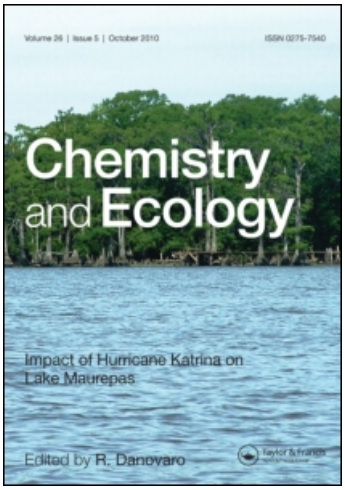

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SCANNING ELECTRON MICROSCOPY OF HUMIC SUBSTANCES PRODUCED DURING CELLULOSE DECOMPOSITION

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Humic substances (HS) produced during the aerobic decomposition of polysaccharides still need to be recognized as such and characterized. Humic (HA) and fulvic (FA) acids extracted at different time intervals during composting of cotton residues from carding, where no decomposition of lignin occurred, were investigated by scanning electron microscopy (SEM), size exclusion chromatography and infrared spectroscopy. Throughout the experiment, fulvic acids possessed a larger number of carboxyl groups but a lower number of weak acidic groups (negatively charged acid groups at pH 11) than humic acids. The number of carboxyl groups increased with time in both fulvic and humic acids, while that of phenolic groups decreased. Infrared spectra showed that the ratio between carboxyl and methyl groups decreased from 8.5 to 4 in FA after 18 days. This change corresponded with the disappearance of flat sheet structures observed by SEM at pH 6. Our results suggest that the shape of humic molecules as observed by SEM reflects the strength of hydrophilic/hydrophobic interactions with the solvent molecules. About 80% of FA molecules had a molecular weight lower than 3500 as deduced from column calibration with HS standards obtained by ultrafiltration. About 40% of humic acids were composed of small molecules; only 20% HA had a molecular weight larger than 45000. Polysaccharide-derived HS may not be limited to the high molecular weight poorly transformed fraction containing bonded polysaccharide structures, but may also consist of low molecular weight components with no structural similarity to the original material.

KEY WORDS: Humic substances, SEM, cotton wastes, composting.

INTRODUCTION

Theories about humification in soil include both lignin and cellulose among the principal components of plant residues from which humic substances are formed (Stevenson, 1978). Whereas lignin is refractory to microbial attack, polysaccharides are easily depolymerized by microorganisms and converted to carbon dioxide, cell tissues and synthesized secondary products which are directly or indirectly connected with a comparatively rapid formation of humic substances. Tests with ^{14}C labelled cellulose showed that, after six weeks, the residual ^{14}C activity of the soil was distributed in all the various soil humus fractions with about 38% of the residual ^{14}C activity in humic and fulvic acids (Martin *et al.*, 1974).

The importance of the polysaccharide-derived fraction of soil HS is clearly shown by the fact that after incubation of uniformly labelled wheat straw polysaccharide for two years, 50% of ^{14}C in humus was found in HS, compared with about 65% for labelled lignins (Stott and Martin, 1990) and that 60% of the C in most organic residues consists of cellulose and other polysaccharides. Although there is some spectral evidence of the presence of chemically bonded polysaccharide structures in humic molecules (Gonzalez-Vila *et al.*, 1983; De Nobili *et al.*, 1990a) knowledge about the polysaccharide-derived fraction of humic substances is still scarce. A part of this poorly humified polysaccharide containing fraction can be isolated by electrophoresis (De Nobili *et al.*, 1990a). It is still impossible at present to distinguish between cellulose and lignin derived HS. Almendros and Leal (1990) studied the oxidative degradation of artificial HS derived from the acid catalyzed dehydration of glucose. However, a more natural approach is to study HS from composted cellulosic materials. Lignin is scarcely, if at all, decomposed during composting (Inbar *et al.*, 1991) and HS obtained by composting plant residues must therefore derive for the most part from the polymerization of newly synthesized aromatic substances (quinones) produced by microorganisms during decomposition of polysaccharides such as cellulose and hemicellulose or from the condensation of sugars with amines (Maillard reaction). Even if the conditions under which HS are formed during composting, where cellulose breakdown is carried out mainly by thermophilic cellulosolitic bacteria, are somewhat different than in soil, thermophilic microorganisms have a widespread distribution in soil and their cultures can be readily obtained from soils and manures (Alexander, 1977).

The characterization of HS produced during composting of plant residues has therefore not only an intrinsic interest but can provide us with information about the possible characteristics of the polysaccharide-derived, newly formed, fraction of soil HS.

Scanning electron microscopy of correctly prepared samples allows the observation of structural detail at the level of macromolecular dimensions (Muller, 1988; Stevenson and Schnitzer, 1982). In studies of HS, scanning electron microscopy (SEM) has mainly been used, sometimes with contrasting results, to investigate the morphological conformation of humic and fulvic acids extracted from soil (Chen and Schnitzer, 1989). HS from compost have never been characterized. Visser (1982) investigated changes occurring during the process of humification of a glucose-yeast mixture; his results, however, were strongly affected by the fact that no precautions were taken to freeze samples at low temperature. Attempts to relate the micromorphological features of HS with chemical structure have been few (Lobartini and Tan, 1988; Chen and Schnitzer, 1976).

The aim of this work was to investigate the chemical and morphological changes of polysaccharide-derived fulvic and humic acids produced during composting of cellulosic materials. We also tried to improve the understanding of the meaning of micromorphological features of HS and of their relation to chemical structure.

MATERIALS AND METHODS

Cotton waste used for composting contained 54.2 $\pm$ 1% cellulose, 6.1 $\pm$ 0.2% hemicelluloses and 14.2 $\pm$ 1.5% lignin. The total organic matter content, determined by loss on

ignition at 550°C, was 83.3%. The ash was mostly composed of SiO_3 (25.98%), CaO (26.09%), K_2O (18.43%), Al_2O_3 (4.88%), MgO (6.28%), P_2O_5 (4.08%) and Fe_2O_3 (3.22%).

About 50 kg of residues from cotton carding were composted at room temperature in a 125 dm^3 polyethylene tank after adding water, urea and sodium phosphate to obtain optimal composting conditions corresponding to 70% humidity, C/N = 80 and C/P = 400 (Baca *et al.*, 1992). The composting material was turned manually every two or three days. Temperature was measured daily at a depth of 15 cm and samples were taken at regular intervals to control humidity and measure weight loss. The overall weight loss after 90 days of composting was 51.1%. Analysis of the composting material used before and after extraction of HS with 0.5M NaOH showed that HS produced during the first ninety days of composting (2.8%) originated exclusively from the decomposition of cellulose and hemicelluloses, as no significant alteration of the lignin content had occurred in the course of the experiment (Baca *et al.*, 1992), nor was any part of it extracted by 0.5M NaOH. In fact, taking into account weight loss after 90 days of composting, the material contained 17.2 ± 1.7% lignin, 11.2 ± 1% cellulose and 0.37 ± 0.04% hemicellulose. After extraction with NaOH the residue contained 18.26 ± 2% lignin, 7.81 ± 0.8% cellulose and no hemicelluloses. Lignin, cellulose and hemicellulose content were measured according to the methodology described by Goering and Van Soest (1970).

Humic and fulvic acids were extracted by shaking ten grams of composting material with 100 ml of 0.5M NaOH under N flux for one hour. The samples examined were taken after 3 days (beginning of the thermophilic phase), 18 days (end of the thermophilic phase) and 90 days of composting (end of the curing period). Extracts were immediately treated with hot acid-washed H^+-saturated cation exchange resin (Amberlite IR 120) until a constant pH was reached (2 to 2.5). Humic acids were separated by centrifugation at 2920 g for about 20 minutes. Fulvic acids were purified by adsorption on a small polyvinylpyrrolidone (PVP) column, re-eluted with 0.5M NaOH and treated again with H^+-saturated cation exchange resin. Recovery of extracted organic carbon after chromatography on PVP was checked for every extract by determining the amount of organic carbon in both the absorbed (humic) and non-retained (labile organic C) fractions. Recoveries ranged from 102 to 98%. Both humic and fulvic acid preparations were freeze-dried in acid form.

The elemental composition and ash content of the humic acids and fulvic acids preparations are reported in Table 1.

Determination of negatively charged dissociated acid groups was performed by precipitation titration with CTA + (cetyltrimethylammonium) bromide as described by De Nobili *et al.* (1990b). Freeze-dried humic and fulvic acids were dissolved in dilute sodium hydroxide and distilled water to make a 1 $g l^{-1}$ solution with a pH 11. The pH of aliquots of the solution was then adjusted to pH 9, 8, 7 and 6 by treatment with H^+ -saturated cation exchange resin.

IR spectra were recorded on KBr pellets of finely ground HA and FA preparations which had been freeze-dried at pH 2 to 2.5 and stored in a desiccator over P_2O_5. Molecular weight distributions were calculated from size exclusion chromatography data. Analyses were performed by HPLC with a Waters 590 pump and a 484 UV-Vis

Table 1 Elemental composition of humic and fulvic acids extracted with 0.5 NaOH from composting cotton waste. Fulvic acids were purified by adsorption chromatography on PVP.

Sample	*%C*	*%H*	*%N*	*%Ash*
HA 3 days	50.3 ± 03	6.0 ± 0.1	5.0 ± 0.1	3.0
HA 18 days	50.3 ± 1.3	6.2 ± 0.1	5.8 ± 0.2	1.5
HA 90 days	49.7 ± 0.8	6.0 ± 0.1	6.0 ± 0.1	2.8
FA 3 days	41.8 ± 0.8	4.9 ± 0.1	2.4 ± 0.1	20.0
FA 18 days	43.6 ± 0.8	5.1 ± 0.1	2.7 ± 0.1	15.5
FA 90 days	45.1 ± 0.8	4.3 ± 0.1	3.8 ± 0.1	--

detector set at 400 nm, on a 30 cm long Biosil-tsk-250 column (Biorad) using 0.025M tris-phosphate at pH 7.5 as eluent (Tsutsuki and Kuwatsuka, 1984). The column was calibrated by means of a series of humic acid fractions in the range 100000–50000, 50–30000, 30–10000 and 10000–5000, respectively, obtained from ultrafiltration on Amicon membranes YM 100, YM 50, YM 30, YM 10 and YM 5 of HS extracted from a spodic soil. Under the experimental conditions chosen, no peaks or peak tails were eluted after the void volume of the column. A good linear relationship ($p < 0.0001$) was found between molecular weight and elution volume of the soil HS fractions used as standards for column calibration (De Nobili *et al.*, 1989).

For scanning electron microscopy (SEM) 1 gl^{-1} solutions of FA and HA at pH 11 were made freshly by dissolving 0.01 g of FA or HA in the appropriate amount of diluted sodium hydroxide and distilled water. Aliquots of the pH 11 solutions were treated with H^+-saturated cation exchange resin to obtain FA and HA solutions at pH 6 in order to treat all solutions in the same way. Lower pH values were not considered to avoid precipitation of HA. To prepare samples for SEM, 20 μl droplets of FA or HA solutions were frozen by letting them fall one by one from the tip of a 200 μl Gilson micropipette into small polyethylene vessels filled with freon kept at equilibrium temperature with liquid nitrogen. The vessels with the solidified freon gas and droplets were then placed immediately in the freeze drier. Freeze-drying was complete in about one hour. The small dimensions and the consequently very low thermic capacity of the droplets assure a faster freezing than when a mica sheet or any other materials is used as a support. Although Tan (1985) found that samples could be frozen usefully by a direct liquid N technique avoiding the use of freon, we observed that droplets tend to float on liquid nitrogen and take a considerably longer time to freeze. We therefore chose to adopt the freon liquid N technique.

RESULTS AND DISCUSSION

Brown coloured humic substances could be extracted after only three days from the composting material. The mean E4/E6 ratio of 0.05M $NaHCO_3$ solutions of HS at pH 8 during the first 30 days of composting was 7.87 ± 1.73 and showing a slightly

increasing trend consistent with the progressive depolymerization of part of HS observed by HPLC-SEC.

The molecular weight distributions of FA and HA extracted after 3, 18 and 90 days of composting are reported in Figures 1 and 2. Fulvic acids were characterized by very low molecular weights: about 80 to 90% of FA molecules had a molecular weight lower than 3500. About 40% of humic acids were composed of small molecules (MW < 3500) and only 20% had a molecular weight larger than 45000. There was an increase from about 16 to 20% of the excluded molecular weight fraction > 500000) at the end of the thermophilic phase (18th day). Afterwards the excluded fraction seemed to undergo depolymerization and a corresponding increase was observed in the MW fractions from 12500 to 500000. These data are not comparable with data found in the literature as they were deduced from column calibrations made with proteins or dextran standards and not with humic substances fractions (De Nobili *et al.*, 1989).

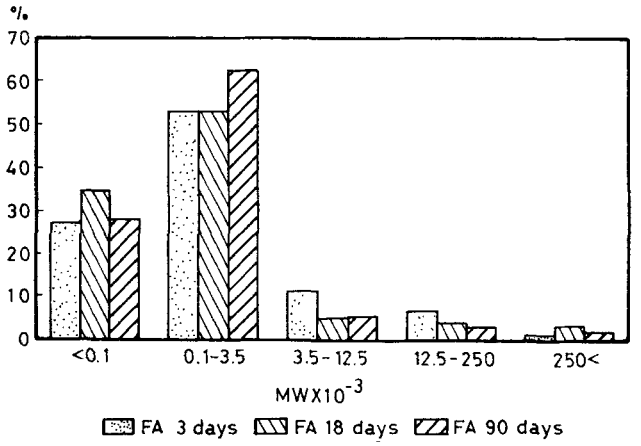

Figure 1 Molecular weight distribution of fulvic acids (FA).

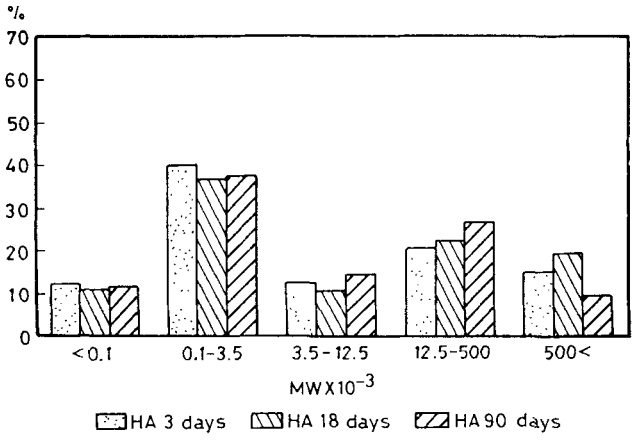

Figure 2 Molecular weight distribution of humic acids (HA).

The infra-red spectra of both humic and fulvic acids (Figure 3 and 4) exhibited all the major infra-red features common to all soil humic and fulvic acids: a strong OH stretching at 3400 cm^{-1}, a CH stretching at 2940 cm^{-1}, a shoulder for C=O stretching and a strong COOH adsorption (asymmetrical stretching of COOH and H-bonded C=O) at 1720 and 1640 cm^{-1}. This band increased with humification together with the corresponding adsorption band of the C—O stretching vibration of carboxyl and phenolic groups (1240–1180 cm^{-1}) and bending of phenolic and alcoholic OH at 1420–1330 cm^{-1}. The trend is consistent with formation of HS by condensation of quinones (Stevenson, 1978). Weak adsorption of all samples in the 900–700 cm^{-1} region can be attributed to the presence of highly substitued aromatic rings (Senesi

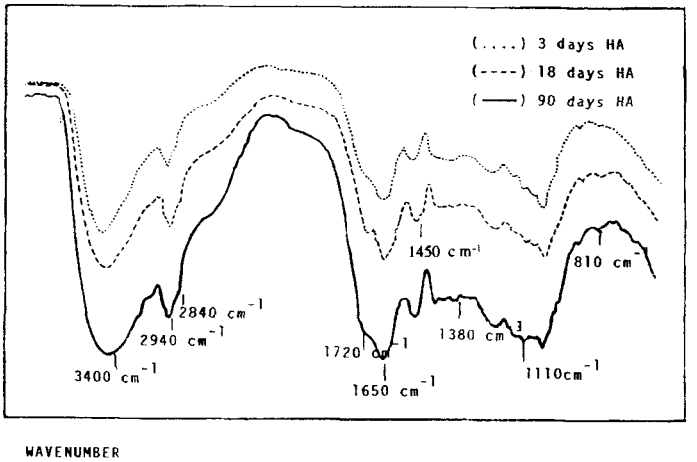

Figure 3 Infra-red spectra of humic acids.

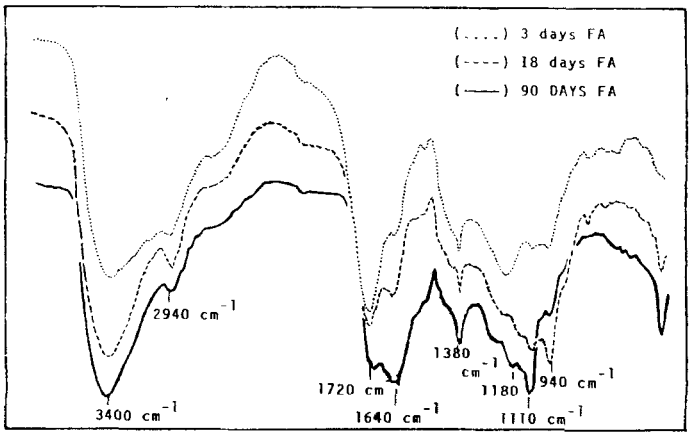

Figure 4 Infra-red spectra of fulvic acids.

et al., 1983). Three day-old FA has an IR spectrum typical of low molecular weight soil fulvic acids (type II B according to Stevenson, 1978). Considerable changes in structure occurred between 3 days and 18 days of composting. The evolutionary trend was towards the formation of FA with spectra similar to type III (18 day-old FA) and type II C soil (90 day-old FA). IR spectra of HA strongly resemble those of type III D soil HA with absorption in regions indicative of proteins and carbohydrate moieties around 1640 cm^{-1} (C=O stretching of amides) and 1520 cm^{-1} (C=N stretching of amides) and in the region 1200–950 cm^{-1} (C—O stretching of polysaccharide-like substances). However infra-red spectra of HA and FA extracted from composting cotton waste showed only a relatively weak absorption in the region 1000–1300 cm^{-1} with respect to other polysaccharide-rich HA fractions isolated from soil (De Nobili *et al.*, 1990a). This would indicate that HS other than those containing carbohydrate-components can derive from polysaccharides.

The curves obtained by precipitation titration of FA with CTA + (Figure 5) showed an increasing similarity with those of HS extracted from soil (De Nobili *et al.*, 1990b). The evolutionary trend is more confused with humic acids as the precipitation curve of 90 day-old humic acids is rather flat with no significant change in the number of dissociated acid groups with pH (Figure 6). Fulvic acids possessed a higher number of

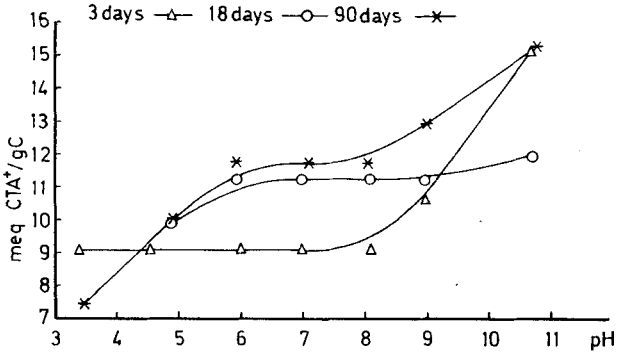

Figure 5 Density of negatively charged dissociated acid groups (meq g^{-1}) on fulvic acid (FA) as a function of pH.

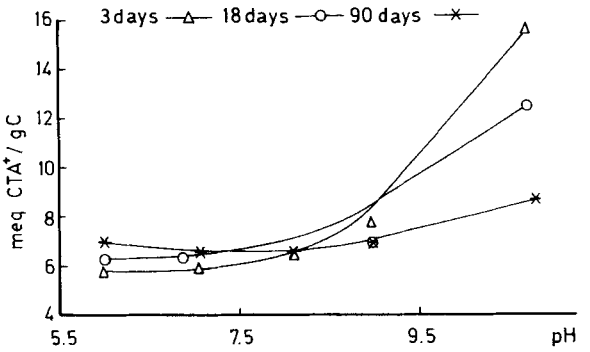

Figure 6 Density of negatively charged dissociated acid groups (meq g^{-1}) on humic acid (HA) as a function of pH.

dissociated acid groups at pH 6 and a lower number of weak acidic groups, which are likely to consist mainly of phenolic groups at pH 11. The negative charge density of FA at pH 6 increased sharply from 3 to 18 days. The amount of carboxyl groups (calculated as the number of dissociated acid groups at pH 7) increased with time in both humic and fulvic acids, passing from 9.05 meq g^{-1} in three day-old FA to 11.42 meq g^{-1} organic C after 18 days for fulvic acids, and from 5.88 meq g^{-1} (three day-old HA) to 7.36 for 18 day-old HA. In contrast, phenolic groups (calculated as the number of dissociated acid groups at pH 11 minus the number of dissociated acid groups at pH 7) decreased sharply during the thermophilic phase for both humic and fulvic acids, changing from 6.10 meq g^{-1} organic C to 0.5 meq g^{-1} in 18 day-old fulvic acids, and from 9.8 to 5.27 meq g^{-1} respectively in 3 day- and 18 day-old HA. Phenolic groups increased again to respectively 2.38 meq g^{-1} in FA and 1.76 meq g^{-1} in HA extracted from the compost after 90 days. The negative charge density of HA at pH 6 did not change appreciably with composting time.

Micromorphological changes of HS during composting investigated by SEM

Despite being frozen repeatedly with great care with solid freon, three day-old FA (not shown in figure) displayed flat, almost featureless, surfaces, which at pH 6 seemed to be composed from a continuous layer of partially fused globular particles ranging from about 100 to 250 nm. At pH 11 these surfaces appeared more corrugated but higher magnifications showed enhanced fusion of globular particles. After 18 days, fulvic acids already showed morphological features which resembled soil fulvic acids: at pH 6 (Figure 7); thin perforated sheets and threads with small globular or elongated bodies were present and merged into a nearly continuous sheet at pH 11 (Figure 7), as reported by Chen and Schnitzer (1976). After 90 days, scanning electron micrographs of FA at pH 6 (Figure 7) showed a loose fibrous structure. Some of the larger fibres showed a very thin ribbon-like structure where a few narrow fibres were grouped along their length. A solid sheet structure was again observed at pH 11, but more perforated and sponge-like than 18 day-old fulvic acids.

Humic-like substances extracted after three days at pH 6 showed a three dimensional loose net structure of fibres (80 nm thick) and thin small sheets (Figure 8). At pH 11, the same humic acid preparation showed the expansion of fibres into bundles of rounded flakes or thick crumpled ribbons (Figure 9). Ribbon-like structures were longer and more numerous in 18 day-old HA. These humic acids, produced during the thermophilic phase when rapid decomposition of the more easily available substrates occurs, showed at pH 6 the coexistence of rounded globular particles with a diameter ranging from 0.4 to 1 μm with 100–200 nm thick tubular fibres. As the predicted molecular size of a molecule with a molecular weight of about 1000 is 15–20 nm, the smaller spheroids could correspond to molecules with a molecular weight of about 200000. This would be in agreement with the fact that globular bodies were found in practically all humic acids. The excluded molecular weight fraction of HA (MW > 500000) reached a maximum after 18 days. The highest molecular weight fraction, which, in number, accounts for only about twenty percent of HA after 18 days, would occupy approximately 70% of the overall volume occupied by HA molecules. Similar spheroids were observed by other authors in fulvic and humic acids extracted

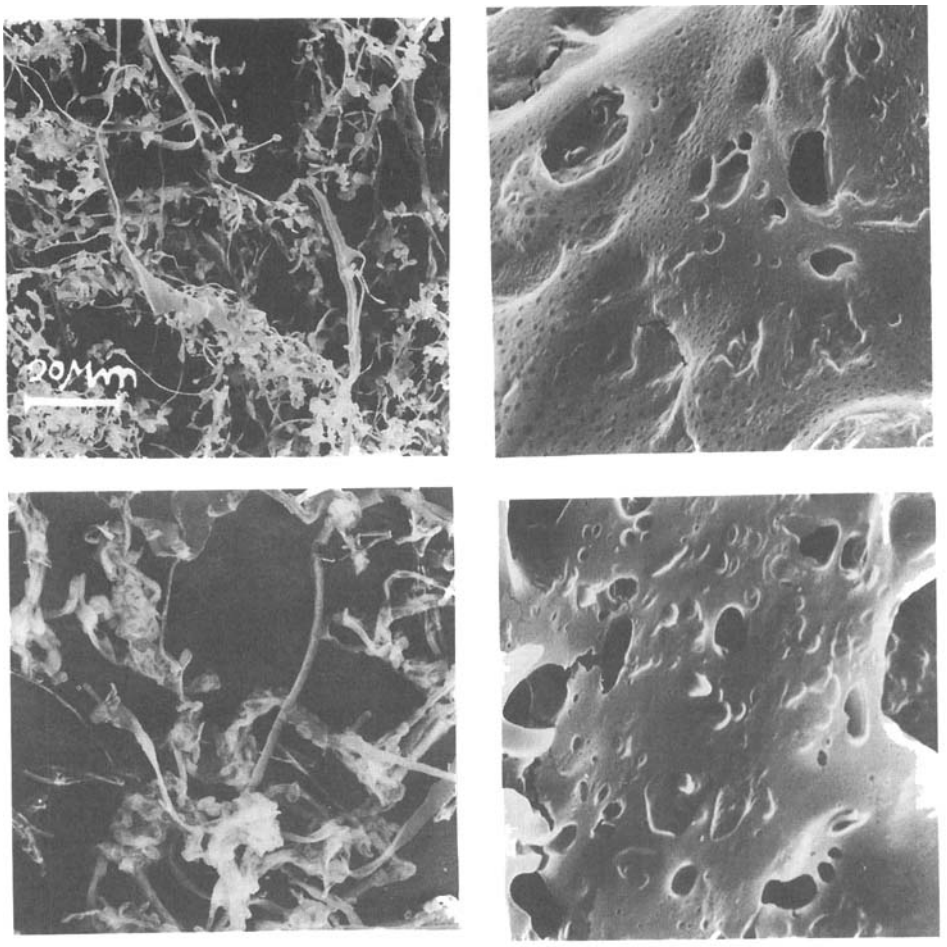

Figure 7 Scanning electron micrographs of fulvic acids extracted from composting cotton waste after 18 days (top) and 90 days (bottom). Solutions (1 mg ml^{-1}) were adjusted to pH 6 (left) and pH 11 (right).

from certain soils (Chen and Schnitzer, 1989; Tan, 1985). According to Tan (1985) these particles are not silica or other inorganic particles (in our work they were found mostly in humic acids which have a much lower ash content than FA) but polysaccharide components. FA preparations used in this work were purified by adsorption chromatography and therefore do not contain free saccharide components, but secondary polysaccharide could be included in the structure of HA. The number of fibres increased greatly in 90 day-old HA; coarse fibres (400 nm) became more numerous, although shorter in length (4–10 nm). Thinner fibres and a consistent number of globular particles were still visible. No relevant change was observed at pH 11 between HA extracted after 18 and 90 days, except that ribbon-like structures appeared thicker and wider.

The influence of pH is clearly visible on the micromorphology of FA and much less so far HA. In the case of FA, the charge density of the molecules increased strongly with

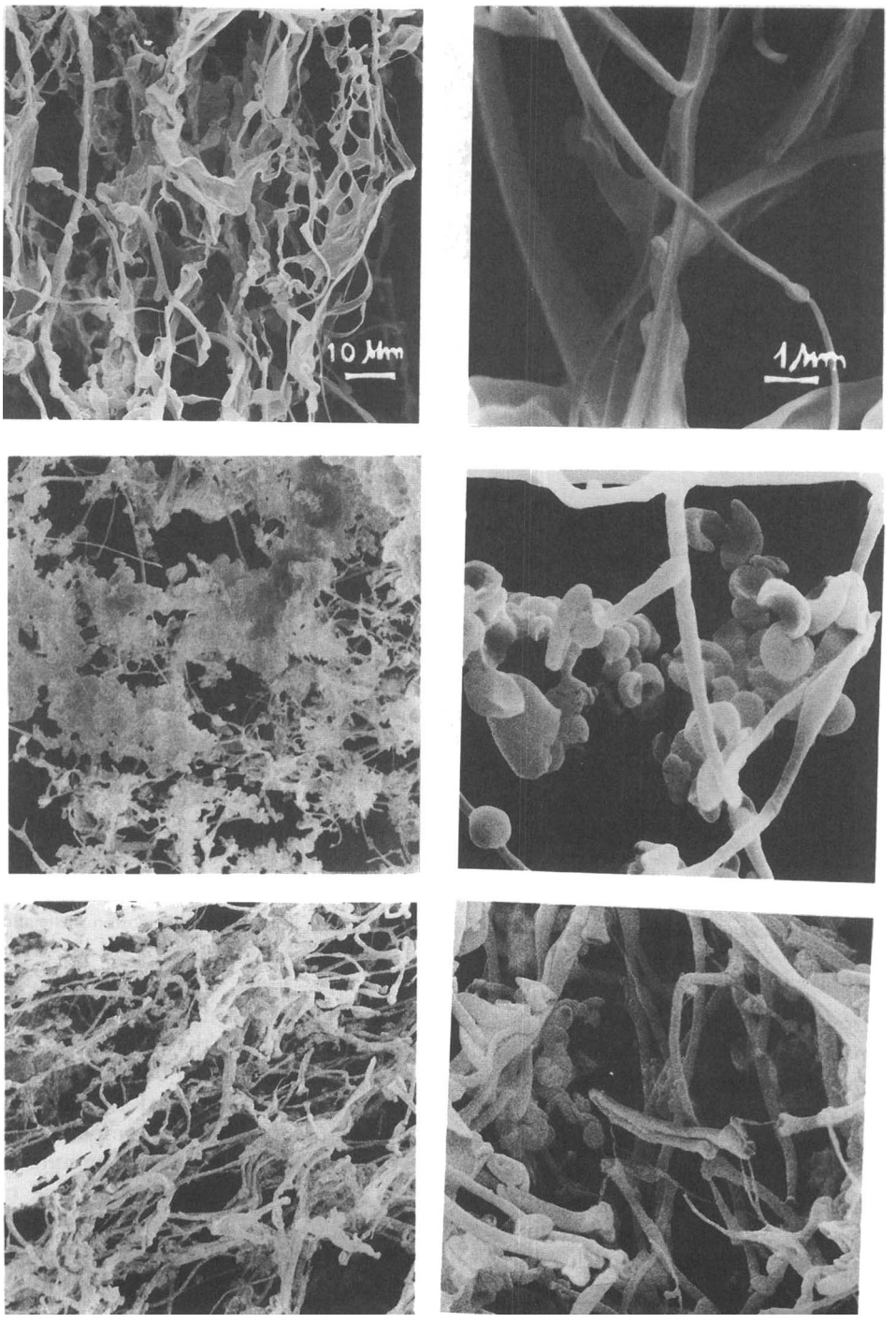

Figure 8 Scanning electron micrographs of pH 6 solutions of humic acids extracted from composting cotton waste after three days (top), eighteen days (centre), and ninety days (bottom). Left: low magnification (× 800) and right: high magnification (× 9000).

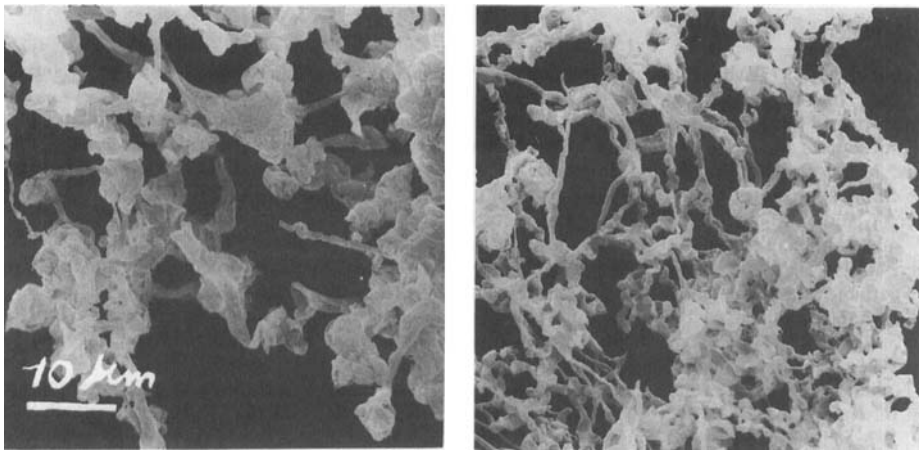

Figure 9 Scanning electron micrographs of pH 11 solutions of humic acids extracted after three days (left) and 18 days (right).

time, but, contrary to expectations, at pH 6 the trend was towards the formation of an increasingly fibrous structure. Although Lobartini and Tan (1988) did not observe any pH effect, they found that humic acids extracted from two mollisols, which formed characteristic solid sheet structures, had NMR spectra characterized by a strong carboxyl signal. In our case, three day-old humic acids, which had the highest negative charge density at pH 11, did not collapse like FA into a flat featureless structure.

Functional groups are more strongly solvated when they are in an ionized form: this means that more water is present in the hydration shells. The presence of a large amount of bound water around humic molecules is a likely cause for the formation of the extended sheets observed at high pH. On the other hand the presence of hydrophobic groups which do not perturb the structure of water (Muller, 1988; Saito, 1969) favours the formation of slender fibres and smaller aggregates. The much higher absorption at 2920 cm^{-1}, found in three day-old humic acids with respect to fulvic acids, supports this hypothesis. The ratio between carboxyl and methyl groups, calculated from the corresponding absorbance at 1720 and 2920 cm^{-1}, has an absolute maximum (8.5:1) in three day-old fulvic acids, while it is only 2.7:1 in three day-old HA. At 18 days the ratio falls to 4:1 in FA. Afterwards, it increases again slightly in both fulvic (5.6:1) and (3.2:1) humic acids without reaching the apparent collapse ratio. The micromorphological structure of HS would therefore reflect the strength of the hydrophilic/hydrophobic interactions with water molecules of hydration shells.

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