Interactions between natural organic matter and organic pollutants as revealed by NMR spectroscopy

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Natural organic matter (NOM) plays a critical role in regulating the transport and the fate of organic contaminants in the environment. NMR spectroscopy is a powerful technique for the investigation of the sorption and binding mechanisms between NOM and pollutants, as well as their mutual chemical transformations. Despite NMR relatively low sensibility but due to its wide versatility to investigating samples in the liquid, gel, and solid phases, NMR application to environmental NOM–pollutants relations enables the achievement of specific and complementary molecular information. This report is a brief outline of the potentialities of the different NMR techniques and pulse sequences to elucidate the interactions between NOM and organic pollutants, with and without their labeling with nuclei that enhance NMR sensitivity. Copyright © 2015 John Wiley & Sons, Ltd.

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Introduction

The prevention of environmental contamination by organic pollutants (OPs) and the cleanup of contaminated sites have become a worldwide priority, requiring an advanced knowledge as to both the pollutants reactivity and fate within environmental matrices. Such an in-depth understanding is essential to design efficient strategies for decontamination, reduction of pollutants toxicity and bioavailability, and prediction of their sequestration/migration in the environment. There are different types of organic compounds of both anthropogenic and natural origin that are potentially toxic to plants and animals, even at low concentrations. Most of anthropogenic organic contaminants are industrial products, such as pesticides, pharmaceuticals, endocrine disrupting compounds, and flame retardants. Depending on their chemical structure and reactivity, they are typically classified as polycyclic aromatic hydrocarbons, polychlorinated biphenyls (PCBs), nitroaromatic compounds, phenols, and anilines. Polycyclic aromatic hydrocarbons are a large group of organic compounds with two or more fused aromatic rings and include molecules such as naphthalene, anthracene, and phenanthrene. They result prevalently from pyrolytic processes and incomplete combustion of organic materials during industrial and other human activities, including the processing of coal and crude oil and the combustion of natural gas. PCBs are persistent OPs differing in the number of chlorine atoms attached to their biphenyl rings. Mixtures of PCB congeners have been widely manufactured from 1930 to the 1970s and used as coolants in transformer oil, dielectric fluids, and lubricants. Although the usage of PCBs has been banned over 30 years ago in many countries, they still exist in old electrical equipment and environmental media to which humans can be exposed. Nitroaromatic compounds, which are organic molecules made of at least one nitro group attached to an aromatic ring, are largely used in chemical manufacturing, oil refining, pesticides, bactericides, explosives, and intermediates in the synthesis of dyes and other high volume. Phenols and anilines are toxic constituents of dyestuff wastewater, and pentachlorophenol, in particular, represents one of the most diffused phenols, as it is widely used as a wood preservative or biocide.

Because of the serious health and ecological risks deriving from the continuous and uncontrolled release of pollutants in the environment and their transformation products of even higher toxicity, more research has been progressively devoted to better understand the environmental reactivity of these persistent OPs. In particular, interactions of pollutants with natural organic matter (NOM) have been increasingly studied. A relevant body of literature has shown that NOM associated with aquifers, soils, and sediments plays a principal role in regulating the transport and fate of contaminants in the environment. NOM is the most abundant form of organic carbon on the earth’s surface, ubiquitous in terrestrial and aquatic environments and widely heterogeneous, depending on the climatic, morphological, and microbiological features of its development. NOM significantly affects the quality of drinking water, the yield of crop production, and the environmental mobility of chemicals and heavy metals. NOM is also an important factor in the carbon biogeochemical cycle, and its abiotic and biotic dynamics in soil is directly related to either carbon sequestration or CO2 emission in the atmosphere.

The multifunctionality of NOM is ascribed to its peculiar chemical nature. In fact, NOM is reckoned to be a supramolecular association of relatively small (<1000 Da) and heterogeneous molecules,
derived from the environmental transformation of biomolecules released from dead cells, and held together in metastable conformations by weak bonds, such as hydrophobic and hydrogen bonds[38] or metal bridges. [34] NOM metastable conformations can be reversibly disrupted by interactions with small amounts of organic acids,[25–38] while the same amount of acids does not alter the conformation of true macromolecules stabilized by covalent bonds.[39] The supramolecular nature of NOM and its response to organic acids has been advocated to explain the bioactivity of humic matter on plants,[40] its molecular dynamics,[41] and the slow release of sorbed contaminants.[42] Furthermore, the recognition that NOM is composed by supramolecular associations rather than macromolecules has allowed the development of a fractionation strategy, called Humeonics, that enables the analytical detection of most of the single molecules that constitute the supramolecular assembly.[43–46]

Despite these recent achievements on the NOM chemical understanding, the mode of interactions between pollutants and NOM is still unclear and needs further studies.[47] The following principal mechanisms proposed to explain such interactions are non-covalent: (i) adsorption driven by weak forces, such as hydrophobic (van der Waals, π–π, CH–π and hydrogen bonds, and (ii) electrostatic linkages, such as charge transfer, ion exchange, or ligand exchange.[21] However, OPs are also believed to form covalent bondings with NOM, as enabled by specific environmental biotic or abiotic agents, such as microbial or enzymatic activity.[48–51]

Several different analytical techniques have been applied to elucidate the pollutants–NOM interactions, although no single analytical technique enables a complete structural or functional clarification of such interactions. However, NMR spectroscopy is certainly one of the most powerful techniques for the investigations of the sorption mechanisms occurring between NOM and pollutants.[52] NMR is a nondestructive technique that is capable to unravel the molecular complexities of the environmental heterogeneous matrixes through the large versatility of its applications in the solid, semisolid, and liquid state.[53,54] Despite its relatively low sensitivity, NMR offers a number of unique advantages, including the possibility to use different, complementary, and multidimensional experimental strategies and pulse sequences, which can be combined to show specific intermolecular and intramolecular interactions.[16,55–58]

NMR spectroscopy in environmental research

The NMR phenomenon occurs when the nuclei of magnetically active atoms (spin number ≠ 0) are immersed in a static magnetic field and exposed to a second oscillating magnetic field. Following the pulse interaction between the applied electromagnetic radiation and the dipolar moments of the nuclei subjected to the static field, the resonance energy is relaxed, producing an FID that contains detailed information about the structure, dynamics, reaction state, and chemical environment of the molecular material under study. The combination of phase-modulated and length-modulated pulses specific of the selected NMR pulse sequences enables a controlled manipulation of nuclear spins that ultimately provides structural dynamics information and reveals through-bonding or through-space correlations among nuclei, in either monodimensional or multidimensional systems. A number of pulse sequences are commonly available to perform single or multiple selective excitations of NMR signals and, when required, to suppress specific undesired signals without incurring in relevant spectral distortions.[53,54]

Several and diversified NMR pulse sequences are today available for both solution-state and solid-state NMR, which allow to face specific acquisition problems in environmental studies as a function of samples and reaction conditions. In particular, 1D and multidimensional NMR experiments have been employed to structurally characterize OPs and functional groups of NOM[59,60] as well as to study the relationships between NOM and organic contaminants.

NMR works dealing with organic compounds involved directly or indirectly in environmental processes are based on magnetically active nuclei with spin number of one half, such as 1H, 13C, 15N, 19F. Basically, the most common drawbacks rise from the following: (i) the scarce natural abundance of the magnetically active nucleus being examined, (ii) the low gyromagnetic ratio (γ) of the latter (responsible for a relatively weak NMR sensitivity), and (iii) the intrinsic low concentration of certain nuclei in natural systems. Fortunately, a number of techniques have been developed in order to circumvent most of these problems. For example, in case of 13C or 15N nuclei, whose natural isotopic abundance corresponds only to 1.1% and 0.366%, respectively, a solution is to employ 13C or 15N-labeled compounds.[49,61–63] This strategy to enhance NMR sensitivity is exploited in structural biology, wherein very complex biomacromolecules, such as proteins, are biosynthesized and overexpressed by microorganisms that have been fed with 13C or 15N-labeled compounds.[64,65]

In the case of NOM, this principle was first applied to verify by broadband decoupled 13C-NMR, the occurrence of covalent binding between a 13C-labeled 2,4-dichlorophenol (DCP) and peat humic molecules, upon the action of a peroxidase enzyme.[66] The alteration of the DCP chemical structure due to coupling with humic matter resulted in changes in the 13C chemical shifts of the labeled carbons, which were indicative of the site and type of bonding interaction. Similarly, Piccolo and coworkers[68] applied high-resolution NMR to follow the transformation of a 15N-labeled phenoxymalonic acid (2,4-D) herbicide in the presence of both humic matter and horseradish peroxidase. They showed for the first time that the abiotic degradation of 2,4-D can be catalyzed by dissolved humic substances at neutral pH. In fact, NMR spectra revealed that three different humic acids (HAS) catalyzed the abiotic splitting of 2,4-D into DCP and acetic acid at pH 7 but not at pH 4.7. Conversely, peroxidase did not catalyze the oxidative degradation of 2,4-D at any pH and even inhibited the effect of humic substances (Fig. 1). Catalytic degradation by humic matter was attributed to free-radical reactions enhanced by the stereochemical contribution of large conformational structures formed by the association of heterogeneous humic molecules at neutral pH. Inhibition of 2,4-D degradation, when humic substances were combined with peroxidase, was explained by modification of both chemical and conformational humic structure due to peroxidase-promoted oxidative cross-coupling among humic molecules.[67]

An increased importance of NMR in environmental studies is provided by 19F tagging in halogenated compounds that allows to understand and predict the behavior of chlorinated pollutants. The advantage consists in the high sensitivity of the fluorine nucleus and its typical large chemical shift range, which helps to identify site-specific interactions.[68–70] An example is represented by the use of a 19F-labeled pentachlorophenol (PCP) to ascertain its oxidative copolymerization with NOM due to a biomimetic oxidative catalyst, such as a water-soluble iron porphyrin.[71] The 19F-NMR spectra showed that after the catalyzed oxidative reaction in the presence of NOM, the 19F signals were more abundant and scattered over a larger chemical shift interval than for the 19F-labeled PCP alone (Fig. 2). This was explained by the covalent...
coupling that occurred between PCP and humic molecules and consequently varied the chemical surroundings of $^{19}$F nuclei in respect to PCP alone, thus generating a large number of NMR resonances.

Similar studies were conducted by Thorn and coworkers by using $^{15}$N-labeled dinitrotoluene and trinitrotoluene and their derivatives, in order to follow their degradation by $^{15}$N-NMR spectroscopy in the presence of NOM and in different experimental conditions. Among these experiments, the horseradish peroxidase was again found to promote the covalent binding of 4-methyl-3-nitroaniline-$^{15}$N to NOM. Liquid-state $^{15}$N-NMR spectra of a HA solution with the $^{15}$N-labeled nitroaniline showed that this pollutant became covalently bound to HA both before and after addition of the enzyme to solution (Fig. 3).

In case of nuclei with low gyromagnetic ratio, different strategies have been developed to enhance their NMR sensitivity. One of these relies on the transfer of magnetization from a very sensitive nucleus, such as hydrogen, to an insensitive nucleus. A number of different pulse sequences have been designed to induce heteronuclear magnetization transfer via through-bonding (intramolecular polarization) or through-space (both intramolecular and intermolecular polarization) correlations. In particular, an important application of through-space magnetization transfer is represented by the cross-polarization (CP) technique that is widely applied for solid-state experiments, especially for studies on NOM in soils and sediments.

NMR sensitivity may also be improved by special probes and equipment, such as cryoprobes and dynamic nuclear polarization (DNP) systems. Because the NMR noise is predominantly generated by electrical resistance occurring in the receiver coil, the cryoprobe is designed to reduce noise by cooling the NMR coil and preamplifier with liquid helium but still maintaining the sample at room temperature. DNP is a recent technique applicable for both liquid-state and solid-state NMR that has revolutionized many aspects of NMR spectroscopy. It consists in a substantial nuclear hyperpolarization achieved through a magnetization transfer from microwaves-excited electrons to nuclei, whose NMR sensitivity may be enhanced up to 400-fold or more in particular conditions.

**Chemical shift and signal broadening in solution-state NMR**

Although modern solution-state NMR spectroscopy provides a number of sophisticated monodimensional or multidimensional pulse sequences, in many cases, traditional 1D spectra may be sufficient to show reliable evidence of intermolecular interactions. In fact, a ligand–sorbent association perturbs the chemical environment of interacting nuclei, whose NMR signals are varied depending on several factors such as ligand affinity, extent of bound ligand, and chemical exchange kinetic. Because the interactions between NOM and other molecules are mostly driven by weak forces, such as hydrophobic (van der Waals, π–π, CH–π) and hydrogen bonds, chemical shift drifts or signal broadening of pollutants signals may reveal such associations, assuming that the alteration in solution viscosity may be ignored. The chemical shift drifts account for the change in the overall magnetic field.
The drift of $^{19}$F chemical shifts was also followed to verify the occurrence of associations between the 4′-fluoro-1′-acenaphthone organic contaminant and the Suwannee River fulvic acid. A progressive downfield drift of fluorine signals was detected when the fluorinated contaminant was dissolved in a 55% CH$_3$OD/45% D$_2$O solution at an increasing fulvic acid concentration. Moreover, the occurred interactions between a forest soil HA and several $^{13}$C-labeled PAHs, such as naphthalene, 1-naphthol, and quinoline, were shown by Simpson and coworkers by means of $^{13}$C-NMR spectroscopy. In particular, both proton and carbon signals of the studied pollutants showed an enhanced line broadening in the presence of HA, whereas no change in pollutants chemical shifts could be detected.

### Relaxation times

NMR relaxation is the process by which the total energy of a nuclear system, acquired after excitation by a radio-frequency pulse, is progressively lost and the equilibrium state is recovered. The importance of measuring the relaxation velocity of a nucleus is due to the fact that, given the absence of interfering factors, it is related to either structural variations or interactions undergone by the nucleus. Generally, NMR relaxation may be divided into longitudinal and transverse components, which generate spin–lattice (T$_1$) and spin–spin (T$_2$) relaxation times, respectively. These NMR parameters are very sensitive and reliable in revealing indirectly the changes in the overall molecular mobility, including translational, rotational, and vibrational motion. Therefore, NMR relaxation times are excellent means to assess the non-covalent association between a small sorbate molecule and a larger sorbent through the consequent reduction of the sorbate translational and rotational motion. T$_1$ and T$_2$ values can also be employed to extrapolate the molecular correlation time $\tau_c$ that is defined as the average time taken by a nuclear spin to rotate through one radian. The $\tau_c$ correlation time may provide a further indication on the change in ligand molecular mobility, because the shorter the tumbling rate of a ligand involved in the interaction, the longer becomes its correlation time. However, it is also to be noted that in a mixture that still contains the free ligand, the extrapolated T$_1$ or T$_2$ relaxation times correspond to an intermediate value ranging between that of the free ligand and that of the sorbed form. Furthermore, relaxation times remain also dependent on solution viscosity, magnetic field strength and homogeneity, and temperature.

In the late 1990s, a number of works have related the interactions between organic contaminants and NOM with the help of NMR relaxation data. Bortitaysnis and Hatchell employed a $^{13}$C-labeled phenol at the C-1 position in order to follow its presumed association with a HA. These authors showed that the T$_1$ relaxation time of the $^{13}$C-labeled phenol varied linearly with the concentration of humic matter, and on this basis, they characterized and quantified non-covalent interactions in the humic–phenol association. Analogously, Nanny et al. proved in different solvents, such as chloroform, methanol and methanol/water, the occurrence of non-covalent interactions between $^{13}$C-labeled acenaphthenone, a microbial metabolic by-product of acenaphthene, and the Suwannee River fulvic acid. Interestingly, these authors observed an enhanced solubilization of acenaphthenone that was accounted to the encapsulation of the pollutant in the three-dimensional fulvic acid structure, predominantly solvated with methanol.

Deuterated compounds were also employed to investigate pollutants interactions with NOM. In fact, application of $^2$H-NMR
Spectroscopy is advantageous because deuterium is a relatively sensitive nucleus for NMR experiments, while its relaxation mechanism is predominantly quadrupolar, thereby greatly simplifying interpretation of T1 data. The interaction between benzene-d6, pyridine-d5, and phenol-d5 and three different HAs was shown by liquid-state 2H-NMR to be non-covalent and significantly influenced by solution pH, degree of aromaticity, and functionality on the aromatic ring.[91] Interestingly, this work also reported the calculation of the spin correlation time (τc) for a quadrupolar nucleus in monoaromatic compounds, by taking into account the changes in solution viscosity. Furthermore, the NMR measurements of 19F relaxation and correlation times were also applied to assess the nature of interactions between 4′-fluoro-1′-acetonaphthone and the Suwannee River fulvic acid.[82]

A further approach based on nuclear relaxation parameters and useful to identify the mechanisms behind pollutants–NOM interactions is related to the use of paramagnetic molecular probes, which induce an enhanced nuclear relaxation of excited spins through interactions between electrons and nuclear dipoles.[85] The efficiency of such paramagnetic relaxations derives from the fact that the electron magnetic moment is 658 times larger than the nuclear magnetic moment and results in long-range interactions capable to relevantly influence both nuclear spin–lattice and spin–spin relaxation rates.[87] Paramagnetic probes have been largely employed as contrasting agents in magnetic resonance imaging studies, especially in medical research, with the aim to enhance detection of as well as the derived binding constants, showed that DCP was more strongly complexed by humic than fulvic acids. The authors attributed this phenomenon to the predominant hydrophobic character of HA that favored a more effective trapping of hydrophobic DCP molecules, thereby establishing host–guest associations.[60]

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dead tumor cells in vivo or to assess early tumor responses to a drug treatment. [92,93]

An application of this technique to environmental studies was proposed by Chien et al. [94] who investigated the interaction of trifluoromethylated atrazine with a soil HA by measuring the relaxation of ${}^{19}$F-NMR signals in the presence of two well-known paramagnetic probes, namely, gadolinium-ethylene-diaminetetraacetate (Gd-EDTA) and 2,2,6,6-tetramethyl-1-piperidinyloxyl (TEMPO). The F-enriched atrazine was dissolved in aqueous humic solutions, and the changes in ${}^{19}$F atrazine relaxation induced by paramagnetic probes were then followed. While the hydrophilic anionic Gd-EDTA did not exert any significant paramagnetic relaxation in atrazine, the hydrophobic neutral TEMPO induced a rapid change in the nuclear relaxation rate of the herbicide. The authors thus inferred that humic molecules formed ‘micelle-like’ aggregates containing hydrophobic cores into which nonpolar organic compounds may partition together with the hydrophobic TEMPO, thereby leading to an efficient paramagnetic relaxation of the fluorinated atrazine.

Recently, two paramagnetic spin probes, such as TEMPO and 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl, were subjected to interactions with several different NOM samples in order to determine whether the sorption of these probes depended on specific domains or functional groups. [95] In particular, solid-state $^{13}$C-NMR spectra of NOM were used to identify a specific molecular region in which $T_2$, $T_1$, and $T_1C$ relaxation times were selectively reduced as a function of the proximity of fluctuating magnetic fields produced by the unpaired electrons of probes. They found that the paramagnetic effect was distributed along all functional intervals of NOM spectra and no selective interactions were observed, thereby failing to reveal any preferential sorption on specific molecular structures of NOM. [96]

In binding studies between small ligands and large sorbents, signals of the large molecule frequently overlap those of the ligand, thus preventing a clear detection of the latter. This problem is successfully overcome by applying pulse sequences, which introduce different NMR filters and enable simplification of mixture spectra by selectively attenuating the undesired signals. [97] The most common strategies to achieve this purpose are based on $T_1$, $T_2$, or on their combination and may be used in both one-dimensional and two-dimensional NMR spectroscopy. [98] Indisputably, these filters may represent an efficient method to reach a clear detection of small contaminants signals, when they are masked by the intense and broad resonances produced by NOM.

**Molecular diffusion**

Diffusion-ordered NMR spectroscopy (DOSY-NMR) is an important tool for studying the changes in molecular dimension of biomolecules and small compounds when in complexes with NOM. [99]

DOSY-NMR is based on a pulsed field gradient sequence that enables measurement of translational diffusion (conventionally defined as self-diffusion) of dissolved molecules and provides direct information on molecular dynamics, including intermolecular interactions [100,101] and conformational changes. [102,103] Following the Einstein–Stokes theory, the diffusivity of a molecule decreases when its hydrodynamic radius increases, and information on intermolecular associations may be obtained by measuring the diffusion coefficient in a molecular system. [104,105] The diffusion rate of a small ligand thus changes considerably when it becomes associated with a large molecule. As a result, the diffusion coefficients of bound and unbound ligands differ substantially, thus enabling the diffusion NMR technique to selectively detect the bound ligand signals. [106]

The processing of DOSY spectra is unique in enhancing the resolution of the diffusions of molecular components in complex samples, because it provides a direct correlation of translational diffusion to the chemical shift in a second NMR dimension. [107] As discussed by Simpson, [108] there are different methods to process DOSY spectra. The CONTIN technique was introduced by Morris et al. [109] in NOM studies as a means to solve the inverse of the Laplace transform and provide a distribution of diffusivities for a number of soil fulvic acid and HA. [110] Thus, for mixtures containing NOM and a contaminant, DOSY-NMR has the potential to map the changes in diffusion of both the contaminant and the surrounding molecules, thus providing a direct evidence of the occurred interactions. [111] However, analogously to relaxation parameters, also in case of self-diffusion, one has to take into account that the self-diffusion measured by DOSY-NMR corresponds to an intermediate value between those of the free and bound ligands.

DOSY-NMR has been up to date largely employed in different research fields to show the occurrence of ligand–sorbent interactions and conduct molecular screenings based on ligand affinity. The technique was also efficiently applied in environmental studies and focused on interactions between NOM and organic molecules. [28,60,86,88,98] An early example was the application of DOSY-NMR to compare the extent of interactions between a peat HA and both the methyl tert-butyl ether, an important petroleum additive, and chlorsulfuron, a widely used and toxic herbicide. [109] In this work, the two contaminants were mixed with HA, and a series of 1D bipolar pulse pair longitudinal eddy-current delay diffusion experiments at increasing gradient strength were then acquired. The strongest interaction of the contaminant with humic matter was then directly related to the rapidity by which the pollutant signals were suppressed by the gradient strength. Moreover, the same work showed that DOSY may also efficiently reveal the associations between NOM and environmentally hazardous metals, such as cadmium. [110]

Remarkably, useful thermodynamic parameters may also be obtained by DOSY-NMR spectroscopy. [104] The self-diffusion constants of pollutants in interaction with NOM were employed to determine the fraction of contaminant bound to NOM and calculate both the association constants and Gibbs free energy for a number of pollutants such as DCP, [60] simple phenol and a number of halogenated phenols, [111] and the ionic and hydrophilic glyphosate herbicide. [112]

Diffusion-edited techniques are among the NMR filters employed to simplify the spectra of mixtures composed by molecules with different characteristics and dimensions. Differently from relaxation-edited filters, diffusion-based filters exploit the rapid diffusion of small molecules, thus enabling the isolation and characterization of signals due only to large molecules or aggregates. [110,111] Interestingly, some diffusion-edited sequences were also developed to isolate signals of small molecules. In particular, the pulse sequence called gradient modified spin-echo-bipolar gradient pulse pairs stimulated echo, introduced by Otto and Larive, [112] aimed to isolate diffusion signals of pollutants in a mixture with NOM components. This $^1H$-NMR pulse sequence was capable, by first using a spin echo to null the resonances of the coupled spins, to elegantly neglect the spectral resonances of coupled spins and detect only those of singlets.

A recent pulse sequence based on a diffusion filter is represented by reverse diffusion. [99] It consists in the subtraction of a control spectrum from another one achieved with suppression of signals of small molecule by means of a diffusion filter. It is advocated that
this sequence may be even superior to a traditional relaxation-based filter, because the extent of losses for small molecule signals becomes less influenced by relaxation times.\[59\]

**Saturation transfer difference (STD) methods**

Dipole–dipole coupling is one of the NMR processes through which the relaxation of a nuclear spin can occur. The excess of energy acquired by a nucleus subjected to a radio frequency may be transferred from one to another spin system, provided that internuclear distance is lower than 5 Å.\[53\] Such a through-space magnetization transfer is identified as the nuclear Overhauser effect (NOE), and it has been largely employed as follows: (i) to identify intermolecular interactions, by detecting changes in the relaxation rate of a molecule bound to a larger macromolecule; (ii) to build up a detectable magnetization in a partner spin system; and (iii) to enhance signal intensity for relatively insensitive nuclei.

Because NOE is due to dipole–dipole couplings, it is highly sensitive to internuclear distances and can be used not only to ascertain the existence of intermolecular interactions but also to measure both intramolecular and intermolecular distances. An NMR experiment based on NOE through-space transfer was applied to study the interactions between a fluorinated acetonaphthone and a Suwannee River fulvic acid.\[82\] In this work, a NOE-difference experiment has been conducted to selectively irradiate fulvic acid resonances in the presence of the contaminant. Such irradiation induced a heteronuclear 1H−19F NOE effect, and the 19F signal of acetonaphthone resulted intensively enhanced.\[82\]

The saturation transfer difference (STD) is one of the most robust and reliable methods among all through-space NMR experiments to investigate NOM–pollutants interactions. This technique, originally introduced by Mayer and Meyer (1999)\[112\] to evaluate the extent of non-covalent protein–ligand interactions, permits the fast detection of ligand bindings to large molecules, such as proteins, macromolecules, and aggregates, and allows to easily discriminate ligands from nonbinding molecules. In STD experiments, the frequency of large molecules, which are generally characterized by short tumbling rate, is saturated by a selective shaped-pulse irradiation. The ligand interacting with the irradiated molecule receives a partial saturation transfer through the NOE effect, and it is NMR detected. A second similar pulse sequence is then applied by an off-resonance irradiation to avoid any saturation of the large molecule. Finally, the subtraction of the second spectrum from the first one generates a difference spectrum that shows only signals for the protons involved in non-covalent interactions. Only the ligand protons in interaction with the large molecule can experience a polarization transfer and produce an enhanced signal intensity. The efficiency of the STD technique largely depends on the chemical exchange between the ligand bound and free states, which should be faster than the relaxation time. While only a relatively small amount of sorbent is sufficient for this experiment, a large excess of ligand is recommended in order to magnify the binding process and ensure sufficiently intense NMR signals.\[57,113\]

The STD technique has been already employed to elucidate the interactions occurring between NOM and xenobiotics.\[114,115\] Recently, the interactions between the glyphosate (N-phosphonomethylglycine) herbicide and soluble fulvic acid and HA have been studied at both pH 5.2 and 7, whereby the herbicide is present in different ionic forms.\[99\] The NOM–glyphosate association was also studied by the 1H-NMR STD technique that revealed that the non-covalent interactions were significantly enhanced only at pH 5.2 for both humic materials (Fig. 5). This result was explained with the stabilization of the NOM–glyphosate complex by the H-bonds established between the herbicide, protonated at the phosphonate group at pH 5.2, and the complementary oxygen-containing functions in humic matter.

A saturation transfer double difference (STDD) technique has been introduced as a variation of normal STD experiment for NOM–pollutants studies.\[114\] It consists in obtaining a clear spectral subtraction of the background signals due to NOM, thereby reaching an accurate quantitative STD estimation of epitope maps for the pollutant. This STDD technique was successfully employed to quantitatively describe the interactions between a pea HA and several water-soluble pesticides, such as diflufenoxpyr, imazapyr, acifluorfen, and chlorfluoruron.\[114\]

Later, a 1H19F reverse heteronuclear STD technique was applied to investigate the sorption of a perfluorinated organic compounds on a pea HA and other biomolecules, such as lignin and albumin.\[115\] Such further modification of the original STD technique comprises a selective irradiation of 19F nuclei in the perfluorinated compound, whose magnetization is then transferred to NOM nuclei only in the case of direct interaction with the 19F-labeled molecule. The final output of the application of this NMR experiment is a 1H spectrum that shows only those NOM protons that received the through-space magnetization transfer due to the direct association with the irradiated fluorinated compound.\[115\] A combination of the reverse-heteronuclear and forward-heteronuclear STD experiments was attempted by Longstaffe and Simpson.\[116\] They employed the former technique to identify the binding pattern of structurally similar xenobiotics that interact with NOM, while the latter sequence was used to

**Figure 5.** 1H saturation transfer difference (STD) spectra of glyphosate with humic matter at pH 5.2 and 7: (A) reference STD spectra acquired without irradiation, (B) STD difference spectra of a sample treated with soil fulvic acids, and (C) STD difference spectra of a sample treated with lignite humic acid. A vertical expansion (64x) was applied to the difference spectra. (Reprinted with permission from Mazzei and Piccolo,\[99\] Copyright 2012 by American Chemical Society).
reveal the binding orientation of fluorinated xenobiotics during the interaction.

Solid-state NMR

The MAS technique enables NMR analysis of crystalline or amorphous solid samples. MAS NMR has been conceived to minimize the processes that prevent the acquisition of meaningful NMR spectra in the solid state, such as strong homonuclear dipolar interactions, chemical shift anisotropy, quadrupolar interactions, and enhanced magnetic susceptibility. However, relevant advantages in NMR studies may originate from dipolar interactions. In fact, strong heteronuclear dipolar couplings can lead to efficient through-space magnetization transfer that is called cross-polarization and is capable to induce significant signals enhancement. The CP-MAS technique is widely applied in environmental studies, because it increases the sensitivity for less abundant nuclei with low gyromagnetic ratio, such as $^{13}$C, $^{15}$N, and $^{29}$Si, via polarization transfer from the large $\gamma$ and most abundant $^{1}$H nucleus. These advantages make the CP-MAS technique mostly appropriate to study the composition of NOM and its interactions with OPs.

A number of NOM samples with different chemical composition were studied by solid-state MAS NMR to evaluate their interactions with phenanthrene. This work showed that NOM nonpolar aliphatic components had a relevant role in phenanthrene retention. In particular, the authors acquired solid-state $^{13}$C-NMR spectra by both a Bloch decay experiment (direct polarization) and CP, in order to investigate the correlations between the Freundlich sorption coefficient of phenanthrene and the characteristics of NOM samples such as aromatic, nonpolar aliphatic [including amorphous poly(methylene)] and polar components. Interestingly, also the $^{1}$H inversion recovery detected by $^{13}$C was used to differentiate the poly(methylene) domains from the branched nonpolar aliphatic segments and correlate their distribution with the phenanthrene sorption capacity by NOM.

Moreover, some advanced solid-state experiments, such as $^{1}$H wide-line spectra (coupled to $T_{1}$/$H$ filters to remove the contribution of mobile components) and inversion recovery to measure $^{1}$H $T_{1}$ relaxation times, were employed to relate the domains of different mobility in several humic substances with their capacity to adsorb OPs. The different mobility of humic domains was also assessed from the broadening of signals obtained with the 2D $^{1}$H–$^{13}$C wide-line separation (WISE) technique. This sequence enabled a relationship between the molecular mobility of humic domains and the polarization transfer that was considered that the more efficient and faster, the broader was the signal in the WISE spectrum. As a confirmation of such result, 2D $^{1}$H–$^{13}$C heteronuclear correlation (HETCOR) spectra were also acquired by varying the mixing times and, thus, exploring the rigidity of humic domains as a function of the $^{1}$H–$^{13}$C spin-diffusion rate. It was found that both mobile and rigid domains of different compositions coexist in humic matter, although mobile domains represent the principal site whereby organic contaminants may partition.

As for solution-state NMR, the quality of solid-state NMR was improved by the application of spectral filters. For example, cross-polarization/total side band suppression (CP/TOSS), chemical shift anisotropy (CSA), and dipolar dephasing filters were applied to characterize in details the chemical composition of NOM prior to its interactions with DCP, phenanthrene, and 1,2,4,5-tetrachlorobenzene. The CP/TOSS sequence selectively removes spinning sidebands from the spectrum, thus enabling a concomitant lower rotor spinning rate and an enhancement of the CP efficiency. The CSA filter is designed to only show $sp^{3}$-hybridized carbons in the spectrum, by selectively removing anisotropic signals, such as those for aromatic or carbonyl carbons, while increasing the detection of anomic carbons. By varying the dephasing delay, the dipolar dephasing filter allows to selectively suppress carbon signals as a function of their protonation, whereby the length of filter delay is generally dictated by the following order: $CH_{2} < CH < CH_{3} < C_{quaternary}$. Nevertheless, it should be recalled that these techniques may also lead to the loss of observable signals, depending on other factors, such as filter duration and specific nuclear relaxation times.

Recently, an advance of CP-MAS, referred to as the multiCP technique, has introduced a simple and robust method that can provide quantitative 1D $^{13}$C-NMR spectra of NOM with an improved larger signal-to-noise ratio than previously achievable by quantitative direct-polarization NMR. It consists in long (>10 ms) CP contact from $^{1}$H, without any significant magnetization loss due to relaxation and with a moderate duty cycle of the radio-frequency irradiation. This is followed by multiple 1 ms CP times, alternated with $^{1}$H spin–lattice relaxation periods to repolarize protons. The quantitative reliability of this pulse sequence can be appreciated by the comparison (Fig. 6) of $^{13}$C spectra for a prairie soil HA acquired by either a direct polarization or a multiCP technique, with and without the application of a dipolar dephasing filter.

The advanced multiCP pulse technique was applied to characterize different NOM types, which were then investigated by two-dimensional $^{1}$H–$^{13}$C HETCOR correlation NMR spectroscopy after their interactions with a fully deuterium-exchanged, carbonyl-$^{13}$C-labeled benzophenone. The full deuteriation for benzophenone had the objective to eliminate intramolecular $^{1}$H–$^{13}$C correlations, thus allowing the benzophenone $^{13}$C-carbonyl to interact exclusively with the nearby protons in NOM. The spin diffusion from benzophenone carbons to NOM protons was followed by HETCOR experiments, which employed three different mixing times (0.01, 0.3, and 10.25 ms). As for the interactions with a Pakoee peat, the three HETCOR experiments indicated the molecular domains with which benzophenone preferentially interacted (Fig. 7a–c). The signals related to benzophenone carbonyl, at 198 ppm, and aromatic, at 137 ppm, were further shown by $^{1}$H HETCOR vertical 1D slices, at different mixing times, in Fig. 7e.

![Figure 6](image)

**Figure 6.** Quantitative $^{13}$C-NMR spectra of a standard prairie soil humic acid, measured at 14 kHz MAS within 5 h each, after 11.5 ms (10 × 1.1 ms + 0.5 ms) multiCP with $\tau_{Z} = 0.4$ s (thick black line) and after direct polarization (DP) with 30 s recycle delays (dashed red line), scaled to match peak intensities. Thin continuous (green) and dashed (blue) lines: corresponding multiCP and DP spectra, respectively, of nonprotonated C and mobile segments selected by recoupled dipolar dephasing. (Reprinted with permission from Johnson and Schmidt-Rohr, Copyright 2014 by Elsevier).
and $T_f$, respectively, whereas the proton projection for the HETCOR experiment at the longest mixing time is reported in Fig. 7g. Interestingly, $^{13}$C-detected $^1$H inversion-recovery spectra were also acquired for the Pahokee peat sorbed with benzophenone (Fig. 7d), in order to detect the spatial proximity of NOM domains and predict their availability for interactions.$^{[127]}$

The $^{19}$F spectroscopy was also employed in the solid state to investigate the role of lipid composition in geosorbents, such as coastal sediments, humin, and HAs, on the adsorption of phenanthrene.$^{[70]}$ The authors used the 2,2,2-trifluoroethyl laurate as a probe to show the relevant contribution of the lipid components of the selected geosorbents. In fact, they observed that a significant change occurred in $^{19}$F spectra after adding phenanthrene to a humic matter that was previously sorbed with the fluoroprobe.

The $^{19}$F spectroscopy was also applied to study the association between organofluorine xenobiotic compounds and an unmodified peat.$^{[128]}$ In particular, the $^{19}$F nuclei of organofluorine compounds were used to induce observable interactions.$^{[127]}$

Figure 7. Two-dimensional HETCOR NMR spectra of the Pahokee peat sorbed with benzophenone-$^{13}$C=O-$d_{10}$ with (a) 1 ms LG-CP ($T_{m,e} = 0.01$ ms), (b) 1 ms HH-CP ($T_{m,e} = 0.3$ ms), and (c) 1 ms HH-CP with 10 ms spin diffusion ($T_{m,e} = 10.25$ ms). (d) $^{13}$C-NMR-detected $^1$H inversion recovery of Pahokee peat sorbed with benzophenone-$^{13}$C=O-$d_{10}$ after the indicated recovery delays (ms). The $^1$H spectra extracted at the $^{13}$C chemical shifts of $^{13}$C=O (197 ppm, panel e) and nonprotonated aromatic C (137 ppm, panel f) at different mixing times. (g) Proton projection along 0–220 ppm at $T_{m,e} = 10.25$ ms. (Reprinted with permission from Cao and coworkers,$^{[127]}$ Copyright 2014 by American Chemical Society).
transverse magnetization to the $^1$H nuclei in the peat molecular components, which were in direct interaction with the sorbed xenobiotics. This experiment was conducted with the recently conceived comprehensive multiphase MAS probe that involves a spectrometer lock. The latter device provides an enhanced line shape for $^1$H detection, as compared to a traditional solid-state hardware, and also includes gradients that allow to conduct NMR diffusion experiments in the solid state.[129,130]

**HR-MAS NMR**

The versatility of NMR spectroscopy was further enlarged by the introduction of the high-resolution MAS (HR-MAS) technique that enables NMR investigation on semisolid or gel-phase samples.[131] The sample is placed in a locked-up rotor and added with a small amount of a deuterated solvent that helps to reduce dipolar interactions, which are further minimized by spinning the rotor at the magic angle. The HR-MAS probes are generally provided with a deuterium lock and a gradient system. The latter device permits experiments (originally designed for solution-state NMR), such as diffusion-based and diffusion-edited experiments, or those exploiting the selection of spoil gradients blocks for both 1D and multidimensional spectroscopy. A further singular advantage of HR-MAS is that it may distinguish between the free and bound ligand forms, because the technique detects the latter only by fully removing anisotropy through MAS rotation.

Simpson and coworkers[28] early applied HR-MAS NMR to study samples of environmental interest. They showed that the structure of a whole soil at the solid–aqueous interface may be characterized by HR-MAS spectra, by combining one-dimensional and two-dimensional solution-like experiments, including T$_2$ filters when necessary. In addition, they observed variations in spectra induced by specific solvents, such as deuterated water, that produced broader signals, and dimethylsulphoxide-d$_6$ that conversely led to an improved resolution, prevalently because of the solvent-induced displacement of H-bonds. When the herbicide trifuralin was put in contact with the soil, new signals appeared in the spectra and were attributed to the herbicide bound to the external soil matrix.[28]

The role of the physical conformation of organic matter in the processes of pollutants sorption was also explored by HR-MAS.[132] A number of organo-clay complexes were formed by coating montmorillonite and kaolinite with humic matter and phenanthrene in Na$^+$-dominated or Ca$^{2+}$-dominated solutions at different pH and ionic strength values. The resulting HR-MAS spectra suggested that the sorption of the pollutant onto the organo-clay complexes occurred as a function of the distribution of hydroxyl sites on the mineral surfaces. In fact, these sites determined the interfacial configuration of the humic coating through the bonds formed with the complementary carboxyl groups in humic molecule, thus varying the accessibility of sorptive sites to phenanthrene.[132] The HR-MAS spectroscopy was also applied to study the interactions between toluene and HA.[133] This work showed that a correlation existed between the molecular motion of humic aliphatic components and the attenuation of self-diffusion for toluene through the humic matrix.

**Perspectives**

The NMR spectroscopy has so far largely contributed to a molecular understanding of the interactions between OPs and NOM in the environment. However, much is left to be done in this sector, especially because of the complex properties of NOM, which may vary in different environmental compartments, as well as in different geographical and climatic conditions. Future efforts should be devoted to elucidate further not only the molecular content of NOM but also how the molecular components are stereochemically arranged in water and soil. To such an aim, a great contribution to the unraveling of the molecular composition of NOM can be again provided by NMR spectroscopy with its fast advances in novel and highly performing pulse sequences or additional hardware devices (powerful magnets, cryosystems, new probes, hyphenation with chromatographic separations, etc.). Most of the techniques described here will be made more efficient in the near future with the advance of NMR technologies.

Nevertheless, coupling of NMR spectroscopy with high-resolution and ultra-high-resolution liquid-chromatography/mass-spectrometry techniques should also enhance considerably the structural precision in identifying the humic molecules responsible for the binding and environmental transport of pollutants in different conditions. It is also believed that the large amount of refined data that can be obtained in the future by advanced NMR and mass-spectrometry instrumentation will have to undergo extensive elaboration by intelligent software and multivariate analyses in order to reduce statistical uncertainty in data interpretation. Finally, the stereochemical understanding that is required to predict the adsorption on and diffusion through NOM of pollutants of different polarity will be achieved by handling the vast amount of molecular data with computational chemical software. This computational support will become fundamental to increase the significance of collected NMR data, such as those acquired with DOSY or through-space correlations techniques, which can be used to feed the predictive models of pollutant behavior in the environment.

**References**
