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**CHARACTERIZATION OF HUMIC SUBSTANCES IN HIGHLY POLLUTED
RIVER SEDIMENTS BY PYROLYSIS METHYLATION – GAS
CHROMATOGRAPHY – MASS SPECTROMETRY.**

Laurence Mansuy¹, Yassine Bourezgui², Evelyne Garnier-Zarli², Emilie Jardé¹, Véronique Réveillé²

¹G2R UMR7566 UHP, BP 239 – 54506 Vandœuvre Cedex,
²LBSE Université ParisXII Créteil, 61 avenue du Général de Gaulle – 94000 Créteil

ABSTRACT

Humic substances were extracted from two sediment cores sampled in a river on the outskirts of a large city (Deûle near Lille, France) and in its highly polluted oxbow-lake. Humic acids (HA) were characterized by infrared spectroscopy and *in situ* pyrolysis/methylation-gas chromatography-mass spectrometry. The main results are the important aliphaticity of HA in the oxbow-lake at all depths, mainly due to the presence of a lipid fraction associated with the HA structure. The abundance of sterols in this fraction implies that lipids could originate from the accumulation of sewage in the oxbow-lake. Humic acids of the Deûle sediments show a different trend ; their evolution is characterized by a decrease of the pollutant inputs with increasing depth. The results demonstrate the efficiency of Py-GC-MS in identifying the different organic contributions to HA and in distinguishing natural from anthropogenic sources.

INTRODUCTION

Pollutant discharge, morphological and hydraulic modifications of rivers, deforestation and urbanization of watersheds, induces disturbances of the physical, chemical and biological equilibrium of river systems. This leads to the gradual silting of aquatic systems. In urban areas, this phenomenon is even more marked since rivers receive abundant industrial and

domestic inputs and rain water from urban run-off. Thus, organic and inorganic deposits can constitute an excellent adsorbent phase for organic and inorganic micropollutants. More specifically, the presence of humic substances in sediments has a significant influence on the immobilization and the possible remobilization (during dredging for instance) of pollutants (Khan & Schnitzer, 1972; Schlautman & Morgan, 1993; Gaffney *et al.*, 1996).

In order to further our understanding of the interaction of humic substances with pollutants, it is first essential to characterize them. They have a complex structure reflecting the various origins of organic matter : autochthonous (organic matter synthesized in the aquatic system), allochthonous (terrestrial debris of higher plants) or anthropogenic (that can be the main input in urban areas) (Ishiwatari, 1985; Leenher, 1991; Saiz-Jimenez, 1996). The complexity of these molecules also results from the multiple processes involved in the chemical and biological degradation of the original organic material (Flaig, 1988; Andreux & Meunier-Lamy, 1994).

A variety of analytical methods have been employed to characterize humic substances and there is now a growing interest for molecular characterization using degradative methods such as pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS) (Schnitzer & Schulten, 1992; Hatcher & Clifford, 1994; Saiz-Jimenez, 1994; Schulten *et al.*, 1995; Schulten & Leinweber, 1996). Its main advantage lies in the identification of molecular markers characterizing the different organic contributions in soils or sediments. The method is important in the study of polluted soils or sediments since it allows identification of anthropogenic agents (de Leeuw *et al.*, 1986). This technique has been improved with the development of *in situ* methylation using tetramethylammonium hydroxide (TMAH) which favors the volatilization and the chromatography of polar compounds abundant in humic substances (Challinor, 1989; Hatcher & Clifford, 1994; Saiz-Jimenez, 1995; McKinney *et al.*, 1998).

In this paper, we present the results of an investigation on the molecular characterization of organic matter of aquatic sediments in two different systems : a river and its highly polluted oxbow-lake. The aims of the study were to evaluate the ability of *in situ* methylation Py-GC-MS to characterize humic substances in highly polluted sediments, to determine the difference in the organic composition of the river and oxbow-lake sediments and to correlate the Py-GC-MS signatures with their environment.

SAMPLES AND METHODS

Sampling site

The sediments of a channeled river, the "Deûle", were used as a reference for this study. The Deûle river, located in the North of France, belongs to the Artois-Picardie watershed, and flows through the outskirts of Lille. Because this region is highly industrialized and is extensively used for agriculture, the water quality is poor and the sediments are polluted primarily with cadmium and lead.

A second sediment core was collected in the oxbow-lake of the Deûle river. This 850 m long and 20 m wide body of water crosses a downtown section of Lille and an industrial area. It is connected to the Deûle river by a narrow channel that allows a reciprocal connection between the two environments depending on the hydrodynamic conditions (Figure 1). The oxbow-lake is fed by the Deûle river at one end and by a spillway which drains the wastewater network of the city at the other end. It also collects the rain water coming from the motorway and the rail-road that overhang the oxbow-lake. Usually, the current goes from the Deûle towards the oxbow-lake and the low run-off speed favors accumulation of sediments and pollutants in the oxbow-lake. However, during heavy showers, the current goes towards the Deûle river since most of the run-off spills into the oxbow-lake.

Sampling and sample preparation

Sampling was performed with a core drill in order to get a vertical profile with minimum disturbances. The cores were frozen and cut into 10 cm layers. Six layers were obtained from the Deûle core (60 cm long) and eight from the oxbow-lake core (80 cm long). After defrosting, the sediments were dried, sieved at 2 mm, and crushed.

Humic acids (HA) were isolated from the sediments, purified and freeze-dried according to the procedure defined by McCarthy et al. (1979) and Thurman and Malcolm (1981). HA were ultrasonically extracted with dichloromethane in order to recover the lipids often associated with this fraction. The extraction was performed several times until the solvent was colorless. A sample of sewage sludge was also extracted with dichloromethane and the lipid fraction recovered in order to compare its composition with those from the sediment samples.

Analytical methods

The sediments samples were analyzed for total organic carbon (Walkey & Black, 1934). In order to characterize the functional groups within the HA, they were analyzed by Fourier transform infrared microspectroscopy. An aliquot was placed between two diamond windows (2 mm diameter, 1mm thick) of a compression cell (7.5 x 5.1 cm², SpectraTech Int.). After compression of the sample, the top window was removed. The area to be analyzed was selected under the microscope with visible light and a x10 objective. Micro-Fourier transform infrared (FTIR) analysis was performed on a Bruker IFS-88 equinox spectrometer coupled to a Bruker multipurpose infrared microscope which was fitted with a 250 µm narrow band MCT detector cooled to 77K. The standard analytical conditions were x15 objective, 40-60 µm diameter infrared spot, 60 scans (30s) and spectral resolution of 4 cm⁻¹. Spectra were subtracted from the background measured on a clean diamond window under the same

analytical conditions. The use of diamond cells avoids the adsorption of water and the spectral shifts often observed when using KBr pellets [Ruau, 1997 #53].

Humic acids were then subjected to Py-GC-MS analysis. The samples were methylated *in situ* with TMAH based on the method of Challinor (1989), Hatcher and Clifford (1994) and Saiz-Jimenez (1995). An approximate 10:1 weight ratio of TMAH:sample was placed in a quartz tube and pyrolyzed at 620°C for 15s in a CDS 2000 pyroprobe. The pyrolysis products were analyzed by gas chromatography-mass spectrometry (HP 5890 Series II coupled to a HP 5972 mass spectrometer). The temperature of split/splitless injector was 280°C. The GC was fitted with a 60 m J&W DB-5ms, 0.25 mm i.d. and a 0.1 µm film fused silica column. After cryofocusing, the GC oven was programmed to 300°C at 5°C/min. The final temperature was held for 15 min.

Lipid analyses were performed with an HP5890 Series II GC connected to a HP5972 mass spectrometer ; the same GC column was used. The oven temperature was programmed from 40°C to 300°C at 5°C/min then held at 300°C for 15 min. Prior to GC-MS analysis, these fractions were derivatized with bis (trimethylsilyl) trifluoroacetamide (BSTFA+TMS).

RESULTS

Characteristics of the sediments

Morphological as well as *in situ* physicochemical analyses were performed on the two sediment cores. The oxbow-lake core shows a uniform black color all along the profile due to a high total organic carbon (TOC) content ranging from 10 to 15%. The reducing conditions, as revealed by the negative redox potential, are the result of high O₂ consumption induced by the microbiological and chemical degradation of organic matter and the insufficient renewal of oxygen from the overlying water.

The color of the Deûle sediment core varies from gray black greenish in the upper layer (10 cm) to an ochre green color in the deeper layers (up to 60 cm). The TOC content varies from 2.5% in the upper layer to 1% in the 60 cm layer. The oxidizing conditions in the river allow a good turnover of the sedimentary organic matter.

Infrared analysis

Infrared spectra of HA, extracted from the top and bottom sediments of the two cores, are shown in Figure 2. Their interpretation is based on the work of Garcia *et al.* (1992) and Piccolo *et al.* (1992). The main absorption bands are : a broad band at 3100-3600 cm^{-1} (H-bonded OH and NH groups), a slight shoulder at 3000-3100 cm^{-1} (aromatic C-H stretching), a band at 2800-3000 cm^{-1} (aliphatic C-H stretching), a shoulder or a peak at 1710 cm^{-1} (C=O stretching of COOH and ketonic carbonyls), a peak or a shoulder at 1590-1700 cm^{-1} (aromatic C=C stretching, H-bonded C=O, NH_2 deformation), a peak or a shoulder at 1490-1590 cm^{-1} (NH_2 deformation of amides), a band at 1300-1500 cm^{-1} (aliphatic CH deformation).

The infrared spectra of the oxbow-lake HA are similar whatever the depth. They are always characterized by intense aliphatic CH bands and a significant contribution of N-containing compounds, such as peptides, as revealed by the intense band at 1590-1700 cm^{-1} concomitant with the band at 1490-1590 cm^{-1} . The broad band at 3100-3600 cm^{-1} maximizes at 3300 cm^{-1} due to a significant contribution of N-H stretching due to peptides. These features were observed by Piccolo *et al.* (1992) in HA of sludge-amended soils.

The infrared spectrum of HA from the Deûle upper layer is similar to that of the oxbow-lake although the aliphaticity and the N-containing functions are slightly less prominent. These parameters both substantially decrease with increasing depth. The strong absorption at 3400 cm^{-1} is shifted towards lower frequencies (3300 cm^{-1}) resulting from the decreasing

The main feature of the pyrograms of the oxbow-lake HA is the significant contribution of steroids (Figure 3). Fatty acids also contribute to the lake HA pyrograms : their distribution is dominated by n-C₁₆ and n-C₁₈ fatty acids, and to a lesser extent by n-C₂₀, n-C₂₂, n-C₂₄ fatty acids. Lignin-derived pyrolysis products are poorly represented. As observed in the infrared spectra (Figure 2), no change in the distribution of the pyrolysis products is observed with the depth.

In contrast, the pyrograms of the Deûle HA show changes with increasing depth (Figure 4). While present, steroids are less abundant than the n-fatty acids. Unlike the oxbow-lake samples, the contribution of lignin-derived products is greater and includes a wide variety of compounds. In the uppermost layer (10 cm deep), the P-containing compounds (phosphoric and phosphonic acids) are prominent. With increasing depth, there is a much lower contribution from steroids and n-alkanes. In the deeper layer, the main compounds are phosphoric and phosphonic acids, lignin-derived products and n-C₁₆ and n-C₁₈ fatty acids. This pyrogram is less complex than that of the oxbow-lake but contains many compounds not yet identified.

Lipid analyses

Extraction of lipids from the HA yields, on average, 20% in the oxbow-lake HA and the upper layer of the Deûle HA and less than 8% from the 80 cm fraction of the oxbow-lake HA.

The lipid fraction mainly contains n-fatty acids with the same distribution as in the pyrograms of HA and abundant steroids that have been identified as C₂₇ to C₂₉ 5 β -stanols, and C₂₇ and C₂₉ stanones (Smith *et al.*, 1982). The lipid fraction of the 60 cm Deûle humic acids contained very low concentrations of steroids.

The 5 β -stanols (more specifically 5 β -cholestan-3 β -ol ; coprostanol) are used as common markers of manured soils (Bull *et al.*, 1998), or domestic waste or human fecal material

contribution of N-H stretching of peptides over the O-H stretching of oxygen-containing functional groups.

In situ Py/methylation-GC-MS

Figures 3 and 4 show the total ion current (TIC) chromatograms of the pyrolysates of HA of the upper and lower parts of the Deûle and oxbow-lake cores. Compounds were identified from their mass spectra (comparison with standards), retention times and literature data (Table 1).

The identified pyrolysis products can be grouped according to their possible origin or source. The two main groups represented in the pyrograms originated from lignin and lipids. The pyrolysis products from lignin, and degraded lignin, include styrene, methoxybenzenes, di- and trimethoxybenzenes, methoxybenzoic acids, di- and trimethoxybenzoic acids, (methoxyphenyl)-propenoic acids, dimethoxybenzaldehydes (Hatcher et al., 1995; Hatcher and Minard, 1996; Klap et al., 1998). Compounds originating from lipids are n-alkanes, n-alkenes, fatty acids, dicarboxylic acids and steroids. Fatty acids are mainly n-alkanoic acids ranging from C₆ to C₂₆ with a strong even carbon-numbered signature in the C₁₄-C₂₄ region. The C₁₄-C₁₈ distribution reflects an input from bacteria and plants whereas long chain n-alkanoic acids (C₂₂, C₂₄, C₂₆) are major components of wax coatings on land plant leaves (Cranwell, 1974; Cranwell, 1982; Rieley *et al.*, 1991). Steroids have been tentatively identified based on their mass spectra and literature data (Smith *et al.*, 1982; Bull *et al.*, 1998). The steroid distribution is dominated by 5 β -cholestanol and C₂₉ 5 β -stanol. This class of compound contributes to the aliphaticity of the humic acids observed by infrared microspectroscopy. N-containing compounds identified include an alkyl-indole, C₁₆ and C₁₈ alkylamines. S- and P-containing pyrolysis products were also identified (Table 1).

(Leeming *et al.*, 1997; Sherblom *et al.*, 1997). Coprostanol is produced by the microbial reduction of cholest-5-en-3 β -ol in the mammalian gut (Macdonald *et al.*, 1983).

Figure 5 compares the steroid fraction extracted from the upper layer of the oxbow-lake humic acid and that extracted from sewage sludge. The sterol chromatograms are virtually identical strongly suggesting that the lipids have originated from sewage accumulated in the oxbow-lake.

DISCUSSION

IR analyses of the oxbow-lake humic substances suggest that the HA are highly aliphatic. This is attributed mainly to the presence of abundant sterols and fatty acids. This special feature of the oxbow-lake HA can be considered anomalous. Indeed, comparison of the infrared spectra of humic acids and fulvic acids from each sample (not reported in this study) leads to the conclusion that humic acids are much more aliphatic than their fulvic counterparts. This result contradicts the usual definition of humic and fulvic acids based on a difference of aliphaticity and reactivity, fulvic acids being more aliphatic, less aromatic and more reactive than humic acids (Schulten & Schnitzer, 1995; Gaffney *et al.*, 1996). Highly aliphatic HA have been reported in sewage sludge and in organic-waste-amended soils (Garcia *et al.*, 1991; Piccolo *et al.*, 1992; Garcia *et al.*, 1992). The infrared spectra presented in the cited studies are very similar to those of the oxbow-lake humic acids with an intense aliphatic band and a large contribution of N-containing compounds (amides).

The second interesting feature of HA from the oxbow-lake sediments is the absence of changes in compound distributions with depth. This agrees with the core description, which shows a uniform black color and a high TOC content at all depths. This can be explained by the hydrodynamic conditions of this environment which is subjected to substantial sediment movements during heavy rains since the oxbow-lake is used as a spill-way. Moreover, the

reducing conditions due to the abundant accumulation of organic matter could stop the diagenetic processes.

The Deûle HA are different. Humic acids from the upper layer have a composition very similar to those of the oxbow-lake. However, the aliphaticity and the nitrogen content decrease with increasing depth mainly due to the decrease in the contribution of sterols and fatty acids and an increasing contribution of lignin-derived compounds. The molecular evolution is also associated with a decrease in TOC content with depth.

The similarity in composition of the upper Deûle HA can be explained by the fact that the two systems are connected by a channel that allows sediment inputs from the oxbow-lake towards the river. Thus, organic pollutants issuing from the oxbow-lake can accumulate in the upper layers of the Deûle, especially during rainy weather. The decrease in pollutants, with depth, indicates that the inputs are recent or that there is a better turnover of the organic matter in the river sediments.

This contaminating fraction can be partly extracted with dichloromethane and should not be considered part of the structure of HA. Analysis of the lipid fraction allows us to conclude that the occurrence of 5β -stanols, which are markers of sewage, indicates a significant input of domestic wastes to the sediments. This is consistent with the use of the oxbow-lake as a spillway for the waste water basin of Lille.

The presence of a lipid fraction in HA is questionable. It may be easily explained by the fact that steroids are hydrophobic and concentrate in HA fractions during the procedure of extraction of humic substances. Many studies report the retention of hydrophobic organic compounds by HA (Khan & Schnitzer, 1972; Saiz-Jimenez & de Leeuw, 1986; Mills *et al.*, 1996; Saiz-Jimenez, 1996). Extraction of HA with organic solvent is not efficient enough and the analyses of oxbow-lake HA, after solvent extraction, still indicate a significant amount of sterols in the pyrograms and aliphaticity in the FTIR spectra. The fact that the extraction was

not fully efficient suggests that these lipids are trapped in the structure of HA and inaccessible to the solvent but pyrolysis can release them. This fraction is then the predominant signature in the pyrograms overlapping the humic signal mainly constituted of lignin-derived units.

CONCLUSION

The characterization of the Deûle river sediments and those from its oxbow-lake by Py/methylation-GC-MS and GC-MS allowed us to differentiate compounds having a natural origin (terrestrial inputs, such as lignin-derived compounds) from anthropogenic compounds (sterols and to a lesser extent fatty acids). The former belong to the structure of humic acids while the latter are mainly free lipids trapped in the HA structure that cannot be fully recovered by solvent extraction. Detailed analysis of these extracted lipids allowed us to identify the abundant presence of 5β -stanols which we interpret to be sourced from the accumulation of sewage in the oxbow-lake. These sterols can be used as molecular markers of a specific pollutant (sewage) to organic matter in the sediments and allow us to define the extent of this input in the sediments at different depths in the oxbow-lake and in the connected system (Deûle river). These results show the importance of characterizing the lipid fraction that can provide crucial information on the nature and origin of pollutants, as well as showing that humic substances can incorporate some of the contaminants into their structure.

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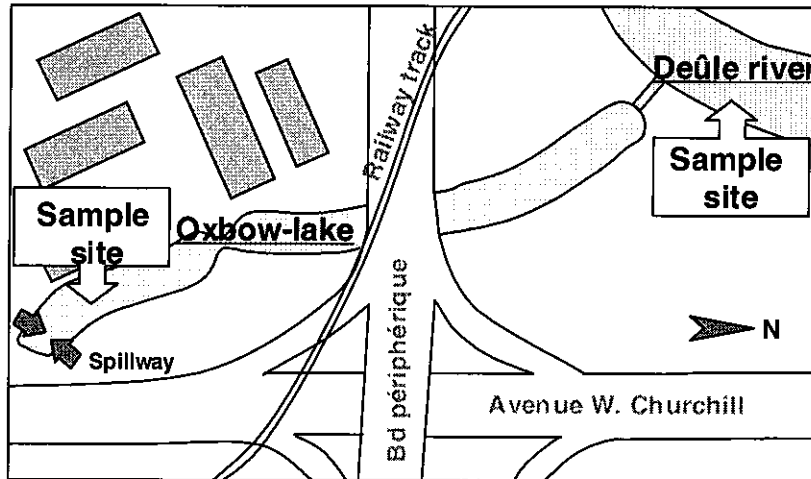
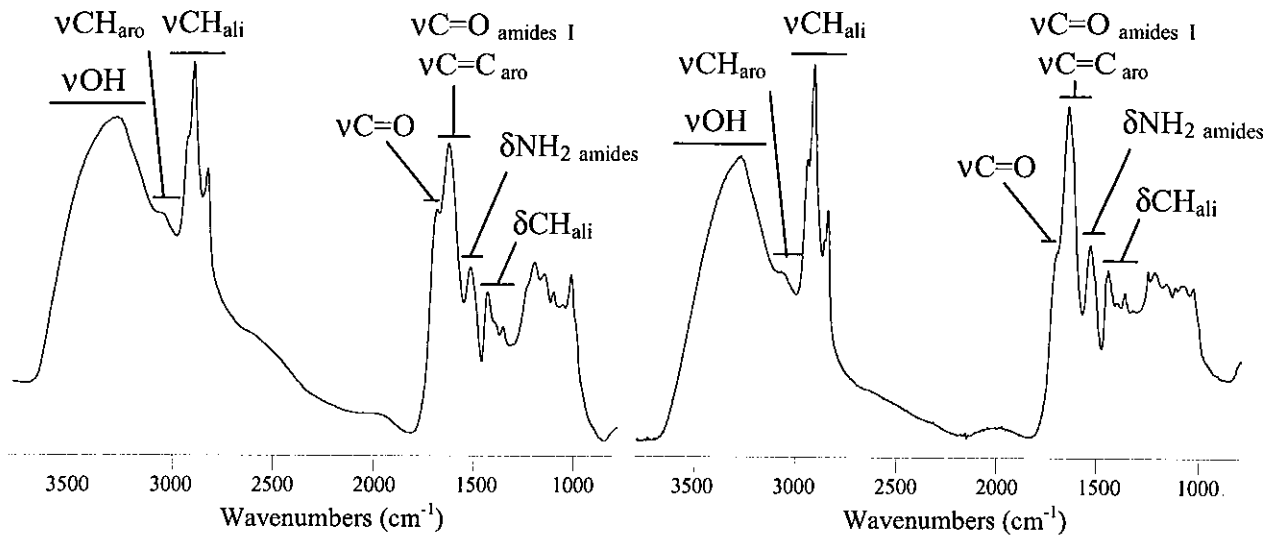
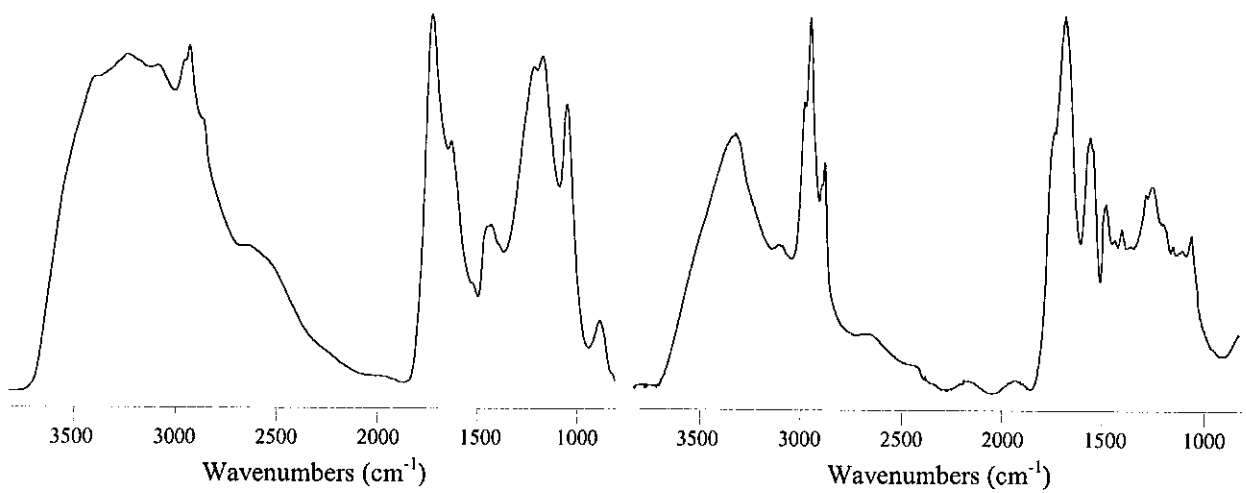


Figure 1



a. Deûle 10 cm layer

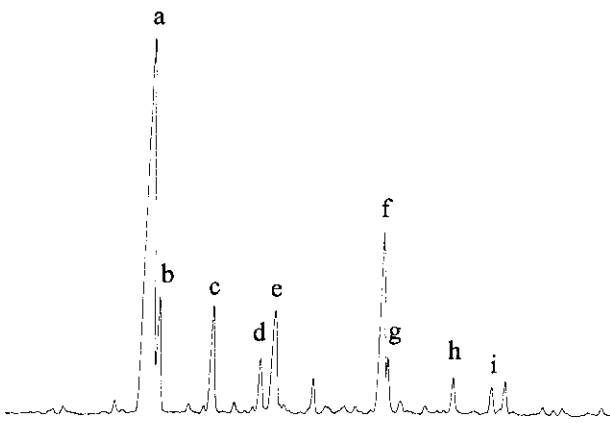
c. Oxbow-lake 10 cm layer



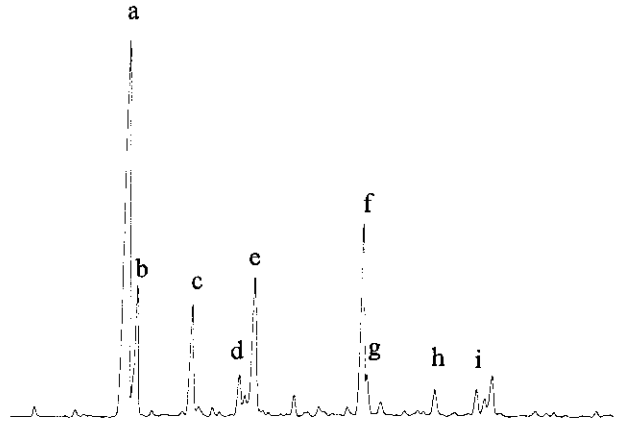
b. Deûle 60 cm layer

d. Oxbow-lake 80 cm layer

Figure 2



A. Steroids in the extract of humic acids from the oxbow-lake sediments



B. Steroids in the extract of a sewage sludge.

Figure 5

Peak	Compound name	Peak	Compound name
1	Phosphonic acid, dimethyl ester	46	Pentadecanoic acid, methyl ester
2	Styrene	47	1-Octadecene
3	Phosphoric acid, trimethyl ester	48	1-(Dimethoxyphenyl)-1,2,3-trimethoxypropane
4	Diethylene glycol dimethyl ether	49	Tetradecanoic acid, 12-methyl-, methyl ester
5	Benzaldehyde	50	1,2,4-Benzenetricarboxylic acid, trimethyl ester
6	Methylstyrene	51	2-Propenoic acid, 3-(3,4-dimethoxyphenyl)-, methyl ester
7	(Methoxymethyl)benzene		
8	1-Methoxy-4-methyl-benzene	52	Hexadecanoic acid, methyl ester
9	2-Butenedioic acid, dimethyl ester	53	N, N-Dimethylhexadecylamine
10	Butanedioic acid, dimethyl ester	54	Heptadecanoic acid, methyl ester
11	Benzoic acid, methyl ester	55	Octadecenoic acid, methyl ester
12	Octanoic acid, methyl ester	56	N, N-Dimethyloctadecylamine
13	1,4-Dimethoxybenzene	57	Octadecanoic acid, methyl ester
14	Decanal	58	Tetracosane
15	Triethylene glycol dimethyl ether	59	Eicosanoic acid, methyl ester
16	3,4-Dimethoxytoluene	60	Dehydroabiatic acid ?
17	4-Methoxy-benzaldehyde	61	Pentacosane
18	1-Methylindole	62	Heneicosanoic acid, methyl ester
19	Lignine derivative (m/z = 129)	63	Hexacosane
20	Decanoic acid, methyl ester	64	Docosanoic acid, methyl ester
21	Proteinaceous derivative (m/z = 98)	65	Heptacosane
22	1,2,4-Trimethoxybenzene	66	Tricosanoic acid, methyl ester
23	4-Methoxy-benzoic acid, methyl ester	67	Docosanoic acid, 2-methoxy-, methyl ester
24	2-Propenoic acid, 3-phenyl-, methyl ester	68	Octacosane
25	Dodecanal	69	Tetracosanoic acid, methyl ester
26	3-methoxy-4,7-dimethyl-1H-isoindole	70	Tricosanoic acid, 2-methoxy-, methyl ester
27	1,3,5-Trimethoxybenzene	71	Sterol 1
28	1,2-Benzenedicarboxylic acid, dimethyl ester	72	Sterol 2
29	3,4-Dimethoxybenzaldehyde	73	Nonacosane
30	1,4-Benzenedicarboxylic acid, dimethyl ester	74	Tetracosanoic acid, 2-methoxy-, methyl ester
31	Dodecanoic acid, methyl ester	75	Triacontane
32	Nonanedioic acid, dimethyl ester	76	Hexacosanoic acid, methyl ester
33	Ethanone, 1-(3,4-dimethoxyphenyl)-	77	5 β -Cholestanol
34	3,4-Dimethoxybenzoic acid, methyl ester	78	C27-stanol
35	1-Hexadecene	79	3 β -Methoxycholest-5-ene
36	3,4,5-Trimethoxybenzaldehyde	80	Sterol 3
37	Benzeneacetic acid, 3,4-dimethoxy-, methyl ester	81	5 α -Cholestanol
38	4,5-Dimethoxy-2-(2-propenyl)phenol	82	C28 stanol
39	Ethanone, 1-(3,4,5-trimethoxyphenyl)-	83	Octacosanoic acid, methyl ester
40	2-Propenoic acid, 3-(4-methoxyphenyl)-, methyl ester	84	Sterol 4
41	3,4,5-Trimethoxybenzoic acid, methyl ester	85	C29 5 β ,3 β , 3 α -stanol
42	Tetradecanoic acid, methyl ester	86	C29 stanol
43	2-Propenoic acid, 3-phenyl-, ethyl ester	87	24S-stigmast-5-en-3.beta.-ol
44	4-Acetyl-2,3,6-trimethoxytoluene	88	Sterol 5
45	1-(Dimethoxyphenyl)-1,2,3-trimethoxypropane	89	Sterol 6

CAPTIONS

Figure 1 : Map of sample sites showing the connections between the Deûle river and its oxbow-lake.

Figure 2 : Infrared spectra of humic acids extracted from the Deûle river sediments at depths of 10 cm (a) and 60 cm (b) and from the oxbow-lake at depths of 10 cm (c) and 80 cm (d).

Figure 3 : Chromatograms of pyrolyzates of HA extracted from the oxbow-lake sediments at depths of 10 cm (a) and 80 cm (b). Peak assignments are reported in Table 1.

Figure 4 : Chromatograms of pyrolyzates of HA extracted from the Deûle river sediments at depths of 10 cm (a) and 60 cm (b). Peak assignments are reported in Table 1.

Figure 5 : Distribution (TIC) of the steroids extracted from oxbow-lake humic acids (A) and extracted from a sewage sludge (B). (a) 5β -cholestan- 3β -ol, TMS ; (b) C27- 5β -stanol, TMS; (c) Coprostan-3-one ; (d) Cholest-5-en- 3β -ol, TMS ; (e) C28- 5β -stanol, TMS ; (f) 24-ethyl-coprostanol, TMS ; (g) C29 stanol, TMS ; (h) C29- 5β -stanone ; (i) 24-dimethylcholest-5-en- 3β -ol, TMS.

Table 1 : Identification of peaks in the pyrograms shown in Figure 2.