Bull. Environ. Contam. Toxicol. (2003) 70:761–768

DOI: 10.1007/s00128-003-0048-7

Bulletin of Environmental Contamination and Toxicology

Interaction of Formaldehyde with Soil Humic Substances: Separation by GFC and Characterization by ^{1}H-NMR Spectroscopy

C. M. Cooke,[1] N. J. Bailey,[2] G. Shaw,[1] J. N. Lester,[1] C. D. Collins[1]

[1] Department of Environmental Science and Technology, Faculty of Life Sciences, Imperial College of Science, Technology, and Medicine, South Kensington, London SW7 2BP, United Kingdom
[2] Biological Chemistry, Faculty of Medicine, Imperial College of Science, Technology, and Medicine, South Kensington, London SW7 2AZ, United Kingdom

Received: 30 June 2002/Accepted: 5 December 2002

Humic substances (HS) are major components of soil organic matter. They are structurally complex, containing a wide variety of functional groups, depending on such factors as their age and the parent material from which they are derived (Wershaw 1999). Despite their extensive distribution (in soils, lakes, rivers and seas) much remains to be discovered about their specific chemical structure and their subsequent interactions with chemicals in the environment. The question of how HS affect the activity, sorption, bioavailability and survival rate of pesticides becomes a major issue as these compounds are applied to an ever-increasing area of agricultural land (the area treated in the UK has increased four-fold from 1974 to 1996 according to Thomas and Wardman (1999)). Formaldehyde is present in the environment both as a result of natural processes (decomposition of plant residues) and from man-made sources (industrial production and emissions), and is considered an environmental contaminant due to detection at levels higher than background concentrations. Formaldehyde has a variety of uses in many industries but direct contact with the soil occurs due to agricultural application of formaldehyde as a fumigant, fungicide or bactericide (Tomlin 2000).

Gel filtration chromatography (GFC) provides a simple and reliable method of directly separating HS into fractions based on differences in their molecular sizes as they pass through a column packed with a gel matrix with a specific pore size range. Co-elution of radiolabelled compounds with the GFC-separated HS fractions can provide direct evidence of chemical interactions. This method is therefore a useful tool for evaluation of the interactions of HS fractions with chemicals of ecological interest (Lee et al. 2001), but it does not seem to have been used widely in studies of pesticide binding to HS. Characterisation by proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy of HS fractions will also increase the understanding of HS structure and aid explanation of the nature of chemical interaction (Lee et al. 1998).

MATERIALS AND METHODS

Soil was collected from the top 20 cm of a disused apple orchard at Imperial College's field station, Silwood Park [51° 24.6' N, 0° 39.4' W], air-dried and sieved (pH 4.7; sand 81.7%, silt 12.7%, clay 5.7%; organic carbon 1.8%). Samples of 20 g were placed into centrifuge tubes and wetted with 6 ml of de-ionised water. Acetone, 5 ml, was added with 100 µl of ^{14}C-formaldehyde (Sigma-Aldrich Ltd. UK). Tubes

Correspondence to: C. D. Collins

were capped, attached to an end-over-end shaker for 2 hr to allow thorough mixing, and then samples were transferred to 75 ml plastic pots, with acetone washings. The acetone was allowed to evaporate in a fume cupboard before incubation under controlled conditions at 20°C.

Soil samples were analysed over a six-month period using three extractants selected for their differing abilities to extract both HS and ^{14}C-formaldehyde from the soil. Extraction times were chosen for convenience of sample handling and analysis: (A) deionised water: 1, 5, 12 and 21.5 weeks; (B) 0.5*M* sodium hydroxide solution (NaOH(aq)): 5.5, 8, 12 and 19.5 weeks; and (C) dimethylsulphoxide-HCl 0.6*M* (8:2 v/v) (DMSO-HCl): 9.5, 12, 16 and 21.5 weeks. At these times, 4g sub-samples were weighed into centrifuge tubes and an extractant solution was added (ratio of 1:5 soil/extractant). Samples were shaken for 2 hr to ensure thorough mixing, and centrifuged in a Denley BS400 benchtop centrifuge for 15 min at 5000 g. The supernatant was poured off and 1 ml was taken for injection onto a gel filtration column. Samples were frozen to reduce microbial action if there was a delay between extraction and analysis. For samples extracted with NaOH(aq) or deionised water, GFC apparatus consisted of a C26/40 column attached to a Pharmacia GradiFrac system and a HiLoad pump, with a matrix of Toyopearl HSW-40 gel and a flowing phase of 10% methanol. For the organic extractant, DMSO-HCl, refinements to the method were necessary: a Pharmacia solvent resistant column (SR 10/50) was employed with Sephadex^{TM} LH20 gel media and a flowing phase of pure DMSO. An injection of 1 ml of sediment-free extracted HS was made onto the gel columns, the pump speed set at 1 ml min^{-1} (SR 10/50 0.1 ml min^{-1}) and the eluent collected as 1 ml samples. UV absorbance readings and ^{14}C activity of the eluent samples were measured as described in Lee et al. (2001). Control samples containing spiked solutions of ^{14}C-formaldehyde alone (no HS) were also injected onto the gel filtration column to determine the free elution pattern.

Further blank soil samples (^{14}C-formaldehyde-free) were extracted with NaOH(aq) and DMSO-HCl as described, for NMR spectroscopic analysis. HS fractions were separated by GFC, with 1 ml eluent samples from six separate injections collected and bulked in glass vials corresponding to specific peaks. Bulked peaks were freeze-dried, resuspended in deuterated water (D_2O) and freeze-dried again. Before analysis, 700 µl of solvent were added (D_2O for NaOH extracts; fully deuterated DMSO for DMSO extracts). Samples were centrifuged for 5 min, the supernatant taken off, and 650 µl was taken for ^{1}H-NMR spectroscopy (Bruker DRX 600 NMR spectrometer), operated at 600.13 MHz for the ^{1}H nucleus. The spectra for NaOH extracts (summation of 256 transients) were referenced to trimethylsilylpropionic acid (TSP) at 0.00ppm or formate at 8.46ppm, while spectra for DMSO extracts (summation of 128 transients) were referenced to DMSO at 2.50ppm. A standard pulse sequence was used to pre-saturate the residual water (HOD) resonances to reduce the signal intensity in the spectra.

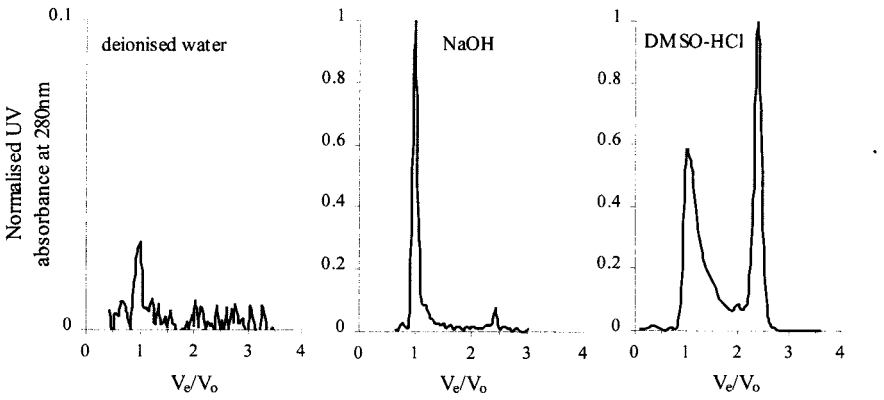

Figure 1. UV absorbance of humic substances (HS) separated by gel filtration chromatography (GFC) after extraction from the orchard soil with deionised water, 0.5*M* NaOH(aq), and DMSO-HCl 0.6*M* (8:2 v/v). (Note different scale for water extraction; V_o = column void volume and V_e = eluted peak position).

RESULTS AND DISCUSSION

The GFC elution behaviour of HS and ^{14}C-formaldehyde was recorded in chromatograms, where individual peaks (V_e) were normalised to the column void volume (V_o), the latter determined using Dextran Blue. The large size of Dextran Blue molecules (2MDa) ensured that they were fully excluded from the gel particle matrices, thus providing a reference for GFC column void volume. Figure 1 illustrates typical chromatograms of GFC elution behaviour, as measured by UV absorbance at 280nm, of HS extracted from the orchard soil with the three extractants. UV absorbance measurements were normalised to the highest value (except for water) and qualitatively indicated the presence of HS. Extraction with NaOH and DMSO were more effective at removing HS from the soil than deionised water (Figure 1). The low recovery of HS by deionised water extraction caused difficulty in measuring the UV absorbance values (and subsequently ^{14}C activity), although a HS peak could typically be distinguished from the background at the void volume (V_e/V_o=1).

Extraction with NaOH(aq) resulted in a clear separation of HS into two peaks: a high molecular weight (HMW) fraction (eluted at V_e/V_o=1, void volume) and a smaller lower molecular weight (LMW) fraction (V_e/V_o=2.4). Thus, the major proportion of extracted HS had an average molecular weight greater than the upper exclusion limit of the gel (10MDa). The minor peak was referred to as a LMW fraction because there was no evidence to suggest that it was retarded on the column (e.g. as a result of hydrophobic interactions) and so must represent HS of lower molecular weight, which would take longer to pass through the column than the larger material of the major peak. Figure 1 also shows the typical elution pattern of DMSO-HCl-extracted HS using the SR column, exhibiting again a clear separation of HS into two peaks: a HMW fraction (V_e/V_o=1, void volume) and a LMW fraction eluted where V_e/V_o=2.4. In contrast to the NaOH-extracted HS, DMSO-HCl extraction resulted in a greater proportion of HS being eluted as a LMW peak.

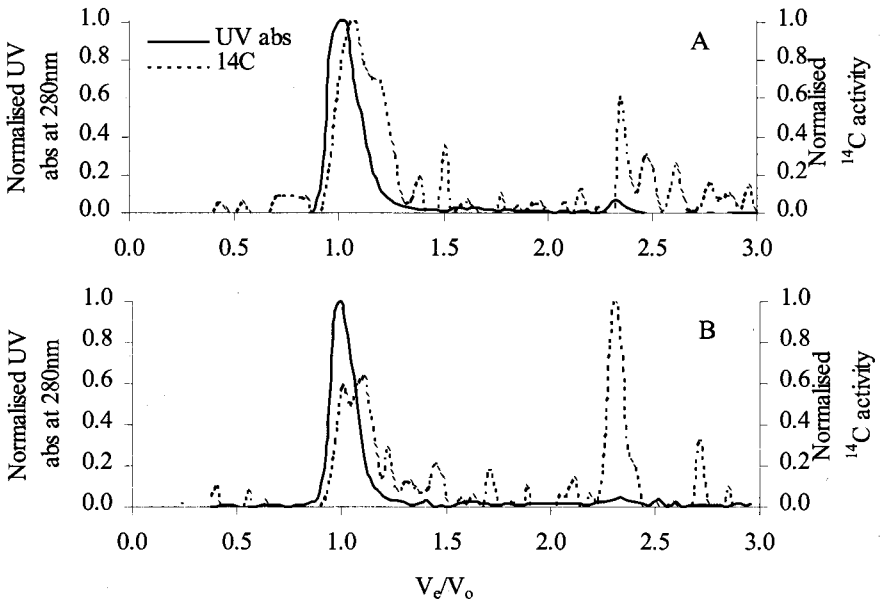

Figure 2. Association of ^{14}C-formaldehyde (on secondary *y* axes) with HS separated by GFC (primary *y* axes) after extraction from the orchard soil with 0.5*M* NaOH(aq) after (A) 12 weeks and (B) 19.5 weeks incubation.

A linear regression through the origin ($R^2 \geq 0.998$) was used to describe the relationship of UV absorbance with standards of known humic acid concentration (data not shown), thus intensity of UV absorbance can be used to indicate the quantity of HS extracted, for comparison of relative peak heights of GFC-separated fractions extracted from the same soil. For the extractants: DMSO-HCl > NaOH > deionised water.

A radiolabelled compound was used to investigate the association of formaldehyde with extracted HS. Resolution of the association with NaOH- and DMSO-HCl-extracted HS (Figures 2 and 3, respectively) was achieved by overlaying ^{14}C activity traces and UV absorbance readings. Figure 2 displays the overlaid traces for NaOH(aq) extraction after 12 and 19.5 weeks incubation. The chromatograms were normalised in relation to their respective maximum values. Replicates exhibited reproducible separation characteristics for both HS and ^{14}C activity, and control samples (not shown) illustrated that free ^{14}C-formaldehyde eluted as a broad peak at V_e/V_o=2.5. Figure 2A shows that for soil extracts, a large proportion of ^{14}C activity was eluted just after the void volume at V_e/V_o=1.1. This does not indicate a direct association between the ^{14}C activity peak and the HMW HS peak because a) the two traces are not exactly synchronous and b) the ^{14}C peak tails off with a shoulder that lags behind that of the HMW HS. Instead, this could represent loose or partial association of ^{14}C-formaldehyde with HMW HS, either intact or in partially degraded form. The fate of formaldehyde in soils is not fully understood, but it is biodegradable to CO_2 and H_2O or formic acid under aerobic and anaerobic

conditions. It is also biologically active and reacts readily with phenol, amine, amide, sulfide, purine, and pyrimidine molecular groups (Barker et al. 1996), each of which can be found in HS. Furthermore, formaldehyde is subject to spontaneous polymerisation (IPCS 1989), forming units of paraformaldehyde ($(CH_2O)n$, molecular weight = 600Da) which could account for the first ^{14}C activity peak.

A peak of ^{14}C activity was observed superimposed over the elution position of the LMW HS peak, V_e/V_o=2.4 (Figure 2). In contrast, this does suggests a direct association of formaldehyde with LMW HS because the peak is well defined and synchronous with the LMW HS peak. A small degree of activity eluted after this peak, possibly due to ^{14}C-formaldehyde that was free from association with HS, or degraded. Differences were evident between the two sampling periods shown in Figure 2. After 19.5 weeks there was a shift to greater ^{14}C activity associated with the LMW HS peak than with the peak at V_e/V_o=1.1, compared with at 12 weeks. The ^{14}C activity peak was again narrow and well defined, characteristics that indicated a direct association of ^{14}C-formaldehyde with the LMW HS peak. There was also less ^{14}C activity detected after the LMW HS-associated peak, indicative of free, unassociated formaldehyde that had become increasingly associated with LMW HS, perhaps representing a more stable degree of binding over time. Absolute values of ^{14}C activity indicated that the amount associated with the LMW peak had increased by ~50% at 19.5 weeks compared with at 12 weeks. The ^{14}C activity detected close to the void volume (V_e/V_o=1.1) clearly did not follow the shape of the HS UV absorbance trace and was most likely the result of association of ^{14}C-formaldehyde (or degradation products) with the carbon structure of the HMW HS.

Figure 3A illustrates the association of ^{14}C-formaldehyde with DMSO-HCl-extracted HS after 16 weeks. The UV absorbance and ^{14}C activity traces were normalised and overlaid. A predominant peak of ^{14}C activity was evident at V_e/V_o=1, corresponding to the void volume and HMW fraction, and a second but smaller ^{14}C activity peak eluted at V_e/V_o=2.4 overlapping with the LMW fraction. The ^{14}C activity peaks were distinct and synchronous with the elution positions of HS peaks, strongly suggestive of a direct association between ^{14}C-formaldehyde (or its degradation products) and the HS fractions. Replicated control samples identified the elution position of free ^{14}C-formaldehyde (no HS) (Figure 3B), using Dextran Blue as the void volume marker. The ^{14}C-activity profile reproducibly exhibited two peaks: a predominant peak at V_e/V_o=2.5 which rose sharply but was very broad with a significant tail, and a minor peak which eluted with the void volume. This minor peak was consistently 9% of the height of the major peak in all replicates, and while its nature is unknown, it was most likely due to chemical impurity or polymerization as described above. The elution position of free ^{14}C- formaldehyde was close to the LMW HS fraction. However, the character of the compound peak in the presence of HS (peak width at half peak height = 0.075 ± 0.04) was sufficiently different from the free compound in the controls (peak width at half peak height = 0.33 ± 0.12) that conclusions relating to HS associations can be drawn with confidence without confusing the free and associated material.

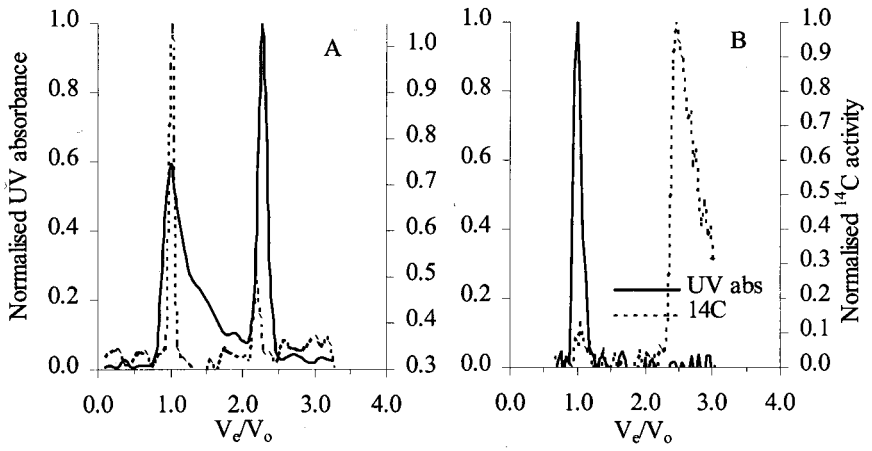

Figure 3. (A) Association of GFC-separated-HS and ^{14}C-formaldehyde, extracted from orchard soil after 16 weeks with DMSO-HCl. (B) Control sample of free ^{14}C-formaldehyde (no HS), overlaid with Dextran Blue void volume marker (V_e/V_o =1).

^{1}H-NMR spectroscopy of the HS fractions was carried out (Figure 4) with the aim of characterising the HS for two reasons. Firstly, extraction with NaOH and DMSO seemed to result in different HS separations, and secondly because organic chemical associations over time appeared to favour the LMW fraction over the HMW fraction after NaOH extraction. Figure 4 displays the ^{1}H-NMR spectra for the HMW and the LMW HS fractions separated by GFC after extraction with NaOH (C1 and C2, respectively), and after extraction with DMSO-HCl (SR1 and SR2, respectively). The sharp resonance in C1 and C2 at 4.8ppm resulted from residual water (HOD), formed by exchange of acid protons in the HS and from deuterated water. This signal was reduced as far as possible without distorting the signals from humic protons in the vicinity of the HOD peak. ^{1}H-NMR spectra firstly confirmed that C1 (HMW fraction) contained more macromolecular material than C2 (LMW fraction), and that the two fractions differed in character. There was very little signal in the aromatic hydrogen region, 6-10ppm, in C1 suggesting that phenols and polycyclic aromatics did not comprise a major fraction, whereas there was more signal at this point in C2. The sharp resonance at 8.46ppm in both C1 and C2 was identified as the formate ion, postulated to be a decomposition product of HS when treated with sodium hydroxide (Wilson et al. 1988).

A predominant feature of C1 was the intensity of the region extending from 3-5ppm, present to a lesser extent in C2. This region commonly contains species of a slightly electronegative (or more oxygen rich) nature, such as carbohydrate. Signal at 2.1-2.2ppm (aliphatic region) in C1 and C2, may have arisen from protons attaching aliphatic carbon to carboxylic groups or aromatic rings. The sharp singlet at 1.9ppm would be consistent with an acetyl/acetate methyl functionality, and the resonances at 1.2-1.7ppm would have arisen from a variety of aliphatic species. The sharp resonance at 1.4ppm (and 4.1ppm) was consistent with lactate, which along with other compounds (i.e. succinate, acetate, aryl methoxyl compounds), has previously

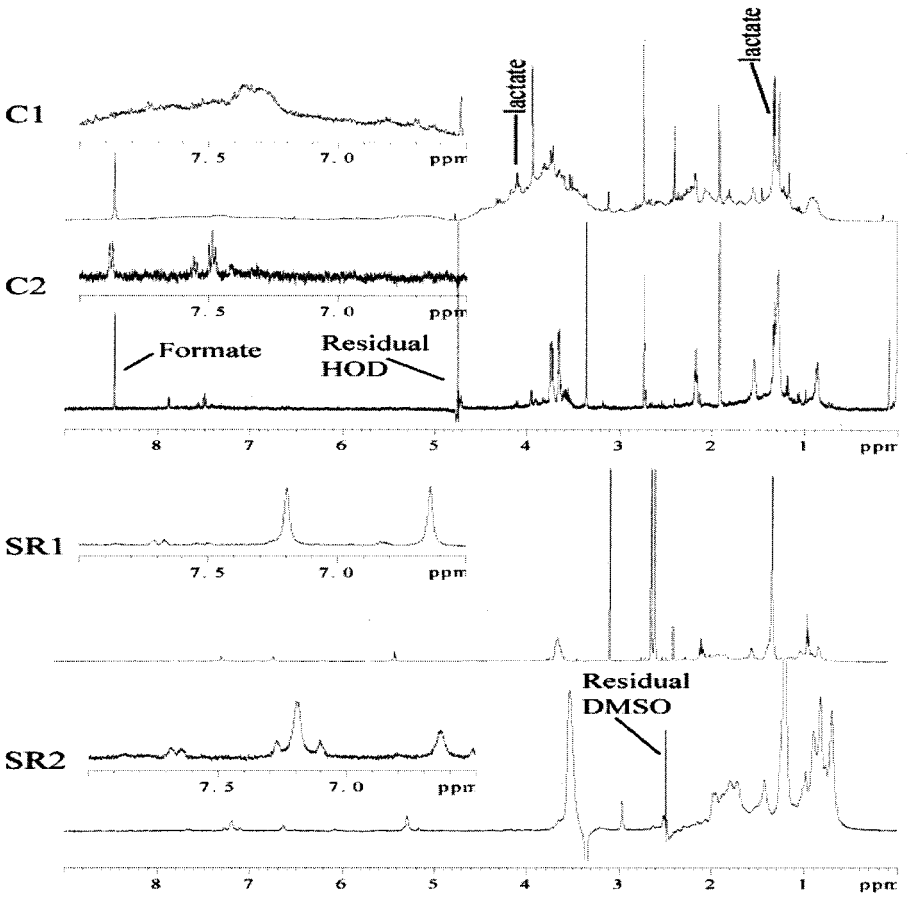

Figure 4. ^{1}H-NMR spectra of HS separated by GFC after extraction from the orchard soil with 0.5*M* NaOH(aq) (fractions C1 and C2), and extraction by DMSO-HCl (fractions SR1 and SR2).

been detected in HS extracts (Wilson et al. 1988) and ruled out as contaminants, but were likely to be present as small fragments that were originally bound to larger molecular species and had been hydrolysed and/or oxidised by alkali. C2 was found to contain much less macromolecular material than C1, with resonances that are consistent with small species (e.g. benzaldehyde, acetophenone, 7.4-7.9ppm) (Suzuki et al. 1998).

In SR1 and SR2 the resonance at 3.5-3.6ppm resulted from residual HOD, and at 2.5ppm from residual deuterated DMSO; these were reduced as far as possible. The spectra of the DMSO extracts were very different to those of the NaOH extracts, the major difference being the absence of carbohydrate material (3-5ppm), but this was due to treatment of soil with HCl rather than HS structure itself. No formate was detected in the DMSO extracts, which confirms formate as a degradation product of HS treated with NaOH. DMSO extracts contained some aliphatic material (1-2ppm), and both SR1 and SR2 appeared to contain more aromatic material (6-8.4ppm) than

NaOH extracts, consistent with reports by Piccolo et al. (1998), and strong UV absorbance readings (Chen et al. 2002). The HS spectra clearly show differences in molecular structure due to the different extractants: alkaline NaOH extraction is based on an electrostatic repulsion mechanism, while the dipolar aprotic solvent of DMSO in aqueous acidic solution, exerted a mechanism based on disruption of intermolecular hydrogen bonds. The fact that different mechanisms are capable of extracting HS of different chemical composition and complexity implies that each HS fraction may have different interactions with chemicals in soils, underlying the importance of specific molecular structure of HS in explaining interactions with pesticides in the environment. Understanding these associations may potentially lead to development of methods for remediation of chemical-contaminated soils.

Acknowledgments. This research was funded by a studentship from the UK Department for Environment, Food and Rural Affairs (DEFRA, formerly MAFF). We also thank Prof. J. Nicholson, Biological Chemistry, Imperial College, London, UK, for access to NMR spectroscopy facilities.

REFERENCES

Barker JR, Herstrom AA, Tingey DT (1996) Formaldehyde: environmental partitioning and vegetation exposed. Water Air Soil Poll 86:71-91.

Chen J, Gu B, LeBoeuf EJ, Pan H, Dai, S (2002) Spectroscopic characterization of the structural and functional properties of natural organic matter fractions. Chemosphere 48:59-68.

IPCS (1989) Environmental Health Criteria 89: Formaldehyde. International Programme on Chemical Safety, World Health Organisation.

Lee GSH, Wilson MA, Young BR (1998) The application of the "WATERGATE" suppression technique for analysing humic substances by nuclear magnetic resonance. Org Geochem 28:549-559.

Lee RT, Shaw G, Wadey P, Wang X (2001) Specific association of ^{36}Cl with low molecular weight humic substances in soils. Chemosphere 43:1063-1070.

Piccolo A (1988) Characteristics of soil humic extracts obtained by some organic and inorganic solvents and purified by HCl-HF treatment. Soil Sci 146:418-426.

Suzuki Y, Imai S, Kawakami M, Masuda Y, Akasaka K (1998) Identification and determination of low-molecular weight organic compounds in contaminated fog water using proton nuclear magnetic resonance spectroscopy. Bull Environ Contam Toxicol 60:355-362.

Thomas MR, Wardman OL (1999) Pesticide Usage Survey Report 150: Review of usage of pesticides in agriculture and horticulture throughout Great Britain 1986-1996. MAFF Publications, London UK.

Tomlin CDS (2000) The pesticide manual. Twelfth Edition. The British Crop Protection Council, UK.

Wershaw RL (1999) Molecular aggregation of humic substances. Soil Sci 164:803-813.

Wilson MA, Collin PJ, Malcolm RL, Perdue EM, Cresswell P (1988) Low molecular weight species in humic and fulvic fractions. Org Geochem 12:7-12.