Kinetics of rapid covalent bond formation of aniline with humic acid: ESR investigations with nitroxide spin labels

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ABSTRACT

The bioavailability of many soil contaminants depends on their interaction with the soil organic matter. The paper presents a new approach of using stable paramagnetic spin labels for investigating the kinetics of covalent binding of specific xenobiotic functional groups with humic acids, a major organic matter fraction. Leonardite humic acid (LHA) was incubated with the nitroxide spin labels amino-TEMPO (4-amino-2,2,6,6-Tetramethylpiperidin-1-oxyl) and anilino-NO (2,5,5-Trimethyl-2-(3-aminophenyl)pyrrolidin-1-oxyl), respectively, which contain an aliphatic or aromatic functionality susceptible to interaction with LHA. Electron spin resonance

(ESR) spectra of LHA samples without and with the enzyme laccase were recorded at X-band frequency (9.43 GHz) at room temperature and neutral pH. Binding was detected by a pronounced broadening of the spectral lines after incubation of LHA for both spin labels. The development of a broad signal component in the spectrum of anilino-NO indicated the immobilization due to strong binding of the aniline group. The reorientational correlation time of bound anilino-NO is more than two orders of magnitude greater than that of the free label. The ratio of the amount of bound to the unbound species was used to determine the kinetics of the covalent bond formation. Reaction rate constants of 0.16 min^{-1} and 0.01 min^{-1} were determined corresponding to half-times of 4.3 min and 69.3 min, respectively. Treatment of LHA with laccase enhanced the amount of the reacting anilino-NO species by a factor of 7.6, but left the reaction rate unaltered. Oxidative radical coupling was excluded by using the spin trap agent n-tert-butyl-alpha-phenylnitrone.

Keywords:

ESR; spin labeling; nitroxide radical; organic xenobiotics; humic acid; covalent binding; bound residues; aromatic amines

INTRODUCTION

Human activities, e.g., waste deposition, mining, fertilizing with manure, or application of chemicals for pest and weed control, have led to contamination of soil systems. Many of these anthropogenic compounds are synthetic organic chemicals, to which soil has never been naturally exposed and which are thus called xenobiotics. In biodegradation simulation studies, many xenobiotic chemicals exhibit a large fraction of residues, which cannot be further extracted even by harsh methods (non-extractable residues, NER) [1]. Various interaction processes in soil such as sorption, sequestration (entrapment) or immobilization via binding to soil organic matter (SOM), clay minerals, and organo-clay complexes [2] govern the extractability and thus their availability for leaching to groundwater, volatilization, abiotic and biotic degradation, or uptake by living organisms. NER, also called "bound" residues, have been extensively reviewed, in particular for pesticides but also for many other organic chemicals [3, 4, 5].

Covalent binding of xenobiotics and its metabolites to soil components is of particular interest because it ultimately withdraws the xenobiotic and its metabolites from any adverse environmental effect. It is known since many years that functional groups of xenobiotics such as aromatic amino groups are involved in covalent binding to SOM, in particular to humic substances (HAs) [6, 7]. Aromatic amines, e.g. aniline (aminobenzene), are the building blocks of many pesticides, veterinary pharmaceuticals, textile dyes and other classes of synthetic chemicals and comprise an important class of environmental contaminants [8]. They are also reductive transformation products of nitro-aromatic explosives such as 2,4,6-trinitrotoluene (TNT) and received a great deal of attention in remediation of contaminated manufacturing sites [9 – 11].

^{15}N NMR studies with aniline and other aromatic amines have revealed nucleophilic addition of the aromatic amino group to quinone and other carbonyl groups of humic substances [6, 8, 11 - 14]. A fast, reversible 1,2-nucleophilic addition and a slower irreversible 1,4-nucleophilic addition of the aromatic amino group to the carbonyl group of quinones have been proposed resulting in the formation of Schiff base (iminoquinone) and Michael amine-carbonyl adducts (anilinoquinone), respectively. Reaction kinetics of aromatic amines with humic substances has been studied using ultrafiltration techniques and HPLC for analytic determination [12, 15]. Fast and slow reaction rate constants were observed in the order of 10^{-2} and 10^{-3} h^{-1}, i.e. half-times of 50 and 500 h, respectively, depending on pH, substitution of the aniline ring, initial aniline concentration and humic substance [8, 15]. Rapid initial sorption could also be dominated by irreversibly covalent binding. However, the method applied did not allow determining very fast reaction kinetics in the first few hours after incubation. Oxidative reaction mechanisms resulting in the formation of aniline radicals, which couple with radical species, have also been proposed [16]. Thorn et al. [13] pointed out that radical reactions involving aniline radicals and semiquinone radicals are possible in the presence of peroxidase or Mn-oxides, as these govern one-electron oxidation initiated polymerization reactions of aromatic amines and of quinones, but an open question remained, whether a large pool of quinones is available in soil readily able to add such a weak nucleophile.

The extractability of sulfonamides, which contain an aniline functional group as part of its antimicrobial active moiety, decreased rapidly in soil incubation studies, even if harsh extraction methods were applied [17 – 19]. The strong sorption behavior is attributed to the formation of non-extractable residues (NER). Bialk et al. [20, 21] concluded that the rapid NER formation of sulfonamides in soil may be due to Michael adducts (anilinoquinones) and Schiff base formation

79 (iminoquinones) of the aromatic amino group, which can only partially be cleaved during
80 fractionation and vigorous extraction procedures. Bialk and Pedersen [22] observed fungal
81 peroxidase-mediated covalent coupling of sulfonamide with HA. Gulkowska et al. [23]
82 proposed a two-step process with an initial formation of imine and anilinoquinone followed by
83 incorporation into the soil polymer structure. They ruled out oxidative radical coupling with
84 constituents of SOM as an alternative reaction pathway.

85 ESR spin labeling with stable nitroxide spin labels has successfully been applied since many
86 years for investigating the structure and dynamics of biological macromolecules and assemblies,
87 particularly membranes and proteins [24, 25]. Stable nitroxide radicals can also be used for
88 studying the interaction of xenobiotics in soil and sediment systems. The signal of the
89 paramagnetic NO group is influenced by its soil microenvironment and can be recorded by ESR
90 spectroscopy. Using ESR of spin-labelled organic macromolecules such as polysaccharides,
91 Steen et al. [26] could monitor the sorption to natural sediment surfaces. Sorption specificity and
92 sorption mechanisms to SOM have been studied by Lattao et al. [27] with paramagnetic probes,
93 where nitroxide compounds of different polarity were used as relaxation agents for NMR
94 spectroscopy. By means of spin relaxation they could show that nitroxide spin labels exhibit
95 “little or no preferential sorption in SOM based on functional group chemistry or putative micro-
96 domain character”. However, they did not investigate the interaction of nitroxide compounds
97 containing functional groups such as aniline with SOM.

98 We applied the method of nitroxide spin labeling to investigate the interaction of the functional
99 amino group to soil humic acid. Signals of nitroxide spin labels, substituted with a functional
100 group such as aliphatic or aromatic amine, depend on the molecular environment, e.g. polar or
101 non-polar, and the binding state, e.g. covalently bond. The property of nitroxide radicals to act as

good hydrogen bond acceptors [28] does not compromise their use. Moreover, oxidative radical coupling reaction as a mechanism for covalent binding can be investigated by using a spin trapping agent [29]. The objectives of our work are to (i) develop the method of nitroxide spin labeling for the investigation of covalent binding of typical xenobiotic functional groups (here: aniline) to humic acid as a model for complex soil matrices (proof of concept); (ii) apply the method for the determination of reaction kinetics of aniline to soil humic acid; (iii) investigate the influence of phenoloxidase enzyme on the reaction kinetics; and (iv) verify or falsify the assumption of a radical reaction for the covalent binding of aniline.

EXPERIMENTAL SECTION

Chemicals. All chemicals were purchased from Sigma-Aldrich (Munich, Germany) if not otherwise noted. They were analytic grade products and used without further purification.

Humic acid. Leonardite humic acid (LHA) was purchased from the International Humic Substances Society (http://www.humicsubstances.org). Stock solution of 15 mg/mL was prepared by dissolution in aqua bidest. and adjusted to pH = 7.0 with 1 M NaOH.

Phenoloxidase. Extracellular fungal laccase from *Agaricus bisporus* with an activity of 5.6 U mg^{-1} was used without further purification. Stock solutions of 21.4 U/ml were prepared and stored at 4° C. Aliquots of 10 µL were added to 60 µL of LHA solution and incubated for three days at room temperature.

Spin labels. Stock solutions of 3 mM 4-amino-2,2,6,6-Tetramethylpiperidin-1-oxyl (amino-TEMPO) and 2,5,5-Trimethyl-2-(3-aminophenyl)pyrrolidin-1-yloxy (anilino-NO), synthesized according to Gadanyi et al. [30], were prepared by dissolution in EDTA-phosphate buffer and stored at – 80 °C.

ESR Spectroscopy. ESR spectra were recorded at X-band frequency (9.43 GHz) at room temperature using a Magnettech Mini Scope MS 200 (Magnettech GmbH, Berlin, Germany). The microwave power was adjusted to 10 mW and the magnetic field modulation amplitude was set to 0.2 mT. Samples of 15 µL of 15 mg/mL LHA solution were mixed with 5 µL of 3mM spin label solution to achieve a final concentration of 750 µM and filled into glass capillaries with an inner diameter of 0.9 mm (Hirschmann Laborgeräte, Germany). Spectra were recorded in intervals of four minutes for the first half hour after label addition and afterwards at 1, 2,...5 hours after label addition. Three replicates of each experimental setting were made and fitted with Origin 7 (OriginLab Corp., USA) to derive the reaction kinetics. All spectra and graphs were plotted with Origin 7 and CorelDraw X4 (Corel Inc., Canada).

Radical scavenger. N-tert-butyl-alpha-phenylnitrone (PBN) was used as a spin trap agent. Stock solution of 300 mM PBN was in turn added to aqueous solutions of LHA, LHA plus laccase, LHA plus aniline, and finally LHA plus laccase and aniline. Mixtures were made for a final PBN concentration of 50 mM and a volume of 20 µL. For the laccase settings, LHA and laccase were mixed in a 6:1 ratio (60 µL LHA + 10 µL laccase) and incubated at room temperature for three days. A 3 mM aniline stock solution was prepared from anilinium-HCl, adjusted to pH 7, and mixed with LHA in a 1:4 ratio to obtain a concentration of 750 µM. Fenton's reaction was used for producing oxygen radicals and testing spin trapping by PBN [31]. For the reaction, 8.5 µL of 200 µM iron sulfate solution and 8.5 µL of 40 mM hydrogen peroxide were mixed. ESR spectrum was recorded immediately after adding 3.3 µL of 300 mM PBN to this mixture providing a final concentration of 50 mM.

Spectra simulation. To analyze the rotational correlation times of the spin labels and the ratios of free and bound spin labels in the ESR spectra, experimental spectra were fitted using the

147 software Multicomponent774
148 (http://www.biochemistry.ucla.edu/biochem/Faculty/Hubbell/software.html). Spectra were
149 interpolated using Origin 7, to reduce the number of data points from 4.096 to 512 as required by
150 Multicomponent774. Simulated spectra were fitted to experimental ESR spectra of amino-
151 TEMPO in absence and presence of LHA using a model of isotropic Brownian rotational
152 diffusion. Fitting parameters were the rotational correlation rate, the hyperfine tensor component
153 A_{zz}, and a Gaussian line width to account for not resolved hyperfine interaction with the methyl
154 protons. All other parameters were fixed according to typical values for TEMPO derivatives in
155 aqueous solution ($g_{xx} = 2.0080$, $g_{yy} = 2.0058$, $g_{zz} = 2.0023$, $A_{xx} = A_{yy} = 0.6$ mT). For fitting of the
156 spectra of anilino-NO axial symmetry of the reorientational diffusion had to be assumed to
157 achieve reasonable agreement between simulation and experiment. Thus rotational diffusion was
158 accounted for by using the two values of the rotational correlation rates parallel and
159 perpendicular to the symmetry axis, R_{II} and $R_{\perp}$ All other parameters were chosen identical to
160 those given above. For the simulation of a two component spectrum originating from two
161 fractions of differently immobilized spin labels a superposition of two spectra was calculated
162 with two different sets of rotational correlation rates. Fittings were performed of such simulated
163 two component spectra to the experimental ones with the ratio of the two fractions and the
164 rotational correlation rates as fitting parameters.

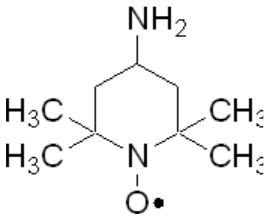
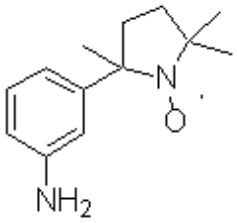
165 **RESULTS AND DISCUSSION**

166 **Physical-chemical properties of amino spin labels.** Amino-TEMPO and anilino-NO represent
167 molecules with amino moieties typical for organic chemicals, which are susceptible to
168 interaction with humic substances. Among others, the two compounds differ in their lipophilicity
169 expressed as logarithm of the octanol-water partition coefficient, Log K_{OW}, and the acid-base

dissociation constant, pK_a, of the amino group. Measured Log K_{OW} of amino-TEMPO are 3.50 [32] and 3.64 [33] at pH 12 and 0.04 at pH 7 [32]. pK_a values of amino-TEMPO are 8.99 [33] and 9.10 [33], respectively. No measured Log K_{OW} and pK_a were available for anilino-NO. With KOWWIN of the EPISuite v4.11 package from the U.S. Environmental Protection Agency [34] only Log K_{OW} values of the hydroxylamine form, i.e. the protonated non-radical N-OH form, could be calculated. EPISuite provided values for Log K_{OW} of 0.56 for NOH-forms of amino-TEMPO and 1.91 of anilino-NO, respectively. We assumed that the difference of 0.52 between the measured Log K_{OW} of the radical NO-form and the estimated non-radical NOH-form of amino-TEMPO represents the fragment of the O-radical and used it to adjust the Log K_{OW} value of anilino-NO accordingly, which provided a Log K_{OW} of 1.39 (Table 1). A pK_a of the conjugate acid of the amino group of anilino-NO of 4.73 (Table 1) was estimated with the SPARC Online Calculator [35]. At pH 7, anilino-NO exist in its neutral form, whereas amino-TEMPO is protonated at the amino moiety, i.e. it is in the cationic form. This explains the low Log K_{OW} of amino-TEMPO at pH 7. We thus expect that amino-TEMPO mainly interacts at neutral pH via ionic interactions with humic acid. On the other hand, the pK_a of a non-ionic substance is related to its nucleophilic reactivity [15]. Thus, anilino-NO is susceptible to covalent binding due to nucleophilic addition reaction with humic acid.

Table 1. Structure and properties of nitroxide spin labels;

Nitroxide spin label	Amino-TEMPO	Anilino-NO
CAS-No.	14691-88-4	328000-24-4
IUPAC name	4-amino-2,2,6,6-Tetramethylpiperidin-1-oxyl	2,5,5-Trimethyl-2-(3-aminophenyl)pyrrolidin-1-oxyl

Chemical structure		
log K_{OW}	3.50 (pH = 12)[1] 0.04 (pH=7)[1] 3.64 ± 0.07 (pH=12)[2]	1.39[3]
pK_a of amino group	9.10 ± 0.10[1] 8.99 ± 0.01[2]	4.73[3]

[1]) [32]; [2]) [33]; [3]) estimated, see text

LHA incubated with amino-TEMPO. The relationship between the nitroxide reorientational motion and the line shape of the continuous wave (cw) spectrum recorded at X-band frequencies (9 GHz, 0.3 T) has been extensively reviewed [36], thus these properties are summarized in brief. The term "mobility" is used in the following in a general sense and includes effects due to the rate, anisotropy and amplitude of the nitroxide reorientation. Weak interaction of a nitroxide with its micro-environment in solution of low viscosity results in a high degree of mobility. In this case, the anisotropic components of the hyperfine interaction of the electron with the nitrogen nuclear spin magnetic moment are averaged out and the spectrum consists of three equally spaced lines of small width in the order of 0.2 mT. The splitting between the lines is given by the isotropic component of the hyperfine interaction. In turn, strong interaction of the nitroxide group with the micro-environment, which restricts unhindered reorientation motion, or high viscosity of the solvent, are characterized by increased apparent hyperfine splitting and line widths. The effect of line broadening and corresponding amplitude decrease due to motional restriction is most pronounced for the high field resonance line. In the limiting case of

completely hindered reorientational dynamics the spectrum shows a so-called powder spectrum line shape [36]. The ESR spectra of amino-TEMPO in aqueous solution and upon incubation with LHA are shown in Fig. 1.

Fig. 1 ESR spectra recorded at X-band (9.4 GHz) in the B-field region between 333 and 339 mT: amino-TEMPO in aqueous solution (grey continuous line) and upon incubation with LHA (broken line).

Three sharp hyperfine lines of nearly equal amplitude reveal unrestricted fast reorientational motion of amino-TEMPO in aqueous solution. Fitting of simulated ESR spectra to the experimental ones using the model of isotropic Brownian rotational diffusion yields the value of the rotational correlation time of 20 ± 5 ps. Upon incubation with LHA the amplitude of the high field hyperfine absorption signal is significantly reduced (Fig. 1) revealing a decreased mobility of the nitroxide. Fitting of a simulated spectrum yields a value of the rotational correlation time of 95 ± 10 ps. Since the viscosity of the used LHA solution is approximately 1.3 times the viscosity of pure water [37] the observed fivefold increase of the rotational correlation time of amino-TEMPO must be due to an interaction of the spin label with components of LHA which restrict its mobility. Possible mechanisms include transient bonding of the amino group via an ionic interaction to LHA, e.g. cation-exchange sorption[38] or hydrogen bonding of the nitroxide group to hydrogen donors[39]. Since the experimental spectra do not provide any indication for two components the weakly bounded and free nitroxide fractions may be in fast equilibrium resulting in a spectrum with average rotational correlation time.

LHA incubated with anilino-NO. A different picture evolves for anilino-NO (Fig. 2). Compared to the EPR spectrum of anilino-NO in aqueous solution (Fig. 2, upper panel) the

reduction of the amplitudes of the low field and high field hyperfine absorption signals with respect to the center line reveal a decreased mobility of anilino-NO in the presence of LHA (Fig. 2, middle panel).

Fig. 2 ESR spectra recorded at X-band (9.4 GHz) in the B-field region between 332 and 340 mT: Anilino-NO in aqueous solution (upper panel) and upon incubation with LHA (measured, middle panel and simulated, lower panel). The inset in the middle panel highlights the low field resonance line of the signal of the bound label. For comparison with the experimental spectrum the simulated spectra for the free and bound components are superimposed in the lower panel. The concentration of the immobilized component was enhanced by a factor of 2.7 compared to the experimental case for better visibility.

Reasonable fitting of simulated spectra to the experimental ones required a model of axial symmetry of the rotational diffusion with the symmetry axis being parallel to the nitroxide y-axis. From the reorientational rates parallel and perpendicular to this axis, R_{II} and $R_{\perp}$, an effective reorientational correlation time

$$\tau = \frac{1}{6\sqrt{R_{\perp} R_{II}}} \qquad (1)$$

was calculated. For anilino-NO in aqueous solution and in the presence of LHA we determined correlation times of 50 ± 23 ps and 219 ± 53 ps, respectively. Similar to the finding with amino-TEMPO the increase of the rotational correlation time by a factor of four cannot be explained by the increase of the viscosity of the solution upon incubation with LHA. Thus, we conclude that a transient weak interaction of anilino-NO with components of LHA, e.g., hydrogen bonding to the nitroxide group or hydrophobic interaction, is responsible for the decrease of reorientational motion. Axial symmetry of rotational diffusion with the symmetry axis parallel to the y-axis of

249 the nitroxide can be readily explained by the shape of the molecule with its longest axis oriented
250 nearly parallel to the nitroxide y-axis (Fig. 3).

251 **Fig. 3** Structure of anilino-NO with orthogonal coordinate system

252 In addition to this weakly immobilized spin label, a broad spectral component is present which
253 indicates strongly immobilized anilino-NO. This component is most clearly resolved in the low
254 and high field regions of the spectrum (see Fig. 2, insert for the low field region). Thus, a
255 fraction of anilino-NO is immobilized due to strong bonding to LHA, which restricts the
256 reorientational dynamics of the nitroxide moiety. A simulated spectrum with two components of
257 weakly and strongly immobilized spin labels is shown in Fig. 2, lower panel, with the component
258 for the strongly immobilized spin label enlarged compared to the experiment spectrum for
259 clarity. Reorientational correlation times of weakly and strongly immobilized anilino-NO
260 determined from fittings of simulated two component spectra to the experimental ones yielded
261 219 ± 53 ps and 6.2 ± 0.9 ns, respectively.

262 The ESR spectrum of LHA incubated with anilino-NO leads to the conclusion that humic acids
263 play a significant role in binding of aromatic amino-groups of xenobiotics. It is known, that
264 oxidizing catalyst, e.g. phenoloxidase enzymes or metal oxides, enhance the covalent binding of
265 functional aniline group to humic acids [20, 22]. Therefore, we additionally used laccase as an
266 oxidizing enzyme to investigate the activating effect on the reaction kinetics (see below).

267 **Spin number ratio.** For sake of simplicity the weakly and strongly immobilized spin labels will
268 be labeled “free” and “bound” in the following. The ratio of the spin number of the bound to that
269 of the free species, N_{bound}/N_{free} , is used for the determination of the reaction kinetics. It is

calculated from the corresponding ratio of the peak height (amplitude) of the low field resonance line of the signal of the bound to that of the free, unbound label according to:

$$\frac{N_{bound}}{N_{free}} = 45.9 \cdot \frac{amplitude_{bound}}{amplitude_{free}} \qquad (2)$$

The factor 45.9, which relates the ratio of spin numbers to that of spectral amplitudes, was determined from the simulated spectrum shown in Fig. 2, lower panel. Here, the ratio of the spin numbers was calculated from the ratio of the double integrals of the spectra of the two components, the ratio of the amplitudes was determined from their values at 330.0 and 334.0 mT.

Kinetic studies. Spectra were recorded immediately after mixing of the spin labelled molecules with LHA and repeated every few minutes. Intervals were increased with reaction time. Fig. 4 shows the peak of the strongly bound anilino-NO within the first five hours.

Fig. 4 ESR signal of the bound species of anilino-NO incubated with LHA

The spin number ratio is plotted against time in Fig. 5. The narrow variance of the three replicates demonstrates complete mixing and the precision and reproducibility of the measurements. Note that the right amplitude axis belongs to the data recorded without laccase. The first data point, measured after a few minutes mixing time, was set to zero although the signal of the bound species was already visible. This was done for all settings because the starting point of the reaction is undefined. A mono-exponential (2) and a bi-exponential model (3) were tested for fitting the experimental data:

$$f(t) = a_1 \cdot (1 - e^{-b_1 t}) \quad (3)$$

$$f(t) = a_1 \cdot (1 - e^{-b_1 t}) + a_2 \cdot (1 - e^{-b_2 t}) \quad (4)$$

The coefficients a_i denote the relative amount of anilino-NO reacting with LHA and b_i are the pseudo-first-order reaction rate constants. Table 2 shows the fitted model parameters for both models. The mono-exponential model reveals values of 0.18 ± 0.004 for a_1 and 0.03 ± 0.002 min^{-1} for b_1, corresponding to a half-time of 24 min. However, a better fit was achieved with two parallel first-order reactions. The two reactions have pseudo-first-order rate constants b_1 and b_2 of 0.01 ± 0.001 and 0.16 ± 0.017 min^{-1}, which corresponds to half-times of 69.3 and 4.3 min, respectively. The relative amount of the reacting species a_1 is 0.14 ± 0.002 and thus 2.8 times greater than a_2, which is 0.05 ± 0.003. Thus, the very fast reaction determines the overall increase of the bound anilino-NO label only at the very beginning. Colon et al. 2002[15] determined kinetic data for the reaction of ortho-, meta- and para-substituted anilines with sediment and found two pseudo-first-order rate constants depending on the substitution pattern. Corresponding half-times were around 20 h and 500 h and thus larger than those found with the spin labeling method. After t = 4 h incubation time of aniline in a pond sediment, Weber et al. 2001[8] found sorbed aniline fractions of 0.18 and 0.16 at pH = 6.82 and 7.37, respectively. They attributed them to rapid covalent binding of the neutral form of aniline but could not determine the rate constants. They observed a longer term sorption rate (t > 4 h) with pseudo-first-order rate constants of around 0.005 h^{-1}, which correspond to the findings of Colon et al. [15]. Hennecke (personal communication) investigated the aerobic and anaerobic transformation of aniline in the water-sediment simulation system according the OECD Test Guideline 308 [40] and found very rapid formation of NER in sediment with high organic carbon content up to 62 % of the applied radioactivity immediately after adding. However, they could not determine the rate constant of the very rapid NER formation.

Fig. 5 Plot of the spin number ratio versus time of the bound species of anilino-NO incubated with LHA without (black, right y-axis) and upon incubation with (red, left y-axis) laccase; bold and dashed lines are fitted curves according to the bi- and mono-exponential kinetic model.

Table 2 Fitted model parameters for mono- and bi-exponential reaction kinetics of anilino-NO with LHA and with LHA incubated with laccase.

Setting	Relative amount of fast reacting species a_1	Rate constant fast reaction b_1 [min^{-1}]	Relative amount of very fast reacting species a_2	Rate constant of very fast reaction b_2 [min^{-1}]	Coefficient of determination, r^2
LHA (bi-exp)	0.14 ± 0.002	0.01 ± 0.001	0.05 ± 0.003	0.16 ± 0.017	0.999
LHA (mono-exp)	0.18 ± 0.004	0.03 ± 0.002	-	-	0.988
LHA+ Laccase (bi-exp)	1.07 ± 0.015	0.01 ± 0.001	0.06 ± 0.016	0.27 ± 0.29	0.998
LHA + Laccase (mono-exp)	1.09 ± 0.022	0.01 ± 0.001	-	-	0.999

Laccase is known to catalyze the formation of covalent bonds by oxidizing unreactive hydroquinone moieties in humic substances to electrophilic quinone moieties [6, 8, 23]. We thus incubated LHA with extracellular fungal laccase from *Agaricus bisporus* for three days at room temperature and mixed it with anilino-NO. Fig. ~~4~~ 5 shows the spin number ratio plotted against time. Note that the left y-axis belongs to the setting with laccase. A larger amount of anilino-NO reacting with LHA was observed than in the experiments without laccase. Again, a mono- and bi-exponential model was used for fitting. Obviously, both models give almost the same data fit.

The kinetic model parameters a_1 and b_1 are thus also almost identical, i.e. 1.09 ± 0.022 and 0.01 ± 0.001 min^{-1} for the mono-exponential and 1.07 ± 0.015 and 0.01 ± 0.001 min^{-1} for the bi-exponential model, respectively (Table 2). The very fast reaction with $b_2 = 0.27 \pm 0.29$ min^{-1} has a neglecting influence on the covalent binding of anilino-NO to LHA due to the low value of $a_2 = 0.06 \pm 0.016$, which is 17.8 lower than a_1. The treatment with laccase has obviously activated only the slower reacting LHA sites. The rate constants for the reaction are identical for the settings with and without laccase, whereas the relative amount of reacting species is considerably enhanced from 0.14 to 1.07, which is factor of 7.6. This finding is in line with the observations of other authors [10, 21, 23], who showed the increase of reactive sites of humic substances mediated by the phenoloxidase enzyme laccase. We conclude from our kinetic study with laccase that the broadened signal of anilino-NO can be attributed to covalent binding to LHA, presumably a nucleophilic addition to quinones or other carbonyl moieties.

Radical coupling reaction. Radical reactions involving free radical intermediates and semiquinone radicals have also been proposed as covalent binding mechanism [2]. We thus added the spin trap agent PBN, which displays a characteristic ESR spectrum if free radicals are present in a sample. For comparison, Fenton's reaction was used to produce free radicals, which react with PBN to stable spin adducts. Fig. 6 shows ESR spectra of PBN incubated with LHA and 750 µM aniline (bottom), with laccase and 750 µM aniline (middle), and with Fenton's reagent (top). ESR spectra of LHA without and with laccase show the unchanged broad signal of the organic radicals, which are typical for humic acids [41] but no additional peaks from free radicals or spin adducts with PBN. If free radicals would have been produced in the LHA experiments, i.e. radicals intermediates or/and radical semiquinones, similar spectra as with Fenton's reagent would have been recorded. From the absence of the characteristic PBN spin

adduct spectrum we conclude that oxidative radical coupling can be excluded as a mechanism of the covalent binding of anilino-NO to LHA, even in the presence of laccase.

Fig. 6 Plot of experimental spectra of LHA with aniline (bottom), aniline and laccase (middle) and Fenton's reaction (top) in presence of 50 mM PBN. Top spectrum displays characteristic PBN spectral lines produced by radical reaction. Ordinate axis of top spectrum is enhanced by 1.8 relative to the other two spectra.

CONCLUSION

Our ESR experiments with labelled aniline as a model compound have shown in a proof-of-concept approach that the method can be used to identify strong binding of aromatic amines and determine its reaction kinetics to humic acid, an important soil constituent. Derivatives of nitroxide radicals are stable enough to study the interaction of specific functional groups to isolated humic substances as model compounds. We conclude from the spectral differences of the two amine substituted nitroxide radicals that the aromatic amino group covalently binds to humic substances at neutral pH, while aliphatic amines presumably interact via cation exchange or hydrogen bonding of the nitroxide group to hydrogen donors of LHA. Covalent binding of labelled aniline was evidenced by adding laccase. Oxidative radical coupling could be excluded by using the spin trapping agent PBN.

Our experiments demonstrated the suitability of nitroxide labels to investigate the interaction of xenobiotic chemicals with humic acids. In particular, the fast reaction of aniline with HA could not yet be revealed with other methods so far. It could explain the rapid loss of the extractability of sulfonamides after application to natural soil [17 – 19]. Humic acids have a wide distribution of potential reaction sites, which might be activated by treatment with laccase or other oxidizing

enzymes. The fast reaction of aniline with LHA observed with the nitroxide spin label might be a first step in a series of subsequent binding processes [23]. Covalent binding can unambiguously distinguished from other sorption processes such as sequestration, which would less restrict mobility of the spin labelled molecule. The specificity of the interaction process coupled with the sensitivity of the spin label signals make the method also suitable for more complex systems like soil and sediment. Recently, nitroxide spin probing experiments with natural soil [42] have demonstrated the potential of the method for investigating the interaction of xenobiotic functional groups with complex soil systems.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGEMENTS

We kindly acknowledge the lab-assistance of Elena Bondarenko. Support of the Hungarian National Research Fund OTKA K104956 is greatly acknowledged.

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Figure captions

Fig. 1 ESR spectra recorded at X-band (9.4 GHz) in the B-field region between 333 and 339 mT: amino-TEMPO in aqueous solution (grey continuous line) and upon incubation with LHA (broken line).

Fig. 2 ESR spectra recorded at X-band (9.4 GHz) in the B-field region between 330 and 340 mT: Anilino-NO in aqueous solution (upper panel) and upon incubation with LHA (measured, middle panel and simulated, lower panel). The inset in the middle panel highlights the low field resonance line of the signal of the bound label. For comparison with the experimental spectrum the simulated spectra for the free and bound components are superimposed in the lower panel. The concentration of the immobilized component was enhanced by a factor of 2.7 compared to the experimental case for better visibility.

Fig. 3 Structure of anilino-NO with orthogonal coordinate system

Fig. 4 ESR signal of the bound species of anilino-NO incubated with LHA

Fig. 5 Plot of the spin number ratio versus time of the bound species of anilino-NO incubated with LHA without (black, right y-axis) and upon incubation with (red, left y-axis) laccase; bold and dashed lines are fitted curves according to the bi- and mono-exponential kinetic model.

Fig. 6 Plot of experimental spectra of LHA with aniline (bottom), aniline and laccase (middle) and Fenton's reaction (top) in presence of 50 mM PBN. Top spectrum displays characteristic PBN spectral lines produced by radical reaction. Ordinate axis of top spectrum is enhanced by 1.8 relative to the other two spectra.

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484 Fig. 1

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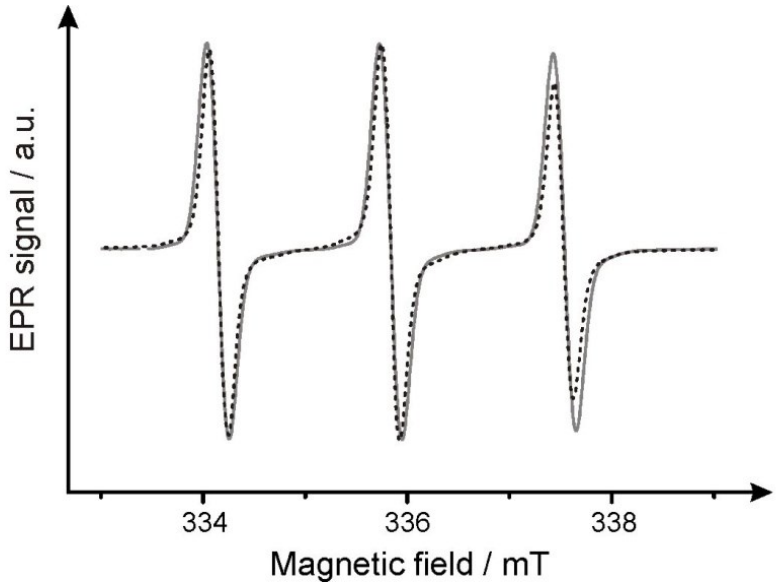

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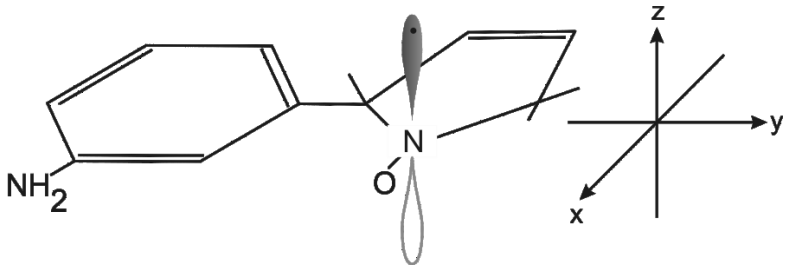
Fig. 2



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499 Fig. 3

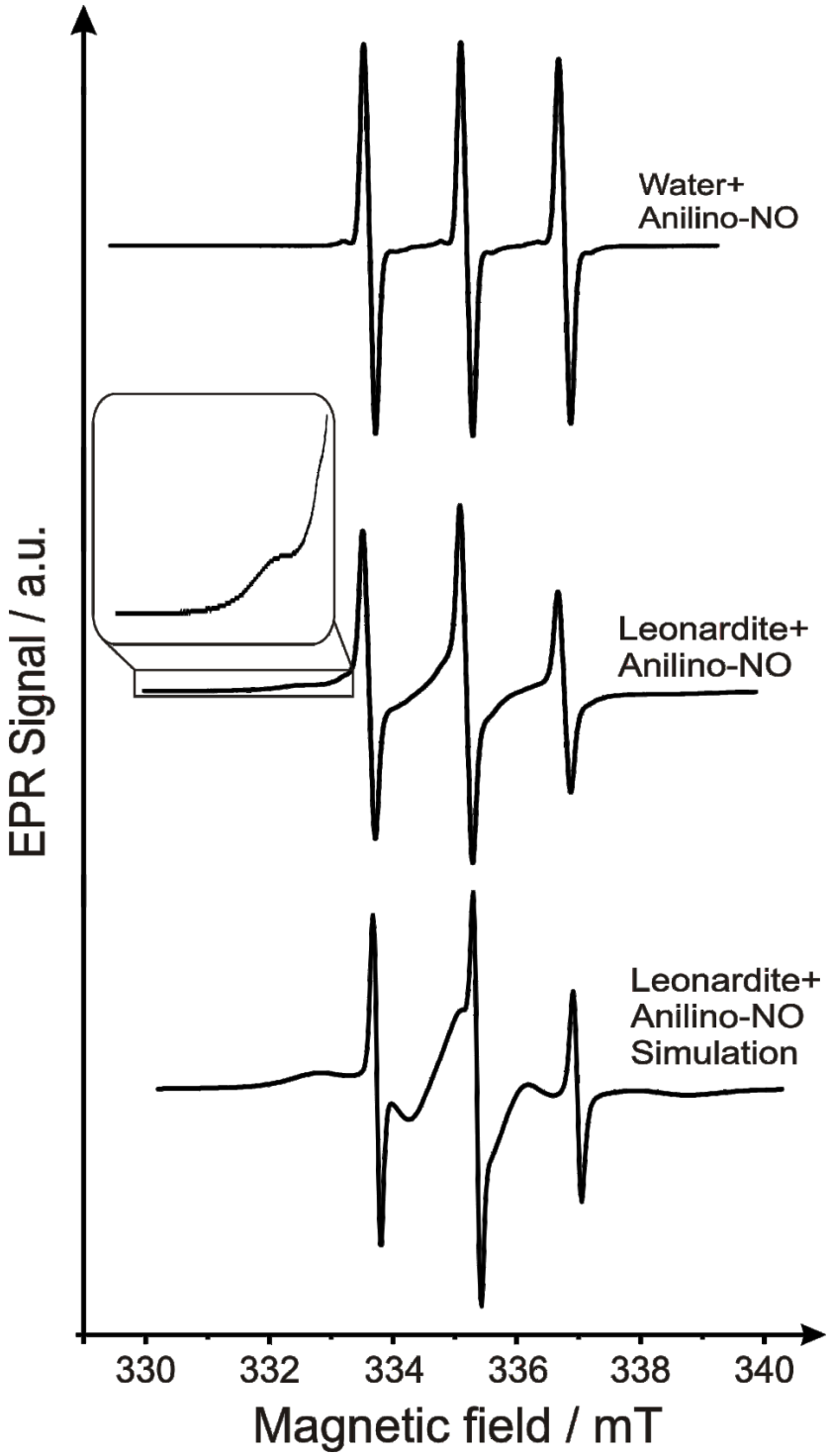

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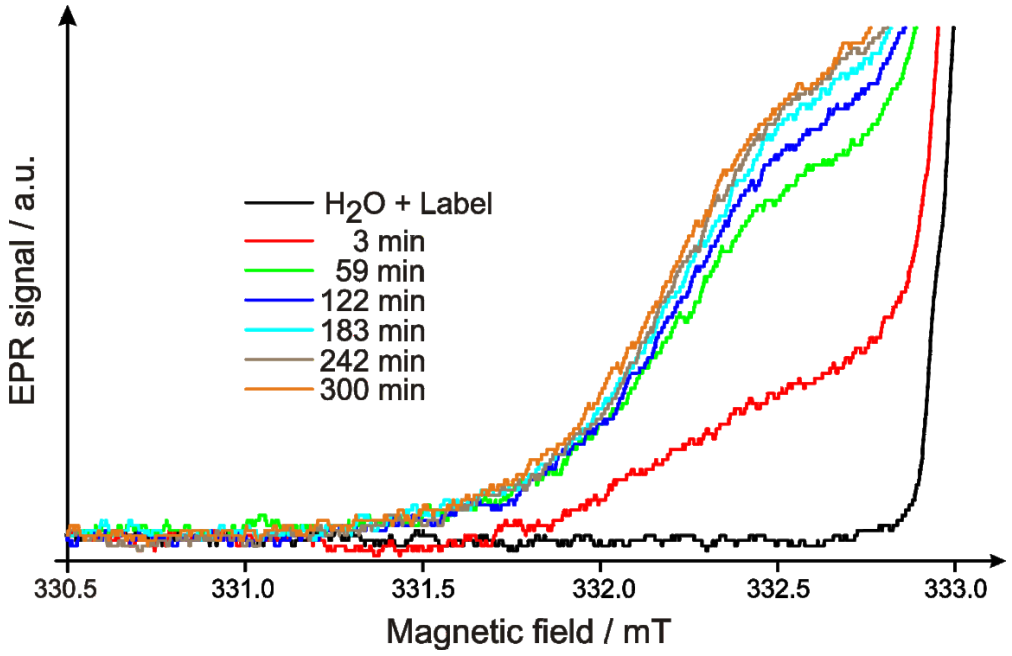
Fig. 4


EPR signal / a.u.
H2O + Label
3 min
59 min
122 min
183 min
242 min
300 min
330.5
331.0
331.5
332.0
332.5
333.0
Magnetic field / mT

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509 Fig. 5
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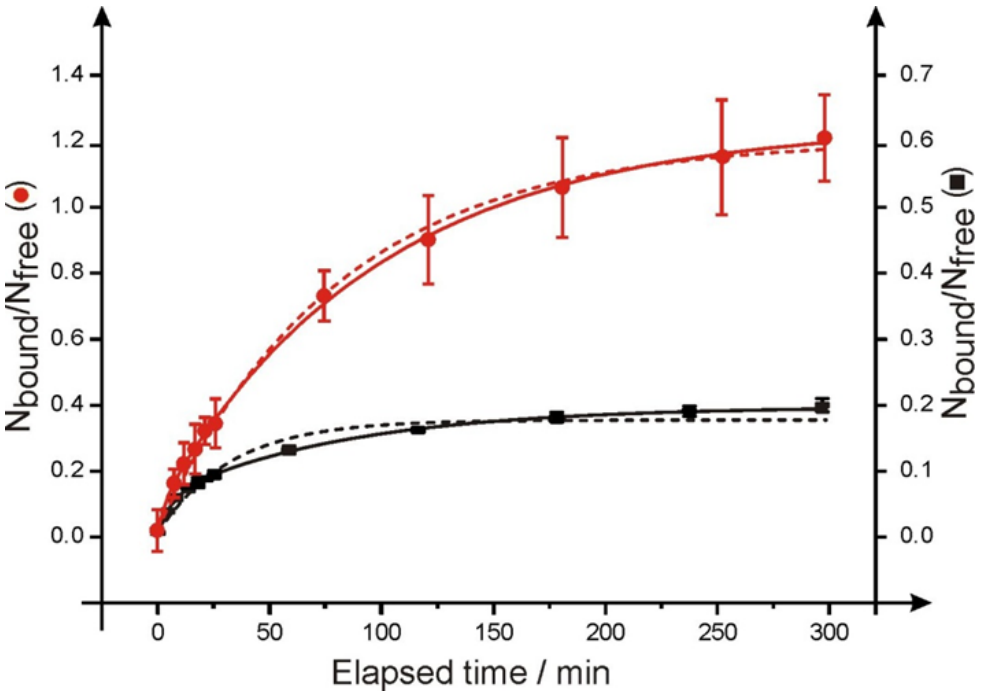

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515 Fig. 6
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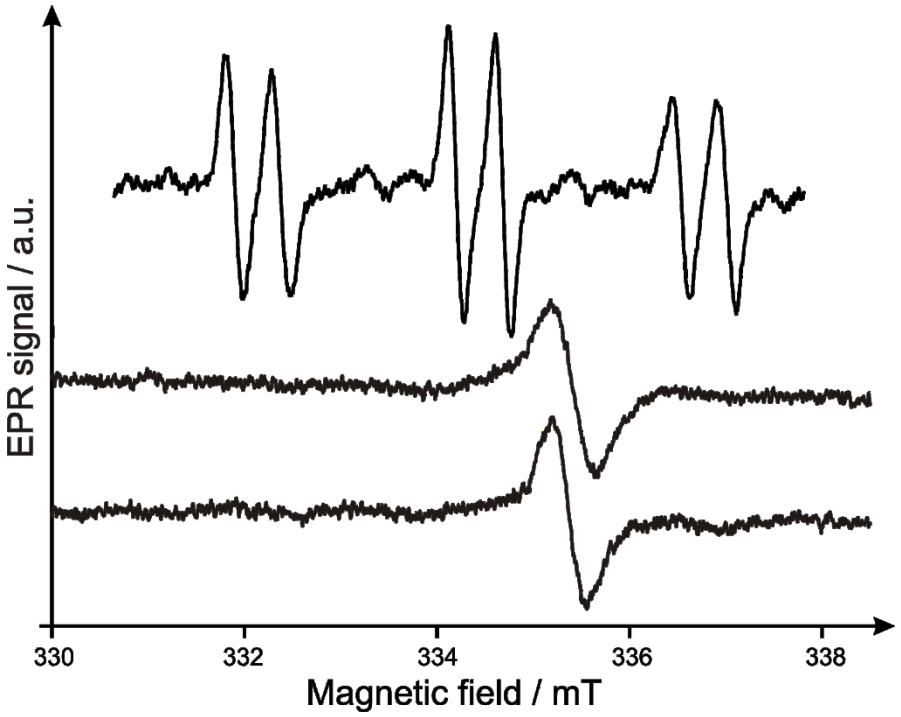

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