Waste Management 99 (2019) 1-11

Contents lists available at ScienceDirect

Waste Management

journal homepage: www.elsevier.com/locate/wasman

Characterization of humic acids in a continuous-feeding vermicomposting system with horse manure



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ARTICLE INFO

Article history: Received 6 June 2019 Revised 16 August 2019 Accepted 19 August 2019

Keywords: Humic acids Chemical and spectroscopic techniques Vermicomposting Continuous-feeding system Horse manure

ABSTRACT

The increasing numbers of kept horses create problems with processing horse manure as important local waste. This work was focused on horse manure vermicomposting in a real-field continuous-feeding system under controlled conditions, and on the complex study of the maturity and stability of the produced vermicompost. Commonly used simple indicators such as the C/N ratio, N-NH⁴/N-NO₃ ratio, DOC or ion exchange capacity, and also more advanced spectroscopic and thermoanalytic techniques were used and applied on the humic substances isolated from the vermicompost during its maturation (12 months in total). When compared with the original horse manure, vermicomposting decreased the aliphatic, protein-like, and polysaccharide humic components, whereas vermicomposting increased the aromatic-ity and contents of oxygen-containing functional groups. The typical tryptophan-like fluorophores in the manure, corresponding to the freshly produced organic matter of biological or microbial origin, were progressively transformed to humic-like fluorophores during vermicomposting. The most thermally labile humic fraction disappeared quickly during the very early vermicomposting stages. The results of spectroscopic and thermogravimetric analyses suggest that stable and mature vermicompost was produced after 6–9 months of vermicomposting, which was also supported by biologically-based maturity indicators.

1. Introduction

The global horse population is about 58 million (FAOSTAT, 2018). The increasing numbers of horses kept within or near urban areas creates environmental problems, as there is a lack of arable land for spreading manure and insufficient methods or capacity for manure storage (Hadin et al., 2016). Manure is typically applied to soils as fertilizer for agricultural production due to its favorable physico-chemical properties. However, manure is also considered as an environmental pollutant (He et al., 2016). Leaching or runoff of nitrogen and phosphorus impair both ground - and surface waters (Hooda et al., 2000). Manure can also increase global climate change via emissions of methane and nitrous oxide (Leytem et al., 2011).

Manure has little stability (Moral et al., 2005), but it is very suitable for composting or vermicomposting. Garg et al. (2005) studied the effect of various animal wastes (manures) on the growth and reproduction of an epigeic earthworm *Eisenia foetida* under identical laboratory conditions. The net biomass gain/earthworm in dif-

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ferent animal manures was in the order of: sheep > donkey > buffalo > goat \approx cow \approx horse > camel. The number of cocoons produced per earthworm per day in different wastes was in the order: sheep > cow \approx horse \approx goat > camel > donkey > buffalo. The increase in the number of earthworms was 39.5-fold in horse manure and 26-fold in cow manure. Stability can be distinguished as temporary (caused by, for example, lack of water in the raw material) or permanent (creation of stable humic substances). The amount and especially the quality of organic substances in products of biomass recovery are considered as important indicators of their biological maturity and chemical stability (Peev et al., 2017). The humified fraction of organic matter is resistant to microbial degradation, and thus is important for maintaining long-term soil fertility (Bernal et al., 2009).

Immature and slightly stabilized "compost or vermicompost" can cause many problems when stored and used. Oxygen-free spaces are formed, which in turn spontaneously smell and produce substances toxic to plants. Continued decomposition of these unstable materials after their application to the soil has a negative influence on plant growth due to decreasing oxygen and/or available nitrogen with associated phytotoxic effects (Jeong et al., 2017). They also release nutrients more quickly and can promote the re-development of pathogenic microorganisms. Immature



composts can contain high levels of ammoniacal nitrogen and ammonia, organic acids, or slightly soluble compounds that can reduce seed germination and root plant growth. On the other hand, a stable and mature compost or vermicompost exhibits complete organic matter decomposition, and has significant sorption and ion exchange properties, and releases nutrients gradually, and thus optimizes the soil solution composition. In addition, mature vermicompost is not an odor problem without access to the air and during long-term storage (Wichuk and McCartney, 2010).

The stability can be precisely assessed using instrumental techniques such as elemental analysis (CHNS/O), ultraviolet-visible spectroscopy (UV/Vis), Fourier-transform infrared spectroscopy (FTIR), and fluorescence spectroscopy or thermogravimetric analysis (TGA). Elemental analysis is a basic chemical characteristic of humic acids that quantitatively determines the contents of C. H. N. O. S in their molecules. The content and ratio of each element reveals the specialties of humus substances (Droussi et al., 2009). The UV/vis technique is used to determine the maturity of compost by measuring the rate of humification in wavelength ranges from about 200 to 800 nm or greater (Gieguzynska et al., 1998). The FTIR spectroscopy is a qualitative technique for differentiating functional groups of organic substances, and is usually utilized in the investigation of soil organic matter, organic wastes, and their compost (Amir et al., 2010). Fluorescence Excitation-Emission Matrix (EEM) spectroscopy is among the most promising tools for characterising heterogeneous organic matter by providing comprehensive information on the composition, properties, and behaviour of the samples (Coble et al., 2014; Ohno and He, 2011). Given its sensitivity, selectivity, and non-destructive nature, EEM is widely used to differentiate the changes and transformations of OM in natural environments (Albrecht et al., 2015; Wu et al., 2009). TGA is an easy method which aids in illustrating the end product by providing chemical characteristics of samples (Wu et al., 2011).

Humification of organic matter during composting is manifested by the formation of humic substances which consist of a humic and fulvic acid fraction (Inbar et al., 1990). Several different structural models of humic acids (HAs) have been suggested (Stevenson, 1994; Al-Faiyz, 2017). HAs are synthesized as the most important by-product during composting, because they are widely utilized for the sorption of nutrients and remediation of environmental pollution (Yuan et al., 2017).

The aim of the study was to evaluate the process of vermicomposting of horse manure in a continuous-feeding system with a focus on the quality of HAs produced. Based on the results, the optimal time of vermicomposting and the use of the vermicompost can be predicted. The novelty of the work consists in more accurately determining the stability of vermicompost from horse manure using advanced spectroscopic techniques, unlike commonly used simple indicators such as the C/N ratio, N-NH₄/N-NO₃⁻ ratio, or cation exchange capacity. Thus far, experimental data on humic acids isolated from vermicompost of horse manure are missing and this is the first study. Diffuse Reflectance Infrared Fourier Transform (DRIFT) technique was used for characterization of humic acids, although this method is routine. Infrared spectra obtained by means of a DRIFT were chosen as it required no mechanical treatment of the sample (transmission technique, e.g. tablet pressing) and therefore less opportunity for analyst-induced variation between samples can be presented.

2. Material and methods

2.1. Feedstock and vermicomposting set-up

The experiment was set up at a family vermicomposting company in Kyjov, Czech Republic (N 49°1.61737', E 17°7.32838').



Fig. 1. Diagram of the continuous-feeding vermicomposting system with horse manure at a family vermicomposting company in Kyjov, Czech Republic.

The concrete container for vermicomposting occupied a ground plan 4×1.5 m, and was placed in a stone room to avoid the influence of weather conditions, especially precipitation (Fig. 1). The temperature of the vermicomposted material partially copied the outside temperature of the temperate climate. However, the high temperatures in the summer and the low in the winter were moderated by being placed in a container in the room even though it was without air conditioning. The bedding layer consisted of mature horse manure with earthworms (*Eisenia andrei*), with a density of about 50 earthworms per liter (10 g of earthworms per kilogram), and was placed first. Subsequent 5 cm layers of mature horse manure were added every week to avoid high temperature above 35 °C that is lethal to earthworms. Selected physico-chemical properties of the feedstock used are shown in Table 1.

Besides the raw horse manure samples, other samples were taken up from the cross profiles of different depths. Depth placement and the average age of each layer were as follows:

V: 0–13 cm, 5 weeks IV: 13–26 cm, 3 months III: 26–39 cm, 6 months II: 39–52 cm, 9 months I: 52–65 cm, 12 months

Potential earthworms were separated, counted, and weighted. The resulting vermicompost sample without earthworms was divided into 3 parts and treated as required for laboratory analyses. One part of the vermicompost sample was stored at 4 °C until the pH and electrical conductivity (EC) could be determined. The second part was dried at 30 °C to a constant weight and ground. This was then used for analyses of the total and available contents of elements and the ion exchange capacity (IEC). The third part of the vermicompost sample was frozen at -20 °C and then lyophilized for subsequent determination of the groups of microorganisms by the phospholipid fatty acid (PLFA) method.

2.2. Chemical and biological analyses

Measurements of active pH and EC (1:5 w/v) were conducted with use of a WTW pH 340 i and Testo 240, respectively according to EN 13037. The CHNS Vario MACRO cube analyzer (Elementar Analysensysteme GmbH, Germany) was used for determination of total carbon (C_{tot}) and nitrogen (N_{tot}). The total contents of P, K, and Mg were determined by decomposition utilizing a wet method in a closed system with microwave heating using an Ethos 1 microwave system (MLS GmbH, Germany). The contents of

Table 1Selected physico-chemical properties of the horse manure used in the experiment.

	Dry matter [%]	Volatile solids	pH/H ₂ O	EC [μ S cm ⁻¹]	C/N	P_{tot} . [mg kg ⁻¹]	K _{tot} . [mg kg ⁻¹]	Mg _{tot} . [mg kg ⁻¹]
Horse manure	26.7 ± 2.0	80.9 ± 1.3	7.9 ± 0.1	1039 ± 202	33.1 ± 1.3	5200 ± 400	20800 ± 1200	4400 ± 300

Values are means \pm SD (n = 3).

ammonium nitrogen (N-NH⁴₄), dissolved organic carbon (DOC), and the available portions of P, K, and Mg were determined in calcium chloride/DTPA (CAT) solution (0.01 mol l⁻¹ CaCl₂ and 0.002 mol l⁻¹ diethylene triamine pentaacetic acid (DTPA)) at a ratio of 1:10 (w/ v) according to the International BSI Standard EN 13651. The NH⁴₄-N and NO³₃-N contents in the extracts were measured colorimetrically using a SKALAR SANPLUS SYSTEM[®]. The element concentrations were determined using inductively coupled plasma optical emission spectrometry (ICP-OES, VARIAN VistaPro, Varian, Australia) with axial plasma configuration. The ion-exchange capacity (IEC) was determined conductometrically (Váchalová et al., 2014). Samples for the PLFA analysis were extracted in triplicate using a mixture of chloroform, methanol, and phosphate buffer (1:2:0.8; v/v/v). Detailed description of the method is shown in Šnajdr et al., (2011).

2.3. Extraction of humic acids

Humic acids were extracted from horse manure before and after the vermicomposting process using a modified procedure recommended by the International Humic Substances Society (Swift, 1996). Briefly, air-dried samples were demineralized with 0.1 M HCl at a ratio of 1:10 (sample/solution), and the suspensions were then washed with distilled water until an almost neutral pH was achieved. All samples were shaken overnight with 0.1 M NaOH and 0.1 M pyrophosphate solution under N₂ using a sample/extractant ratio of 1:10. After centrifugation at $2700 \times g$ for 30 min, humic acids were precipitated from the supernatant by means of acidification to pH < 2 with 6 M HCl, and further separated by centrifugation at $2700 \times g$ for 20 min. Precipitated humic acids were treated (twice) with a mixture of 0.5 vol% HCl-HF over a period of 24 h, and subsequently separated in the centrifuge. Humic acids were purified and dialysed using a Spectrapore membrane (MWCO 1000) until free Cl^- and F^- ions were washed out and finally freeze-dried. The samples were left to equilibrate in the ambient laboratory atmosphere at about 25 °C, which resulted in the final equilibrium moisture. Strictly speaking, that which is isolated from vermicompost and/or compost is not a true humic acid, such as HAs extracted from coal, soil, and peat (Stevenson, 1994). In this work we will use this designation for our isolated samples, which were obtained by the standard extraction method. The designation of extracted humic acids is as follows: humic acid isolated from raw horse manure - HARHM; humic acid isolated after 5 weeks of the vermicomposting process - HA5W; humic acids isolated after 3, 6, 9, and 12 months of the vermicomposting process -HA3, HA6, HA9, and HA12.

2.4. Humic acids analysis

2.4.1. Elemental analysis

The elemental composition of all humic acids was determined using an EA 3000 CHNS/O analyser (Euro Vector, Pavia, Italy). Samples (1–1.5 mg) were packed in tin capsules and placed in an oven for combustion at 980 °C using pure oxygen as the combustion gas and pure helium as the carrier gas. Calibration curves for carbon (C), hydrogen (H), nitrogen (N), and sulphur (S) were obtained using sulphanilamide as a reference standard sample. The percent oxygen content was calculated by the difference, and the data obtained were corrected for moisture and ash content. All samples were incinerated at a heating rate of 10 °C/min from room temperature to 900 °C under air atmosphere. The weight loss occurring at 105 °C was measured and taken as the moisture content. The final residue, after heating to 900 °C, was measured and taken as the ash content.

2.4.2. UV/Vis spectrometry

UV/Vis spectra were obtained on a Hitachi U-3900H UV/Vis spectrometer (Hitachi, Tokyo, Japan) by recording the absorption spectra between 200 and 900 nm. Samples were measured in a 10 mm quartz cuvette and with a standard phosphate buffer blank (0.1 M NaH₂PO₄, Na₂HPO₄).

2.4.3. FTIR spectrometry

FTIR spectra were obtained by means of a Diffuse Reflectance Infrared Fourier Transform (DRIFT) technique using a Nicolet *i*S50 spectrometer (Thermo Fisher Scientific, Waltham, USA). Approximately 4 mg of powdered HA samples were homogenized with 400 mg of KBr in an agate mortar, and then transferred to the sample holder cup of the diffuse reflectance accessory. All spectra were recorded over the 4000–400 cm⁻¹ range at a resolution of 4 cm⁻¹ and were the average of 512 scans. The KBr infrared grade spectrum was used as the background for DRIFT measurement.

2.4.4. Fluorescence spectroscopy

For fluorescence analysis, HAs were dissolved at a concentration of 10 mg L⁻¹ of organic carbon in standard phosphate buffer. The pH-value of solutions was adjusted to seven using phosphate buffer. All spectra were obtained on a FluoroLog fluorescence spectrometer (Horiba Scientific, New Jersey, USA) with a scan speed of 600 nm min⁻¹, using excitation and emission slit bandwidths of 5 mm. Total luminescence spectra (TLS) were obtained by scanning the emission and excitation wavelengths over the range 300-600 nm and 240-550 nm, with the emission and excitation increment set at 5 nm. The sample cell with humic acid solution was kept at a temperature of 20 °C during each measurement. The EEM spectrum of ultra-pure water (Mili-Q) was obtained and subtracted from the EEM spectra of all samples examined to decrease the influence of 1st- and 2nd- order Raman scattering. Primary and secondary inner filter effects were corrected. The fluorescence intensity values of samples (in counts per second, CPS) were corrected using the method devised by Lakowicz (Lakowicz, 2006).

2.4.5. Thermogravimetric analysis

Thermal degradation of the isolated HAs was performed using a Q5000 TG analyzer (TA Instruments, New Castel, Delaware, USA) with ± 1 °C temperature accuracy and $\pm 0.1\%$ mass accuracy. Approximately 3–5 mg of powdered HA was introduced into a platinum pan. The sample was heated at a heating rate of 10 °- C min⁻¹ from room temperature to 1000 °C under a flow of nitrogen (20 cm³ min⁻¹).

2.5. Statistical analysis

Statistical analyses were performed using STATISTICA 12 software (StatSoft, Tulsa, USA). Due to the inhomogeneity of the data, a non-parametric statistical analysis of the comparison of several independent groups using the Kruskal-Wallis test was used. Spearman's correlations were explored between the parameters at the 0.05 probability levels.

3. Results and discussion

3.1. Selected physico-chemical properties

As is evident from Table S1 (Supplementary material), the greatest dry matter content was found in the top layer. The increase of humidity with the age of the layers could be explained by vermicomposting in the concrete container without runoff to maintain a closed loop. Dry matter content in the bottom layer statistically differed from the top layer. Volatile solids decreased gradually caused by the loss of CO₂. In the oldest layer, there were 80% and 58% of volatile solids of the vermicomposted material in the top layer and of the original horse manure, respectively. The pH values ranged from 7.75 to 8.74 which corresponds to Hanc et al. (2019) who vermicomposted distillery residues together with straw. EC initially increased over time and later decreased. The highest value was found in the 3rd layer. Some authors dealing with vermicomposting reported an increase trend of EC during the process due to degradation of the organic matter and the release of the mineral salts (Hanc and Chadimova, 2014). Nonetheless, the other studies have shown a decreasing trend for EC (Domínguez et al., 2018). The nitrogen content gradually increased with the age. This was due to the loss of organic matter causing a relative increase in the content of elements. Value of the C/N in the top layer (19.3) was significantly lower compared to the feedstock (33.1) due to a rapid transformation of organic matter through the earthworms. The decrease in the C/N ratio towards the lower layers is in agreement with the study of Hanc et al. (2017) who vermicomposted household biowaste under large-scale vermicomposting conditions. The decrease of C/N during one year ranged from 16.1 to 10.4. The C/N ratio of vermicompost mainly depends upon rate of C and N mineralization in waste stuff during the decomposition process (Negi and Suthar, 2018).

Table S2 shows parameters of maturity such as N-NH₄⁴, N-NH₄⁴/ N-NO₃⁻, DOC, IEC and IEC/C_{tot}. With increasing age and depth, the maturity of vermicompost should increase. This was confirmed for all maturity parameters, although the differences were nonsignificant between the layers due to the use of strict nonparametric statistical test. The most marked decrease by 60% in N-NH₄⁺ was observed between layers V and IV. The differences between the other layers were lower. The proportion of the most active fraction of carbon expressed as DOC in the C_{tot} fluctuated between 2.6 and 2.9%. A very strong correlation was found between the two parameters (R = 0.98, p < 0.05). Similarly, DOC positively correlated with C/N ratio (R = 0.87, p < 0.05). The processes of mineralization and humification during vermicomposting may result in changes in ion-exchange properties, which can be expressed and quantified by IEC. The IEC increased from 22 mmol₊ 100 g⁻¹ (layer V) to 28 mmol₊ 100 g⁻¹ (layer II and I). It was lower than in mentioned experiments of Hanc et al. (2017, 2019), where the values ranged from 52 to 60 mmol₊ 100 g⁻¹ and 55 to 71 mmol₊ 100 g⁻¹, respectively. The IEC/C_{tot} ratio that takes into account the mineralization of organic matter increased to 1.4 in the oldest layer but it was lower than in vermicompost from household biowaste (3.3) and distillery residues (2.0) found in studies of Hanc et al. (2017, 2019). Changes in total and available phosphorus, potassium, and magnesium contents in layers are illustrated in Fig. 1S.

3.2. Earthworms and microorganisms

Most earthworms were in the youngest layer (Table S3) which corresponds to Castkova and Hanc (2019). The number of earthworms in this layer accounted for 73% of the total number. There were only 3 earthworms on average in the oldest layer of vermicompost, which made up 0.14% of the total number. It can be explained by a lack of food substrate for earthworms, probably the accumulation of some transformed compounds which might be toxic to the earthworms, as well as the lack of aeration. Similarly, the highest earthworm biomass proportion was found in top layer V (73%), followed by layer IV (25%), layer III (0.10%), layer II (0.5) and layer I (0.03%). Due to the lack of suitable food for earthworms, the average weight of earthworms in the bottom layers was lower by 30% compared to upper layers.

The total microbial biomass decreased usually directly proportional with the ages of the layers (Fig. 2S). The difference between the youngest layer (156 µg PLFA g^{-1} dw) and the oldest layer (72 µg PLFA g^{-1} dw) was 45%. With the increase in manure conversion into vermicompost and hence with a decrease in heterogeneity, the standard deviation among the taken samples also decreased. Microbial biomass positively correlated with number and biomass of earthworms (R = 0.85, p < 0.05 for both parameters) which demonstrates that vermicomposting is the cooperation of earthworms and microorganisms. The lowest bacterial/fungal ratio (11) was found in the youngest layer and gradually increased to 28 in layer II.

3.3. Yields, ash contents and elemental analysis

The extraction yields, elemental composition, ash contents, and atomic ratios of HAs isolated from raw and vermicomposted horse manures are summarized in Table 2.

Table	2
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Extraction yields, ash contents, elemental compositions, and atomic ratios of HAs.

Origin of HA	Yield (g kg ⁻¹) ^a	Ash (wt. %) ^a	C (at. %) ^b	Н	Ν	0	H/C	O/C	C/N
HARHM1 ^c	13.1 ± 1.8	1.4 ± 0.1	36.69 ± 0.15	46.39 ± 0.16	2.67 ± 0.10	14.25	1.26	0.39	13.72
HA5W ^d	25.7 ± 3.3	1.7 ± 0.3	38.16 ± 0.22	43.36 ± 0.10	2.14 ± 0.06	16.34	1.14	0.43	17.82
HA3 ^e	22.2 ± 1.4	1.7 ± 0.4	38.29 ± 0.16	43.01 ± 0.08	2.22 ± 0.05	16.88	1.12	0.44	17.28
HA6 ^f	26.6 ± 0.9	1.4 ± 0.1	38.44 ± 0.45	42.97 ± 0.15	2.17 ± 0.02	17.15	1.12	0.45	17.70
HA9 ^g	26.2 ± 0.9	1.6 ± 0.4	38.44 ± 0.65	42.67 ± 0.16	2.15 ± 0.04	17.18	1.11	0.45	17.90
HA12 ^h	28.7 ± 2.9	1.6 ± 0.9	38.42 ± 0.47	42.36 ± 0.07	2.29 ± 0.08	17.94	1.10	0.47	16.76

Values of extraction yield, ash content and elemental compositions are means ± SD (n = 3).

^a On moisture-free basis.

^b On moisture-free and ash-free basis.

^c HA isolated from raw horse manure.

^d HA isolated from vermicompost of average age 5 weeks.

^e HA isolated from vermicompost of average age 3 months.

^f HA isolated from vermicompost of average age 6 months.

^g HA isolated from vermicompost of average age 9 months.

^h HA isolated from vermicompost of average age 12 months.

The yield of HAs ranged from 13.1% to 28.7% depending on the time of the vermicomposting process. The greatest yield (28.7%) was obtained for HA isolated from vermicompost which was composted for 12 months. In contrast, the least yield (13.1%) was determined for HA extracted from raw horse manure. In addition, yields depend on the procedures selected for isolating and purifying the HAs (Doskočil et al., 2018). Similarly, the ash content of HAs can depend both on the origin of the source material as well as on the sample purification procedure. The ash content of HAs isolated from raw horse manure and our sample set of vermicomposts was similar. Slightly greater ash contents were observed for HAs originating from vermicomposts of average ages 5 weeks and 3 months.

The elemental compositions are shown in Table 2 in atomic percent (at. %), because, in contrast to weight percent (wt. %), it better reflects the content of hydrogen in the structures of humic substances. The carbon content increased slightly during the vermicomposting process, while the hydrogen content decreased. The nitrogen level decreased in the first stages of vermicomposting and then very slightly increased at the end of this process. A greater increase in oxygen content was observed for the HAs extracted from vermicomposts compared with HAs isolated from raw horse manure. The elemental composition of HAs from our sample set was similar to that of HAs extracted from different composts and/or vermicomposts from other studies (Enev et al., 2018; Li et al., 2011).

The H/C atomic ratio is considered as an indicator of the degree of aromaticity, and, for our samples, lay within a range (i.e. from 1.10 to 1.26). The aromaticity of HAs isolated from raw horse manure was the least of all the samples, whereas it increased during the vermicomposting process. The H/C level decreased with the vermicomposting process, which could be attributed to an increase of the aromatic and/or a decrease of the aliphatic structures. In the studied HAs, the H/C atomic ratio is similar to values published in previous studies (Senesi, 1989; Zhang et al., 2015). The O/C atomic ratio reflects the amount of oxygen-containing functional groups (e.g. carboxylic and phenolic) in humic substances. The O/C atomic ratio significantly increased during the composting process. These results suggest that during vermicomposting, a progressive transformation of the polysaccharides and polysaccharide-like compounds to other oxygenated compounds such as aromatic carboxylic acids and phenols occurs. The C/N atomic ratio reflects the amount of nitrogen in humic substances. The C/N ratio values were in the range from 13.72 to 17.90. The C/N ratio obtained in this study for the HA isolated from raw horse manure was relatively small, indicating that the organic matter of raw material contained a large amount of nitrogenous substances. The C/N level significantly increased at the first stage of the vermicomposting

Table 3

UV/Vis and FTIR calculated ratios.

process, which could be attributed to a decrease in the amounts of nitrogen-containing compounds, such as protein and proteinlike substances.

3.4. UV/Vis spectroscopy

The UV/Vis absorbance spectra of HAs isolated from all studied samples were typically featureless and characterized by the absence of identifiable maxima and/or minima with decreasing absorbance at increasing acquisition wavelength (not shown).

In our study, HAs absorbed light significantly at wavelengths ranging between 200 and 700 nm. Absorption in this UV/Vis region indicates that the vast majority of the chromophores include aromatic rings with various degrees and substitution types, such as monosubstituted and polysubstituted phenolic compounds and various monoaromatic and/or polyaromatic acids (Korshin et al., 1997). A number of studies have shown that the analysis of UV/ Vis spectra by means of absorption ratios may provide valuable information on the chemical structure and properties of HAs, that may also complement the information obtained using other analytical methods, such as potentiometric titration, size exclusion chromatography, NMR etc. (Asakawa et al., 2011; Ephraim et al., 1995; Novák et al., 2015) The E_{ET}/E_{Bz} ratio (the ratio of absorbance at 253 nm to that at 220 nm) is small for compounds in which the aromatic rings are largely substituted with aliphatic functional groups, whereas greater E_{ET}/E_{Bz} ratios are associated with the presence of oxygen-containing functional groups e.g. hydroxyl, carbonyl, ester and especially carboxyl groups on the aromatic ring (Korshin et al., 1997; Enev et al., 2018). In our study, the E_{ET}/E_{Bz} ratio values ranged from 0.50 to 0.54 (Table 3). These results suggest that the substitution of aromatic rings with polar functional groups was comparable for the vermicompost HAs. The E_{ET}/E_{Bz} ratio for HA isolated from raw horse manure was less than that for any vermicompost HAs. The frequently used E_4/E_6 ratio (the ratio of absorbance at 465 nm to that at 665 nm) reflects the degree of aromaticity and the molecular weight of humic substances of different origins (Li et al., 2011). For the HAs studied, this ratio demonstrated less sensitivity (Table 3), because absorption in the 500-800 nm range was approximately zero. For this reason, it is appropriate to utilize the E_2/E_4 ratio (the ratio of absorbance at 265 nm to that at 465 nm), as it is consistent with the E_4/E_6 . In our study, the E_2/E_4 ratio values ranged from 9.31 to 11.11 (Table 3). The E_2/E_4 ratio for HAs isolated from raw horse manure was much less than those for vermicomposted HAs. The E₂/E₄ values followed an upward waving trend with increased composting time. It is apparent from the data in Table 3 that non-humic substances (e.g. lignin, polysaccharides, and protein-like substances)

Origin of HA	UV/Vis ratios		FTIR intensity ra	itios	
	E_{ET}/E_{Bz}	E_4/E_6	E_2/E_4	$I_{\rm Ar}/I_{\rm Al}^{\rm a}$	$I_{\rm Ar}/I_{\rm COOH}^{\rm b}$
HARHM ^c	0.50 ± 0.00	7.77 ± 0.03	9.31 ± 0.14	1.10	1.08
HA5W ^d	0.52 ± 0.00	8.56 ± 0.13	9.62 ± 0.05	1.14	1.02
HA3 ^e	0.53 ± 0.00	8.66 ± 0.05	10.11 ± 0.31	1.14	1.01
HA6 ^f	0.52 ± 0.01	8.40 ± 0.38	9.87 ± 0.18	1.28	0.99
HA9 ^g	0.52 ± 0.01	8.69 ± 0.17	10.91 ± 0.24	1.30	0.99
HA12 ^h	0.54 ± 0.00	8.53 ± 0.40	11.11 ± 0.10	1.39	0.97

Values of UV/Vis ratios are means \pm SD (n = 3).

^a FTIR ratio calculated as ratio of intensity at 1510 cm⁻¹ to intensity at 2940 cm⁻¹.

^b FTIR ratio calculated as ratio of intensity at 1510 cm⁻¹ to intensity at 1420 cm⁻¹.

^c HA isolated from raw horse manure.

^d HA isolated from vermicompost of average age 5 weeks.

^e HA isolated from vermicompost of average age 3 months.

^f HA isolated from vermicompost of average age 6 months.

HA isolated from vernicompost of average age o months

^g HA isolated from vermicompost of average age 9 months.

^h HA isolated from vermicompost of average age 12 months.

were transformed into humic substances during vermicomposting, thus increasing the humification and stability of the vermicompost. This observation is in good agreement with published studies regarding HAs derived from composts (Li et al., 2017).

3.5. FTIR spectroscopy

The DRIFT spectra of HAs are shown in Fig. 2, and interpretation of their absorption bands was conducted according to the literature data (Stuart, 2004).

Table S4 shows overview of the main absorption bands which were identified in the FTIR spectra. All the spectra of vermicompost HAs contain a sharp and intensive band at 1710 cm⁻¹ corresponding to carboxylic groups, whereas it is absent in the sample isolated from raw horse manure. The carboxylic groups are also indicated by the broad band centered at about 2640 cm⁻¹ resulting from the O-H stretching vibrations of the hydrogen-bonded COOH which forms dimers. A band at about 1420 cm^{-1} , preferentially ascribed to C-O-H in-plane bending of carboxylic groups, is apparent in all sample sets. The degree of carboxylic content was determined using the I_{Ar}/I_{COOH} ratio (the ratio of the intensity at 1510 cm⁻¹ to the intensity at 1420 cm⁻¹). The content of the carboxylic groups is greatest for the HA isolated from horse manure after 12 months of vermicomposting. Further, the content of COOH groups increases with the composting time. Another significant band occurring at 1600 cm⁻¹ can be assigned to stretching C=C groups in aromatic rings, eventually C=O stretching of H-bonded conjugated ketones and guinones. This band was observed only as a weak shoulder in the spectrum of HA isolated from raw horse manure. Another aromatic band is apparent at 1510 cm⁻¹, due to the aromatic C=C stretching of lignin residues, the relative inten-



Fig. 2. DRIFT spectra of HAs isolated from raw horse manure and vermicomposts of different ages.

sity of which decreases with the vermicomposting process. This is supported by the I_{Ar}/I_{Al} ratio (the ratio of the intensity at 1510 cm^{-1} to the intensity at 2940 cm⁻¹), which was used to estimate the degree of aromaticity. The intensity ratio (Table 3) shows that the aromaticity of HAs increases during the vermicomposting process. These results are in good agreement with the elemental analysis (and H/C ratios) listed in Table 2. Two out-of-plane C-H deformation bands were observed in the 850–760 cm⁻¹ region and were assigned tri-substitution per ring (840 cm⁻¹), and disubstitution per ring (768 cm^{-1}). The first of these bands was observed as a sharp and intensive band in the all spectra, while the second of these bands was found as a weak band and/or shoulder. The broad band centered at about 3310 cm⁻¹ corresponds to the O-H stretching of various functional groups, which are connected with an intermolecular hydrogen-bond. The appearance of aliphatic chains is observed in the $3000-2800 \text{ cm}^{-1}$ range. The presence of aliphatic groups is revealed by the band at 2940 cm⁻¹, which is attributed to asymmetric C–H stretching in methylene groups. A band at about 2850 cm⁻¹, attributed to symmetric C-H stretching of -CH₂- groups, is evident exclusively in HA isolated from raw horse manure. The deformation vibrations of the --CH₂-- and --CH₃ groups occur in the spectra at 1454 cm^{-1} . All the spectra of HAs contain a sharp and intensive band at 1662 cm⁻¹ corresponding to secondary amide groups (amide II). Many authors consider the band at 1660 cm^{-1} to be an indicator of protein-like substances (Calderón et al., 2011; Enev et al., 2018). The main differences among the spectra of HAs appear in the fingerprint regions 1280–1200 cm⁻¹ and 1145–1000 cm⁻¹. The first region is characterized by a weak band and/or shoulder at 1260 cm⁻¹ and a relatively intense band at 1230 cm^{-1} corresponding to the C–O vibration in aryl ethers, and to the C-O stretching and O-H bending of phenols. In comparison with other samples, the HAs isolated from raw horse manure did not contain the vibration at 1260 cm⁻¹. The relative intensity of this band increased with the vermicomposting time. The latter region is characterized by a sharp band at 1130 cm^{-1} and a shoulder and/or weak band at 1090 $\rm cm^{-1}$ corresponding to the C-O-C vibration in various alkyl ethers, and to the C-O stretching of secondary alcohols. In the spectra of all samples, the band at 1090 cm⁻¹ gradually reduces with increasing vermicomposting time and its relative intensity decreases. The band at 1037 cm⁻¹ is dominant in the spectrum of HAs isolated from raw horse manure, mainly due to the large polysaccharide substance content. The other samples contain this band with a lesser intensity. Bands and shoulders at wavenumbers less than 650 cm⁻¹ are attributed to mineral matter such as kaolinite, from which bands at 644 cm^{-1} , 536 cm^{-1} and 500 cm^{-1} are the most obvious.

3.6. Fluorescence spectroscopy

The fluorescence spectra or EEM contour maps of the studied HAs (see Fig. 3) were not corrected for Rayleigh scattering peaks, which appear in the form diagonal bands.

The Rayleigh scattering corresponds to the line in the EEM spectra where excitation wavelength equals emission wavelength. The first observation from the EEM contour maps is that humic substances contain fluorophores which are responsible in the fluorescence of composted and/or vermicomposted organic materials (Benítez et al., 2000; Mobed et al., 1996; Xing et al., 2012). The excitation-emission wavelength pairs of the main peaks in the EEM spectra and their fluorescence intensity values from our sample set are listed in Table 4.

The maxima are located at excitation wavelengths in the ultraviolet region (e.g. UVA, UVB, and UVC region) in the 250–390 nm range and at emission wavelengths in the 340–480 nm range. Several fluorophores have been reported previously in the literature,



Fig. 3. EEM spectra of HAs isolated from raw horse manure and vermicomposts of different ages. Individual fluorophores of the HAs samples are marked with the corresponding symbols (A, T, C1, C2) and arrows.

such as peak A at 240–280/400–500 nm (excitation/emission wavelengths pair), peak C at 300–380/400–500 nm, peak M at 290–310/370–410 nm, peak B at 270–280/300–315 nm, and peak T at 270–280/345–360 nm (Coble 1996; Birdwell and Engel, 2010). Non-humic fluorophores indicative of biological activity and/or protein-like material include tyrosine-like peaks and tryptophan-like peaks (Birdwell and Engel, 2010). From the domains described (Fig. 3) the fluorescence of raw horse manure and vermicomposted materials lies primarily in the peak A region,

which is characteristic of humic substances. However, there are maxima having excitation wavelengths greater than 400 nm (including those for terrestrial HAs). The fluorescence region (400–480/460–550 nm), referred to as V (Alberts and Takács 2004; Rodríguez et al., 2013, Enev et al., 2014), usually occurs in coal and/or soil HAs, and was not determined in the spectra from our samples. The absence of the peak V in the examined samples can be explained by the lesser average age of the vermicomposts, which did not exceed one year. The characteristic C domains (des-

Table 4

Positions of excitation-emission wavelength pairs for fluorescence peaks and values of fluorescence intensities for HAs isolated from raw horse manure and different vermicomposts.

Origin of HA	Fluorescence peak region								
	A		С				Т		
			C1		C2				
	Ex/Em (nm)	I _F (CPS) ^a	Ex/Em (nm)	I _F (CPS) ^a	Ex/Em (nm)	I _F (CPS) ^a	Ex/Em (nm)	I _F (CPS) ^a	
HARHM ^b	250/460	0.70					275/335	0.55	
HA5W ^c	255/480	0.84					275/340	0.40	
HA3 ^d	250/475	1.01					280/335	0.50	
HA6 ^e	250/460	0.94					275/340	0.40	
HA9 ^f	250/445	1.06	335/440	0.61	390/435	0.35			
HA12 ^g	270/450	1.10	330/440	0.68	385/440	0.52			

^a $I_{\rm F} \times 10^6$ CPS (counts per second) of fluorescence peaks.

^b HA isolated from raw horse manure.

^c HA isolated from vermicompost of average age 5 weeks.

^d HA isolated from vermicompost of average age 3 months.

^e HA isolated from vermicompost of average age 6 months.

^f HA isolated from vermicompost of average age 9 months.

^g HA isolated from vermicompost of average age 12 months.

ignated as humic-like fluorophores) for humic substances originating from soils, peats, lakes, and rivers, with fluorescence maxima corresponding to excitation/emission wavelength pairs of 300-380/400-500 nm, were observed in HAs after 9 and 12 months of the vermicomposting process. Because HA9 and HA12 have a greater contribution from fluorophores C compared to humic substances originating from soils and peats, we chose the designation C1 and C2 and for all maxima located in this region. Peaks C1 and C2 (330-335/440 nm and 385-390/435-440), which occur only in HAs after 9 and 12 months of the vermicomposting process, exhibited the greatest excitation wavelength of all the EEM spectra and a lesser fluorescence intensity than the peaks in region A. We conclude that the maximum position is probably related to single aromatic systems and substituents. Some authors (Sierra et al., 2005; Rodríguez et al., 2013) have suggested that the C domain can be attributed to the presence of hydroxybenzoic acids and other substituted phenolic units originating from lignin, hydroxycoumarinlike structures, xanthone and/or quinoline derivatives which originated from degraded plant materials. According to previous studies, peak C may be interpreted such as fluorophores from autochthonous microbial processes (McKnight et al., 2001). The comparison of EEM spectra of HAs from vermicompost and HAlike fractions (HAL) from vermicomposted winery and distillery wastes (Romero et al., 2007) suggests that HAL fractions may also participate in the fluorescence of HAs in the C region. Moreover, the presence of peaks with an emission wavelength greater than 470 nm is in agreement with previous studies which suggest such maxima originate from intramolecular charge-transfer states, rather than from independent fluorophore families in humic substances (Boyle et al., 2009). This assumption is consistent with the values of the absorption coefficients E_{ET}/E_{Bz} and I_{Ar}/I_{COOH} obtained from the spectrometry experiments. It indicated that the time of the composting process could increase the content of humic-like materials in the HAs from the vermicomposting product. For most of the HAs (HA isolated from raw horse manure and HAs extracted from vermicomposts younger than 9 months), typical maxima lie within the T region and are thus restricted to wavelengths in the 275-280/335-340 nm range, suggesting they all have similar structures and components. According to this T domain, protein-like compounds were decomposed during the first nine months of vermicomposting (Fig. 3). In contrast, the fulviclike compounds increased slightly during the vermicomposting process. The location of the C1 and C2 domains were independent of the excitation and emission wavelengths without any trend with respect to the composting time. Compared with the T domain location of protein-like compounds (peaks T at 276–281/340–370 nm) (Baker, 2001; Lannan et al., 2013), the locations of tryptophan-like peaks of the HA samples all showed a blue shift in terms of emission wavelengths. Fluorophore families at the intermediate excitation wavelengths (250–280 nm) and shorter emission wavelengths (<380 nm) are related to soluble microbial by-product-like materials (Coble, 1996). Protein-like fluorophores were found to occur at enhanced levels in compost originating from municipal solid waste (MSW), sewage sludge (SS), and/or wastewater (WW) (Benítez et al., 2000; Wei et al., 2007; Pan et al., 2018). Further, the tryptophan-like compound may have originated by different manures where tryptophan is present as 'free' molecules or bound in proteins and/or humic substances (Wang et al., 2014).

Using the differences in the location of peaks (fluorophores) and fluorescence intensity, various structural units in the humic substances can be elucidated. At nearly the same excitation wavelength, some peaks in region A, fluorophores which correspond to the fulvic-like compounds, shift toward a lesser emission wavelength, while the fluorescence intensity increases. The shorter emission wavelengths and greater fluorescence intensity measured in the EEM spectra can be associated with simple aromatic structures and low average molecular weight components. In contrast, the longer emission wavelengths can indicate the presence of condensed aromatic rings and electron-withdrawing functional groups such as carbonyl-containing substituents, and hydroxyl and alkoxyl groups. It is well known that polycyclic aromatic hydrocarbons (PAHs) fluoresce at emission wavelengths from 400 to 500 nm when subjected to excitation at 250-380 nm. Hence, the emission wavelengths ranging from 340 to <430 nm could be associated with three-ring and/or four-ring aromatic structures (Doskočil et al., 2018). The PAHs with four or more aromatic ring per fluorophore emit light in the 400-500 nm wavelengths range.

3.7. Thermogravimetric analysis

Subsequently, all the studied HAs were subjected to detailed thermogravimetric analysis. The respective thermograms (TGA and DTG) are shown in Fig. 4. It can be clearly seen that the weight of all the HAs samples drops in a stepwise manner.

TGA curves separated into two main groups. The first group contained only one member – the humic acid isolated from the original manure, whereas all composted HAs were concentrated in the second group. More detailed information can be seen in the DTG curves (Fig. 4b). The DTG curve for the HA isolated from original manure indicates three principal weight-loss steps are located around 240,



Fig. 4. Thermogravimetric analyses (a) TGA; (b) DTG) and results for HAs isolated from raw horse manure (wine curve), vermicompost of average ages 5 weeks (black curve), vermicompost of average ages 3 months (blue curve), vermicompost of average ages 6 months (green curve), vermicompost of average ages 9 months (orange curve), and vermicompost of average ages 12 months (red curve). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

330, and 410 °C. The second step represents the largest weight-loss step and will be called the main peak; in fact, its dominating shape distorted the other two peaks in shoulders. The DTG curves obtained for HAs isolated from vermicomposts also demonstrate the first two peaks, while the last peak practically disappeared. Moreover, the temperatures of the peak maxima were shifted to higher temperatures when compared with the DTG record of the HA extracted from original manure. The differences among the vermicomposted HAs are not significant. The differences among the HAs isolated from various vermicomposts are not significant. The shifts indicate that the corresponding compounds in HAs obtained from vermicomposts are of greater thermal stability than in the HA isolated from the original manure. In other words, the most thermally labile components seemed to be consumed during the composting process. On contrary, the disappearance of the third peak points to the degradation of the most stable thermal fractions.

The thermal behaviour of organic matter from natural compost with recycled compost in this study was somewhat different from the behaviour reported for composts prepared from agricultural wastes where only two peaks in the 200-600 °C range were reported (Dell'Abate et al., 2000). Three peaks are reported for some types of soil HAs or soil organic matter (Capel et al., 2005; Montecchio et al., 2006). The overlapping character of our TG records points to the continuous complex mixture without components of sharply different thermal stability. The first peak (shoulder) is attributable to the loss of organic volatiles and decomposition of the least stable fractions (e.g., decarboxylation of organic acids); loss of residual (bound) moisture also cannot be excluded. The main degradation is produced by the combustion of polysaccharides or aliphatic compounds (Montecchio et al., 2006). The last peak (shoulder) could be attributable to the decomposition of aromatic moieties. Because it was not distinct in the HAs isolated from vermicomposts it could also represent original polymers depolymerized during the composting process into less stable oligomers or monomers. Further, it can be hypothesized that the shoulder disappearance is due to the release of (labile) organics from the organo-mineral fractions by the action of agents released by decomposing microorganisms. In any case, it is seen that the most thermally stable fraction disappears during the first stages of the composting process.

4. Conclusion

In this study, advanced spectroscopic techniques and thermogravimetric analysis combined with the routine measurements of chemical indicators such as the content of biogenic elements, C/N ratio, N-NH⁴₄/NO³ ratio, DOC, and ion exchange capacity brought new information on manure vermicomposting in a real-field continuous-feeding system, and enabled the complex characterization of HAs isolated from original horse manure and vermicomposts of different ages. Fluorescence spectroscopy (TLS) is a relatively simple and effective technique to study the maturity and stability of the produced vermicompost.

Horse manure vermicomposting resulted, over time, in an increase in pH, IEC, IEC/C_{tot}, N_{tot}, P_{tot}, K_{tot}, Mg_{tot}, K_{avail} and a decrease in dry matter, volatile solids, C/N ratio, N-NH⁴₄, N-NH⁴₄/N-NO³₃, DOC, P_{avail}, Mg_{avail}, number and earthworm biomass, and microbial biomass including fungi and bacteria. Thus, the maturity of the vermicompost improved.

The results suggest that vermicomposting decreased the aliphatic, protein-like, and polysaccharide humic components, whereas vermicomposting increased the aromaticity and contents of oxygen-containing functional groups (e.g. carboxylic, phenolic, and etheric groups in the HAs). The typical tryptophan-like fluorophores lie within the T region (275/335 nm), which can be ascribed to the freshly produced organic matter of biological or microbial origin. This non-humic component was progressively transformed to humic-like fluorophores during vermicomposting. Humic-like fractions with Ex/Em wavelength pairs of 330-335/400 nm and 385-390/435-440 nm were observed after nine months of vermicomposting. These fluorophores lie in the EEM C region, which implies that the components are of the same form, or from very similar fluorophore families. Fulvic-like fluorophores were observed in the EEM spectra of HAs throughout the vermicomposting period. EEM spectra are comparable to fingerprints for HAs during the vermicomposting, and they can be regarded as indicators to assess the maturity of vermicomposts.

Spectroscopic characterization is a useful and relatively quick way of obtaining new knowledge on vermicomposting of manure in a real-field continuous-feeding system.

Acknowledgement

The authors would like to thank Ladislav Palecek and his wife for experiment maintenance and Christina Baker Starrman for revision of the English text.

Funding

This work was supported by the Ministry of Agriculture of the Czech Republic [grant number QK1910095]; and the Ministry of

Education, Youth, and Sports [grant numbers LO1211, and SV19-02-21140].

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.wasman.2019.08.032.

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