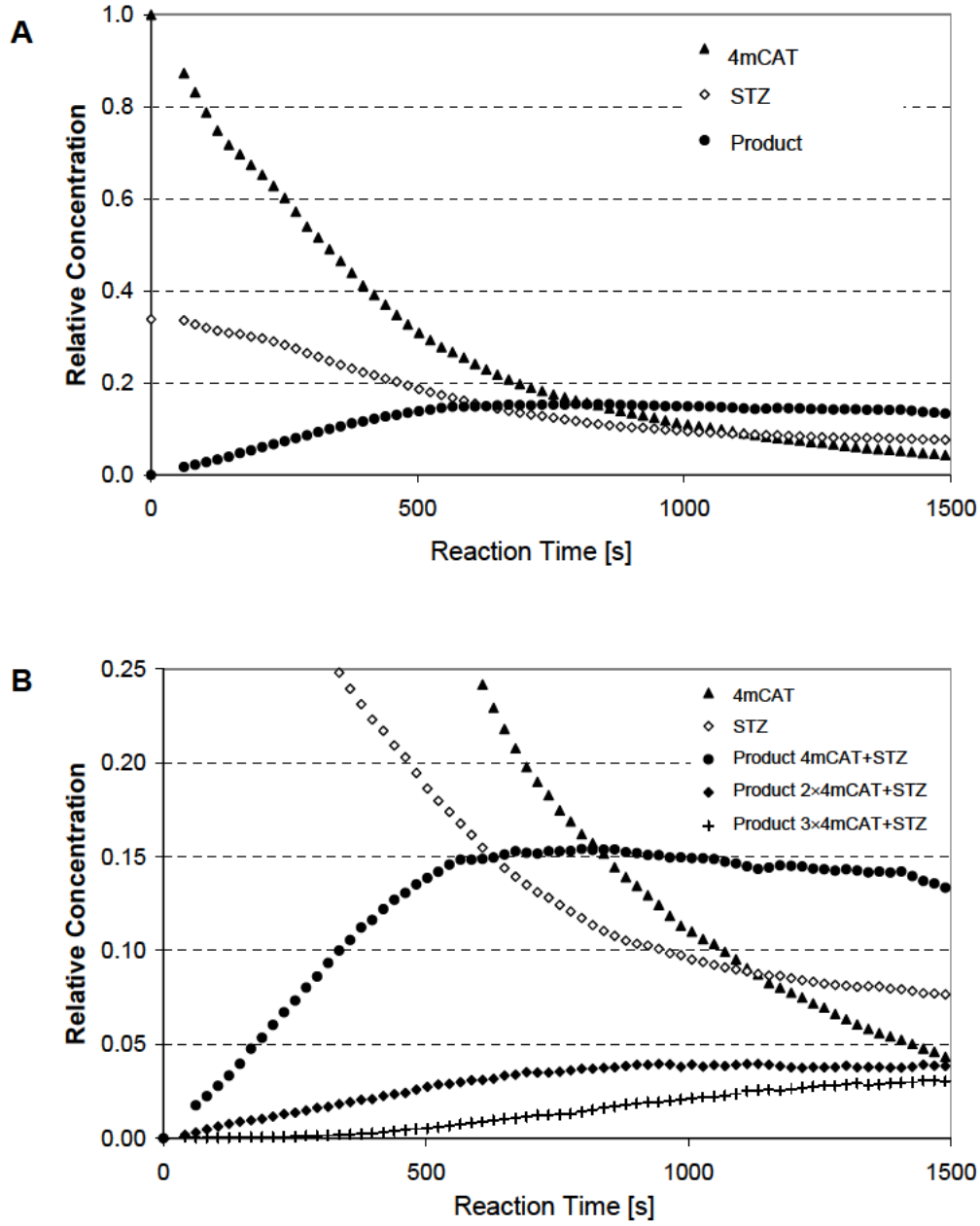


Figure S1. (A) Short-term kinetic assay of the reaction of sulfathiazole with 4-methylcatechol in the presence of laccase. Molar concentrations were normalized to 4mCAT for comparison. (B) Enlargement of the same kinetic assay showing oligomerization products. The relative concentrations of reaction products were estimated assuming the same MS ionization efficiency as for sulfathiazole for all monitored compounds.

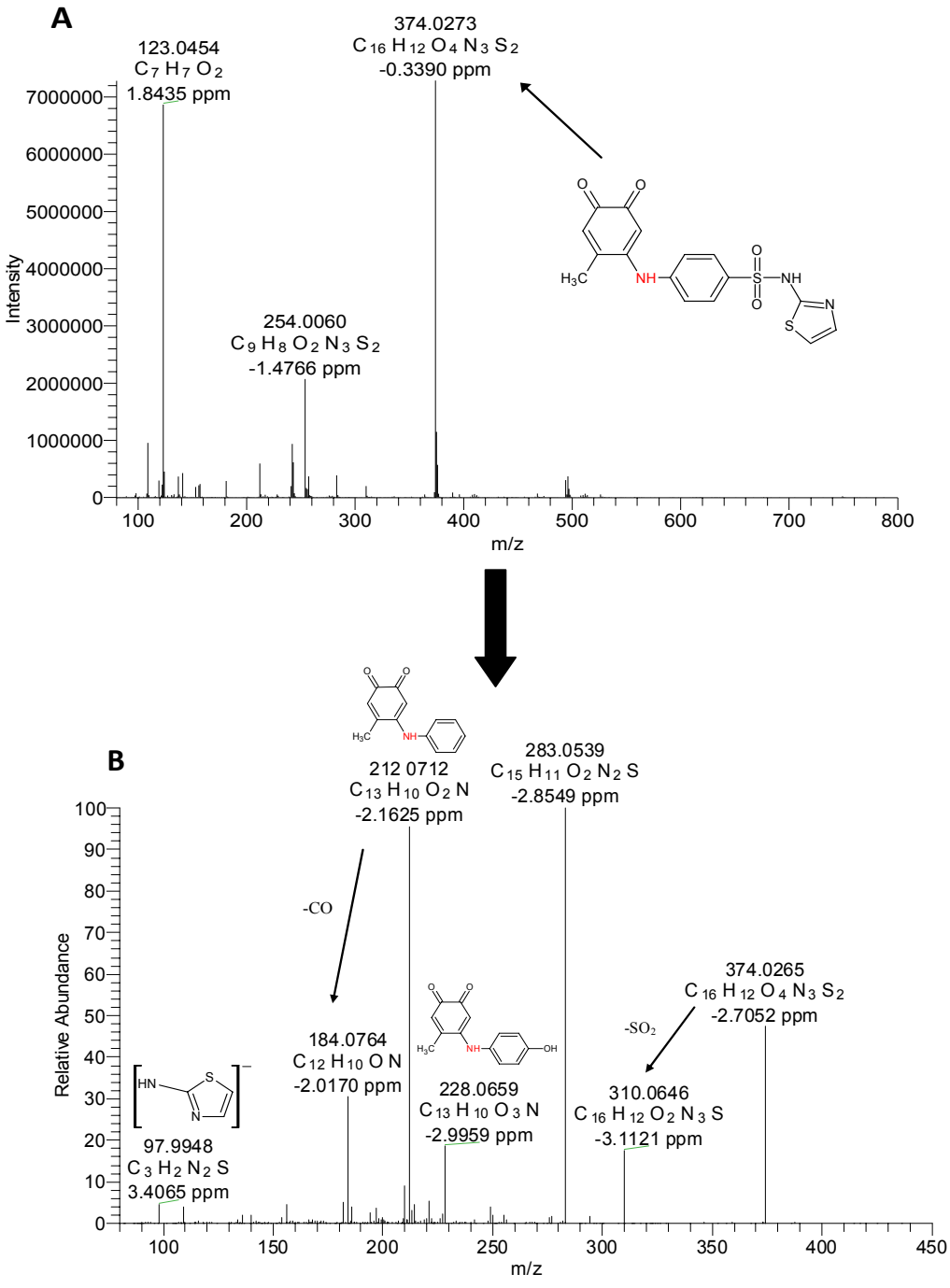


Figure S2**.** (A) High-resolution full scan mass spectrum (negative mode) of the reaction mixtures between sulfathiazole and 4-methylcatechol. (B) High resolution MS/MS product ion spectrum of the 1,4-nucleophilic addition product of sulfathiazole with 4-methylcatechol (m/z 374.0273).

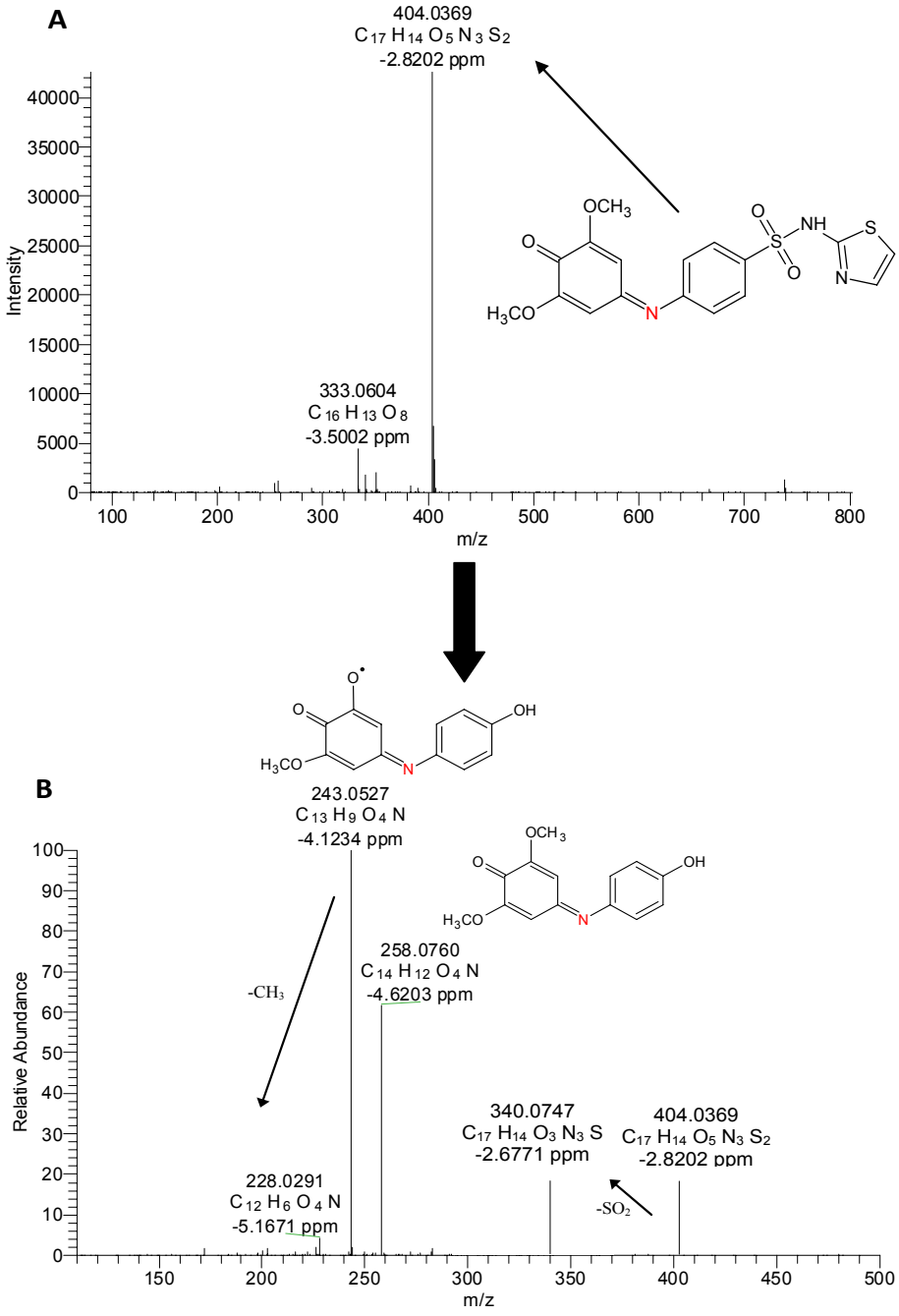


Figure S3. (A) High-resolution full scan mass spectrum (negative mode) of the reaction mixtures between sulfathiazole and syringic acid. (B) High resolution MS/MS product ion spectrum of the 1,2-nucleophilic addition product of sulfathiazole with syringic acid (m/z 404.0369).

^{1}H and ^{15}N NMR analysis of ^{15}N aniline reaction products with CAT

The temporal changes in laccase mediated reaction systems of ^{15}N aniline-catechol and ^{15}N aniline-syringic acid were monitored by ^{1}H-^{15}N HMBC NMR spectra. In both systems the correlation signals from both, reacted and unreacted aniline were observed. Two aromatic protons (δ^{1}H at 6.7 and 7.0 ppm) of unreacted aniline correlated with the nitrogen at 58 ppm. The 2-hour long incubation of aniline with catechol and laccase yielded three major correlations to nitrogens with main resonances δ^{15}N at 101, 109, and 317 ppm (Figure S4 a). The reaction product of STZ with CAT in the presence of laccase was identified earlier as an anilinoquinone (see main text). Thorn *et* al. (1996)[4] reported that reaction of aniline with humic substances resulted in anilinoquinone formation based on tentative peak assignment in the δ^{15}N range of 100-122 ppm. Thus, the resonances at 101 and 109 ppm were assigned primarily to two different types of anilinoquinones. Although the δ^{15}N range of aromatic amides overlaps with those of anilinoquinones (100-110 ppm), a nucleophilic acyl substitution resulting in an amide bond is unlikely due to lack of carbonyl compounds in the aniline-catechol reaction system. The third resonance at 317 ppm mentioned above is in a typical range of chemical shifts for imines.[10] Simmons *et* al. (1987)[11] assigned this signal to an iminodiphenoquinone nitrogen observed in a product mixture obtained from the laccase-catalyzed reaction of guaiacol with 4-chloroaniline. The ^{15}N chemical shifts of quinoline are normally expected in the same spectral region as those of imines. Owing to the absence of correlations to aromatic protons around δ^{1}H ≈ 8.5 - 9.5 ppm (expected from ^{1}H chemical shift calculations) the presence of this class of compounds is rather unlikely.

Significant changes in nitrogen chemical shifts (range of 0-200 ppm) were observed after longer incubation times of 2 hours to 20 days, with a significant increase of the signal of anilinoquinones at 101 and 109 ppm (Figure S4 a-b). A weak signal appeared at δ^{15}N 70 ppm corresponding to anilinohydroquinone. The resonances of anilinoquinone nitrogens are usually shifted to higher frequencies compared to those of anilinohydroquinones owing to a deshielding induced by deprotonation.[12] The oxidative self-coupling of anilines may result in a wide range of products such as aminodiphenylamine, 1,2-diphenylhydrazine, benzidine, azobenzene, azoxybenzene.[13] Phenazine and other diimine-derived structures reported by Simmons *et* al. (1987)[11] may also occur in the region of imine nitrogens. The signal observed at δ^{15}N 95 ppm could correspond to hydrazine adducts, which serve as precursors in azobenzene formation. However, we observed no correlations that could possibly be assigned to azobenzenes in the recorded spectra. Thorn *et* al. (1996)[13]

reported that the kinetically controlled reaction of aniline with fulvic acid was favored over coupling with aniline itself in the presence of peroxidase. Thus, we assume that the contribution of self condensation products of aniline to the spectra is negligible.

We were unable to assign the signals at δ^{15}N 162 ppm. A tentative assignment reported by Thorn *et* al. (1996)[4] suggests that this resonance may correspond to pyrroles, indoles or similar heterocyclic nitrogens.

New correlations were observed also in the region of imine nitrogens around 320 and 338 ppm. Possible assignments of these resonances could include quinoline or phenazine nitrogens and in fact, phenazines have been reported to appear in early stage of aniline oxidation.[14] The weak signal at δ^{15}N 331 ppm may be assigned to quinone imines or iminediphenoquinones.[4]

The ^{1}H-^{15}N HMBC NMR experiment of the 20 day sample was analyzed again after 60 days of storage in solution. Some NaOD solution was added to fully dissolve the sample. A significant new signal was observed at δ^{15}N 196 ppm (Figure S4 c). At the same time the relative intensities of both, anilinquinones and imines, significantly decreased. The outstanding resonance at 196 ppm may be assigned to imidazoles or benzimidazoles.[15] With continuing incubation time the relative signal intensity of the aniline nitrogen resonance continuously decreased and in the spectrum recorded after 2 months the major signal was observed in the region of heterocyclic nitrogens.

^{1}H and ^{15}N NMR analysis of ^{15}N aniline reaction products with SYR

Incubation of aniline with syringic acid and laccase yielded correlation signals in the ^{1}H-^{15}N HMBC NMR spectra that indicate covalent bond formations (Figure S5 a). A weak resonance at δ^{15}N 103 ppm was assigned to anilino-1,4-quinone nitrogens. As already reported for the catechol incubation experiments we also observed a strong correlation at 338 ppm assigned to imines. The 1,2-addition of aniline nitrogen to sterically hindered quinones results in quinone imine formation (Schiff bases). As reported earlier the reaction of STZ with SYR in the presence of laccase resulted in the formation of imines following the 1,2-addition reaction pathway. We assume a similar mechanism for aniline but, unfortunately, adequate chemical shift references for deprotonated quinone imines are difficult to obtain.[16] However, the tentative assignment of δ^{15}N 338 ppm to quinone imines reported by Thorn *et* al. (1996)[4] reasonably fits to our results. In this chemical shift region also the nitrogens of phenoxazinones are expected. However, we did not observe any correlations to highly deshielded protons expected from ^{1}H chemical shift

calculations for this class of compounds. The weaker correlation signals observed in the spectral region of imines ($\delta^{15}N \approx 350$ ppm) may originate of hydrazones or oximes.

As observed for the incubation of catechol, the distribution of nitrogen changed with time in the syringic acid series. After 2 days of incubation, significantly increased resonances corresponding to anilinoquinones were observed (Figure S5 b). In contrast to the reaction carried out in the presence of catechol, the resonance at 70 ppm assigned to anilinohydroquinones did not appear in this series of spectra during the entire incubation time. Additionally to the quinone imines ($\delta^{15}N$ 338 ppm), new signals at 321 and 354 ppm indicated the presence of hydrazones and oximes, respectively. The weak signal at 344 ppm may originate from azoxybenzenes, also explaining the presence of a new weak correlation observed at 95 ppm assignable to hydrazines. No significant deviations of relative signal intensities were noticed between day 2 and 5 (Figures S5 b and c). After 20 days, however, the resonance of the quinone imine at 338 ppm decreased beyond the detection limit, whereas a new resonance at 145 ppm appeared in the region assigned to anilides, enaminones, quinolones and/or indoles by Thorn *et* al. (1996).[4]

Figure S5 e shows the ^{1}H-^{15}N HMBC NMR spectrum of the reaction mixture of the day 20 sample remeasured after 4 weeks in solution. The signals of hydrazones at 321 and anilinoquinones at 100 ppm significantly decreased. Instead, two major resonances corresponding to free (unreacted or released) aniline at 58 ppm and a new signal of anilide or acetanilide nitrogens at 125 ppm were observed. According to Thorn *et* al. (1996),[4] the signal at 127 ppm may also correspond to enaminones.

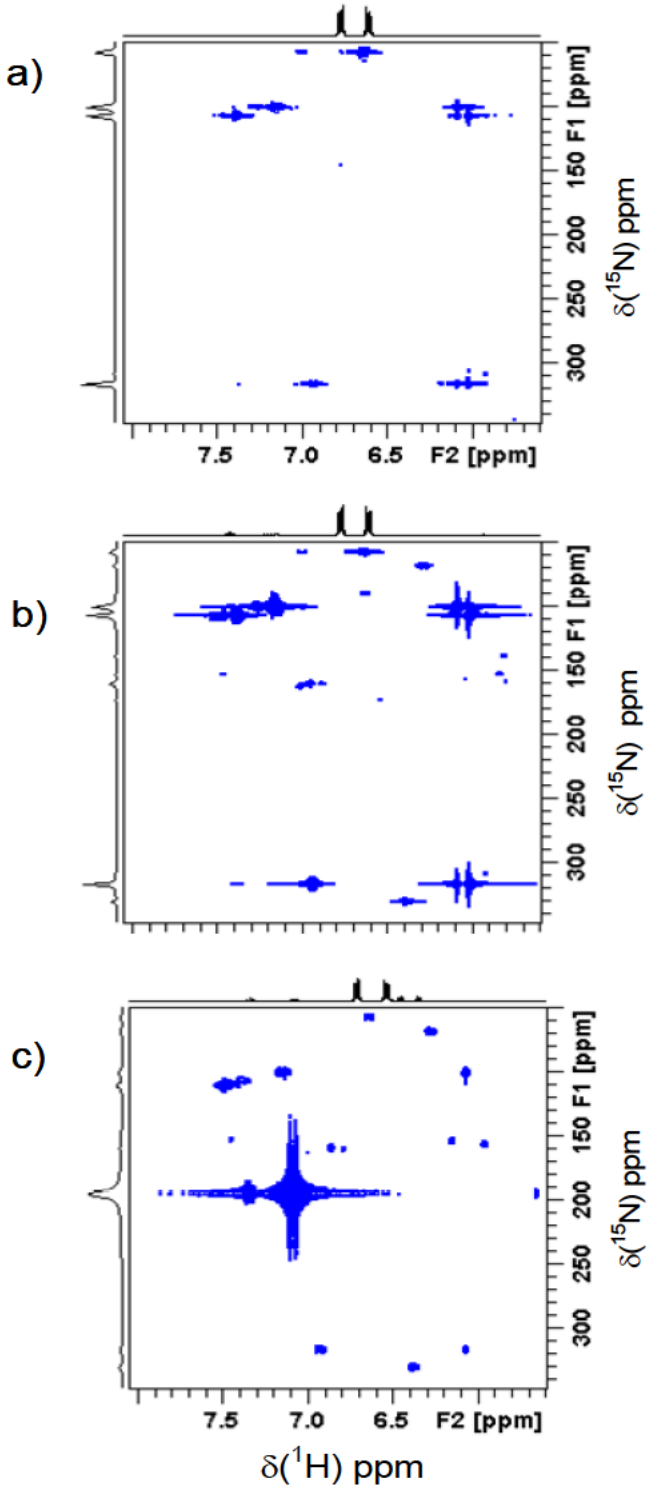


Figure S4. ^{1}H-^{15}N HMBC NMR spectra of ^{15}N-aniline transformed in the presence of catechol and laccase after a) 2 hours and b) 20 days of incubation. Spectrum c) was recorded after storing the solution for additional 30 days at room temperature.

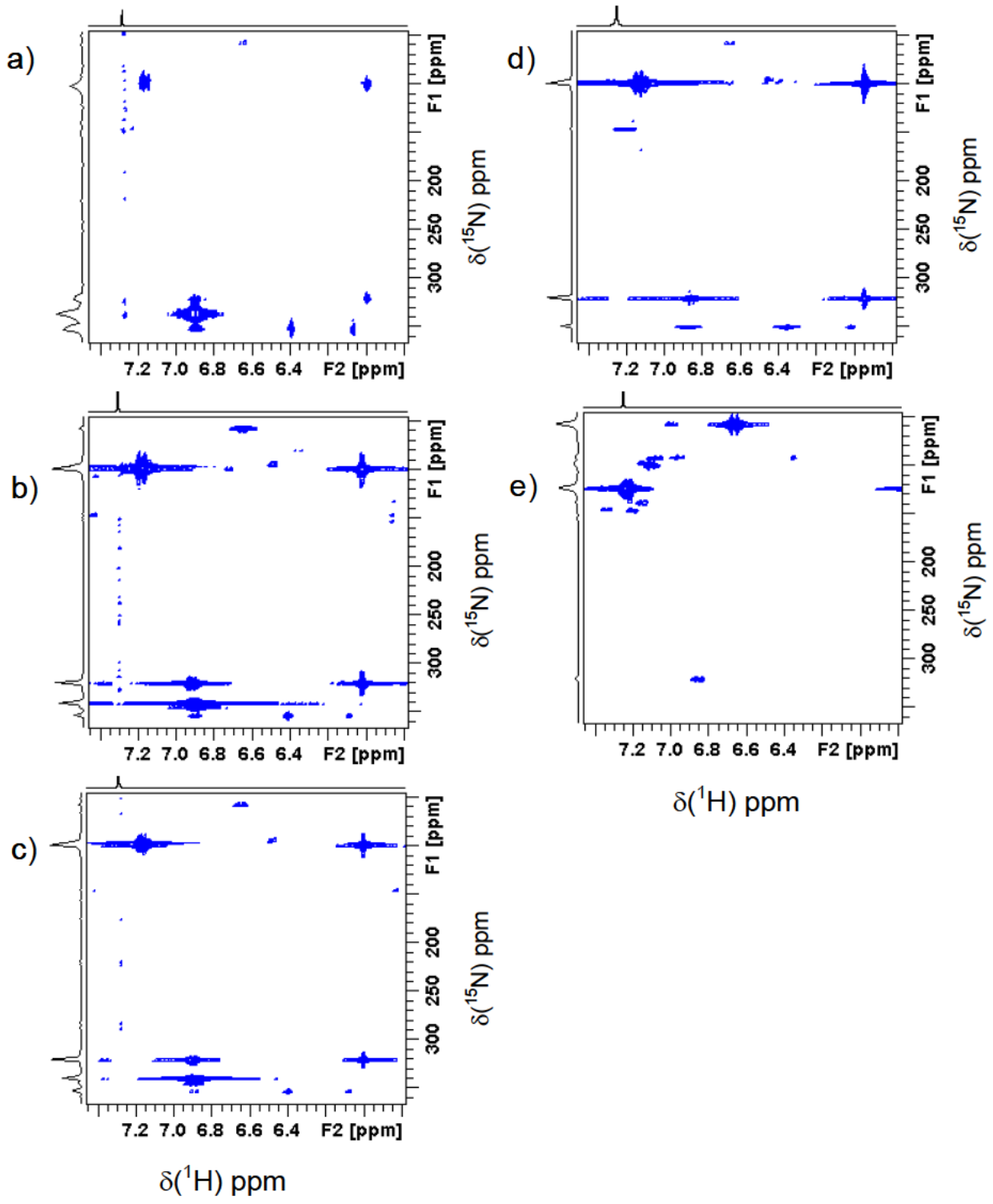


Figure S5.^{1}H-^{15}N HMBC NMR spectra of ^{15}N-aniline transformed in presence of syringic acid and laccase after a) 2 hours, b) 2, c) 5 and d) 20 days of incubation. Spectrum e) was recorded after storing the solution for additional 30 days at room temperature.

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Chapter 3

Stability of sulfonamide in dissolved organic matter

Stability of sulfonamide antimicrobials in organic matter: Linking extractability to covalent bonding
Gulkowska, A.; Krauss, M.; Hollender, J.
to be submitted to Environmental Science and Technology

Introduction

Sulfonamides are a class of antimicrobials which are used in animal husbandry practices worldwide in large amounts.[1] A specific concern regarding sulfonamides is their persistence in the environment, as their residues were detected in various environmental compartments.[2-6] The environmental spread of sulfonamides is causing concerns due to the possible risk of adverse effects on microbial communities[7] and the development of sulfonamide-resistant pathogens, which the World Health Organization regards as a major threat.[8] Sulfonamides are quite water soluble and show a pH-dependent speciation suggesting a high mobility in the environment. Studies investigating losses from grasslands and arable soils revealed very small sorption coefficients, $K_{d,}$[9] indicating a significant potential for sulfonamides losses to water bodies. Yet, the overall losses of sulfonamides to the surrounding surface water via leaching and surface runoff amounted to 2.5%.[6,10,11]

Despite the low soil/water partition coefficients, a large fraction of sulfonamides is retained in soil in the form of nonextractable residues (NERs).[12-14] NERs are operationally defined as parent compounds or metabolite(s) which are not released from the soil matrix by extraction methods that do not change the compounds themselves and/or the structure of soil matrix.[15] Thus, the extent of NER formation depends strongly on the extraction procedure used, which is particularly evident for sulfonamides. Stoob *et* al. (2006)[16] showed that the extractable fraction of different sulfonamides increased up to a factor of six if the extraction temperature in PLE was increased from 100°C to 200°C. While the NER fraction of ^{14}C-sulfadiazine amounted to 54-69% at day 0 for a soxhlet extraction using ethanol:water 9:1[13] it was 10-20% for the same soil samples if a microwave-assisted extraction with acetonitrile:water 1:4 at 150°C was applied.[13,14] After this initially rapid formation, NERs increased only slowly with time in the studied soils, amounting to > 90% for the soxhlet extraction[13] and to 40-50% for the microwave-assisted extraction after 218 days.[14]

These observations suggest that the initially fast formation of NERs occurs by covalent bonding of the parent compounds or reactive transformation products. Based on literature on other aromatic amines[17-19] mechanistic studies utilizing model systems demonstrated conclusively that the covalent bonds between sulfonamides and model humic constituents (substituted phenols) or humic acids occurs by nucleophilic addition of the aniline nitrogen to quinones.[20-22] The latter are formed from the corresponding phenols by oxidative enzymes or manganese oxide. It has been shown in Chapter 2 that the initially formed imine and anilinoquinone bonds are partly released

by exhaustive extraction procedures such as PLE in experiments with substituted phenols. The bonding strength increased with incubation time due to a further incorporation of the initial reaction products, resulting in an increased stability against pressurized liquid extraction. Furthermore, the results of the study in Chapter 2 revealed that a formation of covalent bonds between sulfonamides and substituted phenols could also occur during the PLE at high temperatures, which might result in an additional NER formation by the extraction procedure.

To which extent covalent bonds with soil organic matter are stable against extraction procedures commonly applied to assess NERs in soils is up to now unknown. As a starting point to answer this question, our objective was to link the macroscopic observations from extraction and desorption procedures to the mechanisms of sulfonamides covalent bonding with humic acids. To this end, the extent of covalent bonding between sulfamethazine (SMZ) as a model sulfonamide and different humic acids was studied in single solute and binary competitive solute sorption experiments. Experiments were carried out over three ranges of sorbate concentrations as well as in the presence and absence of laccase, an oxidative enzyme. The Leonardite humic acid (LHA) and four synthetic humic acids (SHAs) with different properties were used as a model for SOM. The stability of the reaction products against desorption and PLE was assessed.

Materials and methods

Chemicals

Sulfamethazine (SMZ; 4-amino-*N*-(4,6-dimethyl-2-pyrimidynyl)-benzenesulfonamide) was obtained from Sigma-Aldrich (Buchs, Switzerland), *p*-ethoxyaniline (EXA) from Merck (Hohenbrunn, Germany), and SMZ ^{14}C-labeled at phenyl ring (^{14}C-SMZ; 3.7 MBq/mL, specific activity 0.3034 GBq/mmol, 99% in ethanol) from American Radiolabeled Chemicals (St. Louis, MO). Stock solutions of SMZ, ^{14}C-SMZ, and EXA were prepared in methanol. The latter was diluted in buffer to the desired concentration and was directly used in sorption experiment. A mixture of labeled and unlabeled SMZ standard (thereafter addressed as SMZ) was prepared in ammonium acetate buffer to obtain a starting SMZ concentration of 40 µg/mL, equivalent to 27.14 kBq/mL. HPLC grade methanol, acetonitrile and water (Acros Organics, Geel, Belgium) were used as solvents for extraction and as liquid chromatography eluents. Unless otherwise specified, a 0.1 M ammonium acetate buffer at pH 5.0 was used throughout the study. Extracellular fungal laccase from *Trametes versicolor* (oxygen oxidoreductase E.C. 1.10.3.2.) with a reported activity of 22.4 U/mg was obtained from Sigma-Aldrich. Fresh enzyme stock solution was prepared at a concentration of 1 mg/mL in 0.1 M ammonium acetate buffer.

Preparation and use of HA solutions

Leonardite humic acid (LHA) was purchased from the International Humic Substances Society (IHSS) (St. Paul, MN). Prior to use, LHA was purified involving cation exchange and dialysis as described in Supporting Information (SI) based on references 23 and 24.[23,24] Four synthetic humic acids (SHA) were used in this study: an enzymatically synthesized nitrogen free HA (EnHA), chemically polymerized nitrogen-free HA (NaHA) and nitrogen containing HA (AmHA), as well as HA with hydrophobic moieties obtained by incorporation of nonylphenol (NLHA). The detailed description on the different synthesis procedures, purification and characterization of the obtained HAs can be found in the SI. Required amounts of purified humic acids were dissolved in acetate buffer. The pH was adjusted to 5.0 with ammonia (25%w/w) or acetic acid (100%) followed by adjustment to the final desired volume with acetate buffer. The pH was checked the following day and re-adjusted if required. The HA concentrations in stock solutions were 3.2 g/L. The SHAs were well soluble in buffer except AmHA, which remained in suspension at given pH.

Sorption experiments

Sorption experiments were carried out in pH-equilibrated solutions of acetate buffer at pH 5.0 in duplicate. Single solute SMZ experiments and binary competition experiments for the pair SMZ-EXA were carried out in 20 mL brown glass vials. Aliquots of HA stock solutions were transferred to incubation vials to achieve a concentration of 1.6 g/L. The initial aqueous SMZ concentrations for the single solute sorption experiment were 0.04, 0.4, and 4 µg/mL. Binary solute experiments were carried out at one initial SMZ concentration of 4 µg/mL and a molar ratio of SMZ: EXA of 1:3. Additionally, single solute and binary solute sorption experiments with the initial aqueous SMZ concentrations of 4 µg/mL were carried out in the presence of 0.8 U/mL laccase. All four SHAs were tested at one initial SMZ concentration of 4 µg/mL in the presence and absence of laccase. Single solute samples with buffer and 4 µg/mL SMZ were run as controls. Vials were shaken horizontally (170 rpm) at 19°C in the dark.

Desorption and extraction procedures

After 36 d of sorption HA-SMZ mixtures were sampled and sample aliquots were processed using a combination of desorption and extraction procedures as outlined in Figure 1. For LHA samples, a desorption experiment was initiated for 42 days in custom-made dialysis systems described in detail by Richter *et* al. (2009).[25] Briefly, each system consisted of two 4 mL half-cells separated by a dialysis membrane (Spectra/Por Biotech, Cellulose ester, 500, MWCO, Socochim. Lausanne, Switzerland) impermeable for HA, but permeable for SMZ. Dialysis systems were loaded by pipetting 3.5 mL-aliquots of the LHA-SMZ solutions to one of the half-cells under intensive stirring of the LHA-SMZ solution, and by adding 200 ± 10 mg of Oasis HLB solid-phase extraction sorbent (Waters, Rupperswil, Switzerland) as an infinitive sink material to the other half-cells. The latter were filled with 3.5 mL of acetate buffer. Cells were shaken horizontally at 170 rpm at 19°C to desorb SMZ from LHA. The pH remained constant during the whole experiment (pH 5.0 ± 0.2). After 14 and 28 days, the Oasis material was exchanged for fresh one. On day 14, 28 and 42 the complete content of the Oasis half-cells was transferred to 6 mL filtration tubes with polyethylene frits placed in the bottom (Supelco, Bellefonte, PA). To ensure a complete transfer, these half-cells were flushed six times with 3 mL of HPLC grade water and the water was transferred to the filtration tubes. The cartridges were dried under vacuum. Dried Oasis

material was transferred to 2 mL vials and stored at 4°C until solid combustion analysis. After 42 d of desorption, also the HA- SMZ solutions of the second half cell were sampled and directly measured using analytical procedures described below. Control experiments in dialysis cells were run to assess the kinetics of SMZ diffusion through the membrane, the attainment of equilibrium and sorbate losses to the membrane (see SI for details).

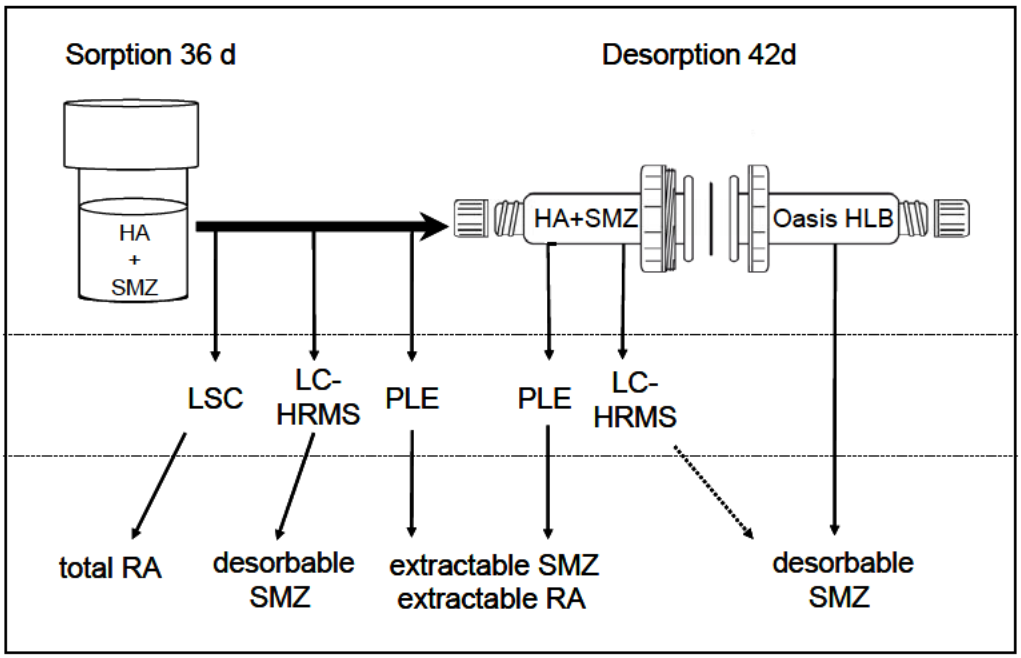

Figure 1. The work flow applied in this study for the assessment of SMZ extractability from dissolved OM and evaluation of stability of covalent bonds formed. Desorption with infinite sink was done for LHA only, not for the synthetic HAs. (HA: humic acid; SMZ: sulfamethazine; LSC: liquid scintillation counting; LC-HRMS: liquid chromatography-high resolution mass spectrometry; PLE: pressurized liquid extraction; RA: radioactivity).

A second approach to determine the desorbable fraction was direct LC-HRMS analysis. 20 µL of the HA-SMZ mixture solution were withdrawn and transferred to an LC vial along with 180 µL of water and injected into the autosampler of the LC-HRMS as described below.

For all HA-SMZ reaction mixtures, the extractable fraction of SMZ and total radioactivity (RA) was determined after the sorption and after the desorption experiment by PLE using an ASE 350 (Dionex, Sunnyvale, CA). Aliquots of 1 mL HA-SMZ mixture were dripped into a stainless steel cell prefilled with diatomaceous earth (Restek Corporation, Bellefonte, PA) and extracted at 140°C using water/acetonitrile 85:15 (v/v) adjusted with ammonium acetate buffer to pH 5.6 (see Chapter

2). An aliquot of 1 mL extract was withdrawn from the collection vial and filtered into a 2-mL LC vial using a 0.2 µm PTFE syringe filter. Compounds were analyzed by LC-HRMS. To check for thermal degradation of SMZ, control samples containing only the SMZ were spiked to a cell filled with diatomaceous earth. Blank samples consisting of acetate buffer were run to assess cross-contamination.

Instrumental Analysis

SMZ was quantified by liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS). Details of the conditions and quality control measures are given in the SI.

To determine the total RA in HA-SMZ reaction mixtures liquid scintillation counting (LSC) was used. Sample aliquots were added to 10 mL of Hionic Fluor liquid scintillation cocktail (PerkinElmer, Waltham, MA) and analyzed with a 2200 CA TriCarb Liquid Scintillation Analyzer (Packard). Samples were counted three times for 10 min and counts were corrected for background activity by blank controls. To determine the RA in the infinitive sink used in the desorption experiment 200 mg of dried Oasis sorbent were placed in a cellulose thimble, and combusted using a Perkin Elmer Sample Oxidizer, Model 307. The CO_2 evolved was trapped in 5 mL of Carbosorb E and mixed with 15 mL of Permafluor E+ LSC cocktail. All samples were analyzed with a Perkin Elmer Liquid Scintilation Analyser, TriCarb 2800TR. Every ten samples one control sample with a defined RA and one blank sample were run for quality control.

Selected samples were analyzed by LC coupled to a diode array detector (Agilent 1100) followed by an flow scintillation counter (FSC; Packard 500TR series) to asses whether transformation products were formed during long-term sorption experiments by laccase oxidation or chemical reactions. Simultaneously, the chromatographic elution profile of the humic acids was obtained based on the UV response at 270 nm. For chromatographic separation a reversed phase Nucleodur C_{18} Gravity column was used. The LC gradient was made using water (A) and MeOH (B), both with 0.1% formic acid starting at 90% of A, increasing B to 95% in 13 min held for 3.5 min, returning back to 90% of A in 1 min and re-equilibration for 10.5 min. The flow rate was 200 µL/min and sample injection volume was 40 µL. After passing the diode array detector, the eluent was mixed with 800 µL/min Ultima Flo-M scintillation cocktail (PerkinElmer, Waltham, MA) prior to the FSC. To check for a complete elution of the reaction mixtures the UV spectra of different HAs were monitored and a recovery test of RA eluting from the column was performed. To this end, the eluent from the whole sample run was collected in 20 mL vial

and analyzed by LSC. The recovery was calculated relative to the RA in the same sample prior to injection as detected by LSC.

Data Analysis

Based on desorption and extraction procedures outlined in Figure 1, three different fractions of SMZ associated with humic acids can be distinguished according to their bonding strength: (i) the desorbable fraction as determined by the direct LC-HRMS analysis, which included also the free SMZ in solution that could not be distinguished (Equation 1). (ii) A fraction that was bound by labile covalent bonds to HA, which was given by the difference between extractable and desorbable SMZ (Equation 2). This is based on the assumption that within the LC system from a dissolved HA all SMZ not covalently bound is released and kinetic limitations of desorption do not apply. (iii) A fraction that was bound by stable covalent bonds to HA, which is given by the difference between the total RA determined by LSC after sorption and the PLE-extractable SMZ fraction (Equation 3).

$$C_{desorbable} = C_{SMZ\ free} + C_{SMZ\ desorbable} = C_{SMZ\ (LC\text{-}HRMS)} \quad (1)$$

$$C_{labile} = C_{extractable} - C_{desorbable} = C_{SMZ\ (PLE)} - C_{SMZ\ (LC\text{-}HRMS)} \quad (2)$$

$$C_{stable} = C_{total\ RA\ (LSC)} - C_{RA\ (PLE)} + C_{RA\ bound\ to\ diatomaceous\ earth} \quad (3)$$

RA and SMZ were recovered completely in the PLE extract of the SMZ in buffer solution indicating no binding of SMZ to diatomaceous earth in the extraction cell. In contrast, recoveries of RA were up to 20% lower for most of the samples containing LHA. Thus, a portion of SMZ was bound to LHA which remains sorbed to the diatomaceous earth despite the harsh extraction conditions and solubility of all LHA in the extraction solvent. Therefore, calculated RA that remained sorbed to diatomaceous earth was added to the stable covalent bond fraction.

Results and Discussion

Characterization of humic acids

Elemental Composition

Table 1 summarizes the results of elemental analysis and compares them to the data of commercially available natural LHA. The SHAs contained no or negligible nitrogen due to the use of N-free precursor substances, except AmHA which contained about 4% of N derived from ammonia present in the solution during synthesis. We notice that the O content of synthetic humic acids is at the upper O-percentage encountered in LHA. The measured values for O are also higher when compared to calculated ones. The oxidative polymerization coincides with a net incorporation of oxygen, as the oxygen content increased relatively to the weighted sum of the precursor compounds. Consequently, SHAs had a higher content of oxygen (38-41%) and a lower content of carbon (54-55%) than LHA (64% C, 31% O).

Table 1. Elemental composition of Leonardite and the four synthesized humic acids (% of a dry, ash-free sample). The calculated values were obtained from the weighted sum of the elemental composition of the precursors that were used for SHA synthesis.

Humic acid	**C**		**H**		**N**		**O**	
mass %	meas.	calc.	meas.	calc.	meas.	calc.	meas.	calc.
LHA	64	64[(a)]	3.5	3.7[(a)]	1.29	1.23[(a)]	31.2[(b)]	31.3[(a)]
EnHA	54	58	4.1	5	0.52	--	41	37
AmHA	54	57	4.5	5	4.2	--	38	38
NaHA	55	58	4.5	5	<0.2	--	41	37
NLHA	55	65	4.6	6	<0.2	--	40	29

[(a)] IHSS reference data for the original LHA sample before clean-up (source: http://www.ihss.gatech.edu/elements.html)

[(b)] not determined

^{13}C CP-MAS NMR spectroscopy

Solid state ^{13}C CP-MAS NMR spectra of natural and synthetic humic acids showed broad signals occurring in well defined spectral regions typical for humic acid samples (Figure 2).[26-28] The ^{13}C CP-MAS NMR spectrum recorded for LHA matches those reported by other authors well.[29,30]

The main resonances found in the SHAs were better resolved than those of LHA. Generally, similar overall line shapes of various relative signal intensities were observed for EnHA, AmHA, NaHA and NLHA showing somewhat sharper resonances located around 10, 21, 40, 56, 116, 129, 145, 156 and 176 ppm. The signals at 10 and 21 ppm could be assigned to aryl and acetyl CH_3 carbons, respectively, and the region at around 56 ppm may be associated with methoxy substituents at aromatic rings of syringyl and guaiacyl units (the structures of precursors units are given in Table S1), or to N-alkyl carbons.[31,32] The strong resonances observed in the region (100-160 ppm)

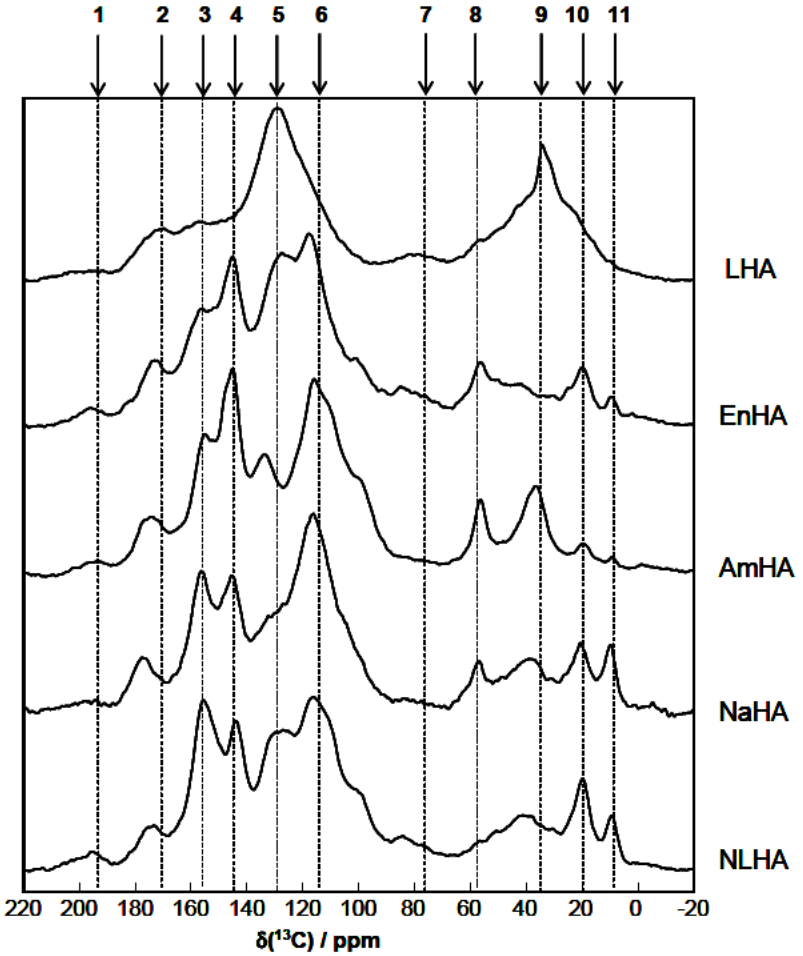

Figure 2. ^{13}C CP-MAS NMR spectra of Leonardite humic acid (LHA) and synthetic humic acids from enzymatic (EnHA) and chemical synthesis with (AmHA) and without (NaHA) nitrogen incorporation, as well as with nonylphenol incorporation (NLHA). The numbering of resonances is according to the spectral regions of selected functional groups given in Table 2.

are characteristic for a large amount of aromatic carbons for all HA spectra, with the prominent resonances at around 116 ppm generally assigned to aromatic methine carbons in *ortho* position to phenolic carbons e.g. in *p*-hydroxyphenol, guaiacyl and syringyl moieties. The broad resonances around 130 ppm could be assigned to aromatic methine carbons with various substituents e.g. in the *p*-hydroxy phenyl ring of cinnamic and/or *p*-coumaric. Precursors with such units were used for HA polymerization (see Tables S1 and S2). The carbons around 145-156 ppm could be assigned to phenolic carbons (present as free OH group or bearing alkyl substituents) and to C or N substituted quaternary aromatic carbons, respectively, and the prominent signal around 176 ppm indicates the presence of carboxyl groups assigned to acids and/or amide groups attached to aliphatic or aromatic compounds (e.g. amino acids or derivatives of benzoic acid). The weak resonances observable in some of the spectra around 195 ppm might be attributed to carbonyl C's of ketones and aldehydes, however, they could also originate from spinning side bands.

Table 2. Chemical shift assignment of selected resonances (see Fig. 2) observed in ^{13}C CP-MAS NMR spectra of the Leonardite and of synthetic humic acids.

Resonance labeled in Fig. S5	Chemical shift range / ppm	Assignment to functional groups
1	185-200 180-190	carbonyl groups of ketones and aldehydes carbonyl groups of quinones
2	160-185	carboxyl groups in aliphatic acids and esters / amide groups of amino acids
3	145-160	phenolic carbons
4	130-145	quarternary aromatic carbons
5	100-135	H-substituted aromatic carbons
6		carbons in *ortho*-position to quarternary phenolic carbons
7	90-110 60-80	anomeric carbons of carbohydrates CH and CH_2 groups of carbohydrates
8	45-60	O-alkyl and N-alkyl groups
9	0-45	
10		CH_2 groups
11		CH and CH_3 groups / quaternary aliphatic carbons

Whereas the quaternary carbons (140-165 ppm) assigned to phenolic groups of precursors used for synthesis appear strong in the spectra of SHAs compared to LHA, the SHAs obviously contain lower amounts of long chain aliphatic methylene groups than LHA (signals around 30 ppm,

Figure 2). Additionally, no resonances of carbohydrates were detected at all, which are not expected from the precursor materials. Although the main resonances were in general similar, there are discernible deviations in relative signal intensities. The increased relative signal intensity of the resonance at 35-45 and 55 ppm observed in the spectrum of AmHA (incorporation of nitrogen during synthesis) may be attributed to N-alkyl groups. The latter seems reasonable as AmHA contained significant amounts of nitrogen as revealed from elemental analysis (Table 1).

In the spectrum of NLHA the resonance of the methoxy group at 56 ppm is almost absent, while methyl groups with relative signal intensity at 21 ppm are relatively higher as compared to spectra of other SHAs.

Mass Spectrometry

NMR yields information about functional groups present in the bulk HA, but fails to give molecular-level details. Thus, for the molecular characterization of humic acids, full scan mass spectra were acquired from direct infusion using the LTQ Orbitrap high-resolution tandem mass spectrometer with electrospray ionization (ESI) in positive and negative ion mode at a pre-set mass resolution of 100,000. The full scan ESI mass spectra of synthetic humic acids appeared in a mass range similar to that of LHA with a maximum intensity at *m/z* 350-600. Ionization of the mixture in the positive mode was weak compared to negative mode, and much more complex, with formation of protonated and various adduct forms, and a few dimers as seen previously.[33] The ionization efficiencies are determined by the type of functional groups present in the sample and their ability to lose or accept proton for negative and positive ionization, respectively. Fact that HA solutions are preferentially ionized in negative mode indicates their relative acidic character due to presence of numerous carboxylic or phenolic groups. However, one should bear in mind that ionization efficiency is influenced by many other factors i.e. solution pH, composition of solvent, freeze-drying, etc.[34,35] Although some researchers reported that ESI produce multiply charged species of NOM, recent investigators confirmed that the overwhelming majority of ions produced by ESI were singly charged.[36-38] This has been recently observed also for terrestrial HA.[35] The mass spectrum of SHA and LHA in negative ionization mode shows several thousand singly charged peaks between *m/z* 200 and *m/z* 800 with a maximum of the peak distribution at around *m/z* 350 (Figure 3). The middle spectra demonstrates that the ions are in fact singly charged simply, because the peaks at the odd masses have their isotopic counterparts located exactly 1.003 mass units higher, the difference between the exact mass of a ^{13}C and ^{12}C. If the isotope peak is observed

at 0.5015 (or 0.3343) higher, then the ion would be doubly (or triply) charged.[34] The mass spectrum of SHAs contained fewer ions than that of LHA, along with a smaller positive mass defect. Thus, SHA has a simpler composition and contains more O, but less C and H. The increase in O_2 series indicates aromatic ring cleavage and therefore a decrease in aromatic nature of the SHA, as was elucidated by NMR (Figure 2). This corroborates previous evidence that the enzymatic and alkaline oxidative polymerization mechanism of phenolics involves ring opening reactions.[29,39]

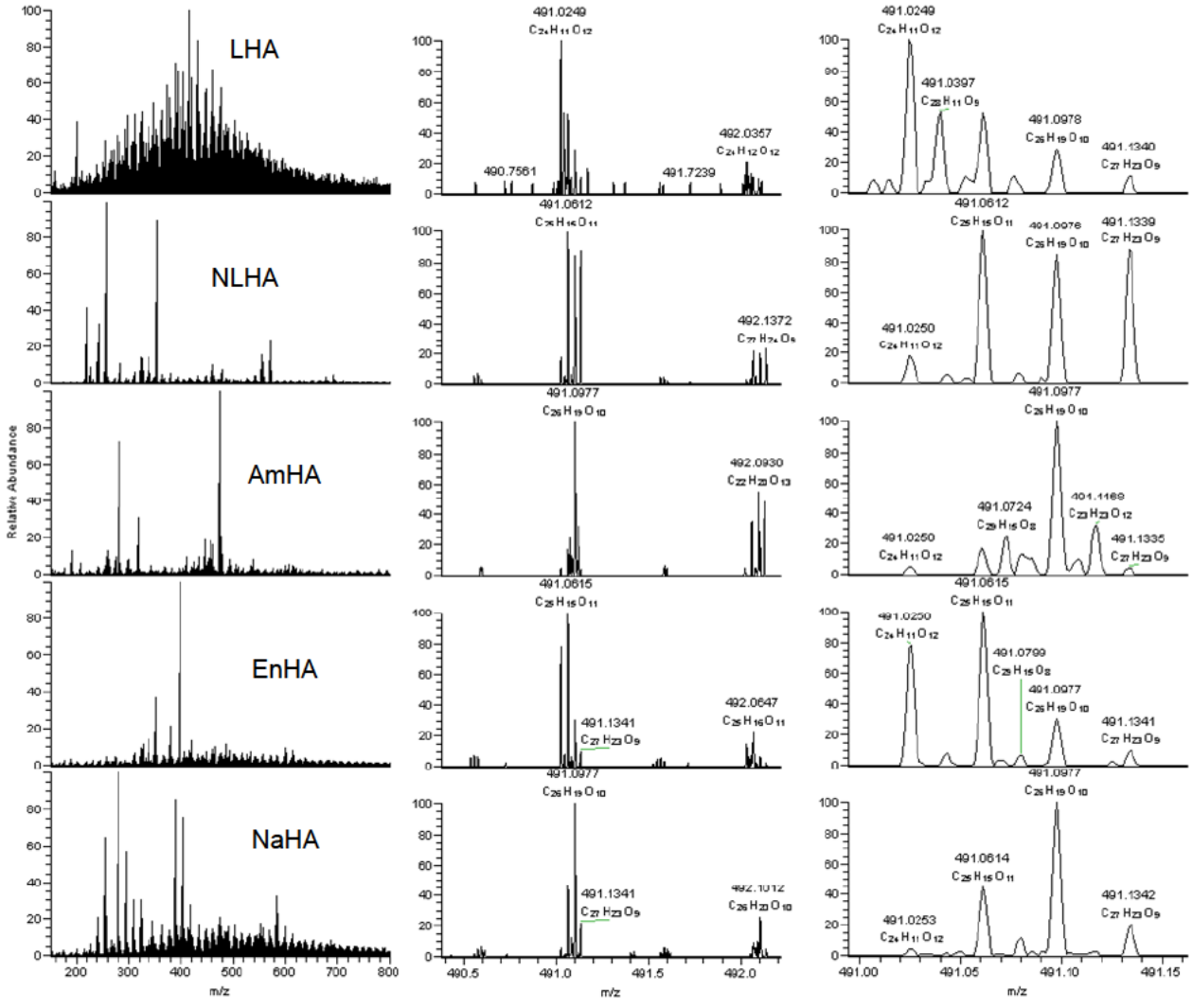

Figure 3. Full scan chromatograms of a Leonardite humic acid (LHA) and different synthetic humic acids (NLHA chemical synthesis with nonylphenol, AmHA chemical synthesis with nitrogen incorporation, EnHA enzymatic synthesis, NaHA chemical synthesis without nitrogen incorporation) acquired by direct infusion on the LTQ Orbitrap in ESI negative ion mode for the mass range 150-2000 Da and the corresponding typical organic matter clusters (section m/z 490.5-492.5 and 491.00-491.15) to indicate ions of identical elemental composition in LHA and SHAs. Proposed elemental compositions of the most abundant ions are given.

The preliminary formula assignment was carried out using the mass calculator option of Qual Browser 2.0.7 (Thermo Electron, Bremen, Germany). The equation was solved using following parameters: $0 \leq C \leq 80$, $0 \leq H \leq 160$, $0 \leq O \leq 40$, and DBE ≥ 0. The sulphur was not included into the formula assignment due to its very low content in the sample and the nitrogen was not included because the HA samples were derived from nitrogen-free precursors.

Comparison of the infinite sink and direct LC-HRMS methods to determine the desorbable SMZ fraction

The total RA in the experimental vials did not change during incubation for 36 d (average 99.7 ± standard deviation 1.5%) indicating that no significant sorption to vessels occurred during the experiment.

For the determination of the desorbable fraction, we compared an infinite sink method with Oasis HLB in a dialysis cell system and a direct LC-HRMS analysis (Figure 4). The Oasis material was not able to take up the complete SMZ from a buffer solution via the dialysis membrane, as indicated by a recovery of about 80%, while all SMZ could be recovered from the buffer solution by LC-HRMS. Partially, an incomplete mass transfer over the membrane was responsible for this lower recovery in the cell-cell-system, as 17% of SMZ remained in the SMZ half cell after 42 days of desorption.

Control experiments described in detail in the SI revealed that the diffusion of SMZ through the membrane was rather slow and an equilibrium concentration between a half-cell containing 4 µg/mL SMZ and a buffer-only half-cell was reached after 20 days (Figure S1). If a 1.6 g/L LHA solution was used instead of the buffer in the second half cell, an apparent equilibrium was also attained after 20 d, but a slow sorption to LHA continued to take place until day 36, resulting in an increase of SMZ mass in the LHA half cell (Figure S2). The time required to reach equilibrium was almost three times longer than that reported for sulfathiazole (255.32 g/mol) in an identical set-up at pH 4.8.[25] The mass balance for both half-cells in Figure S1 reveals further that about 10-13% of the SMZ were sorbed to the membrane, which explains another portion not recovered by the Oasis material. These results suggest that both, mass transfer and sorption to the membrane are artifacts when determining the desorbable fraction in the cell-cell system. The capacity of the Oasis material seemed not to be a limiting factor, as the RA present in the Oasis material decreased from the first to the second and to the third desorption step from 52 to 17 to 9%.

The difference of about 20% in recovered SMZ between the LC-HRMS and the infinite sink method was observed for three other experiments with LHA (Figure 4). In these cases, another process might also contribute to this difference: While the direct LC-HRMS analysis mirrors the degree of sorption after the time of sampling at 36 days, the infinite sink desorption takes

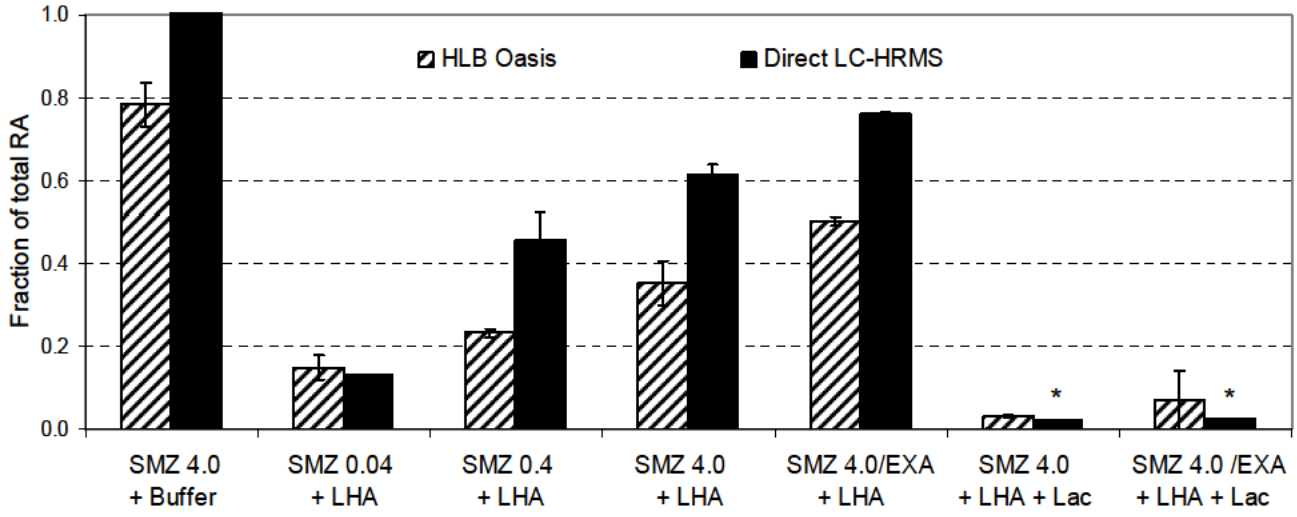

Figure 4. Desorbability of SMZ determined by LC-HRMS direct injection and infinite sink desorption by Oasis HLB sorbent after 36 days of sorption (average and standard deviation of 2 replicates). Control sample with buffer and samples incubated with LHA (single solute system with three different SMZ concentrations (0.04, 0.4, and 4.0 µg/mL) and single and binary solute systems with and without laccase). The asterisks indicate samples in which SMZ was detected below the LOQ in LC-HRMS direct injection; bars at 0.5 LOQ are given for comparison.

another 42 days. During that time an ongoing binding of SMZ to the HA might lower the desorbable fraction during the desorption process. This assumption is supported by the fact that the SMZ concentrations associated with LHA determined in additional LC-HRMS analysis after the desorption experiment were below the LOQ. No difference between the LC-HRMS and the infinite sink method was observed for the lowest SMZ concentration (0.04 µg/mL) and the two incubations in the presence of laccase. For these cases only a rather small absolute amount of SMZ was desorbable and thus mass transfer through the membrane was not limiting desorption by the infinite sink method.

Thus, both approaches to determine the desorbable (including the freely dissolved) fraction yield comparable results for low desorbable concentrations, but the infinite sink method underestimates the desorbable fraction at higher concentrations. The LC-HRMS method yields probably reliable values in both cases, after sorption and desorption, as after injection into the LC eluent a complete desorption of SMZ from HA should occur given the large excess of methanol-containing eluent during the LC analysis.

Desorption and extraction of SMZ from Leonardite humic acid

The distribution of desorbable, labile and stable bound SMZ from the reaction with LHA after sorption (36 days) and desorption experiment (in total 78 days) is shown in Figure 5. The desorbable fraction after 78 days was calculated as a sum of the fraction determined by Oasis material and an LC-HRMS analysis of HA from half cell after desorption.

The covalently bound fraction was > 95% in the samples incubated in the presence of laccase. This is in line with previous findings that the nucleophilic addition of sulfonamides to HAs and small model compounds is driven by the formation of quinone moieties due to an oxidation by laccase (see Chapter 2).[20,22] In that case the presence of the competing nucleophile EXA did not have an impact on the extent of covalent binding of SMZ. But also without the presence of laccase, a considerable fraction of SMZ was bound covalently to LHA and the extent of binding increased with decreasing SMZ concentration. This indicates that reactive sites susceptible to nucleophilic addition were present in LHA, but the relatively low number of these sites was clearly limiting the extent of binding. Similarly, the stronger nucleophile EXA prevented a fraction of sulfonamide from binding resulting in a higher SMZ recovery at SMZ concentration of 4.0 µg/mL.

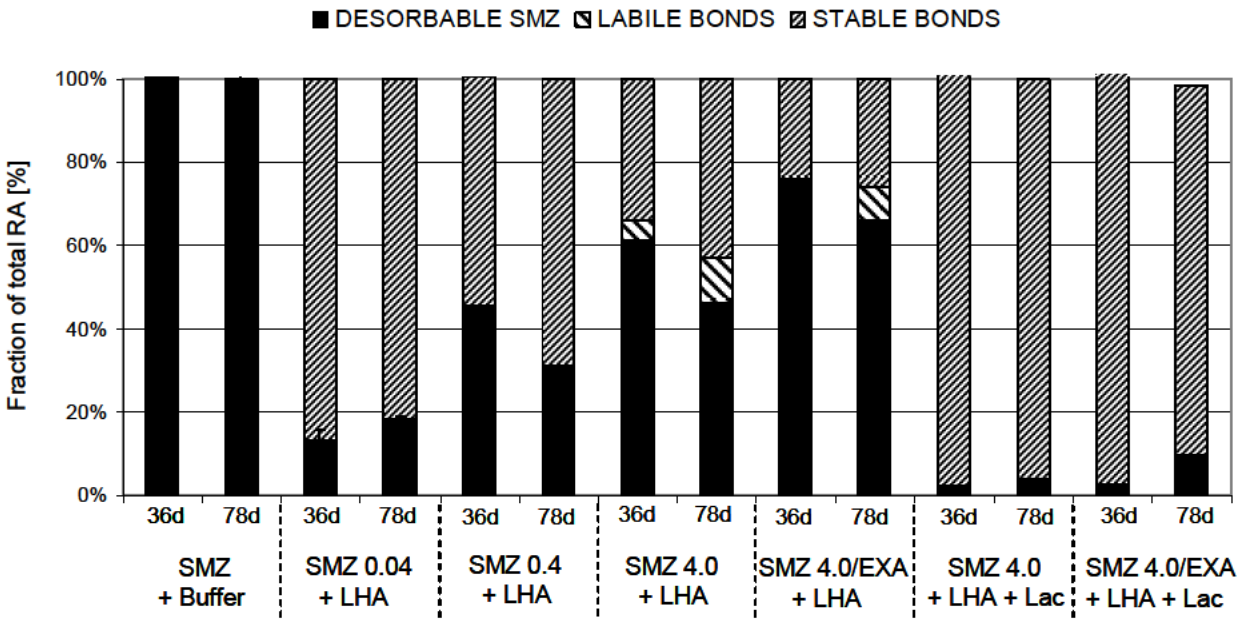

Figure 5. Percentage of desorbable SMZ and labile and stable bound SMZ to LHA after 36 d of sorption and after desorption for another 42 days (total of 78d). See text for definitions of SMZ bond stability.

A fraction of 5% labile bound SMZ of the total RA after the sorption experiment could only be deteted for the reaction of 4.0 µg/mL SMZ with LHA, while it was < 0.1% for all other samples. During the desorption experiment, the fraction of stable bound SMZ increased in all samples. For the reaction of 4.0 µg/mL SMZ with LHA in the presence and absence of EXA also the fraction of labile bound SMZ increased, suggesting that the number of sites in HA able to form stable bonds was limited at the highest SMZ concentration, but not at lower concentrations or if many reactive sites are formed by laccase.

An artificial covalent bond formation in the PLE cell during the extraction procedure between sulfathiazole and small quinone-forming "humic acid constituents" was reported in Chapter 2 and was indicated by a higher concentration of sulfathiazole determined by direct LC-HRMS as compared to PLE. This could not be observed for the extraction of the SMZ sorbed to LHA and is therefore probably less relevant for incubations with humic acids. This is likely due to the higher reactivity of humic acid constituents than LHA.

The larger portion of extractable RA than of extractable SMZ for all samples containing LHA indicates the presence of reaction or transformation products (TPs). No clear peak other than SMZ could be observed in the FSC chromatograms indicating the absence of well-defined SMZ transformation products in all samples (Figure 6). This was confirmed for known or suspected transformation products of SMZ derived from structurally similar sulfonamides (e.g. hydroxylated sulfadiazine, acetylated sulfadiazine and a photolysis product formed by extrusion of SO_2 group),[40,41] which in neither case could be detected by LC-HRMS. This suggests that the difference between extractable RA and extractable SMZ can be attributed to the presence of covalently bound SMZ, which shows up as an unresolved complex mixture in the FSC chromatogram at retention times similar to those of the humic acid as seen from the UV chromatograms obtained simultaneously (Figure 6). It is also evident from Figure 5 that only a fraction of the injected RA could be detected in the FSC chromatograms, about 54% for the LHA-containing samples, and about 14% for the samples in the presence of laccase. The recovery test in which the complete sample eluting from the column was collected and analyzed by LSC revealed that only 59 and 21% of the injected RA eluted from the LC column in the samples without and with laccase, respectively. Thus, the low recovery in FSC chormatograms resulted from both, an incomplete elution of LHA-bound SMZ residues from the LC column and RA distributed over the chromatogram in levels below the LOD of the FSC detector not resulting in a measureable peak.

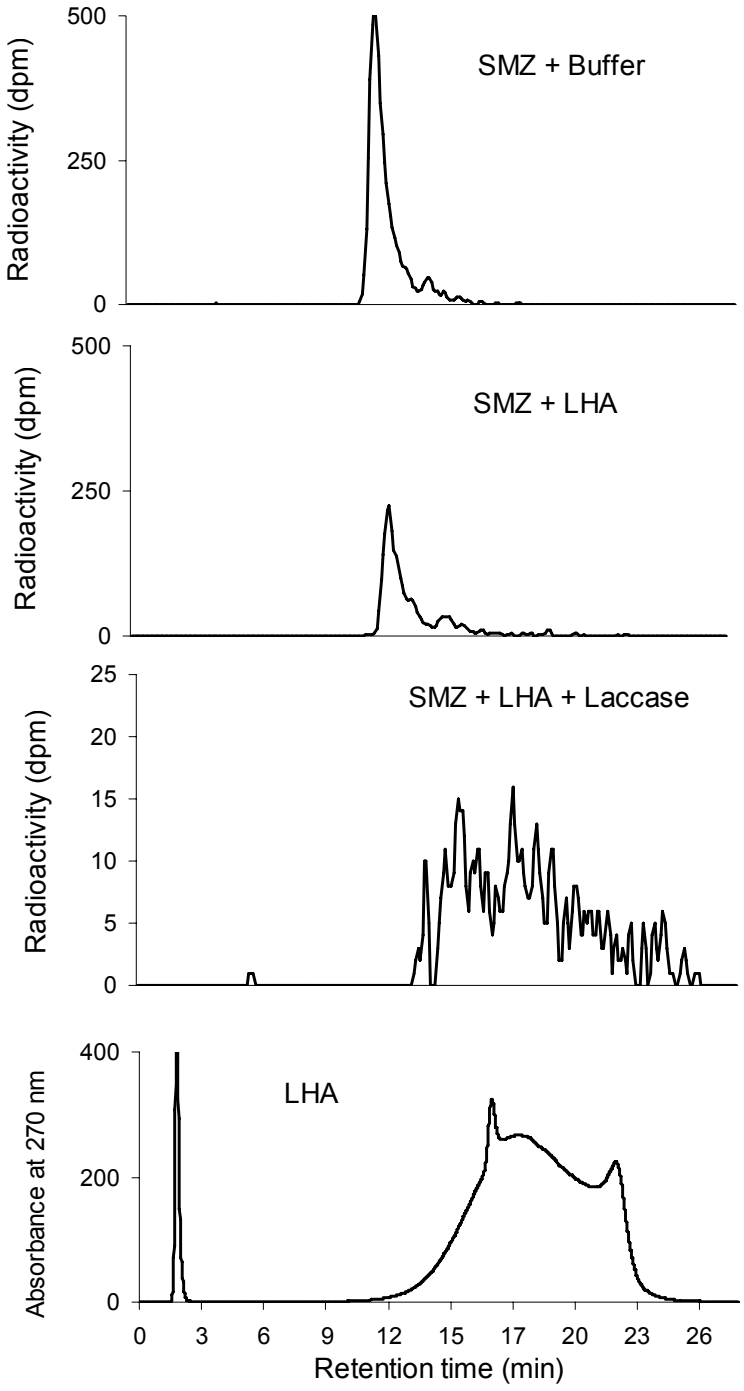

Figure 6. LC-FSC chromatograms of a SMZ standard solution in buffer and samples with LHA after 36 days of sorption with and without the presence of laccase. Note the scale for sample with laccase is 20 times enlarged. The elution profile of LHA from the LC column was monitored by the UV detector at 270 nm.

Desorption and extraction of SMZ from synthetic humic acids

For all SHAs, the desorbable and PLE-extractable SMZ ranged between 13-28% and 33-47% of initially added SMZ, respectively. These numbers indicated the labile bound fraction that accounted for 5-25% of total RA after the sorption experiment (Figure 7) and was, thus, in most cases much larger as for the reaction with LHA. Similarly, a large fraction of covalently bound sulfathiazole released by PLE was found in reaction mixtures with quinone-forming small monomers (see Chapter 2). For EnHA, NaHA and NLHA the covalent bonding of SMZ increased considerably in the presence of laccase and the extractable and desorbable fraction was comparable to that for the reaction with LHA. For AmHA, however, the addition of laccase resulted only in a small decrease of extractability and the PLE-extractable fraction was > 20% after 36 days. The fraction of labile bound SMZ decreased for all but the NaHA sample.

As for LHA there was a considerable difference between extractable SMZ and extractable RA, which amounted to 20 to > 40% in the absence of laccase, while in the presence of laccase this difference was larger except for AmHA. The LC-FSC chromatograms of reaction mixtures between SMZ and SHAs (Figure S3) showed no well-defined TPs, suggesting the presence of SMZ covalently bound to humic acids.

These results indicate that all synthetic humic acids were more reactive towards nucleophilic addition of SMZ than LHA. As the oxidative synthesis involved a formation of quinones from the phenolic precursors, a certain fraction of these quinones was likely preserved in the formed humic acids, which is smaller in the natural LHA. The higher degree of oxidation for synthetic humic acids compared to natural HAs are reported in the literature.[42]

Based on the ^{13}C CP-MAS NMR analysis (Figure 2 and Table 2) the oxygen-containing functional groups could be mainly assigned to phenols, indicated by strong resonances at 116 ppm (aromatic carbons in *ortho* position to phenolic carbons) and at 145-156 ppm (phenolic carbons). These were less prominent in the LHA spectrum, which was dominated by H-substituted aromatic carbons and *O*-alkyl groups. Despite the high oxygen content, a further oxidation of the SHAs was possible, resulting in the covalent bonding of larger fraction of SMZ in the experiments with laccase. This increase was least pronounced for AmHA, which contained about 4% of N derived from ammonia present in the solution during synthesis. Ammonia incorporation into HAs was shown to result in increase of N percentage in HA from 1.15 to 2.28 after reaction.[43] In contrast to all other SHAs, the NMR spectrum of AmHA shows two prominent resonances between 30 and 60 ppm, suggesting the presence of N-alkyl groups. Such functional groups might act

as nucleophiles themselves and compete with SMZ, which would explain the relatively high extractable and desorbable SMZ fraction in the presence of laccase.

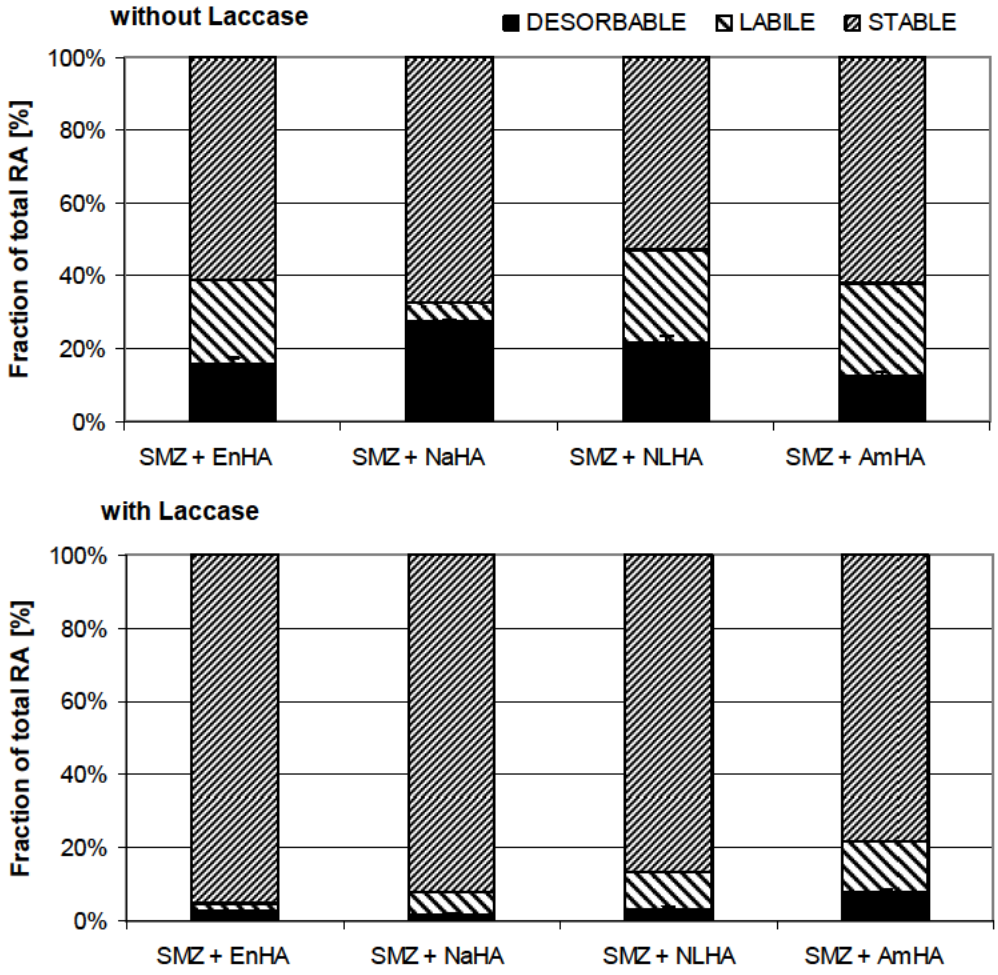

Figure 7. Percentage of desorbable SMZ and labile and stable bound SMZ to different synthetic humic acids after 36 days of sorption without and with laccase. See text for definitions of stability.

Implications for NER formation of sulfonamides in soil

Our study clearly shows that organic matter contains a small pool of reactive quinones able to form stable covalent bonds with sulfonamides, which might be important at low concentrations, but affected by competition with other nucleophiles present. Such a fraction might be responsible for the "instantaneous" NER formation of sulfonamides upon input into soils. The oxidation of organic matter (in this study by laccase) results in a *de novo* formation of a large number of reactive quinones and an extensive covalent bond formation. In that case, competition with other nucleophiles is of less importance.

A fraction of the covalently bound sulfonamides is released by exhaustive extraction procedures such as PLE, and this fraction might explain the large differences in extractability between mild[13] and harsh extraction methods[13,14] already at day 0 of incubation. The increase in bonding strength during the desorption experiment further supports the hypothesis that the slow NER formation with later incubation times could be caused by an increased covalent bonding strength (see Chapter 2). The desorption conditions reflect in this respect the fate in soils, as the (bio)available portion is removed by degradation or translocation in the pore water and replenished by desorption from the solid phase. However, other processes must play a role in soil, as the differences in NER formation based on the mild and harsh extraction methods are large, and the labile covalently bound fraction was rather small in the natural Leonardite HA. Yet, soil HAs might show different reactivities and are only a fraction of the whole soil organic matter pool.

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Supporting Information Available

(1) Text and figures addressing humic acid synthesis and purification. (2) Control experiments in the cell-cell system. (3) LC-HRMS analysis of sulfamethazine. This information is available.

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Supporting information for Chapter 3

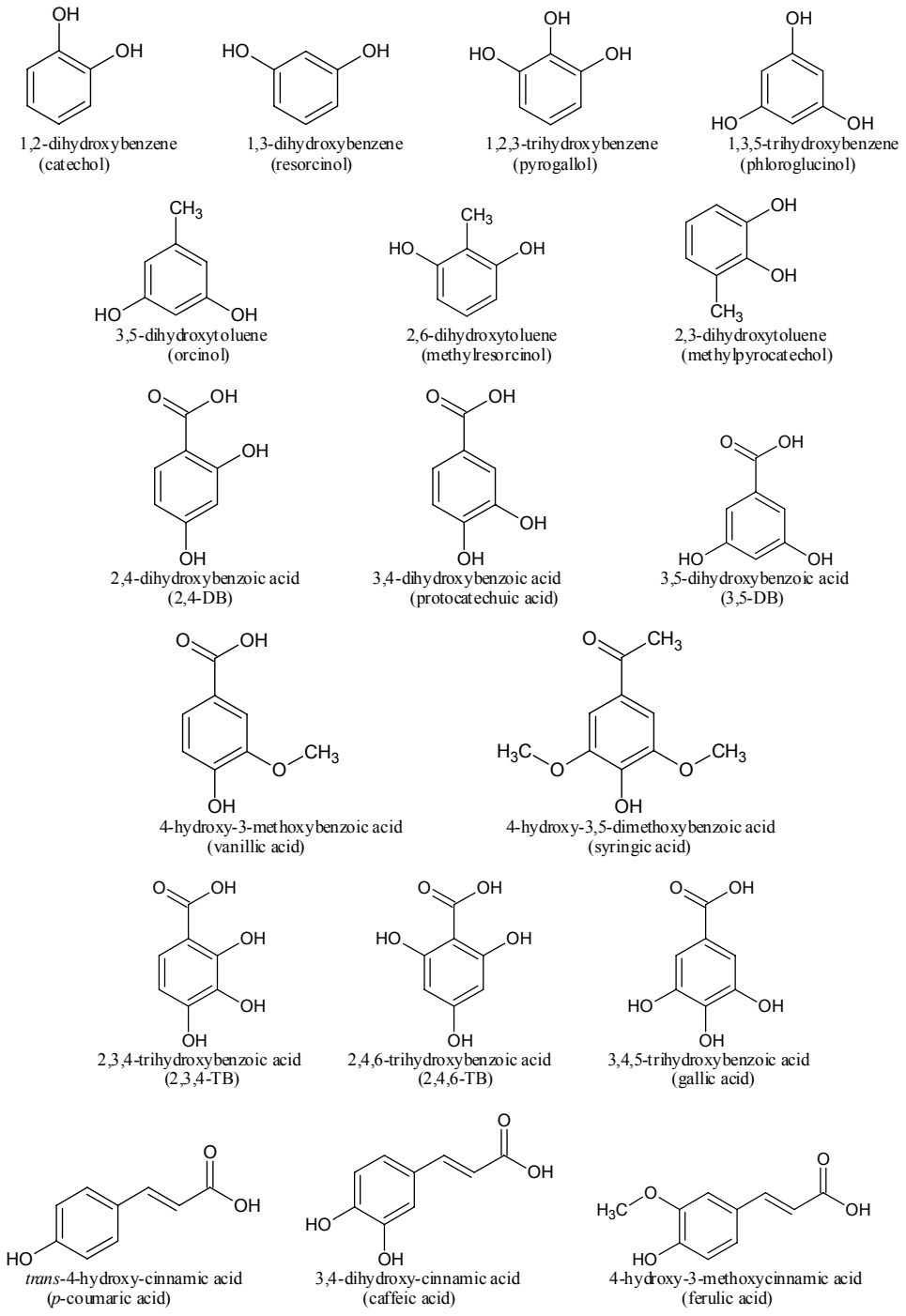
Materials and Methods

Humic Acid Synthesis and Characterization

Chemicals. Eighteen different compounds given in Table S1, such as di- and tri-hydroxybenzenes and-benzoic acids were used for the synthesis of HA-like polymers. All chemicals were at least of *pro analysi* grade and were obtained from Sigma-Aldrich (Buchs, Switzerland), Alfa Aesar GmbH & Co. (Karlsruhe, Germany) or Merck, (Darmstadt, Germany). Nonylphenol (isomer mixture, 99%) and hydrogen peroxide (H_2O_2) (30%) were obtained from Merck. Ammonium acetate, ammonium citrate, sodium acetate, sodium citrate, or potassium phosphate buffers were used (Merck, Darmstadt, Germany). Ammonia (25%), sodium hydroxide (32%), acetic acid (100%), hydrochloric acid (32%), nitric acid (65%), and formic acid (> 98%) from Merck (Darmstadt, Germany) were used to adjust the pH. Unless otherwise stated, de-ionized water purified with a Barnstead Nanopure unit (Skan AG, Allschwil, Switzerland) was used throughout the study. HPLC grade water and methanol were obtained from Acros Organics (Geel, Belgium).

Enzymes. Purified horseradish peroxidase (HRP) (type IX, E.C. 1.11.1.7. with a specific activity of 87 units/mg of solid) was purchased from Sigma-Aldrich. Specific activity is expressed in terms of pyrogallol units. One pyrogallol unit will form purpurogallin from pyrogallol in 20 sec at pH 6.0 and 20 ºC. Peroxidases are enzymes that use H_2O_2 as an electron acceptor and are known for their role in depolymerizing lignin in soils. Extracellular fungal laccase from *Trametes versicolor* (oxygen oxidoreductase E.C. 1.10.3.2.) with a reported activity of 22.4 U/mg was obtained from Sigma-Aldrich (Buchs, Switzerland). Laccases are O_2-depending multicopper oxidase enzymes, widely present in plants, fungi, and bacteria and catalyze oxidation of various phenolic and non-phenolic compounds as well as many environmental pollutants. One unit of laccase activity

Table S1. Structures of precursor compounds used for synthesis of humic acids.



is defined as the amount of enzyme that causes a change in absorbance at 468 nm of 1.0/min in 3.4 mL of a 1 mM solution of 2,6-dimethoxyphenol in citrate phosphate buffer (pH 3.8).

Purification of Leonardite humic acid (LHA). Purification (i.e., homoionization) of LHA was described earlier for the study on sulfathiazole sorption to LHA.[1] Briefly, a 2.5 g of LHA were dissolved in 10 mL of 0.1M NaOH and adjusted to pH 5 with 1 M HCl. The LHA solution was subsequently diluted with de-ionized water to a total volume of 0.5 L. The cation exchange resin (DOWEX 50 WX 8, p.a., 20-50 mesh, Fluka, Buchs, Switzerland) was saturated with Na^+ by washing the resin with 3 M NaCl until the pH in the supernatant remained constant (pH ~5.8). Sodium-saturated cationic exchange resin (~17 g) was added to the LHA solution (0.5 L). The LHA-resin mixture was subsequently shaken for 3 h on a horizontal shaker (SM-30 Control, Edmund Bühler GmbH, Tübingen, Germany) at a low speed of 60 rpm to minimize abrasion of the resin material. Following settling of the resin, the supernatant was decanted and stored. The resin was washed three times with de-ionized water. All supernatants containing Na^+-saturated LHA were pooled, followed by precipitation of LHA by lowering the pH to 1 using 1.0 M HCl. After centrifugation (10.000 × g, 30 min) the LHA precipitate was re-dissolved in 0.1 M NaOH (pH 4-5) and filled into dialysis tubes as described above. Dialyzed LHA was freeze-dried and stored in a desiccator in the dark until further use.

Polymerization of synthetic humic acid (SHA). The following polymerization methods utilized the phenolic precursor mixtures given in Table S2.

Enzymatic synthesis (EnHA and NLHA)

A nitrogen-free synthetic humic acid (EnHA) was prepared by radical polymerization using the peroxidase-H_2O_2 procedure described by Martin and Haider (1980).[2] Briefly, the precursors (1.5 mM each) were dissolved in ammonium citrate buffer (2 mM, pH 5.5), the pH was readjusted with 4 M NaOH, and 6 mg of horseradish peroxidase (HRP) dissolved in the same buffer was added. Simultaneously, the precursor solution and 1000 mL of an aqueous solution of 0.03% H_2O_2 were added slowly under continuous stirring using a peristaltic pump. After addition was complete, the solution was reduced to a volume of 500 mL on a rotary evaporator, acidified to pH 1 with 6 M HCl and left for 24 h to allow precipitation of humic acid.

A humic acid containing hydrophobic moieties (NLHA) was synthesized using a precursor mixture and nonylphenol according to Tanaka *et* al. (2003).[3] The precursor mixture consisting of 10 mM of the phenolic compound and 10 mM of nonylphenol were dissolved in 0.1 M sodium acetate buffer. After adjusting pH to 5.0 with acetic acid, 0.8 U/mL of laccase solution dissolved in the same buffer was added. The mixture was heated at 40 °C for 4 hours. After cooling, the pH was adjusted to 1.0 with HCl and the solution was allowed to precipitate over 1 d.

Alkaline oxidative polymerization (AmHA and NaHA)

This procedure of alkaline oxidative polymerization followed Hanninen *et* al. (1987).[4] Briefly, a precursor mixture (10 mM of each compound) was dissolved in de-ionized water. The pH of one batch of precursor mixture was adjusted to 9.0 with ammonia while NaOH was used for the other batch. After pH adjustment 3 mL of 30 % hydrogen peroxide were added to the mixture. The solution was carried at 50°C for 2 h under continuous stirring and then allowed to stand overnight. The HA was precipitated at pH 1.0 with HCl for 24 h. The synthesis carried out in the presence of ammonia resulted in humic acid with incorporated nitrogen, AmHA. The synthesis carried out in the presence of aqueous sodium hydroxide resulted in a nitrogen-free polymer, NaHA.

Purification of synthetic humic acids

After standing for 24 h, each HA precipitate was separated from the supernatant by centrifugation at 10.000 × G for 30 min and. During repeated centrifugation the precipitated polymers were washed several times with de-ionized water to remove soluble monomer residues during repeated centrifugation. Afterwards the polymers were redissolved in de-ionized water and transferred to dialysis tubes (Spectra/Por; CE, 500 MWCO, Socochim, Lausanne, Switzerland). The samples were dialyzed against de-ionized water until the conductivity and the total organic carbon content of the water did not exceed the values of de-ionized water, which was the case after 12 days. After dialysis was completed, the polymers were freeze-dried and stored in a desiccator.

Table S2. Model compounds used in precursor mixtures for humic acid synthesis.

Precursor	**EnHA**	**AmHA**	**NaHA**	**NLHA**
Concentration of each compound	**1.5 mM**	**10 mM**	**10 mM**	**10 mM**
Catechol	✓	✓	✓ (×2)	✓ (×2)
Resorcinol	✓	✓	✓ (×2)	✓ (×2)
Methylresorcinol	✓	✓	✓ (×2)	✓
Methylpyrocatechol	✓	✓	✓ (×2)	✓
Orcinol	✓	✓	✓	✓
Pyrogallol	✓	✓	✓	✓
Phloroglucinol	✓	✓	✓ (×2)	✓
2,4-DB	✓	✓	✓	---
Protocatechuic acid	✓	✓	✓	---
3,5-DB	✓	✓	✓	---
Syringic acid	---	✓	✓ (×1.5)	✓
Vanillic acid	✓	✓	✓	✓
2,3,4-TB	✓	✓	✓	---
2,4,6-TB	✓	✓	✓	---
Gallic acid	✓	✓	✓	---
p-Coumaric acid	✓	✓	✓	---
Caffecic acid	✓	✓	✓	---
Ferulic acid	✓	✓	✓	---
Nonylphenol isomer mixture	---	---	---	✓

✓ compound used in polymerization procedure

✓ (×2) 20 mM of compound was used instead of 10 mM.

--- compound not used in polymerization procedure

Humic acid characterization

Elemental Analysis

Carbon, hydrogen, nitrogen, and oxygen content of the humic acids were determined with an elemental analyzer (model CHNS-932, Leco, St. Joseph, MI).

Solid State Nuclear Magnetic Resonance (CP-MAS NMR)

The solid-state ^{13}C MAS NMR spectra were recorded at 100.61 MHz on a Bruker AVANCE 400 NMR spectrometer (Bruker BioSpin AG, Fällanden, Switzerland) using a broadband 4 mm cross-polarization magic-angle-spinning (CP-MAS) probe. The experiments were performed at ambient temperature at MAS rates of 12,000 Hz as ^{13}C CP-MAS experiments using contact times of 1 ms with an RF field strength of 47 kHz applying a linear ramp from 100-50% on the ^{1}H channel and with 50 kHz field strength for the 90º ^{1}H excitation pulse and during the SPINAL-64 decoupling sequence (the positions of spinning side bands of the carbonyl resonances in the region of aliphatic carbons were determined by variation of the MAS rates down to 5000 Hz, only a very weak and negligible shoulder was observed around 51 ppm in the ^{13}C CP-MAS NMR spectra).[5] For a reasonable signal-to-noise ratio of the carbonyl resonances we accumulated 15k free induction decays (FID). The chemical shifts were externally referenced to the carbonyl resonance of the glycine CP-MAS setup sample at 176.0 ppm and the FID's were multiplied by an exponential window function of 100 Hz before Fourier transformation.

For the interpretations of the ^{13}C CP-MAS NMR spectra, the overall chemical shift range was divided into the following main resonance regions: alkyl-C (0-45 ppm), methoxyl-C and N-C (45-60 ppm), O-alkyl-C (60-110 ppm), aromatic-C (110-160 ppm), phenolic-C (140-160 ppm), carboxyl-C (160-185 ppm) and carbonyl-C (185-200 ppm).

High Resolution Mass Spectrometry (HRMS)

For the molecular characterization of humic acids, full scan mass spectra were acquired using the high resolution LTQ Orbitrap XL hybrid instrument (Thermo Scientific, San Jose, CA). An amount of 0.5 mg of HA was dissolved in 1 mL of 50% aqueous methanol and samples were directly infused into the ion source using a syringe pump at a flow rate of 20 µL/min. The sheath gas flow was set to 15 arbitrary units, the auxiliary gas flow to 5 arbitrary units;

and a spray voltage of 4.0 kV and a transfer capillary temperature of 350 °C were used. The spectra were acquired in positive and negative ion mode using electrospray ionization (ESI) at a pre-set resolving power of 100,000 at full width-half-maximum (fwhm) referenced to *m/z* 400. The spectra were acquired in two separate runs with the ranges *m*/*z* 100-800 and 150-2000 m/z at maximum ion injection times of 600 ms and processed by averaging scans.

Control experiments in the cell-cell system

The diffusion kinetics of SMZ across the memebrane in the absence of humic acid was determined by adding 3.5 mL of a buffered SMZ solution (4 μg/mL) to one half-cell (SMZ half-cell) and corresponding buffer to the other half-cell (buffer half-cell). The sorption kinetics of SMZ to LHA were determined using the same SMZ solution in one half cell and a 1.6 g/L LHA solution in the second half-cell. Both experiments were run in duplicate. Cells were shaken horizontally (170 rpm) at 19°C in the dark. Sample volumes of 20 μL were withdrawn from both half-cells after 4, 8, and 20 h, 1, 2, 5, 8, 11, 17, 20, 25, 30, and 36 d of equilibration. At each sampling time-point, the SMZ radioactivity was determined in both cells by liquid scintillation counting (LSC). SMZ sorption to the dialysis membrane was determined by subtracting the sum of radioactivity obtained from both half-cells at the end of the sorption experiment after 36 days from the initial radioactivity spiked to the cell-cell system.

LC-HRMS analysis of sulfamethazine

SMZ was quantified by liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) using an autosampler (HTC PAL, CTC analytics, Zwingen, Switzerland), Rheos 2000 LC pumps (Flux Instruments, Basel, Switzerland), and a LTQ Orbitrap XL (Thermo Scientific, San Jose, CA). A sample aliquot of 30 μL was injected into a 20 μL injection loop and separated by gradient elution at a flow rate of 200 μL/min using a Nucleodur C18 Gravity column (125 mm × 2 mm, 5 μm particle size; Macherey-Nagel, Oensingen, Switzerland) equipped with a guard column. The mobile phases consisted of water (A) and methanol (B), both containing 0.1% of formic acid. The composition of the mobile phase was changed linearly from 5% B at the start to 95% B at 14 min before re-equilibration to starting conditions. The MS was operated in full scan mode with a range of m/z 50-600 with the following electrospray ionization (ESI) conditions: positive mode, spray voltage 5 kV, sheath gas flow rate 50 arbitrary units, auxiliary gas flow rate 20 arbitrary units, ion transfer capillary temperature 300°C. The resolving power of the Orbitrap was set to 7500.

Calibration curves were constructed from two rows of calibration standards measured before and after the samples. Quality control standards were measured after each 10 injections to check for instrumental drift. Analysis was stopped and a new calibration curve was constructed if the quality control standard was not within ± 10% of its theoretical value. The limit

of quantification (LOQ) defined by a signal-to-noise ratio of 10:1 was 10 ng/mL; the limit of detection, defined by a signal-to-noise ratio of 3:1, was 1 ng/mL. Instrumental blank samples consisting of water/methanol 90:10 (v/v) were run to check for analyte carryover.

The ion suppression in ESI was investigated by spiking HA-SMZ solution from sorption experiments and PLE extracts with 1 µg/mL SMZ standard solution for two sets of samples in duplicate. Accordingly, two control samples were prepared the same way as either for direct LC-HRMS analysis or for PLE extract analysis. The average values for matrix recovery were 86 ± 3% (n = 2) and 94 ± 1% (n = 2) for direct LC-HRMS and PLE extract analysis, respectively. All reported data were not corrected for these matrix recoveries.

Results and Discussion

Control experiments in the cell-cell system

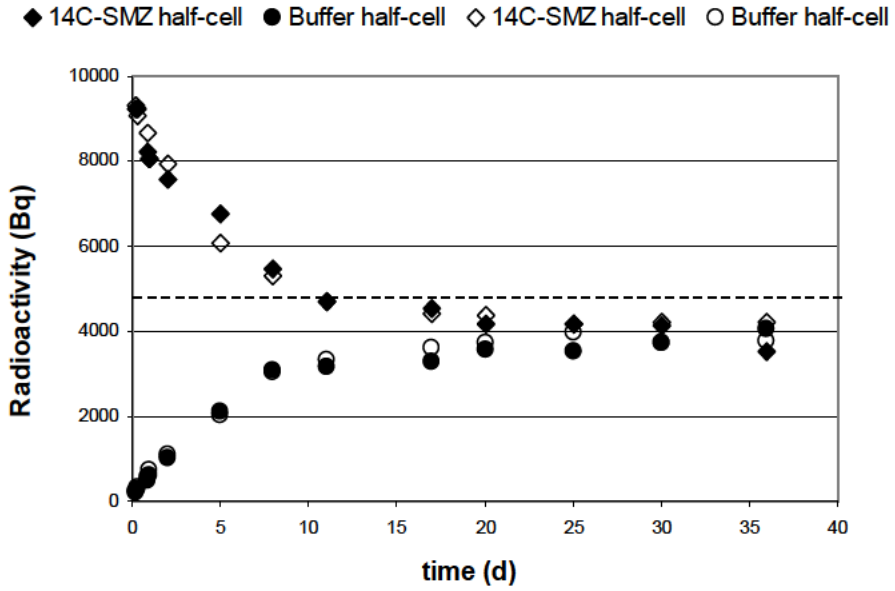


Figure S1. Diffusion kinetics of sulfamethazine (SMZ) across the dialysis membrane in the cell-cell system. The experiment was run in duplicate (open and closed symbols). The dashed line represents the theoretical equilibrium distribution between both half cells if no sorption to the membrane occurred.

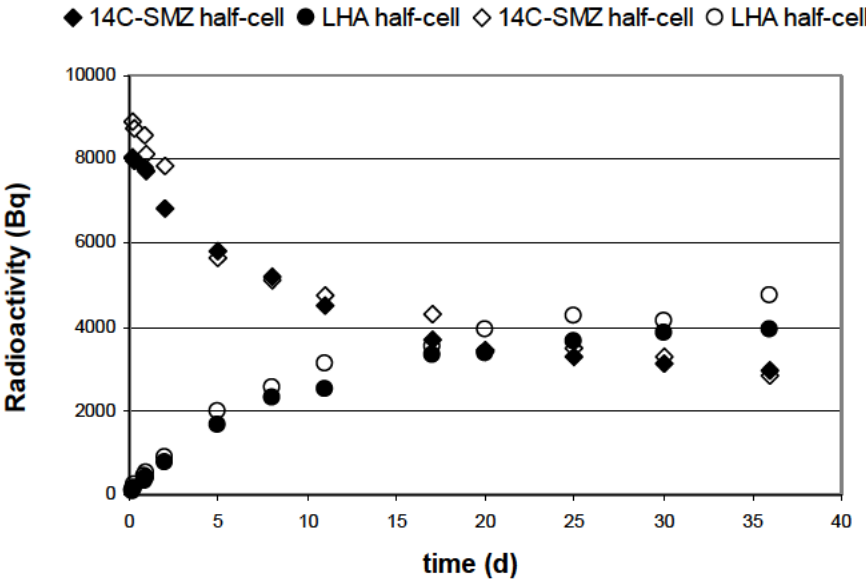


Figure S2. Sorption kinetics of sulfamethazine (SMZ) to Leonardite humic acid (LHA) in the cell-cell dialysis system. The experiment was run in duplicate (open and closed symbols).

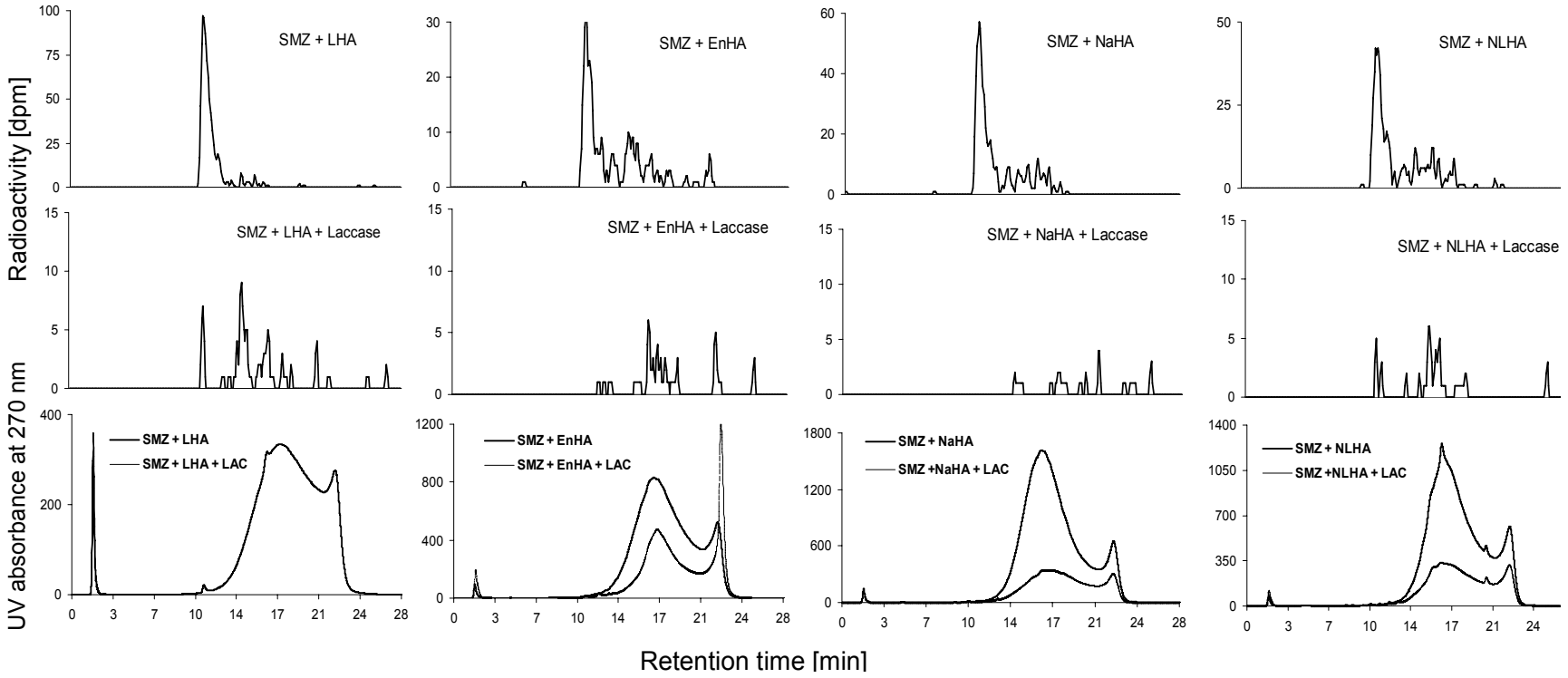


Figure S3. LC-FSC chromatograms of SMZ after 36 d of reaction with Leonardite humic acid (LHA) and different synthetic humic acids with and without the presence of laccase. The elution profile of humic acids from the LC column was monitored by the UV detector at 270 nm.

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Chapter 4

Nonextractable residue formation of sulfonamide in soil

Understanding nonextractable residue formation of sulfonamide antimicrobials by manipulation of agricultural soil
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to be submitted to Environmental Pollution

Introduction

Sulfonamide antimicrobials (SAs) are among the most widely used veterinary antimicrobials worldwide[1] and reach soils mainly by grazing livestock or manure application. Although physiochemical properties of neutral SAs suggest a high mobility in the environment, a large fraction is retained in soil.[2-4]

Long-term studies on the fate of SAs in soils demonstrated that a large portion forms nonextractable residues (NERs) along with transformation products and a very low mineralization.[3,5-7] In these studies sequential extraction and fractionation techniques were applied to monitor extractability of ^{14}C-labelled sulfadiazine in long-term soil incubation experiments. Although the extent of NER formation differed among these studies due to different extraction methods used, a common observation was an initially fast formation of NERs up to 50% of the applied amount within a few days. This is indicative of a chemical reaction rather than slow sorption processes, but further conclusions on mechanisms of NER formation in soils could not be drawn.

The most reactive functional group of SAs is their common aromatic amino group, and direct evidence on the covalent bonding via this group was obtained using humic model constituents and humic acids (see Chapter 2 and 3).[8-10] These studies also showed that nucleophilic addition reactions to quinones are predominantly responsible for covalent bond formation rather than radical reactions based on one-electron oxidation of organic matter or sulfonamides. Most of these reactive quinones are not stable and consequently only a small number of reactive sites is present in humic acids to readily react with SMZ (see Chapter 3). In contrast, the formation of quinones by oxidants such as MnO_2 or oxidative enzymes greatly enhances covalent bonding. This covalent bonding to humic acids and model quinones in the presence of oxidants was also demonstrated for other aromatic amines.[11-14] The studies in Chapter 2 and 3 furthermore indicated that covalent bonds might undergo hydrolysis under typical harsh extraction conditions, thus covalent bonding does not necessarily mean NER formation.

As oxidants such as oxidase enzymes, Mn oxides and possibly Fe oxides are also abundant in soils, these are likely to play a pivotal role in the NER formation of sulfonamides in aerobic topsoils. Studies on other aromatic amines have demonstrated the role of Mn oxides in NER formation,[15] although the reduction of Mn(III)/Mn(IV) was not sufficient to account for all NERs formed.

The objective of our study was to assess the factors governing NER formation of ^{14}C-labelled sulfamethazine in soils. To this end, a selective manipulation of experimental conditions in soil incubation studies as compared to an aerobic control was done. This was achieved by anaerobic

incubation or a removal or addition of manganese oxides and inhibition of oxidative enzymes The addition reaction itself was targeted by addition of a competing nucleophile and addition of selected model hydroquinones to increase the pool of compounds available to form quinones.

Materials and methods

Chemicals

Sulfamethazine (^{12}C-SMZ; 4-amino-*N*-(4,6-dimethyl-2-pyrimidynyl)-benzenesulfonamide; Sigma-Aldrich, Buchs, Switzerland) and the stronger nucleophile *p*-ethoxyaniline (EXA; for synthesis, Merck, Darmstadt, Germany) were used as received. SMZ labeled at the phenyl ring (^{14}C-SMZ; 3.7 MBq/mL, specific activity 2.035 GBq/mmol, 99% in ethanol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). Stock solutions of SMZ, ^{14}C-SMZ, and EXA were prepared in methanol. The latter was diluted in water to the desired concentration and was directly used in incubation experiments. The ^{14}C-SMZ solution was diluted with unlabeled SMZ solution in acetate buffer to achieve a starting SMZ concentration of 97.16 µg/mL and an activity of 33.44 kBq/mL.

HPLC grade methanol, acetonitrile and water (Acros Organics, Geel, Belgium) were used as solvents for extraction and liquid chromatography eluents. All other chemicals were at least of "pro analysi" grade and obtained from Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland).

Soil Sample

An agricultural soil sample was taken in an intensively farmed area in the northern part of the Canton of Zurich, Switzerland. A stainless steel probe with a tube of 5.4 cm diameter was used for soil sampling. 20 sub-samples from 0-5 cm depth were taken randomly across the field and combined into a polypropylene box tightly sealed with a lid for storage. Directly after sampling, the soil was stored at -20°C. Prior to use, the soil was homogenized with a hammer mill at Harlan Laboratories (Itingen, Switzerland) in a frozen state in the presence of dry ice. After milling, the soil was left overnight outside with open lids to allow all the CO_2 to evaporate. Subsequently, the soil sample was again stored at - 20°C until use.

The soil type was classified as a sandy loam Cambisol with a soil organic carbon content of 30 g/kg and nitrogen content of 2 g/kg, and the soil pH 7.9 in water as determined according to method ISO 10390:2005.[16] The maximum water holding capacity of the soil sample was 37%.

Selective manipulation of soil samples

The soil samples were preincubated in the laboratory at room temperture for approximately 30 hours to allow a re-growth of the microorganisms after freezing. Two aerobic samples were incubated as control samples and the following soil treatments were carried out to affect NER formation of added SMZ.

(i) Anaerobic treatment. The soil was incubated under anaerobic conditions in a glove box under N_2 to prevent a re-formation of Mn oxides from Mn^{2+} released during oxidation and/or a re-oxidation of O_2-depending enzymes. Incubation bottles filled with soil were left under vacuum for 12 hours to exhaust the soil gases prior to pre-incubation for 30 hours in the glove box. The SMZ spiking solution and water for water content adjustment were purged with argon before transfer to the glove box.

(ii) Addition of a hydroquinone mixture. A mixture of catechol, resorcinol, 3,4-dihydroxybenzoic acid and syringic acid was added to the soil right before SMZ addition, yielding a final concentration of 425 µg/g of soil.

(iii) Addition of synthetic birnessite (δ-MnO_2). Acid birnessite was synthesized according to procedure outlined by McKenzie (1971)[17] by dropwise addition of 10 M HCl to a boiling solution of 0.4 M $KMnO_4$. After boiling for a further ten minutes, the precipitate was filtered, washed with deionized water and dried at 40°C in an oven. The resulting oxide particles were ground and passed through a 75 µm sieve. The X-ray diffraction pattern (not shown) consisted of 4 lines at *d*-values of 7.27, 3.60, 2.44 and 1.42 Å, which was in good agreement with previously published data.[18-20] An aliquot of birnessite was added to soil yielding a final concentration of 31 mg/g and pre-incubated for 3 days at room temperature.

(iv) Selective removal of Mn oxides from soil was done according to the method of Li *et* al. (2003).[15] 100 g of wet soil were dried at 105°C until the weight was constant. The soil was divided into two 250 mL centrifuge bottles. The removal procedure consisted of the following steps, each of them succeeded by shaking, centrifugation at 5000 × g for 15 min and decanting. All supernatants were combined and stored for further analysis. First, hydroquinone (0.018 M) in ammonium acetate buffer (1.0 M, pH 7.0) was added to dissolve the readily reducible Mn(III)/Mn(IV) fraction. Subsequently, the samples were washed with tap water. Second, a mixture of sodium citrate (0.3 M) and sodium bicarbonate (0.2 M) with sodium dithionite (20.2 g) was added to reduce the Mn(III)/Mn(IV) oxides in more recalcitrant minerals including those

in concretions and accumulations as well as Mn substituted in iron oxide minerals. The soil was washed with tap water twice, dried in oven at 40°C and homogenized. The water content was adjusted to 6.15% WHC. An aliquot of fresh (untreated) soil (23.9 g) was added to the Mn-extracted soil (95.6 g) and incubated for three days at 20°C to allow a re-growth of soil microorganisms. The supernatant collected from centrifugation steps was acidified with nitric acid and the released Mn concentrations were determined by an inductively coupled plasma mass spectrometer (ICP-MS).

(v) Inhibition of natural peroxidase activity in soil was done by adding 57 µL of 1.0 M phenylhydrazine per g of soil one day prior to the addition of SMZ.[21]

(vi) Addition of EXA one day prior to the addition of SMZ at a molar ratio of EXA to SMZ of 3:1.

(vii) Addition of EXA simultanously with SMZ at a molar ratio of EXA to SMZ of 3:1.

Incubation experiment

The batch incubation experiments were carried out in 100 mL glass bottles for 45 days at room temperature and 40% of WHC. For each treatment, 60 g of dry weight equivalent soil were used, from which 5 subsamples of 12 g to be sampled after 1, 3, 10, 24, and 45 days of incubation were distributed into 5 different bottles.

Using a 500 µL glass syringe, SMZ standard solution was added dropwise to the soil surface to obtain a final concentration of (3.8 µg/g soil, corresponding to 1.3 kBq/g soil). The spiked soil was thoroughly mixed using a stainless steel spatula. Finally, the water content in all sub-samples was adjusted to 40% of WHC. The incubation bottles were vented every 7 days (except for anaerobic samples), after which the water content was readjusted, if necessary.

On each sampling day, the 12 g of each soil sample were mixed thoroughly and divided evenly into two vials. Only one vial was further analyzed, while the other was stored at -20°C as a back-up sample. To quantify any non-transferred ^{14}C-SMZ the incubation bottles were rinsed with methanol and the rinsing solutions were analyzed by liquid scintillation counting (LSC). The analysis showed that less than 0.1% of the initial SMZ mass in soil remained in the bottles.

Sequential extraction and fractionation of soil samples

An overview of the procedure used for the assessment of SMZ extractability and NER formation is presented in Figure 1. The total extractable fraction of SMZ was determined by pressurized liquid

extraction (PLE) based on a method for soil samples.[22] The extractable fraction of SMZ was determined by LC-HRMS analysis of these extracts and extractable RA was determined by LSC measurements. The fraction of SMZ that could not be extracted by this PLE method was defined as NER and was determined by solid combustion and trapping of $^{14}CO_2$. Additionally, the PLE-extracted soil at day 45 was fractionated using methyl isobutyl ketone (MIBK) method to obtain a distribution of NERs among fulvic acid, humic acid, humin and residual soil fractions.[23-25]

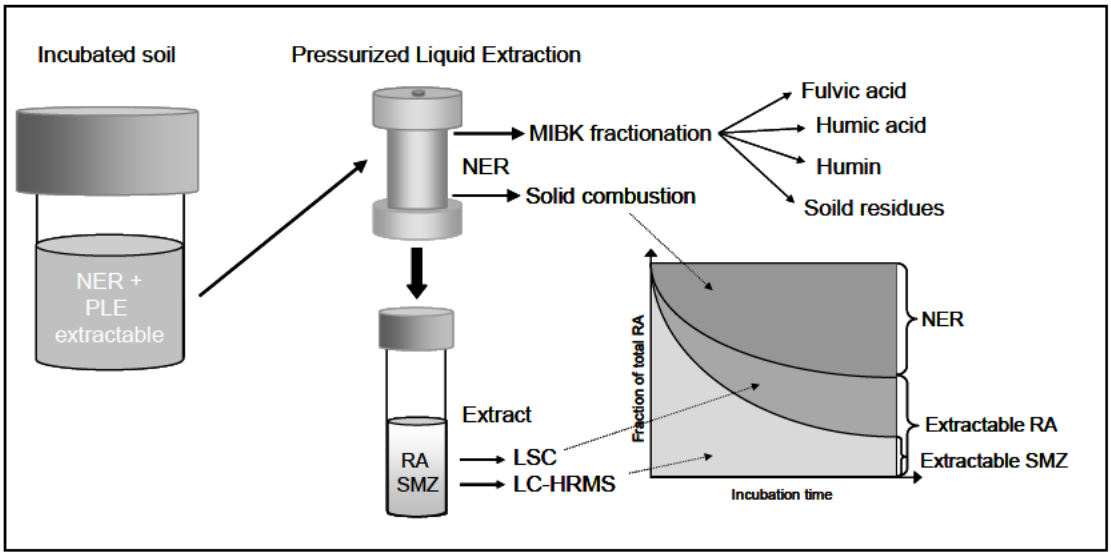

Figure 1. Extraction and fractionation procedure for the assessment of SMZ extractability and distribution among nonextarctbale residue fractions (NER: nonextractable residue; PLE: pressurized liquid extraction; MIBK: methyl isobutyl ketone; RA: radioactivity; SMZ: sulfamethazine; LSC: liquid scintillation counting; LC-HRMS: liquid chromatography coupled to high resolution mass spectrometry.

Desorption

After 45 d of incubation, the weakly sorbed SMZ was removed in a desorption experiment (2×15 d) in the custom-made dialysis systems used to study the sorption-desorption of sulfonamides with humic acid (see Chapter 3).[26] Each system consisted of two 4 mL half-cells separated by a dialysis membrane (Spectra/Por Biotech, Cellulose ester, 500 Da MWCO, Socochim; Lausanne, Switzerland), which was permeable to the SMZ. One half-cell was filled with approximately 2 g of soil followed by 2.5 mL of acetate buffer (pH 5.0). The other half-cell was filled with 200±10 mg of Oasis HLB soild-phase extraction sorbent (Waters, Rupperswil, Switzerland) as infinitive sink material and 3.5 mL of acetate buffer. Cells were shaken horizontally at 170 rpm for 52 days at 19°C to desorb SMZ from the soil. The Oasis suspensions of both desorption steps were combined and transferred completely to 6 mL polyethylene SPE tubes

with polyethylene frits placed on the bottom (Supelco, Bellefonte, PA). To ensure a complete transfer, these half-cells were flushed 6 times with HPLC grade water (3 mL each) and the water was transferred to the filtration tubes. The cartridges were dried under vacuum. The soil suspensions from the other half-cells were dried in oven and both, dried Oasis material and dried soil were transferred to 2 mL vials and stored at 4°C until solid combustion analysis (see below). To account for potential transformation products in desorbable fractions, selected aliquots of dried Oasis material were transferred on top of SPE cartridges (60 mg Oasis HLB 3cc extraction cartridges; Waters), which had previously been conditioned by 2 mL of methanol and 2 mL of water. The cartridges were eluted with 2×5 mL methanol. The eluate was reduced to a volume of 0.1 mL under a nitrogen stream and the final volume was adjusted to 1 mL with water, thoroughly mixed, and then transferred into an LC vial for LC-HRMS analysis.

Pressurized Liquid Extraction (PLE)

Extractable SMZ and RA were determined at every sampling day of the soil incubation experiment. A 10 mL stainless steel cell was filled with about 1 g of diatomaceous earth. A weighted amount of about 3 g of incubated soil was transferred into the extraction cell and slightly compacted. The remaining volume was filled up with diatomaceous earth. Details of the extraction procedure are given in Chapter 2. The mass of the extracts was recorded and an aliquot of 1 mL was withdrawn from the collection vial and filtered into a 2-mL LC vial using a 0.2 µm PTFE syringe filter. SMZ was analyzed by LC-HRMS and total RA in the raw extract by LSC. The PLE-extracted soil was collected in amber glass vials and stored at -20 ºC.

Control samples containing only SMZ were spiked to a cell filled with diatomaceous earth. Blank samples consisting of acetate buffer were run to check for cross-contamination. A loss of free sulfathiazole during PLE with hydroquinones was reported in Chapter 2 possibly due to oxidation to quinones and reaction with SA in the PLE cell. Although this effect was shown to be less pronounced in a similar study with natural and synthetic humic acids (Chapter 3), control experiments were carried out with the reagents used for the manipulation of soil samples (i.e. birnessite, phenylhydrazine, hydroquinones). Diatomaceous earth was spiked with SMZ and the corresponding reagent at the same ratio as in the soil incubation samples, incubated for 16 hours and extracted by PLE.

Fractionation of the nonextractable soil residues derived from ^{14}C-SMZ

Selected PLE-extracted soil samples (about 3 g) with a substantial NER fraction were shaken in NaOH solution (100 mL, 0.5 M) at 180 rpm for 24 h. The mixtures were transferred to separatory funnels to which 75 mL of MIBK were added and acidified to pH 1.0 with 32% HCl. After vigorous shaking and phase separation for 1 h the aqueous phase containing the fulvic acid and soil residual fraction was transferred to 100 ml bottles and the latter was separated from the fulvic acid solution by filtration. A volume of 100 mL of 0.5 M NaOH solution was added to the remaining MIBK phase, shaken vigorously and equilibrated for 1 h. After phase separation the aqueous phase containing the humic acid fraction was discarded to a 100 mL bottle, while the MIBK phase was washed six times with the total of 100 mL of 0.1 M HCl. The washing solution was collected together in a 100 mL bottle. The humin fraction remained dissolved in the organic phase and was transferred to a 100 mL bottle. RA in the humic acid, fulvic acid, and humin fractions was determined by LSC. The soil residue fraction was dried in an oven at 40°C and analyzed by LSC after solid combustion (see below). To quantify the OM removal during the extraction, the solid residual fraction was analyzed for TOC.

Instrumental analysis

To quantify the RA in soil extracts aliquots were added to 10 mL of Hionic Fluor liquid scintillation cocktail (Perkin Elmer, Waltham, MA) and analyzed with an LSC (2200 CA TriCarb Liquid Scintillation Analyzer, Packard). Samples were counted three times for 10 min and counts were corrected for background activity by blank controls. To determine RA in solid samples (soil, Oasis sorbent from desorption experiment) an aliquot of 50 mg was placed in a cellulose thimble and combusted using a Perkin Elmer Sample Oxidizer. The CO_2 evolved was trapped in 5 mL of Carbosorb E, and mixed with 15 mL of Permafluor E+ scintillation cocktail (Perkin Elmer, Waltham, MA). All samples were analyzed with a Perkin Elmer Liquid Scintilation Analyser, TriCarb 2800TR. Every ten samples one control with defined radioactivity and one blank sample were run for quality control.

SMZ concentrations in sample extracts were determined by liquid chromatography high resolution mass spectrometry (LC-HRMS) using an autosampler (HTC PAL, CTC analytics, Zwingen, Switzerland), Rheos 2000 LC pumps (Flux Instruments, Basel, Switzerland), and the high resolution LTQ Orbitrap XL hybrid instrument (Thermo Scientific, San Jose, CA). Details for LC-HRMS analysis and quality control are given in Chapter 3.

To distinguish free ^{14}C SMZ from possible transformation products, PLE extracts from sampling day 45 were analyzed using HPLC (Agilent 1100) coupled to a diode array detector (DAD) followed by a flow scintillation detector (Packard 500TR series). Selected extracts (i.e., from untreated anaerobic, Mn depleted, and peroxidase inhibited samples) were concentrated to 0.5 mL, to which 0.5 mL methanol was added. Samples were filtered through 0.45 μm cellulose filter and aliquots of 40 μL were injected into the LC-FSC system. Separation was done on a reversed phase Nucleodur C_{18} Gravity column (125 mm × 2 mm, 5 μm particle size; Macherey-Nagel, Oensingen, Switzerland) equipped with a guard column with gradient elution of water (A) and MeOH (B) with a flow of 0.2 mL/min for 28 min. The LC program started at 90% of solvent A, increasing solvent B to 95% in 13 min held for 3.5 min, returning back to 90% of solvent A in 1 min and re-equilibration for 10.5 min. After the DAD detector, the eluent was automatically mixed with 0.8 mL/min of Ultima Flo-M scintillation cocktail (Perkin Elmer, Waltham, MA) and pumped through a flow scintillation counter. The UV detector wavelength was set to 270 nm to simultaneously monitor eluting organic matter. After each sample a blank was run to remove OM residues from the column. The retention time of compounds eluting from the extracts was compared to a standard solution of ^{14}C SMZ. A recovery test of the LC method was done by a comparison of the radioactivity injected into the LC system to that leaving the LC column during a single run. Briefly, the capillary that delivers the eluent to the FSC detector was disconnected from this detector and the eluent from the complete LC run was collected into a 20 mL vial. An aliquot was mixed with 10 mL of Hionic Fluor scintillation cocktail and quantified by LSC. The RA was compared to that obtained by LSC of that sample.

Results and Discussion

Extractability in soil under aerobic and anaerobic condition

The level of the total RA did not change during incubation (45 days) for any of the different treatments, indicating that no volatilization or mineralization of SMZ residues occurred during the experiment. This is in line with the literature reporting a mineralization below 2% up to 218 days.[5,6] Blank samples showed no SMZ background concentration in soil at the beginning of experiment and no cross-contamination during the incubation. A comparison of total RA in soil before extraction with the sum of extracted and bound RA for each sample resulted in a RA mass balance in the range of 95-111% for all processed samples, indicating a good recovery of the used methods.

The results obtained for extractable RA, extractable SMZ and NERs are shown in Figure 2. To account for the inhomogeneity of sub-sampling, the data was normalized to the total RA for each sampling day.

The extractability of SMZ decreased rapidly within 3 d for the aerobic control to below 60% of initially applied SMZ (Figure 2A), followed by a continuous slower decrease until end of experiment (45 d). In parallel, a slower decline of extractable RA was observed, resulting in a noticeable difference between extractable SMZ and RA at day 45, at which the extractable SMZ was 22% and the extractable RA was 38% of the initially applied SMZ, respectively. Consequently, the NER fraction increased to 61% at day 45. The distribution among extractable and nonextractable residues largely agrees with results published, although the majority of studies used manure-applied soil.[3,5,6,27] A higher formation of NERs derived from ^{14}C labeled sulfadiazine (> 80% after 14 days) in soil was shown by Kreuzig and Höltge (2005)[3] who used, however, a relatively mild shaking extraction as compared to PLE .

The soil kept under anaerobic conditions showed a rapid NER formation until day 3 (20%), followed by slower increase up to 25% at day 10, and remained at this level until the end of the experiment (Figure 2B). Correspondingly, the extractability of SMZ and RA did not change from day 10 until the end of the experiment and remained at level of 70 and 75% of the initially applied SMZ concentration, respectively. The results clearly support the hypothesis that for covalent bond formation of sulfonamides with organic matter an oxidation step is required. The initial rapid NER formation indicates that a portion of reactive sites were available in soil or could be formed by oxidase enzymes or Mn oxides, which in turn are reduced. However, no new sites were formed due to a lack of oxygen necessary for a re-oxidation of enzymes or formation of Mn oxides from Mn^{2+}.

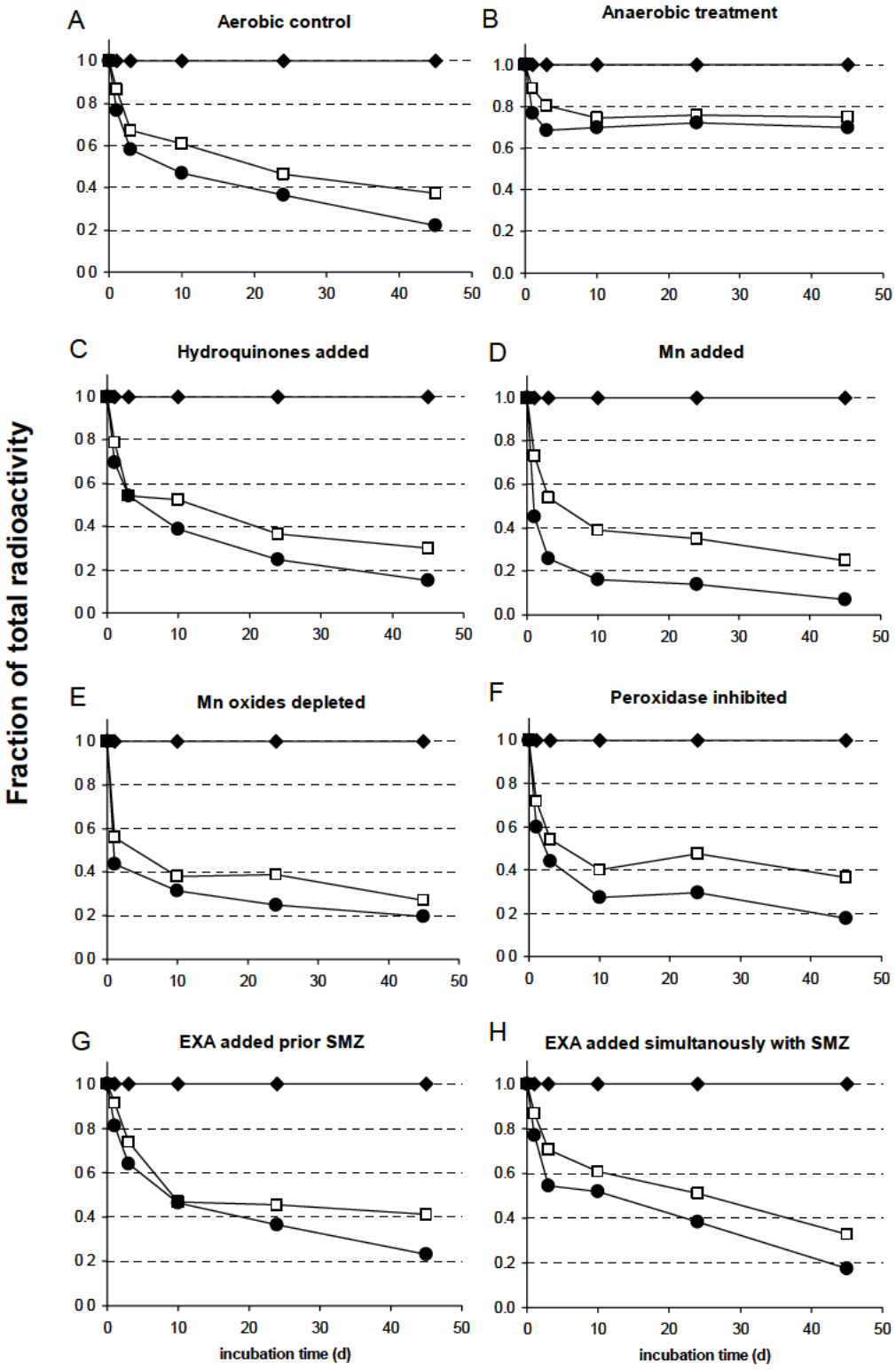

Figure 2. Distribution of sulfamethazine residues in soil during incubation in different treatments or conditions: extractable sulfamethazine (circles) and radioactivity (open squares) as determined by PLE, and total radioactivity (diamonds) as detemined by solid combustion. To account for inhomogeneity of sub-sampling, the data on temporal trends were normalized to the total RA of each sampling day.

Consequently, re-oxidation of organic matter can be considered as a limiting step in NER formation.

The difference between extractable RA and extractable SMZ indicates the presence of transformation products (TPs). These might be acetylated and hydroxylated TPs previously described in the literature.[28-30] To asses whether TPs were formed, selected soil extracts were concentrated, filtered and injected to the LC-FSC system. No new prominent peaks other than SMZ could be detected (Figure 3). Besides ^{14}C-SMZ, however, a fraction of less hydrophilic RA was detected eluting with a retention time in the range of that of the organic matter as seen from UV chromatogram of the extract obtained simultaneously. Similar results were obtained from LC-FSC analysis in incubations of SMZ with dissolved humic acid (see Chapter 3). Thus, the pool of RA in an "unresolved complex mixture" is likely to be covalently bound to humic substances and indicate numerous unknown reaction products of SMZ, but likely no well-defined transformation products, which would result in sharp peaks. LC-HRMS screening analysis for known or suspected TPs of SMZ (i.e., hydroxyl-SMZ, acetyl-SMZ) confirmed that no new prominent peaks other than SMZ were present. Thus, the difference between the extractable RA and the extractable SMZ fraction was defined as extractable fraction of covalently bound SMZ. This finding coincides with that of Förster *et* al. (2008),[27] who also detected a fraction of unknown metabolites in soils that were bound to extractable soil organic matter.

Extractability in manipulated soil

An addition of hydroquinones resulted in an about 10% lower extractability of SMZ and RA and to an about 10% higher extent of NER formation as compared to the aerobic control over the whole incubation period (Figure 2C). The initial faster NER formation indicates that the added hydroquinones increased the pool of reactive sites, which was initially a limiting factor in the aerobic control, but this effect diminished after the first few days. The difference in extractability of SMZ and RA was the same as for the control, suggesting that the added hydroquinones were incorporated into nonextractable organic matter as well.

Addition of Mn oxide to the soil sample entailed a considerable decrease of SMZ extractability to 45% within 1 day as compared to 77% for the aerobic control sample (Figure 2D) and < 10% at day 45 as compared to 22% for the control sample. The RA associated with NERs contributed to nearly 50% of initially applied amount at day 3 and 75% at the end of experiment. This result shows that Mn oxides played a significant role in the NER formation of SMZ. This confirms findings of Li *et*

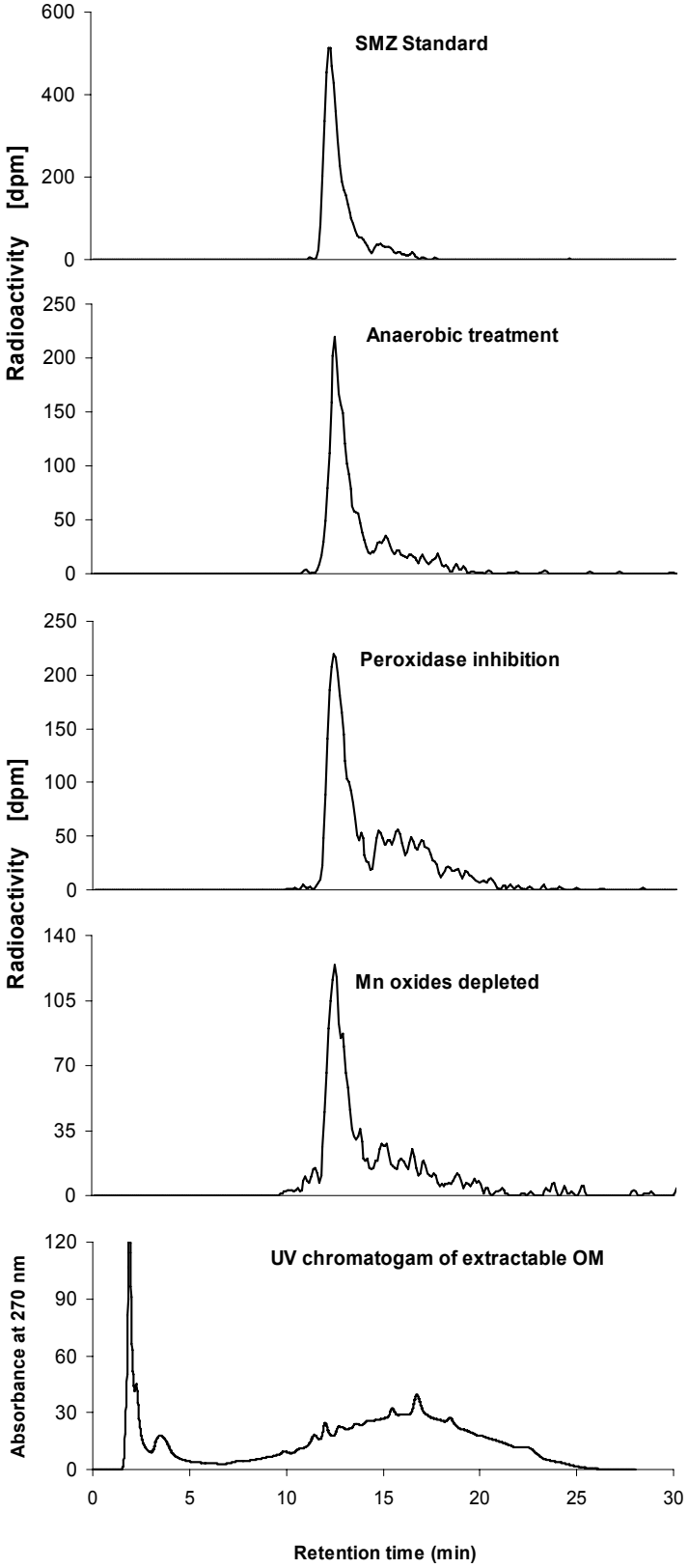

Figure 3. LC-FSC chromatograms of concentrated PLE extracts of selected soil samples compared to a ^{14}C-SMZ standard solution. For comparison, the LC-UV chromatogram at 270 nm of the soil extract from Mn depleted sample is also shown.

al. (2003),[15] who could show that NER formation of naphthylamine and *p*-methoxyaniline in soil coincided with a loss of Mn(III)/Mn(IV) and formation of Mn(II). This sample showed also the largest difference between extractable RA and SMZ, and thus an enhanced formation of reaction products between SMZ and extractable OM.

Preliminary experiments demonstrated that the birnessite we synthesized was not able to oxidize different sulfonamides in solution, although some sulfonamides tested by Bialk *et* al.(2005)[8] were transformed. However, a loss of 58% of SMZ during extraction was observed when SMZ was spiked into PLE cells filled with diatomaceous earth and birnessite. Thus, at the high pressure conditions and elevated temperatures used during PLE birnessite results in a transformation of SMZ and probably a polymerization, as no well-defined TPs were detected. For the birnessite-containing soil, however, this effect should be less pronounced, as during incubation Mn oxides get partially reduced and might become coated by OM.[15]

The sample from which Mn oxides were removed showed, surprisingly, also a decrease in extractability of SMZ and RA with time as compared to the aerobic control (Figure 2E). The reason might lie in the harsh Mn extraction conditions, during which additional reactive sites could have been generated. Extraction of Mn oxides and associated OM could expose previously inaccessible mineral surfaces or organic matter to the surface. Additionally, the first step of the procedure involved a dissolution using hydroquinone, which may not have been completely removed from soil during the washing steps and could form a pool of reactive sites as shown for the sample to which hydroquinones were added. As a result, NERs constituted to 73% of the initially applied RA at day 45 and were only slightly lower than in soils to which Mn oxide was added.

To test whether oxidative enzymes are involved in generating reactive sites, peroxidase enzymes were inhibited in soil before SMZ addition. Figure 2F shows a more rapid decrease of SMZ concentration at the first sampling days when peroxidase was inhibited, but a similar trend and extent of NERs formed as in the aerobic control was observed with further incubation time. Also, PLE extractions from diatomaceous earth in the presence of phenylhydrazine revealed a decrease in SMZ extractability to only 20% while 82% of the RA was recovered suggesting possible side-reactions of SMZ with phenylhydrazine during the extraction procedure or in soil, which could mimic a higher NER formation at the first days of incubation. Given the same extent of NER formation after 45 days, it seems likely that the contribution of peroxidase enzymes was less important as compared to Mn oxides. This seems likely as the relatively high soil pH of 7.9 impedes the activity of phenol-oxidizing enzymes such as laccase.[31] Lertpaitoonpan (2008)[32]

demonstrated that the SMZ concentration decreased in a soil at pH 6.4 sterilized by autoclaving and sodium azide addition to a half as compared to the unsterilized soil indicating that abiotic processes were responsible for approximately 50% of total SMZ dissipation.

The addition of the stronger nucleophile EXA either simultaneously or 1 day before SMZ addition did not impede the formation of NERs and no differences to the aerobic control were observed (Figure 2G and 2H). In experiments with Leonardite humic acid, EXA decreased the covalent binding of SMZ in the absence of laccase due to a limited number of binding sites, while this was not the case when laccase was present, as a large number of binding sites was created (see Chapter 3). Similarly, the number of binding sites seems not to be a limiting factor in the soil incubation, suggesting a rather high generation rate. However, EXA might also react with less electrophilic groups of organic matter or may be transformed more easily by microorganisms.

Desorption of SMZ from soil

The desorbable fraction of SMZ in soil was determined using a dialysis system at the end of the incubation experiment after 45 days. The desorbable fraction of SMZ was determined by solid combustion of the infinitive sink material from one half-cell, while the nondesorbable fraction was determined by solid combustion of the dried soil from the other half-cell. The desorbable fraction was for most samples between 1.1 and 2.0% of the initially applied SMZ, however the anaerobic sample showed a desorbable fraction of 7%, while no SMZ was detectable in the sample which received Mn oxides (Table 1). Thus, the desorbable SMZ concentrations were about an order of magnitude lower than those extractable by PLE, but followed, in general, the same trend. The desorbable fraction was, thus, in the same order of magnitude as the fraction designated as bioavailable in manure-amended soil, which was determined by extraction with 0.1 M $CaCl_2$ (9-10% of RA recovered in $CaCl_2$ fraction after 57 d of incubation).[5]

Distribution of ^{14}C-SMZ NERs among organic matter fractions

The distribution of bound RA derived from ^{14}C-SMZ was determined after fractionation of the soil after PLE at day 45 into fulvic acid, humic acid, humin, and solid residues (Table 1). Due to the low overall NER formation the anaerobic control sample was not included in the fractionation procedure. Substantial portions of RA were detected in the humic acid (7-21%), fulvic acid (10-

20%) and solid residue fractions (20-42%), while a negligible portions of RA were associated with the humin fraction (<3%).

The portions of RA associated with both, fulvic and humic acids in our study were distinctly below those of Schmidt *et* al. (2008),[5] who reported 22-42% and 26-27% RA associated with FA and HA after 57 days, respectively. However, they used a relatively mild extraction as compared to PLE, which in our case might have extracted a portion of humic and fulvic acids and was attributed as the fraction of SMZ covalently bound to extractable organic matter.

Regarding the humin fraction, negligible binding of SMZ occurred and a small portion of RA detected may be due to the "bound humic acid" in humin.[33] The large portion of RA in the solid residual fraction can be explained by (i) a pore diffusion of SMZ in the intra-particle pore system[34,35] or (ii) a strong association of organic matter with the solid residue fraction. Indeed, the elemental analysis revealed that 36-56% of total OC in soil remained in the solid residue fraction. The overall difference of the NER distribution pattern among the different treatments was low. Compared to the aerobic sample, the hydroquinone added sample shows the largest differences, as the largest amount of NERs was detected in fulvic and humic acids and a considerably smaller amount in solid residues. This suggests that the added hydroquinones were mainly incorporated along with SMZ into fulvic and humic acid. On the contrary, the EXA added sample had the lowest fractions bound to humic acid, fulvic acid and humin, but higher fraction bound to the solid residues. An explanation might be that the EXA lowered the binding of SMZ to these fractions and increased the affinity of SMZ molecules to intra-particle pore diffusion.

Table 1. Distribution of radioactivity in extractable and nonextractable residues after 45 days of incubation. Values are given as fraction of total RA in the soil sample.

Sample	Desorbable	Extractable	Extractable	Nonextractable RA					Mass balance
	SMZ	SMZ	RA	Fulvic acid	Humic acid	Humin	Soild residues	Total	
Aerobic control	0.013	0.22	0.38	0.14	0.10	n.d.[a]	0.37	0.61	0.99
Anaerobic	0.070	0.70	0.75	n.a.[b]	n.a.	n.a.	n.a.	0.25	1.00
Hydroquinones added	0.012	0.15	0.32	0.20	0.21	0.03	0.20	0.64	0.96
Mn oxides added	n.d.	0.07	0.25	0.17	0.19	n.d.	0.37	0.75	0.98
Mn depleted	0.013	0.20	0.27	0.16	0.19	0.03	0.37	0.73	1.02
Peroxidase inhibited	0.011	0.17	0.36	0.15	0.11	n.d.	0.36	0.62	0.98
EXA added prior	0.020	0.23	0.41	0.10	0.07	n.d.	0.42	0.59	1.00
EXA added simultaneously	0.016	0.17	0.32	n.a.	n.a.	n.a.	n.a.	0.67	0.99

[a] not detectable

[b] not analyzed

Conclusions

Our results clearly show that the amount of quinones available for nulceophilic addition was a limiting factor as indicated by (*i*) an (initial) increase of NER formation by adding quinone precursors or enhancing their formation by Mn oxide addition and (*ii*) a decrease of NER formation by limiting the formation of quinones under anaerobic conditions. However, the fact that after Mn oxide removal also a higher NER formation was observed suggests that accessibility of sites in organic matter might play a role and maybe other soil minerals (Fe oxides) can contribute to quinone formation as well. These macroscopic observations coincide well with the results from mechanistic studies on model humic constituents and humic acids that covalent bonding by nucleophilic addition is initially the dominant pathway of NER formation and governed mainly by the availability of reactive quinones (see Chapter 2 and 3).

A contribution of pore diffusion to the subsequent slow NERs formation phase might be possible as indicated by their high fraction in the solid residual fraction. However, the slow NER formation with time in aerobic soils could also be explained by covalent bonding. In that case, not the formation of quinones by oxidants would be limiting, but the availability of SAs, which need to desorb from the solid phase prior to reaction. This hypothesis is supported by the fact that no slow NER formation phase was observed in the anaerobic soil incubation.

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Chapter 5

Summary and conclusions

As outlined in Chapter 1, there are several possible mechanisms by which SAs may form NERs and depend on the properties of both, the SA molecule and the soil matrix. Variability in the nature of soil organic matter (SOM) appears to be significant in controlling its reactivity towards SAs and there is no reason to presume that only one mechanism dominates in any particular case. Thus, in real systems more then one process likely contributes to the rate-limited NERs formation complicating the interpretation of macroscopic data.[1] Nevertheless, several findings in this PhD thesis provide a mechanistic explanation for the covalent bonding as the predominant process responsible for the NERs formation of SAs in soils.

The mechanisms were elucidated in Chapter 2 for the model sulfonamide sulfathiazole (STZ) in comparison with the stronger nucleophile *para*-ethoxyaniline in reactions with model phenols forming quinones as well as other carbonyl compounds in the absence and presence of laccase. In agreement with Bialk *et* al. (2005, 2007)[2,3] high resolution mass spectrometry revealed that the initial bonding of STZ occurred by 1,2- and 1,4-nucleophilic additions to quinones resulting in imine and anilinoquinone formation, respectively. An addition to less reactive carbonyl compounds (diketones, esters, ketones) was not observed. The use of the radical scavenger *tert*-butanol enabled us to further elucidate the possible involvement of radicals in the reactions. The same products and similar formation rates as those without scavenger indicated that solely nucleophilic addition reactions and not radical coupling reactions were responsible for the initial covalent bond formation. However, radical coupling is likely important for the further incorporation of the reaction products into polymerization products.

In Chapter 3, the covalent binding of SAs to dissolved humic acid was investigated. The study revealed that a small pool of reactive quinones in OM is available, which might be important at very low antimicrobial concentration, but could probably not account for the observed initial NER formation of a large fraction. This reactive quinone pool, however, increased considerably after oxidation of organic matter resulting in extensive covalent bonding. Thus, quinone formation seems to control the magnitude of NERs formed. Also, the competition with a stronger nucleophile (as it is possible in soils) was not of importance when many quinones were available under these conditions.

In terms of reversibility and possible remobilization the stability of covalent bonds was assessed against desorption and pressurized liquid extraction (PLE). The recovery rates showed no systematic differences in STZ extractability between the two identified product types (namely anilinoquinone and imine) as previously anticipated[4] (see Chapter 2). This suggests that the strength of bonding was not controlled by the initial type of bond, but by the extent of subsequent

incorporation of the reaction product into the formed polymer. This incorporation was monitored for ^{15}N aniline by ^{1}H-^{15}N HMBC NMR spectroscopy. The initial 1,2- and 1,4-addition bonds were replaced by stronger heterocyclic forms with increasing incubation time. Thus, it was possible to distinguish stable and labile (i.e. released by PLE) covalent bonds based on PLE. In the experiments with dissolved humic acids (Chapter 3), only a small fraction of labile bonds was present, which was slightly higher for synthetic humic acid, but the overall covalent bond formation was considerably higher than in natural LHA; furthermore, this labile fraction decreased with contact time.

The work in Chapter 4 contributed to the identification of limiting factors in NER formation of SAs by a selective manipulation of incubation conditions. The results indicated that the amount of quinones was a limiting factor as indicated by (*i*) an (initial) increase of NER formation by adding quinone precursors or enhancing their formation by Mn oxide addition and (*ii*) a decrease of NER formation by limiting the formation of quinones under anaerobic conditions. However, the fact that after Mn oxide removal also a higher NER formation was observed suggests that accessibility of sites in organic matter might play a role and maybe other soil minerals (Fe oxides) can contribute to quinone formation as well. These macroscopic observations coincide well with the results from Chapters 2 and 3 that covalent bonding by nucleophilic addition is initially important and governed mainly by the availability of reactive quinones.

The contribution of intraparticle pore diffusion to subsequent slow NERs formation phase might be possible as indicated by their high fraction in the solid residue after MIBK fractionation (see Chapter 4). However, the slow NER formation with time could also be explained by covalent bonding. In that case, not the formation of quinones by oxidants would be limiting, but the availability of SAs, which need to desorb from the solid phase prior to reaction. The predominance of slow covalent binding over intraparticle pore diffusion is supported by the fact that no slow NER formation phase was observed in the anaerobic soil sample.

Studies described in Chapters 2 to 4 provide a coherent picture on the NER formation mechanisms of SAs from an abiotic homogeneous model system to the soil system. Thus, it was possible to link binding mechanisms obtained from model experiments to operationally defined extraction procedures amendable to soil samples. However, also a combination of these two approaches is inherently incomplete, as model systems can never mimic the complexity of spatially compartmented, heterogeneous soils. On the other hand side, any pretreatment or manipulation

of soil may disrupt the natural state of soil matrix and change its overall properties and reactivity, as it was indicated by the surprisingly enhanced NER formation after Mn oxide removal in the soil incubation experiment (Chapter 4) and the possible artificial NER formation during PLE extraction from the reaction of STZ with model phenols inside the extraction cell (Chapter 2). It has been shown that the physical conformation and layering of organic matter in soils is an important factor in understanding the binding and sorption of contaminants.[5] A way to assess chemical contaminant-soil interactions in its natural state with little or no pre-treatment might be ambient mass spectrometry methods based on desorption electrospray ionization (DESI). By this method, spatially-resolved mass spectra can be obtained from solid samples without sample preparation or pre-separation by creating ions directly from the surface. This powerful technique might be an interesting alternative to hyphenated MS methods in bound residues determination.[6-8] A potential limitation of DESI, however, may be its sensitivity which could be increased with future improvements.[7]

The release of a fraction of covalently bound sulfonamides by exhaustive extraction methods such as PLE might explain the large differences in NER formation based on the mild[10] and harsh extraction methods[10,11] already at the beginning of incubation. Thus, the challenge is to reach an agreement on the experimental methods and/or modeling tools to evaluate NER.[12]

In the end, however, it is not so much important how the residue is defined, but the question of the reversibility between unavailable and available forms of the residues and their biological significance.[12] The results presented in Chapter 3 and 4 showed a decrease in extractability of SAs from humic acid and soil with time. The determined strength of covalent bonds against PLE implies that SAs are unlikely to be remobilized from organic matter to the ambient environment in their original form. Under field conditions however, NERs formation might be controlled by numerous physico-chemical parameters (i.e. temperature, soil pH, water content, redox properties) as well as agricultural practice (i.e. tillage, fertilization, pesticide application).[12] Their impact on possible SA release from NERs remains unrevealed. Hence, more intensive long-term experiments using standardized quantification methods are essential to include NERs in environmental risk assessments.

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