Journal of Industrial Microbiology & Biotechnology (2001) 26, 70–76

www.nature.com/jim

Combined effects of an oxidative enzyme and dissolved humic substances on ^{13}C-labelled 2,4-D herbicide as revealed by high-resolution ^{13}C NMR spectroscopy

A Piccolo[1], P Conte[1], A Cozzolino[1] and M Paci[2]

[1]*Dipartimento di Scienze Chimico Agrarie Università di Napoli "Federico II," 80055 Portici, Italy;* [2]*Diparimento di Scienze e Tecnologie Chimiche Università di Roma "Tor Vergata," 00187 Roma, Italy*

Phenoxyalkanoic acids are a widely used class of herbicides. This work employed high-resolution ^{13}C NMR to study the structural changes induced by humic substances and horseradish perodixase on 2,4-dichlorophenoxyacetic acid (2,4-D) ^{13}C-labelled in the side chain. NMR spectra showed that humic substances chemically catalyze abiotic splitting of [^{13}C]2,4-D into 2,4-dichlorophenol and [^{13}C]acetic acid at pH 7 but not at pH 4.7. Peroxidase did not catalyze the oxidative degradation of [^{13}C]2,4-D at any pH tested and inhibited the effect of humic substances. Catalytic degradation by humic substances was attributed to free-radical reactions enhanced by the stereochemical contribution of large conformational structures formed by 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. Our findings show for the first time that the abiotic degradation of 2,4-D is catalyzed by dissolved humic substances at neutral pH. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 70–76.

Keywords: ^{13}C NMR spectroscopy; humic substances; ^{13}C-labelled 2,4-D; horseradish peroxidase; chemical and enzymatic catalysis

Introduction

Organic chemicals can interact with soil components by a number of mechanisms involving either physical sorption or chemical reactions [28]. Binding of pesticides and other xenobiotics to soil may be environmentally beneficial since immobilized compounds exhibit a reduced bioavailability and their transport to groundwater is greatly restricted [1,2]. Diminished toxicity is also achieved when pesticides are either covalently bound or structurally degraded by the action of oxireductive enzymes present in soil [10,20,21].

Early work with ^{14}C-labelled xenobiotics showed that bound radioactivity was located in all three major fractions of soil organic matter (humic acids, fulvic acids, humin) [13,17]. The rate of binding was a function of the chemical structure of the compound and the type of soil. The labelling of xenobiotics with ^{14}C facilitates quantification of bound residues, but it cannot provide much structural information unless combined with other analytical techniques [3,18]. Recent investigations have demonstrated that further insight in the nature of binding can be achieved by the application of ^{13}C- or ^{15}N-labelled xenobiotics in combination with ^{13}C- or ^{15}N-NMR spectroscopy [12,15,35]. Identification of pesticide residues relied on the intensities and chemical shifts of the labelled atoms.

In the present study, NMR was used to investigate the structural modifications brought about on ^{13}C-labelled 2,4-dichlorophenoxyacetic acid (2,4-D) herbicide by the presence of horseradish peroxidase and dissolved humic substances of different molecular structure. 2,4-D is a widely used herbicide whose precursor and degradation product is 2,4-dichlorophenol (2,4-DCP) [22,25,26]. Horseradish peroxidase, with hydrogen peroxide, is believed to promote the covalent binding of chlorinated aromatic compounds to soil humic material via enzyme-catalyzed redox reactions [11,29]. 2,4-D was found to degrade rapidly in soil but not to be linked to humic-like polymers artificially formed by the action of horseradish peroxidase [34]. The lack of polymerization into soil humus suggested an intracellular degradation of 2,4-D. However, after 1 year most of the residual 2,4-D radiocarbon was not found in the biomass but remained in the hydrolyzable fraction of soil organic matter, suggesting that degradation could also proceed abiotically. Our objective was to apply ^{13}C NMR spectroscopy to investigate whether the combined action of horseradish peroxidase and humic materials could promote abiotic structural changes in 2,4-D.

Correspondence: Dr A Piccolo, Dipartimento di Scienze Chimico Agrarie Università di Napoli "Federico II," 80055 Portici, Italy
Received 9 February 2000; accepted 22 May 2000

Materials and methods

Humic substances

Three humic acids (HAs) were isolated from different sources: HA1, from a North Dakota Leonardite (Mammoth, Int. Chem. Co.); HA2, from an oxidized coal provided by Eniricerche SpA (Italy); and HA3, from a volcanic soil (Typic Xerofluvent) near Rome (Italy). HAs were isolated and purified as described elsewhere [21]. Elemental content of HAs (Table 1) was determined with a Fisons EA 1108 Elemental Analyzer and the ash content, obtained by burning 50–100 mg of the HAs in an oven

Table 1 Average elemental analyses (on ash-free and moisture-free basis) of humic acids and standard error ($n=3$)

HA	C (%)	H (%)	N (%)	C/H	C/N
HA1	45.9 (0.7)	3.7 (0.2)	1.0 (0.2)	12.4	45.9
HA2	48.0 (1.1)	3.0 (0.2)	1.0 (0.2)	16.0	48.0
HA3	53.7 (0.9)	4.9 (0.3)	4.3 (0.2)	11.0	12.5

at 750°C for 8 h, resulted in lower than 5% (w/w) for all humic materials.

^{13}C CPMAS-NMR spectroscopy of humic substances

Cross-polarization magic angle spinning carbon-13 nuclear magnetic resonance spectroscopy (CPMAS ^{13}C-NMR) measurements of humic substances were carried out on a Bruker AMX400 instrument operating at 100.625 MHz on the carbon-13. A recycle time of 1 s and an acquisition time of 13 ms were used. All experiments were conducted with variable contact time (VCT) pulse sequence in order to find the optimum contact time (OCT) for each sample, and to minimize the error on evaluation of the peak areas [8]. OCT ranged between 0.8 to 1.0 ms. A line broadening of 50 Hz was used to transform all the FIDs. The area in the 110–140 ppm region was corrected for the side band of carboxylic groups signal by subtracting the side band area in the 190–230 ppm region from that of the 110–140 ppm region. The areas of each region of the spectra in Table 2 were attributed to nonpolar carbons such as the aliphatic (0–45 ppm) and aromatic (110–160 ppm) ones, and to polar carbons such as the C–O, C–N groups and anomeric carbons (45–110 ppm), and carboxylic carbons (160–190 ppm). The areas of the 0–45 and 110–160 ppm regions were used to calculate hydrophobicity (HB), whereas those of the 45–60, 60–110, and 160–190 ppm regions were used to obtain hydrophilicity (HI) of HAs. The HI/HB ratios are also given in Table 2.

^{13}C-labelled herbicide

The herbicide 2,4-D ^{13}C-labelled by reacting 2,4-dichorophenol with α-ethyl (^{13}C) bromoacetate in anhydrous acetone as previously described [30]. The synthesized molecule of 2,4-D had one carbon-13 α to the carboxyl group and was obtained in a final yield of 84%.

Horseradish peroxidase

Horseradish peroxidase (HRP) was purchased from Sigma Chemical Co. (Milano). HRP activity was assayed by oxidizing ABTS [2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate)]. The reaction mixture (1.5 ml) contained 4 nM (0.75 ml) of ABTS, 4 mM (0.75 ml) of H_2O_2, dissolved in 0.1 M citrate–phosphate buffer (pH 4.5), and 15 μl (0.0044 mg/ml) of peroxidase. Oxidation of ABTS was followed by an absorbance increase at 420 nm (extinction coefficient=24 l mmol^{-1} cm^{-1}). One unit was defined as the amount of HRP that oxidized 1 μmol of ABTS in 1 min at 25°C and pH 4.5. The specific activity of the enzyme was calculated by dividing the activity measured in 1 ml of enzyme by the mass of enzyme contained in the volume.

Interactions of [^{13}C]2,4-D with humic substances and peroxidase

Humic acids (20 mg) were added dropwise with a 0.5 M NaOH solution until dissolution and then diluted to 100 ml with distilled water. A [^{13}C]2,4-D solution (8.4 ml, 0.4 mg/ml) dissolved in the selected buffer was placed in a 100-ml flask together with 10 ml of the humic acid (2 mg) solution and diluted to 75 ml with the same buffer. Three different buffers were used universal buffer (0.2 M acetic acid, 0.2 M boric acid, 0.2 M phosphoric acid, and 1 M NaOH) at pH 7.2; inorganic phosphate buffer at pH 7; and inorganic phosphate buffer at pH 4.7. pH values of 4.7 and 7.0 were chosen on the basis of maximum peroxidase activity and common soil pH, respectively. All buffers contained 4.0×10^{-3} M NaN_3 to suppress microbial activity. The ratio of humic acid to 2,4-D was determined on the basis of the maximum solubility of the humic acid and the minimum amount of 2,4-D that was needed to attain a reasonable signal-to-noise ratio. The humic acid–herbicide mixture was then treated with the required amounts of peroxidase units (2.9, 5.81, and 11.6 ml were added from a peroxidase solution of 1100 units in 50 ml to obtain 64, 128 and 256 enzymatic units, respectively) and 139 μl of 1% H_2O_2 and incubated for 2 h. After incubation, the reaction mixture was acidified to pH 1 with HCl, stored overnight in a cold room for complete precipitation of humic acid, and centrifuged. The pellet was washed several times with acidified water (pH 1) and was then dissolved in 1 ml of 1% NaOD and subjected to NMR analysis.

^{13}C NMR spectra of [^{13}C]2,4-D

The ^{13}C NMR inverse-gated spectra of [^{13}C]2,4-D was obtained using composite pulse decoupling on a Bruker AMX400 NMR spectrometer with a resonance frequency of ^{13}C of 100.625 MHz. A pulse angle of 45° and a relaxation delay of 2 s were used. Preliminary work with pure [^{13}C]2,4-D proved that the adopted relation time did not saturate signals nor distort signal intensities.

Table 2 Average distribution (%) of 13-carbons in resonance intervals (ppm) of CPMAS-NMR spectra and HI/HB ratios of HAs. Standard error of measurements ($n=3$) was less than 0.5 for all ranges

Humic sample	0–45	45–60	60–110	110–160	160–190	HI/HB[a]
HA1	25.3	8.4	18.0	39.4	16.6	0.66
HA2	22.1	9.8	16.4	36.9	23.4	0.84
HA3	35.3	12.8	30.8	16.0	10.3	1.05

[a]HI/HB=[(45–60)+(60–110)+(160–190)]/[(0–45)+(110–160)].

Approximately 22 000 acquisitions were required for suitable spectra.

Results and discussion

The elemental characteristics of humic acids (HAs) used in this study are shown in Table 1 whereas the carbon-13 distribution as revealed by CPMAS spectroscopy is shown in Table 2. HA1 from lignite had the largest content of hydrophobic components, whereas HA3 from a soil was the most hydrophilic (Table 2). The highest C/H values found for HA2 from oxidized coal (Table 1) indicated a larger carbonaceous character of this material than for both lignite and soil HAs.

The lignite HA was used in the first experiment with the universal buffer at pH 7.2 in which humic matter was mixed with [^{13}C]2,4-D and with progressively larger amounts of peroxidase (64, 128, and 256 units). The related NMR spectra are shown in Figure 1. The labelled carbon in the side chain of [^{13}C]2,4-D resonated at 66.8 ppm when the herbicide was dissolved alone in the buffer (Figure 1A). When [^{13}C]2,4-D was mixed with HA1 from lignite the intensity of the signal at 66.8 ppm was substantially reduced, whereas a second and more intense resonance appeared at 29.5 ppm (Figure 1B). The latter could only be attributed to a ^{13}C-labelled acetic acid ([^{13}C]H_3COOH) deriving from the breakage of the ether bond in the herbicide side chain. Addition of 64 units of peroxidase followed by H_2O_2 to the herbicide–humic mixture produced an NMR spectrum with a reversed appearance: the 66.8-ppm signal was more intense than the one at 29.5 ppm (Figure 1C). With higher rates of peroxidase additions (128 and 256 units) the signal at 66.8 ppm increased in intensity while that of the 29.5 resonance was progressively reduced (Figure 1D and E). Variations in signal intensity among spectra are caused by colloidal humic matter [9].

In the second experiment, all three HAs were used in combination with two concentrations peroxidase but in a totally inorganic phosphate buffer at pH 7 (Figure 2). As in the case of the universal buffer, increasing amounts of peroxidase in the presence of HA1 progressively decreased the signal at 29.5 ppm; it was no longer detected with 128 units of peroxidase (Figure 2A and B). The same effect was noticeable in the spectra from the experiment with HA2 from oxidized coal though to a different extent. In fact, the two ^{13}C signals had approximately the same intensity with 64 units of peroxidase while the one at 29.5 ppm seemed to be somewhat reduced relatively to 66.8 ppm when 128 units of peroxidase were present (Figure 2C and D). The spectra of the mixtures with HA3 from soil showed a resonance at 29.5 ppm with a lower intensity than at 66.8 ppm with 64 peroxidase units (Figure 2E). The relatively higher intensity of the 66.8 ppm signal was further increased with 128 units of peroxidase (Figure 2F).

Contrary to what was observed for both buffers at neutral pH, lowering the pH of the phosphate buffer to 4.7 produced spectra in which only a sharp signal at 66.8 ppm for [^{13}C]2,4-D was visible in the presence of different HAs alone (spectra not shown) or when 128 peroxidase units were added either with or without HAs (Figure 3A–D).

These findings suggest that the abiotic degradation of 2,4-D can be induced more by the dissolved humic substances alone (Figure 1B) than when in combination with peroxidase. In fact, in either neutral universal or phosphate buffer hydrolysis of the 2,4-D side chain must have occurred because of the appearance of the 29.5-ppm signal attributable to ^{13}C-labelled acetic acid (Figures 1 and 2). The molecule of 2,4-dichorophenol, also arising from 2,4-D degradation, was not detected in the NMR spectra because it lacked ^{13}C-labelling. The presence of the peroxidase oxidative system at neutral pH seemed to inhibit the degradation reaction (Figures 1 and 2). Moreover, when [^{13}C]2,4-D was dissolved in phosphate buffer at pH 4.7 no degradation was promoted by either the peroxidase system or by humic substances (Figure 3).

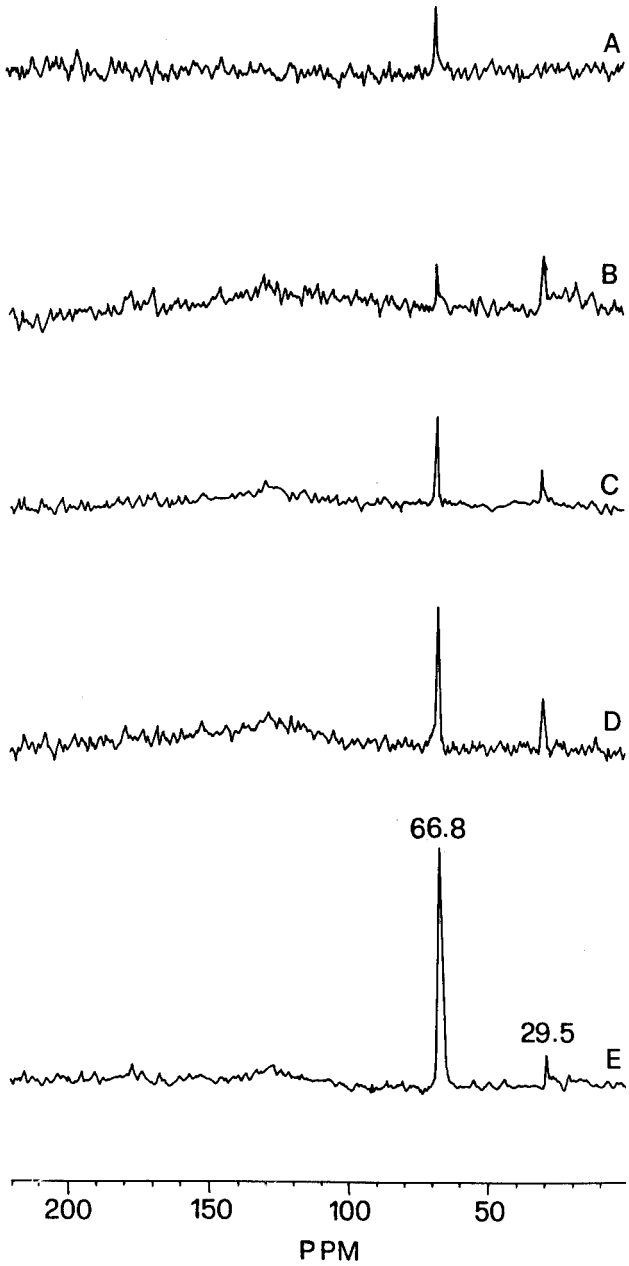

Figure 1 Liquid ^{13}C NMR spectra of reaction mixtures of ^{13}C-labelled 2,4-D in universal buffer at pH 7.2. (A) [^{13}C]2,4-D alone. (B) [^{13}C]2,4-D with HA1. (C) [^{13}C]2,4-D with HA1 and 64 units of HRP (horseradish peroxidase). (D) [^{13}C]2,4-D with HA1 and 128 units of HRP. (E) [^{13}C]2,4-D with HA1 and 256 units of HRP.

The 2,4-D degradation occurring at neutral pH may be based on a free-radical mechanism. Humic substances contain stable free radicals [33] which become significant in number at higher

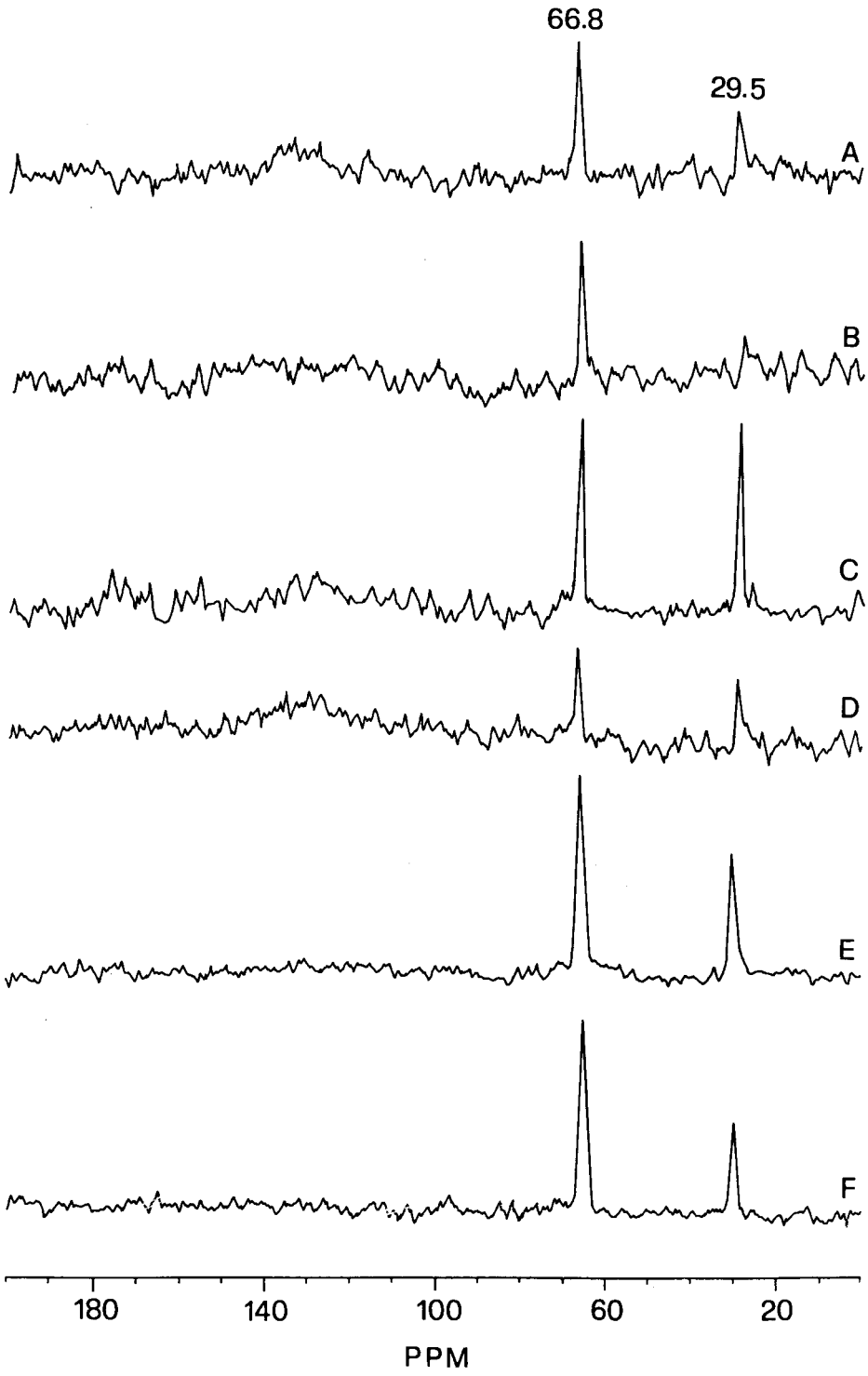

Figure 2 Liquid ^{13}C NMR spectra of reaction mixtures of ^{13}C-labelled 2,4-D in phosphate buffer at pH 7. (A) [^{13}C]2,4-D with HA1 and 64 units of HRP (horseradish peroxidase). (B) [^{13}C]2,4-D with HA1 and 128 units of HRP. (C) [^{13}C]2,4-D with HA2 and 64 units of HRP. (D) [^{13}C]2,4-D with HA2 and 128 units of HRP. (E) [^{13}C]2,4-D with HA3 and 64 units of HRP. (F) [^{13}C]2,4-D with HA3 and 128 units of HRP.

pHs [5], and more free radicals are produced by the addition of H_2O_2 and peroxidase. The high reactivity of humic free radicals may be responsible for 2,4-D degradation. A considerable quenching of free-radical concentration and line broadening was observed in electron spin resonance (ESR) spectra of the products obtained by interaction of soil HAs with aqueous chlorophenoxyalkanoic acids [6,32]. This was attributed to homolytic cross-coupling reactions between humic free radicals and phenoxyl or ariloxyl radical intermediates generated photochemically or by chemical or enzymatic catalysis in the partial

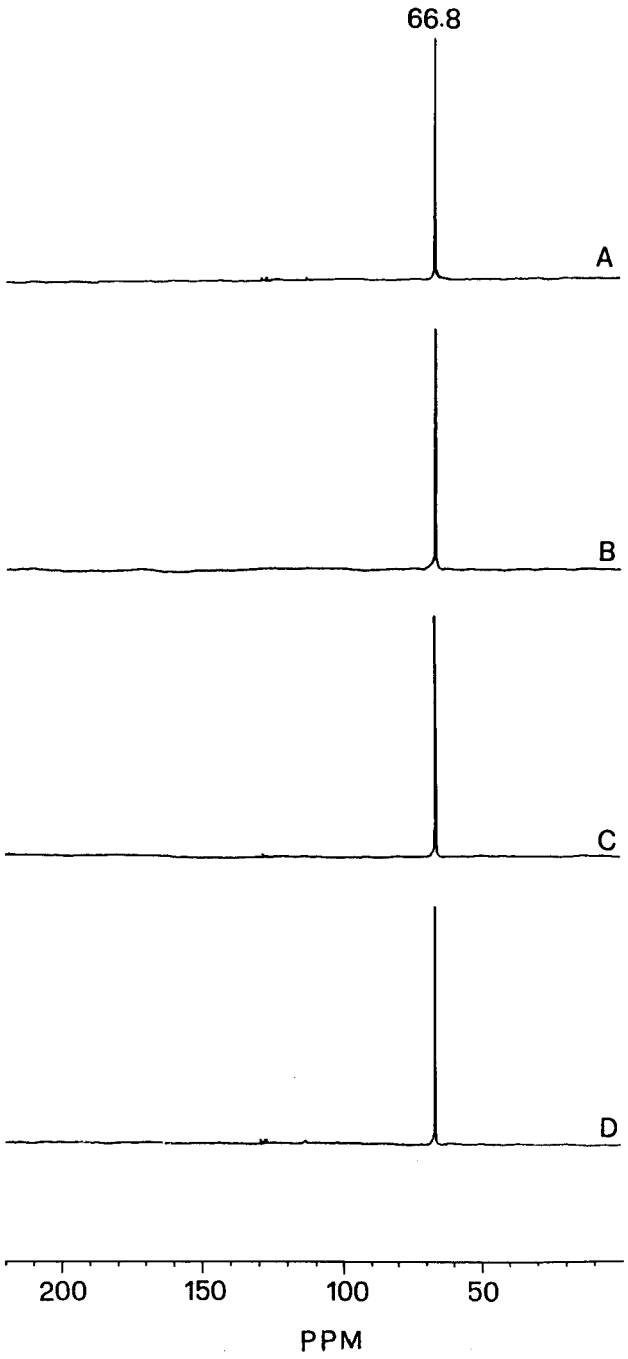

Figure 3 Liquid ^{13}C NMR spectra of reaction mixtures of ^{13}C-labelled 2,4-D in phosphate buffer at pH 4.7. (A) [^{13}C]2,4-D and HRP (horseradish peroxidase). (B) [^{13}C]2,4-D with HA1 and 128 units of HRP. (C) [^{13}C]2,4-D with HA2 and 128 units of HRP. (D) [^{13}C]2,4-D with HA3 and 128 units of HRP.

oxidative degradation of chlorophenoxyalkanoic compounds. It is thus conceivable that both humic materials and the peroxidase system can promote free-radical reactions capable of splitting an otherwise stable ether linkage in the 2,4-D side chain. Our results also indicate that the supposed progressively higher oxidative potential due to increasing amounts of peroxidase appeared less efficacious than humic substances alone in degrading 2,4-D (Figures 1 and 2). This may be explained by the mutual molecular interaction between peroxidase and the conformation of dissolved HAs.

The degree of dissociation of acidic functions at different pH values controls the conformational arrangement and general reactivity of humic substances in solution [33]. It is commonly reported that the size and shape of humic substances vary with pH, assuming elongated shapes at higher pHs and more globular, coiled down, arrangements at lower pHs [4,14]. Recent results have advanced this view by indicating that humic substances behave at neutral pH as supramolecular associations of self-assembling molecules held together by weak dispersive forces (van der Waals, $\pi-\pi$, CH/π) [7,23]. The heterogeneous molecules of HAs can dispose themselves in hydrophobic domains contiguous to hydrophilic domains. Such unstable heterogeneous conformation is broken down in smaller but more stable associations due to formation of energy-richer intermolecular hydrogen bondings when the pH is lowered to the 5.5–3.5 range [7,24].

Increasing quantities of peroxidase may significantly alter the original humic conformation that is able to chemically catalyze the free-radical degradation of 2,4-D [6,32]. Moreover, the additional free radicals generated by the peroxidase–H_2O_2 system which should have enhanced herbicide degradation may have been instead rapidly scavenged by the reducing potential of HAs. The limited oxidative activity of peroxidase in the presence of reducing compounds such as ascorbic acid has already been reported [27]. The peroxidase activity would probably modify the humic chemical composition through oxidative cross-coupling among humic molecules. This would imply an alteration of the humic conformational structure and a consequent loss of the catalytic ability of humic substances.

Both processes of alteration of humic conformational structure by peroxidase and inhibition of peroxidase by humic scavenging would signify a progressive decrease of the abiotic splitting of the [^{13}C]2,4-D ether linkage and account for the changes in NMR spectra with increasing peroxidase–humic ratios (Figures 1 and 2). These explanations would not be in contrast with the slight differences in 2,4-D degradation observed among HAs. Such differences may be accounted for by the variation in chemical compositions of HAs (Tables 1 and 2) which may have influenced the molecular interactions with peroxidase, thereby varying the efficacy in herbicide degradation of the combined humic–peroxidase system, depending on the chemical components which determine humic conformations in solution.

The lack of splitting of the 2,4-D side chain revealed by NMR spectra of samples treated in a phosphate buffer at pH 4.7 can be equally explained by the conformational changes of HAs occurring at lower pH. The humic conformations at pH 4.7 may have lost catalytic activity because they could not offer the same adsorbing sites which appeared to favour free-radical degradation of 2,4-D at about pH 7. This should be partly attributed to the reduced size of the humic molecular associations caused by formation of intermolecular hydrogen bondings at a lower pH [7,24]. Hence, a less favourable stereochemical interaction between humic substances and the herbicide molecule, accompanied by the decrease of free-radicals in HAs at lower pHs [5], contribute to inhibition of the chemical catalysis of 2,4-D degradation exerted by humic matter at pH 7.

The absence of abiotic degradation of 2,4-D when peroxidase was added without HAs (Figure 3A), appears to demonstrate the essential role of humic substances in chemically catalyzing the removal of the 2,4-D side chain. In fact, while peroxidase was

found to be highly active at pH 4 and 5 in catalyzing cross-coupling of anilines [16] and various phenols [19] and chorophenols [31], our results show (Figure 3A) that the enzyme alone cannot degrade phenoxyacetic acid at pH 4.7. A similar conclusion was reached in an earlier microbiological study [34] employing ^{14}C-labelled 2,4-D. This observation is in line with the 2,4-D degradation found at pH 7 which appeared to be promoted by HAs and inhibited by the presence of peroxidase (Figures 1 and 2). The inhibition is likely to be caused by cross-coupling of humic phenolic components catalyzed by peroxidase [31]. Hence, the scavenging of peroxidase activity by HAs [27] modify either the chemical composition and/or the conformational structure of humic associations to the point that the 2,4-D degradation induced by HAs is progressively limited.

Conclusions

This work used an advanced spectroscopic technique, high-resolution ^{13}C NMR, to study structural changes brought about on ^{13}C-labelled 2,4-D, a herbicide still widely used on soils, by humic substances and an enzymatic catalyst such as horseradish peroxidase. Our NMR results show for the first time that humic substances are able to catalyze splitting of the ether bond in the [^{13}C]2,4-D side chain and produce 2,4-dichlorophenol and [^{13}C]acetic acid as degradation products. This degradation was earlier reported to occur only biotically inside the microbial cell. A probable mechanism is related to the free-radical content of humic substances and to their capacity to stereochemically favour 2,4-D degradation by adsorption into large supramolecular conformations. The role of humic conformational structures in the abiotic catalysis was confirmed by the lack of 2,4-D degradation when the extent of humic molecular associations was reduced at lower pHs. Peroxidase did not catalyze oxidative degradation of phenoxyalkanoic herbicide even at the optimum pH of 4.7 for its enzymatic activity. Moreover, peroxidase inhibited the chemical catalysis exerted on 2,4-D by humic substances at neutral pH. This was attributed to a modification of both chemical composition and conformational arrangement of humic associations by oxidative cross-coupling reactions among humic molecules promoted by peroxidase.

The novelty of this work is the demonstration that 2,4-D can be abiotically degraded in the environment in the presence of dissolved humic substances. However, pH controls the rate of 2,4-D degradation, since it affects the conformational structure and molecular dimension of humic matter.

Acknowledgements

This work was partially supported by the Italian Ministry of University and Scientific and Technological Research (MURST) through project no. 9807352092. PC is grateful to the National Inter-University Consortium "Chemistry for the Environment" for a postdoctoral fellowship.

References

1 Alexander M. 1996. How toxic are toxic chemicals in soil? *Environ Sci Technol* 29: 2713–2717.
2 Calderbank A. 1989. The occurrence and significance of bound pesticide residues in soil. *Rev Environ Contam Toxicol* 108: 71–103.
3 Capriel P, A Haisch and SU Khan. 1980. Supercritical methanol: an efficacious technique for the extraction of bound residues from soil and plant samples. *J Agric Food Chem* 34: 70–73.
4 Chen Y and M Schnitzer. 1976. Viscosity measurements on soil humic substances. *Soil Sci Soc Am J* 40: 866–872.
5 Chen Y, N Senesi and M Schnitzer. 1977. Information provided on humic substances by E4/E6 ratio. *Soil Sci Soc Am J* 41: 352–358.
6 Chesire MV and N Senesi. 1998. Electron spin resonance spectroscopy of organic and mineral soil particles. In: Structure and Surface Reactions of Soil Particles (Huang PM, N Senesi and J Buffle, eds.), pp. 350, Wiley, New York.
7 Conte P and A Piccolo. 1999. Conformational arrangement of dissolved humic substances. Influence of solution composition on the association of humic molecules. *Environ Sci Technol* 33: 1682–1690.
8 Conte P, A Piccolo, B van Lagen, P Buurman and PA de Jager. 1997. Quantitative aspects of solid phase ^{13}C-NMR spectra of humic substances from soils of volcanic systems. *Geoderma* 80: 327–338.
9 Conte P, A Piccolo, B van Lagen, P Buurman and PA de Jager. 1997. Quantitative differences in evaluating soil humic substances by liquid- and solid-state ^{13}C-NMR spectroscopies. *Geoderma* 80: 339–352.
10 Dec J and J-M Bollag. 1990. Detoxification of substituted phenols by oxidoreductive enzymes through polymerization reactions. *Arch Environ Contam Toxicol* 19: 543–550.
11 Dec J, KL Shuttleworth and J-M Bollag. 1990. Microbial release of 2,4-dichlorophenol bound to humic acid or incorporated during humification. *J Environ Qual* 19: 546–551.
12 Dec J, K Haider, A Benesi, V Rangaswami, A Schaeffer, U Puecken and J-M Bollag. 1997. Analysis of soil bound residues of the ^{13}C-labelled fungicide cyprodynil by NMR spectroscopy. *Environ Sci Technol* 31: 1128–1135.
13 Fuehr F and W Mittelstaedt. 1980. Plant experiments on the bioavailability of unextracted [*carbonyl*-^{14}C]methanbenzthiazuron residues from soil. *J Agric Food Chem* 28: 122–125.
14 Ghosh K and M Schnitzer. 1980 Macromolecular structure of humic substances. *Soil Sci* 129: 266–276.
15 Hatcher PG, JM Bortiatynski, RD Minard, J Dec and J-M Bollag. Use of high-resolution ^{13}C-NMR to examine the enzymatic covalent binding of ^{13}C-labeled 2,4-dicholrophenol to humic substances. *Environ Sci Technol* 27: 2098–2103.
16 Hoff T, S-Y Liu and J-M Bollag. 1985. Transformation of halogen-, alkyl-, and alkoxy-substituted anilines by a laccase of *Trametes versicolor*. *Appl Environ Microbiol* 49: 1040–1045.
17 Khan SU. 1982. Bound pesticide residues in soil and plants. *Res Rev* 84: 1–25.
18 Khan SU and S Dupont. 1987. Bound pesticide residues and their bioavailability. In: Pesticide Science and Technology (Greenhalgh R and TR Roberts, eds.), pp. 417–420. Blackwell, Oxford.
19 Kim J-E, E Fernandes and J-M Bollag. 1997. Enzymatic coupling of the herbicide Bentazon with humus monomers and characterization of reaction products. *Environ Sci Technol* 31: 2392–2398.
20 Klibanov AM, BN Alberti, ED Morris and LM Felshin. 1980. Enzymatic removal of toxic phenols and anilines from waste waters. *J Appl Biochem* 2: 414–421.
21 Maloney SW, J Manem, J Mallevialle and F Fiessinger. 1986. Transformation of trace organic compounds in drinking water by enzymatic oxidative coupling. *Environ Sci Technol* 20: 249–253.
22 Melnikov NN. 1971. Chemistry of Pesticides, pp. 163–166. Springer, New York.
23 Piccolo A and P Conte. 2000. Molecular size of humic substances. Supramolecular associations *versus* macromolecular polymers. *Adv Environ Res* 3: 508–521.
24 Piccolo A, P Conte and A Cozzolino. 1999. Molecular association of dissolved humic substances as affected by interactions with mineral and monocarboxylic acids. *Eur J Soil Sci* 50: 512–520.
25 Pogany E, K-H Pawlizki and PR Wallnöfer. 1990. Formation, distribution and bioavailability of cell wall-bound residues of 4-choroaniline and 2,4-dichlorophenol. *Chemosphere* 21: 349–358.
26 Porras-Rodriguez M and FI Talens-Alesson. 1999. Removal of 2,4-dichlorophenoxyacetic acid from water by adsorptive micellar flocculation. *Environ Sci Technol* 33: 3206–3209.
27 Primus J-L, MG Boersma, D Mandon, S Boeren, C Veeger, R Weiss and IMCM Rietjens. 1999. The effect of iron to manganese substitution

on microperoxidase 8 catalysed peroxidase and cytochrome P450 type of catalysis. *J Biol Inorg Chem* 4: 274–283.

28 Roberts TR, W Klein, GG Still, PC Kearney, N Drescher, J Desmoras, HO Esser, N Aharonson and JW Vonk. 1984. Non-extractable pesticide residues in soils and plants. *Pure Appl Chem* 56: 945–956.

29 Ruggiero P, JM Sarkar and J-M Bollag. 1989. Detoxification of 2,4-dichlorophenol by laccase immobilized on soil and clay. *Soil Sci* 147: 361–370.

30 Saccomandi F and A Piccolo. 1998. The 2,4-dichlorophenoxyacetic acid (2,4-D) herbicide. Synthesis of a carbon-13-labelled environmental probe. *Ann Chim (Rome)* 88: 443–446.

31 Sarkar JM, RL Malcolm and J-M Bollag. 1988. Enzymatic coupling of 2,4-dichlorophenol to stream fulvic acid in the presence of oxireductases. *Soil Sci Soc Am J* 52: 688–694.

32 Senesi M, TM Miano and C Testini. 1987. Incorporation of water dissolved chlorophenoxyalkanoic herbicides by humic acids of various origin and nature. In: Current Perspectives in Environmental Biogeochemistry (Giovannozzi Sermanni G and P Nannipieri, eds.) pp. 143–156. CNR-IPRA Publ, Rome.

33 Stevenson FJ. 1994. Humus Chemistry. Genesis, Composition, Reaction. Wiley, New York.

34 Stott DE, JP Martin, DD Focht and K Haider. 1983. Biodegradation, stabilization in humus, and incorporation into soil biomass of 2,4-D and chlorocatechol carbons. *Soil Sci Soc Am J* 47: 66–70.

35 Wais A, K Haider, M Spiteller, AA de Graaf, P Burauel and F Füher. 1995. Using ^{13}C NMR spectroscopy to evaluate the binding mechanism of bound pesticide residues in soil. *J Environ Sci Health B* 30: 1–25.