ACTA UNIVERSITATIS AGRICULTURAE ET SILVICULTURAE MENDELIANAE BRUNENSIS
SBORNÍK MENDELOVY ZEMĚDĚLSKÉ A LESNICKÉ UNIVERZITY V BRNĚ

Ročník LII 14 Číslo 4, 2004

MICROBIAL PROCESSING OF HUMIC ACIDS EXTRACTED FROM SOILS OF A LONG-TERM FIELD TRIAL

Z. Filip, J. Kubát

Received: April 13, 2004

Abstract

FILIP, Z., KUBÁT, J.: *Microbial processing of humic acids extracted from soils of a long-term field trial*. Acta univ. agric. et silvic. Mendel. Brun., 2004, LII, No. 4, pp. 137-146

Humic acids (HA) extracted with alkali from non-fertilized and fertilized (NPK + organic manure) soils of field trials established 45 years ago were exposed to activities of a mixed population of indigenous soil microorganisms in the laboratory. The individual cultures were kept on a rotary shaker for 21 days (aerobic conditions) or left standing for 12 months (semi-aerobic conditions) in order to determine the utilization of HA as nutrients, and possible HA structural transformations. In a full-strength nutrient broth HA positively affected the yields of microbial biomass, that, however, was strongly diminished if HA served as the only sources of carbon and nitrogen. In contrary, the utilization of HA was up to 47.3% under semi-aerobic conditions in cultures deficient in easy utilizable sources of carbon (glucose). No substantial differences were observed in the microbial processing of HA from non-fertilized or fertilized soils. Usually, microbial activities resulted in elemental and structural transformations of HA. Aliphatic structural units were preferably utilized as determined by FT-IR spectroscopy, while aromatic structures became rather dominating. Conclusively, independent of the type of microbial processing, HA from soils of a long-term field trial showed rather low resistance to microbial activities, and perhaps they should not account for a resistant fraction of the soil organic matter.

soil organic matter, humic acids, microbial utilization, microbial transformation

Long-term field fertilization trials have been recognized as valuable tools for the prediction of natural and/or human induced alterations in soil quality. In soil plots that undergo individual treatments fate of soil organic matter (SOM) becomes governed by humification and mineralization processes. Among SOM fractions, humic substances comprise for about 60 to 80%, and due to structural complexity they have been mainly considered resistant to microbial attack (Brady and Weil, 2002). This assumption should apply especially for humic acids (HA), the complex molecules of which consists of aromatic lignin derivates, long-chain alkylic compounds, differently modified polysaccharides, and some other structural components (Haider 1999, Haider et al. 1975, Beyer, 1996, Simpson et al., 2002). Studies using radioactive isotopes indicated parts of carbon as converted into humus even thousands years ago and still detectable in soil (Brady and Weil, 2002). On the other hand, in some German soils nearly 30% of the total organic C stored in former times has been apparently mineralized during the last 20 years (Beyer at al. 1999). In a review, we could point out on numerous studies on HA as a regular subject of microbial degradation (Filip et al. 1998). We contributed also several experimental evidences to this processes (Filip et al. 1999, Hertkorn et al. 2002).

Concerning arable soils of long-term field trials, in-

creases or decreases in the content of humic substances have been reported for several differently fertilized plots (Albert, 1999, Schellberg et al. 1999, Baumecker et al. 2002). Thus, there is also an interest in establishing whether HA fractions originated in soils of long-term field trials would primarily differ in their susceptibility to microbial utilization. To elucidate this, we performed laboratory experiments with different HA preparations, and published results related to two soil types, i. e., Orthic Luvisol and Dystric Cambisol (Filip and Kubat, 2001, 2003). In this paper, soil samples from a long-term field trial in Prague-Ruzyně (an Orthic Luvisol soil site) served as a source of HA for which we obtained different new data. First of all, however, we wished to compare and evaluate data on similarities and/or differences in the microbial processing of HA exposed to microbial activities for rather short time in intensively aerated shaken cultures, or kept less aerated, i.e., standing, for a prolonged period of time.

MATERIAL AND METHODS

At the experimental site in Prague-Ruzyne a long-term field trial was established in 1955. The site is located at altitude of 352 m above the sea level, and has average annual temperature of 8.1°C, and precipitation of 450 mm, respectively. Orthic Luvisol, a clay-loam soil, was developed on dilluvial sediments and mixed with losses. Soil samples were taken from Ap horizon (0 to 26 cm in depth) of an untreated control plot (abbreviated as $Soil_{Con}$), and NPK fertilized and manured plot (average N dose 103 $kg.ha^{-1}.a^{-1}$), and abbreviated as $Soil_{Fert}$. The total carbon contents were 1.13% and 1.31%, and the corresponding N contents were 0.126% and 0.141% for the $Soil_{Con}$ and $Soil_{Fert}$, respectively; the value of pH_{KCl} was 6.8 and 7.1.

The extraction and purification of HA, composition of nutrient broth and inoculum, harvest of microbial biomass, and the individual analytical procedures remained the same as described earlier (Filip et al. 1999, Filip and Kubát 2001). In brief, the soil HA were extracted by a mixture (1:1, v/v) of 0.1 M $Na_4P_2O_7$ under N_2 and purified by a dialysis against deionized water and diluted HF. In amounts of 100 mg freeze-dried HA preparations were added to 100 ml sterile nutrient solution in 250 ml Erlenmeyer flasks, which were closed by cotton plugs. A Czapek-Dox nutrient broth containing glucose and $NaNO_3$ as carbon and nitrogen sources was used in a full strength (full broth = FB) or without either glucose (FB – C) or $NaNO_3$ (FB – N) to receive carbon-deficient or nitrogen-deficient solutions, respectively. Control flasks (FB less HA), and samples containing HA were inoculated by 1 ml of 10^{-3} diluted suspension of the respective soil, i.e., samples with HA from $Soil_{Con}$ received inoculum from $Soil_{Con}$, etc. For aerobic incubation triplicate samples were placed on a rotary shaker (100 rev/min) for 21 days in the dark (room temperature). To simulate semi-aerobic conditions, perhaps more typical for soil environments, samples were kept standing for 12 months under the same conditions. After the incubation was terminated, replicates (three in maximum) that did not differe from each other by visual appearance, were combined for analyses which were performed in triplicate. From the liquids, the microbial biomass was harvested by centrifugation for 10 min at 14 344 x g (15 °C). A standard deviation of the analytical data was calculated using a common computer program. Since deviations were less than 5% throughout, mean values of estimated analytic data are presented in the respective tables.

RESULTS AND DISCUSSION

A dominant role of soil microorganisms in processes contributing to the loss of SOM has been recognized a long time ago (Kononova, 1968). Yet, in our experiments, the actual counts of soil microorganisms growing on a Czapek-Dox nutrient medium and possibly acting as HA processors were rather low. The average numbers of colony forming units (CFU) for $Soil_{Con}$ and $Soil_{Fert}$ were about 2.5 x 10^6 CFU for bacteria and 1.5 x 10^5 CFU for fungi per 1 g soil (d.w.). However, under aerobic conditions appreciable amounts of microbial biomass were yielded already from control flasks with no HA added, and the individual yields were enhanced up to 251% if HA were supplementary added (Tab I). Although the incubation was much longer under semi-aerobic conditions of static cultures, distinctly less biomass was harvested after 12 months. If used as nutrient supplements, however, HA strongly enhanced the formation of biomass. The HA preparations from both the $Soil_{Con}$ and $Soil_{Fert}$ exerted similar effects on the formation of microbial biomass. However, the effects were rather low if HA served as the only C or N sources, and especially under aerobic incubation conditions.

From the different SOM fractions, HA have been designated a passive one, the increases or decreases of which occure very slowly (Brady and Weil, 2002). Nevertheless, the capacity of soil microorganisms to force losses of HA was repeatedly demonstrated (Filip et al. 1998, 1999; Gramss et al. 1999). Demkina and Zolotareva (1997) reported a rapid initial mineralization of soil HA that occurs in 30 days and turns into a slow one during a subsequent incubation up to 150 days. Similarly, in our experiments HA added supplementary to a full strength nutrient broth or serving as a sole source of N were less utilized during a long-term static (semi-aerobic) incubation than in 21 days under aerobic conditions (Tab. II). Still, the utilization was more extensive if HA served as the only C or N sources in nutrient solution. Such observati-

I: *Effect of humic acids (HA) on microbial biomass production under aerobic or semi-aerobic incubation conditions*

Biomass from	Yield of biomass			
	Aerobic		Semi-aerobic	
	mg/100 ml	% of contr.	mg/100ml	% of contr.
Controls				
FB*, Inoculum from $SOIL_{Con}$	482.7	100.0	84.7	100.0
FB, Inocullum from $SOIL_{Fert}$	310.7	100.0	115.3	100.0
HA from $SOIL_{Con}$				
FB + HA	630.3	130.6	218.3	257.7
FB – C + HA	26.7	5.5	24.0	28.3
FB – N + HA	33.0	6.8	69.7	82.3
HA from $SOIL_{Fert}$				
FB + HA	780.0	251.0	182.7	158.5
FB – C + HA	23.7	7.6	25.0	21.7
FB – N + HA	28.7	9.2	73.3	63.6

* FB = full-strength nutrient broth; FB – C = FB less glucose; FB – N = FB less $NaNO_3$

ons indicate that HA under testing could be utilized as nutrient sources directly rather then co-metabolically. On the other hand, HA utilization was clearly suppressed if more easily utilizable C or N sources were available to soil microorganisms.

II: *Recovery of humic acids (HA) from microbial cultures and percentages of HA utilization under aerobic or semi-aerobic incubation conditions*

Sample	HA added	HA recovered		HA utilized (%)	
	(mg/100ml d.w.)	aerobic	semi-aerobic	aerobic	semi-aerobic
HA from $SOIL_{Con}$					
FB* + HA	100.0	78.3	83.7	21.7	16.3
FB – C + HA	100.0	55.0	52.7	45.0	47.3
FB – N + HA	100.0	59.7	64.7	40.3	35.3
HA from $SOIL_{Fert}$					
FB + HA	100.0	75.0	82.0	25.0	18.0
FB – C + HA	100.0	59.3	58.7	40.7	41.3
FB – C + HA	100.0	59.0	78.2	41.0	21.3

* See footnote Tab. I

The microbial processing of HA preparations in 21 days under aerobic or 12 months under semi-aerobic cultivation conditions resulted in different elemental changes (Table III). These were mainly correlated with the nutrient status of the respective microbial cultures. Slightly lower contents of C and increased ones of N, e.g., were estimated in HA re-isolated from the full strength nutrient broth (FB) cultures. Perhaps, some HA-like substances rich in nitrogen and possibly produced by microorganisms during the incubation period could contribute to this effect. Rammuni et al. (1987) described such phenomena as side effects of microbial degradation of humic substances.

III: *Elemental composition and some atomic ratios of original humic acids (HA) and those re-isolated from microbial cultures incubated under aerobic or semi-aerobic conditions* (Elements in ash free %)*

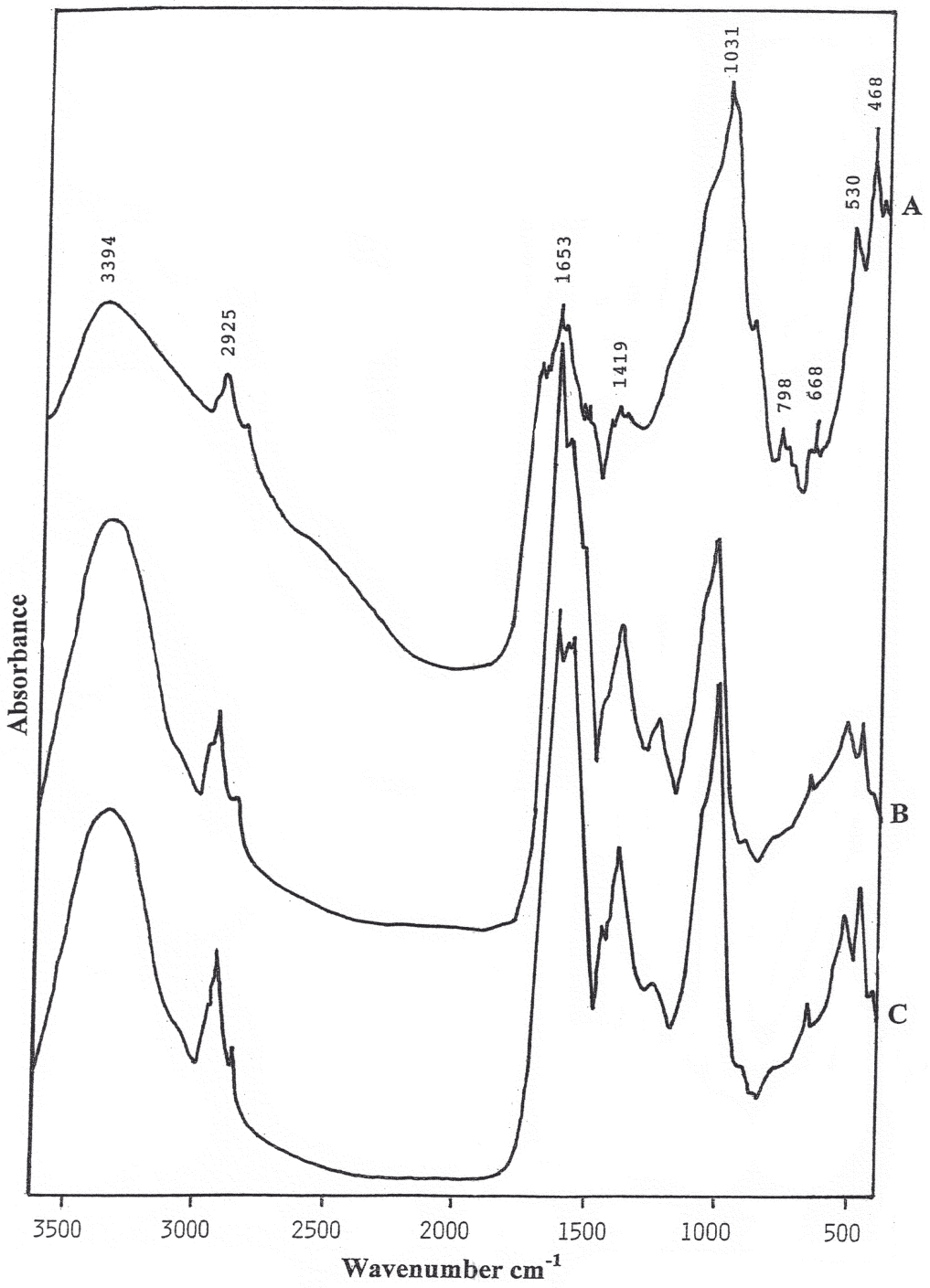
Sample	C		N		H		O		C:N		H:C		O:C	
	Aer.	Semi-aer.	Aer.	Semi-aer.	Aer.	Semi-aer.	Aer.	Semi-aer.	Aer.	Semi-aer.	Aer.	Semi-aer.	Aer.	Semi-aer.
HA from SOIL$_{Con}$														
Original sample		50.6		4.0		5.4		40.0		14.0		1.3		0.6
From FB*	50.7	51.5	7.2	6.0	6.3	6.1	35.8	46.4	8.4	8.0	1.5	1.8	0.5	0.8
From FB – C	52.3	51.6	4.2	3.8	5.4	4.9	38.1	60.3	14.7	15.9	1.2	1.1	0.5	0.9
From FB – N	51.8	49.8	4.2	5.3	5.5	5.5	38.5	39.4	14.3	10.9	1.3	1.3	0.6	0.6
Novel HA		51.7		4.5		5.6		38.2		13.5		1.3		0.6
HA from SOIL$_{Fert}$														
Original sample		53.3		4.4		5.8		36.5		14.3		1.3		0.5
From FB	49.1	52.9	6.6	5.2	5.8	5.7	38.5	36.5	8.2	11.9	1.4	1.3	0.6	0.5
From FB – C	52.8	51.2	4.2	3.9	5.3	5.0	37.7	36.2	14.7	15.2	1.2	1.2	0.5	0.6
From FB – N	50.4	49.8	4.2	5.0	5.3	5.8	40.1	39.9	14.0	11.5	1.3	1.4	0.6	0.6
Novel HA	51.2	54.4	8.3	7.2	7.6	6.6	32.9	31.8	7.2	8.9	1.7	1.4	0.5	0.4

* See footnote Tab. I.

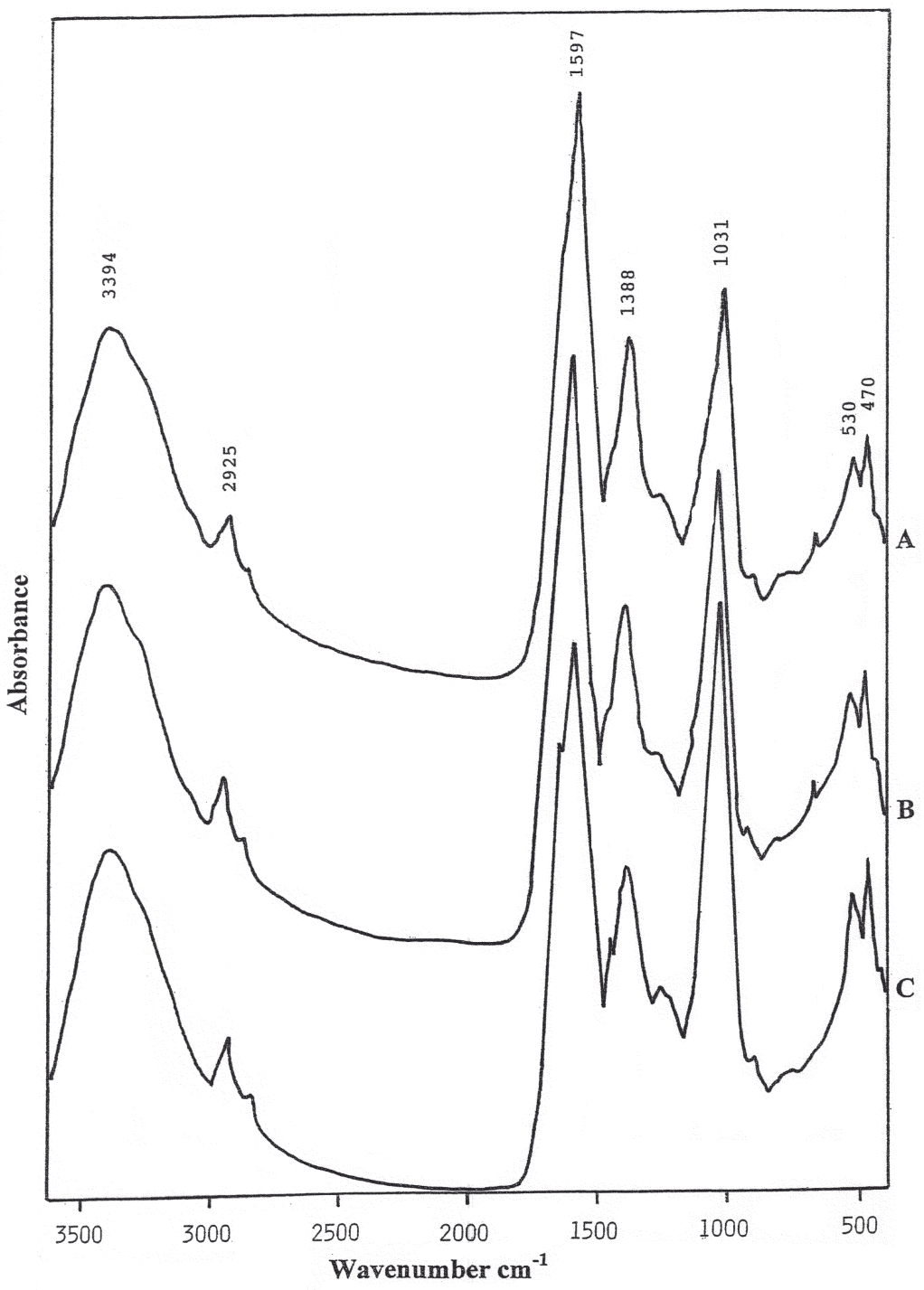
Spectroscopic methods represent a useful tool in the elucidation of structural composition of SOM (Chen et al. 2002, Simpson et al. 2002, Schulten and Leinweber 2000). In our analyses we used Fourier transform infrared spectroscopy (FTIR) for it requires a low quantity of samples (~ 2 mg), and this corresponded well with small amounts of HA recovered from the individuals samples after microbial processing. Fig. 1 indicates different molecular structures as present in the original HA from $Soil_{Con}$ (Fig. 1, A) and their alteration after 21 days aerobic or 12 months semi-aerobic processing in a full-strength nutrient solution (Fig. 1, B, C). By calling to specific references quoted earlier (Filip et al. 1999) the individual absorption bands could be assigned in a following way: At 3394-3363 cm^{-1} a broad band of the O–H (N–H) groups dominates; somewhat weak bands of $–CH_3$ and $–CH_2$ groups appear at 2925 cm^{-1} and 2854 cm^{-1}. At 1720 cm^{-1} a C=O stretch of carboxyles, aldehydes and/or ketones weakly absorbs. At 1653 cm^{-1} an amide I band is dominating, while an amide II band can be detected at 1558 cm^{-1}. An aromatic ring stretch (C=C) absorbs at 1419 cm^{-1}. A strong absorption band at 1031 cm^{-1} could be attributed to C–O stretching vibration in polysaccharides but at least in part also to Si–O groups of mineral moieties of the soil related HA preparations. Similarly, silicate-metal bondings such as Si–O–Me/Al/Mg or Me–OH, and in part C–O–C bondings and deformations could be made responsible for sharp absorption bands at 530 cm^{-1} and 468 cm^{-1} (Fig. 1, A). It should be admitted that the FTIR spectrum of the original HA preparation from $Soil_{Fert}$ demonstrated features very similar to those shown in Fig. 1,A, and therefore it is not presented in a figure here.

The microbial processing of HA incubated under aerobic or semi-aerobic conditions resulted in quite similar structural changes. In the respective FTIR spectra (Fig. 1, B,C) they are indicated by increases in absorption characteristics of aliphatic and nitrogen containing molecular groups such as N–H (3356 cm^{-1}), methyl and methoxyl (2927 cm^{-1}), amide I (1654 cm^{-1}), carboxyl (1394 cm^{-1}), and C–O groups in esters, ethers or phenols or C–N in amide (1240 cm^{-1}).

The FTIR spectra of HA preparations from $Soil_{Con}$



1: *FT-IR spectra of humic acids (HA): A = original HA from SOIL$_{Con}$; B = same HA re-isolated from the full--strength (FB) microbial cultures after 21 days aerobic incubation; C = same HA re-isolated from the full--strength (FB) microbial cultures after 12 months semi-aerobic incubation.*



2: *FT-IR spectra of humic acids (HA): A = HA from $SOIL_{Con}$ re-isolated after 21 days aerobic incubation from microbial cultures deficient in carbon; B = same HA re-isolated after 12 months semi-aerobic incubation from microbial cultures deficient in carbon; C = HA from $SOIL_{Fert}$ re-isolated after 12 months semi-aerobic incubation from microbial cultures deficient in carbon.*

and $Soil_{Fert}$ re-isolated from microbial cultures deficient in carbon (Fig. 2) were clearly dominated by an absorption band at 1597 cm^{-1} that could be attributed to a –C=O stretch in quinons. Some aromatic structures could be responsible also for the IR absorption at 1388 cm^{-1} (OH-deformations and/or C–O stretch in phenolics). The IR absorption at 530–468 cm^{-1}, which is due to mineral and metal moieties in HA, was diminished, and simultaneously, ash content (as high as ~ 40% in original HA preparations) was reduced to 18–25% in the re-isolated HA. This feature apparently indicates splitting of organic and mineral components of the HA preparations to occur during the microbial HA processing. Almost similar changes in the FTIR absorption as discussed in the above paragraphs were observed also in HA preparations that served as sole sources of nitrogen for soil microorganisms (not shown in a figure).

CONCLUSIONS

The HA preparations extracted from non-fertilized or fertilized + manured plots of a long-term field trial established on Orthic Luvisol soil sites undoubtedly served as supplemental sources of nutrients for soil microorganisms. In addition, under both aerobic and semi-aerobic conditions, HA could also serve as only sources of C or N if more easily utilizable nutrient sources are lacking. Thus, these HA should not account for a passive fraction of soil organic matter capable of resistance to microbial utilization and degradation.

SOUHRN

Mikrobiální využití huminových kyselin extrahovaných z půd dlouhodobého polního pokusu

Huminové kyseliny (HA) extrahované alkalickým činidlem z půdy kontrolní a hnojené (NPK + org. hnojivo) parcely dlouhodobého polního pokusu, založeného před 45 lety na půdním typu orthic luvisol, byly vystaveny aktivitám půdních mikroorganizmů s cílem zjistit jejich využitelnost jako živin a také jejich případné strukturální změny. Inkubace tekutých médií zaočkovaných směsnými kulturami mikroorganizmů z pokusných parcel probíhala 21 dnů na kruhové třepačce (aerobní podmínky) nebo 12 měsíců staticky (semi-aerobní podmínky) v laboratoři. V médiu dostatečně zásobeném živinami se přídavek HA projevil výrazným zvýšením tvorby mikrobní biomasy, která byla naopak velmi nízká v podmínkách, kdy HA byly jediným zdrojem C nebo N. Celkové využití HA naproti tomu např. v semi-aerobních kulturách s HA jako jediným zdrojem C, vzrostlo až na 47,3 % jejich původního množství. Mezi využitelností HA z půdy kontrolní nebo hnojené nebyly zjištěny podstatné rozdíly. Účinkem mikrobních aktivit docházelo ke změnám v chemickém a strukturálním složení HA. Spektroskopická analýza (FT-IR) prokázala přednostní využívání alifatických strukturních složek HA mikroorganizmy a naopak zvýšený podíl aromatických struktur v těch preparátech, které byly reizolovány po ukončení inkubace. Souhrnně lze konstatovat, že jak v aerobních, tak i v semi-aerobních podmínkách vykázaly HA z půdy dlouhodobého polního pokusu vcelku malou odolnost vůči mikrobnímu využití a s ním souvisejícími chemickými a strukturálními přeměnami. Neměly by proto být považovány za stabilní složku půdní organické hmoty.

půdní organická hmota, huminové kyselina, mikrobní využití, mikrobní přeměny

ACKNOWLEDGEMENT

Laboratory experiments reported here were accomplished in the Federal Environmental Agency (Umweltbundesamt) Branch at Langen, Germany. The authors thank Mr. W. Pecher for the skill technical assistance. This paper was completed at the Dept. of Soil Science and Microbiology,
Mendel University Brno, Czech Rep. The authors wish to thank Prof. Marta Tesařová for valuable discussions. One of us (Z.F.) thanks the Johann-Gottfried-Herder Foundation, Bonn for granting a Visiting Professorship.

REFERENCES

ALBERT, E.: Wirkung einer langjährlich differenzierten mineralisch-organischen Düngung auf Ertragleistung, Humusgehalte und N-Bilanz. In MERBACH, W. und KÖRSCHENS, M. (Hrsg.) Dauerdüngungsversuche als Grundlage für natürliche Landnutzung und Quantifizierung von Stoffkreisläufen. UFZ-Umweltforschungszentrum Leipzig-Halle: UFZ-Bericht 24, 1999, 59-62.

BAUMECKER, M., ELLMER, F. und CHMIELENSKI, F.-K.: Langfristige Entwicklung der Humusgehalte eines Sandbodens unter dem Einfluss von Düngung und Klima. In Abstracts of the Int. Symp. Nutritional and Environmental Res. in 21st Century – The Value of Long-Term field Experiments. Bad Lauchstädt and Halle, 2002, 24.

BEYER, L.: The chemical composition of soil organic matter in classical humic compound fractions and in bulk samples – a review. Z. Pflanzenernähr. Bodenk., 1996, 159: 527-539.

BEYER, L., SIELONG, R. and PINGPANK, K.: The impact of a low humus level in arable soils on microbial properties, soil organic matter quality and crop yield. Biol. Fertil. Soils, 1999, 28: 156-161.

BRADY, N. C. and WEIL, R. R.: The nature and properties of soils. Upper Saddle River, N. J.: Prentice Hall, 2002.

CHEN, J., GU, E. J., LE BOEUF, H. P. and DAI, S.: Spectroscopic characterization of the structural and functional properties of natural organic matter fractions. Chemosphere, 2002, 48: 59-68.

DEMKINA, T. S. and ZOLOTAREVA, B. N.: Determination of the rate of mineralization of humic substances in soil. Eurasian Soil Sci., 1997, 30: 1085-1089.

FILIP, Z., CLAUS, H. and DIPPEL, G.: Abbau von Huminstoffen durch Bodenmikroorganismen – eine Übersicht. Z. Pflanzenernähr. Bodenk., 1998, 161: 605-612.

FILIP, Z. and KUBAT, J.: Microbial utilization and transformation of humic substances extracted from soils of long-term field experiments. Eur. J. Soil Biol., 2001, 37: 167-174.

FILIP, Z. and KUBAT, J.: Aerobic short-term microbial utilization and degradation of humic acids extracted from soils of long-term field experiments. Eur. J. Soil Biol., 2003, 39: 175-182.

FILIP, Z., PECHER, W. and BERTHELIN, J.: Microbial utilization and transformation of humic acids extracted from different soils. J. Plant Nutr. Soil Sci., 1999, 162: 215-222.

GRAMSS, G., DIEGENHAGEN, D. and SORGE, S.: Degradation of soil humic extracts by wood- and soil-associated fungi, bacteria and commercial enzymes. Microb. Ecol., 1999, 37: 140-151.

HAIDER, K.: Von der toten organischen Substanz zum Humus. Z. Pflanzenernähr. Bodenk., 1999, 162: 363-371.

HAIDER, K., MARTIN, J. P. and FILIP, Z.: Humus biochemistry. In PAUL, E. A. and McLAREN, A. D. (eds.) Soil Biochemistry. New York: Marcel Dekker Inc., 1975, vol 4, 195-244.

HERTKORN, N., CLAUS, H., SCHMITT-KOPPLIN, Ph., PERDUE, E. M. and FILIP, Z.: Utilization and transformation of aquatic humic substances by autochthonous microorganisms. Environ. Sci. Technol., 2002, 36: 4334-4345.

KONONOVA, M. M.: Humus of the main soil types and soil fertility. Pontificus Acad. Sci. Scripta Varia, 1968, 32: 361-379.

RAMUNNI, A., SCIALDONE, R. and FELLECA, D.: Decomposition of humic acids by incubation in a soil water extract under various conditions of oxygen availability. Plant and Soil, 1987, 97: 15-23

SCHELLBERG, J., KÖRSCHENS, M. and HÜGING, A.: Einfluss unterschiedlicher Organischer und mineralischer Düngung auf den C- und N-Gehalt des Bodens in Dauerdüngungsversuch Dokopshof. In MERBACH, W. und KÖRSCHENS, M. (Hrsg.) Dauerdüngungsversuche als Grundlage für nachhaltige Landnutzung und Quantifizierung von Stoffkreisläufen. UFZ--Umweltforschungszentrum Leipzig-Halle: UFZ--Bericht 24, 1999: 45-50.

SCHULTEN, H. R. and LEINWEBER, P.: New insights into organic-mineral particles: Composition, properties and models of molecular structure. Biol. Fertil. Soils, 2000, 30: 399-432.

SIMPSON, A. J., KINGERY, W. L., HAYES, M. H. B., SPRAUL, M., HUMPFER, E., DVORTSAK, R., KERSEBAUM, R., GODEJOHANN, M. and HOFMANN, M.: Molecular structures and associations of humic substances in the terrestrial environment. Naturwiss., 2002, 89: 84-88.

Address

Prof. Dr. Ing. Zdeněk K. Filip, Dr.Sc., Dr.h.c., Hegstrauch 7, 35463 Fernwald, Bundesrepublik Deutschland,
Ing. Jaromír Kubát, CSc., Výzkumný ústav rostlinné výroby, Drnovská 507, 161 00 Praha 6, Česká republika