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How earthworms *Eisenia andrei* interact with sewage sludge-derived biochar

Diana Bošanská[1], Vladimír Frišťák[1]✉, Jana Fančovičová[2], Martin Pipíška[1], Christoph Pfeifer[3], Gerhard Soja[3]

[1]*Department of Chemistry, Trnava University in Trnava, Trnava 91843, Slovakia*
[2]*Department of Biology, Trnava University in Trnava, Trnava 91843, Slovakia*
[3]*Institute for Chemical and Energy Engineering, University of Natural Resources and Life Sciences Vienna, Vienna 1190, Austria*

✉*Corresponding author:* vladimir.fristak@truni.sk

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Abstract

Pyrolysis of sewage sludge is emerging as a promising method for treating this heterogeneous and highly complex waste with increasing research work. One of the main limitations of using sludge-derived biochar as a soil additive is its heterogeneous properties and its content of organic and inorganic contaminants. The main objective of our work was to perform a basic physicochemical characterization of biochar prepared from municipal wastewater treatment plant (WWTP) derived sewage sludge (PSM) at 603 – 615 °C by pH, EC, CHN-S analysis, total concentrations of metals and metalloids (As, Cd, Cr, Cu, Ni, Pb, Zn, Fe and Hg), determination of the presence of polycyclic aromatic hydrocarbons (18-PAHs), polychlorinated dibenzo-p-dioxins and dibenzofurans (17-PCDD/Fs) and polychlorinated biphenyls (7-PCBs). The PCM material was tested by short-term avoidance test and long-term cultivation test with earthworms *Eisenia andrei*. Elemental analysis revealed the concentrations of heavy metals in PSM: Fe (138 g/kg), Zn (2,602 mg/kg), Cu (582 mg/kg), Cr (107 mg/kg), Pb (87 mg/kg), Ni (67 mg/kg), As (<1 mg/kg), Hg (<2 mg/kg), Cd (<1 mg/kg). The total extractable concentrations of 17-PCDD/Fs in PSM were lower than the quantification limits applied for the analysis. The concentration of 7-PCBs in PSM were in case of all studied structures (PCB28, PCB52, PCB101, PCB118, PCB138, PCB153, PCB180) under limit of quantification. There was no relevant evidence of a negative impact of PSM on the behaviour of soil earthworms in the avoidance test. PSM did not cause physiological or biochemical stress to earthworms in long-term cultivation test due to higher reproductive activity. A 10 % PSM amendment caused a more than 3-fold higher concentration of bioaccumulated Fe in *E. andrei* tissues compared to the value of Fe concentration value in the control exposure. Higher concentrations in tissues of earthworms cultured in PSM medium were also observed for Cr and Zn. The differences in the concentrations of accumulated Cd, Cu, Ni, Pb in earthworms from PSM medium were not statistically significant compared to the control. A clear demonstration of the effect of PSM on earthworm behaviour and responses requires a longer-term test, as well as a test that reflects the real environmental conditions in which natural aging of pyrolysis materials occurs.

Introduction

Every year, a large volume of industrial and municipal wastes enters lakes, rivers, oceans, and seas, which have a negative impact on the environment. The risk of wastewater is that it contains, among other compounds, significant concentration of contaminants or pathogens. Dealing with the ever-increasing volume of sludge produced is a huge challenge for our human society. Composting and vermicomposting methods can turn sewage sludge into a biofertilizer rich in nutritionally important elements. In composting, micro-organisms are involved in the conversion of organic waste into compost, while in vermicomposting, the giant nematodes are also involved in the process. Landfilling of sludge is one of the most widespread disposal methods (Yang *et al.* 2017). Landfills designed for sludge must be very well insulated to prevent as much as possible negative contact with the surrounding environment. Among other components, landfills should include a collection tank to collect leachate as well as boreholes to monitoring groundwater pollution. The subsoil of the landfill should be made of a low permeability material such as plastic. For better sludge handling in landfills, it is recommended that the sludge be sufficiently dewatered to a solids content of 25 % (Ogunwumi and Salami 2023). Another method used for sludge disposal is incineration. In incineration reduces the volume of sludge and eliminates pathogens and harmful substances by high temperature. The main problem with this disposal method is the high-water content of the sludge. The sludge should be dewatered to less than 50 % to produce sufficient energy to ensure a self-sufficient incineration process. This level of sludge dewatering, however, requires high energy consumption (Dhote *et al.* 2022).

An alternative method of sludge treatment represents thermochemical conversion. According to Chen *et al.* (2015), pyrolysis sludge treatment represents a sustainable solution for the treatment of sewage sludge from different input sources, as it addresses the issues of energy recovery, nutrient recycling, sludge sanitation, heavy metal immobilization, as well as overall environmental protection. Organic matter is thermally decomposed due to increasing temperature (Rumaihi 2022). The first step before the actual pyrolysis of the sludge is to evaporate the internal moisture at 200 °C. Subsequently, three stages of thermochemical sludge decomposition take place, which have been described by several authors in different ways (Fonts *et al.* 2012; Bonfiglioli *et al.* 2014; Alvarez *et al.* 2015). Fonts et al. (2012) divided sludge pyrolysis into three phases, with the first phase involving the thermal decomposition of viscous compounds at temperatures 200 – 300 °C. In the second phase, at temperatures of 300 – 400 °C, decomposition of organic polymers takes place and in the third phase, at temperatures above 450 °C, decomposition of compounds such as cellulose takes place. However, according to Alvarez *et al.* (2015), the first decomposition of hydrocarbons already occurs at temperatures around 255 °C, the second decomposition of lipids occurs at temperatures around 300 °C, and the third phase involves the decomposition of lignin. Lignin, as a potential component of the input sludge precursor, is characterized by a wide decomposition temperature range, which includes the temperature range of protein decomposition (360 – 525 °C). Different perspectives on the phases of sludge pyrolysis suggest that these differences may be due to heterogeneous experimental conditions and properties of the treatment sludge.

One of the main limitations of using sludge-derived biochar as a soil additive is its heterogeneous properties and its content of organic and inorganic contaminants (Frišták *et al.* 2018). For this reason, detailed guidelines and standards have been developed specifying the appropriate type of input biomass, pyrolysis parameters, permitted contaminant limits, and optimised analytical methods for detailed characterisation of biochar. The list of recommended biomass types for biochar production by EBC (European Biochar Certification 2023) includes sludge from municipal as well as other types of WWTPs from 2023 onwards. In 2021, the European Commission amended Annex II of the EU Fertiliser Regulation 2019/2009 with effect from 16 July 2022. In this legislative change, pyrolysis and gasification products were recognised as possible components of EU products used for fertiliser production. Annex II of the Fertiliser Regulation 2019/2009 also characterised the groups of permitted feedstock precursors, most often based on waste biomass, for the biochar production as a fertiliser, as well as the required process parameters and permitted concentration limits for organic contaminants such as PAHs (polycyclic aromatic hydrocarbons), PCDDs (polychlorinated dibenzodioxins) and PCBs (polychlorinated biphenyls). Thanks to a change in the EU Fertiliser Regulation 2019/2009, biochar derived from waste biomass and sewage sludge as well has uniform criteria across the EU. However, the concentrations of hazardous substances do not take into account the behaviour or response of the soil organisms (e.g. earthworms) to which the sewage sludge-based biochar is to be applied. As is well known, earthworms are an important living component of the soil system and are an essential part of the soil community. However, they are quite often overlooked when assessing the ecotoxicity of selected soil additives.

Earthworms produce a slime-like secretion that helps form stable aggregates that are the basis for the formation of the underlying soil structure. Organo-mineral aggregates that are stable to water are found in the soil in the presence of earthworms. Their important role is to reduce soil compaction and increase soil permeability. The physiology, morphology, and behavioural processes of earthworms are essential to understand their impact on soil functions (Medina-Sauza *et al.* 2019). They are involved in altering the structure of the environment and improving the physical properties of the soil in which they move. They create horizontal and vertical corridors that allow oxygen and water to enter the soil and, conversely, carbon dioxide can escape. They also play an important role in pedoturbation (Ojha and Devkota 2014). The basic ecological functions of earthworms include the decomposition of soil organic matter. They ensure nutrient cycling in the soil, thereby affecting not only the physical but also the chemical properties of the soil. The migration of earthworms and the consequent loss of their beneficial functions in the soil leads to the deterioration of soil properties (Schaefer 2003). Earthworms primarily recycle dead plant material and improve nutrient availability by drawing organic material into their tunnels into deeper soil layers. The ingestion and subsequent digestion of food leads to the formation of humus, earthworm castings contain a mixture of minerals and plant materials containing an abundance of nutrients that are made available to the plants, thereby promoting their growth (Myburgh 2017). Earthworms are an integral part of mixing the different soil layers (Craven and Braken 2016). They are very sensitive on pH values and soil composition.

The main objective of our work was to carry out a basic physicochemical characterization of biochar prepared from sewage sludge of municipal wastewater treatment plant (WWTP). Subsequently, the pyrolysis product was tested as a potential soil additive in terms of earthworm behaviour in acute (avoidance tests) as well as long-term cultivation tests aimed at monitoring mortality or reproduction and monitoring the translocation of selected heavy metals from the sludge derived biochar to earthworm tissues.

Experimental

Pyrolysis material derived from sewage sludge (PSM) was produced in collaboration with the Linz am Rhein municipal association as a test product within the Linz-Unkel WWTP (Linz, Germany). The sewage sludge produced in the Linz-Unkel WWTP or received from the neighbouring treatment plants was firstly anaerobically digested in the two digestion towers. It was then temporarily stored in two secondary thickeners and pumped from there to the dewatering unit of screw press as a new continuous sludge dewatering unit (IEA Derflinger GmbH) with the dewatering performance min. approx. 25 % DS (dry solids). The freshly dewatered digested sludge was fed to the EloDry® dryer (Eliquo Stulz, Germany) directly without intermediate storage via a screw conveyor. Via this dewatered sludge can be fed to the inclined screw conveyor, which transports dried material to the pyrolysis plant. The dryer air circulated by fans was heated to approx. 75 – 80 °C by heat exchangers installed in the dryer. Hot water of approx. 80 – 85 °C was used as the heat transfer medium, which transported the waste heat from the micro gas turbine, the pyrolysis system, and the boiler to the dryer. A large-capacity continuous pyrolysis reactor Pyreka (Pyreg GmbH, Dörth, Germany) with a maximal pyrolysis temperature of 603 – 615 °C and strictly anoxic atmosphere was used for the production. After dedusting in a cyclone separator, the gas formed during pyrolysis was fed to a combustion chamber. Here, the gas was burnt in a controlled manner with the help of a flameless burner according to the Flox process (Pyreg GmbH and WS-Wärmeprozesstechnik GmbH, Renningen, Germany). The intensive mixing of the gas with combustion air (fresh air) resulted in uniform combustion with a low CO content. At a combustion temperature of well below 1,200 °C, only little NO, NO_2 and N_2O was produced. Recirculated flue gas was mixed with the combustion air before it was fed to the Flox burner. A combustion air fan drawn in the fresh air required for combustion and ensured a controllable fresh air volume flow. Flame monitors, pressure transducers and temperature probes were available to monitor the combustion process. The obtained solid pyrolysis material was sieved to get a fraction of 0.5 –1 mm.

Physicochemical characterization of pyrolyzed sewage sludge

The active pH was determined by adding 0.5 g of PSM to 7.5 cm^3 of deionized water (< 0.04 µS/cm) to maintain a 1:15 w/v ratio and the suspension was stirred for 60 min at 45 rpm on a laboratory shaker (Orbital Shaker- Multi-RS 60, Biosan, Latvia). To determine the potential pH of pyrolyzed sewage sludge, 7.5 cm^3 of KCl (1 mol/dm^3) was added to 0.5 g of PSM and stirred similarly for 60 min at 45 rpm on a laboratory shaker. In both cases, the suspensions were allowed to stabilize for 60 min after stirring. The active and potential pH values were measured with a pH meter (pH/EC Multimeter 3420, WTW, Germany). The measurements were performed in triplicate each time. The electrical conductivity (EC) was determined by adding 7.5 cm^3 of deionized water (< 0.04 µS/cm) to 0.5 g of PSM. The conductivity was measured in the supernatant (pH/EC Multimeter 3420, WTW, Germany) after previous centrifugation (EBa 200S, Hettich, Germany). The ash content was determined by the method of Rehrah *et al.* (2014). Carbonate content of PSM was quantified by volumetric method with the application of Janko's lime meter. The total C, H and N contents of the sample were provided by an elemental analyser (CHNS-O EA 1108, Carlo Erba Instruments, Milan, Italy). Total metals and metalloids concentrations in PSM (As, Cd, Cr, Cu, Ni, Pb, Zn, Fe and Hg) were determined by ICP-MS (Perkin Elmer, Elan DRCe 9000, Shelton, CT, USA) and RFS (Spectro-Xepos, Kleve, Germany). To determine the total concentration of 18 structures of polycyclic aromatic hydrocarbons (PAHs) according to United States Environmental Protection Agency (US EPA 2000): naphthalene, acenaphthylene, acenaphthalene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene, benzo(g,h,i)perylene, ideno(1,2,3)pyrene, 1-methylnaphthalene and 2-methylnaphthalene, a modified extraction procedure according to Hilber *et al.* (2012). High performance liquid chromatography (1260 Infinity, AGILENT Technologies) was used for the quantification of PAH compounds using a Restek C18 PAH column (150 × 4.6 mm; 4 µm), acetonitrile-water mobile phase and a diode array detector (DAD) (λ = 280 nm). Sample volume 5 µL and temperature 30 °C. Total concentrations of polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs) and polychlorinated biphenyls (PCBs) were determined by standardized method according EBC guidelines (EBC, 2023). Isotope dilution high-resolution gas chromatography/high-resolution mass spectrometry was employed to determine the levels of 17 PCDD/Fs and 7-PCBs in PSM extracted by accelerated solvent extraction (ASE). 7-PCBs standards represent structures of 2,4,4'-Trichlorobiphenyl (PCB28), 2,2',5,5'-Tetrachlorobiphenyl (PCB52), 2,2',4,5,5'-Pentachlorobiphenyl (PCB101), 2,2',3,4,4',5'-Hexachlorobiphenyl (PCB138), 2,2',4,4',5,5'-Hexachlorobiphenyl (PCB153), 2,2',3,4,4',5,5'-Heptachlorobiphenyl (PCB180) 2,3',4,4',5-Pentachlorobiphenyl (PCB118). Analytical procedures were adopted from the ISO 12884 and powered by Agilent 7890A GC-MS/MS instrument (Agilent Technologies, Santa Clara, CA, USA).

Earthworm avoidance test

The *Eisenia andrei* was used to examine the escape behaviour test. The California earthworm was obtained from a certified breeding facility and is commonly supplied with vermicomposting kits. The selected species is undemanding to raise and easy to handle. It is used in tests due to its rapid reproductive ability and short life cycle (from the newly emerged cocoon through the adult earthworm ranges from 45 to 51 days). We injected the tadpoles into experimental containers (l×w×h, 30×20×17) that were divided by a partition. Each part of the container contained an equal volume of chamber medium, with one part enriched with 1 % and 5 % (v/v) PSM material, respectively. The other portion was unamended and served as a control. Experiments were conducted in 5 replications at an initial soil moisture content of 60 % and a temperature of 22 °C. In each pot, 10 viable individuals were inoculated at the starting line (formed after the divider was pulled). After seeding the individuals, fine soil incorporation was necessary. Subsequently, the experimental containers were covered with foil, placed in a randomized distribution in the laboratory area in a dark/light regime of 16 h/ 8 h. The pH value of the study substrate was 6 at control, after application of PSM there was an increase to 6.8. After the 48-h exposure period, the partition was returned to its original position and the number of earthworms in each partition was counted. In neither experiment was there a rupture nor killing of an individual by the insertion of the dividing septum.

Long-term incubation test

To assess the suitability and toxicological safety of the application of PSM material as a soil additive, we conducted a long-term ecological test using soil invertebrates. Individuals of the California earthworm (*E. andrei*) obtained from a vermicomposting station with certified origin were selected as model organisms. The earthworms were divided into 2 glass containers (l×w×h, 16×14×15) of 10 individuals. First container contained pure substrate (control) and the second contained substrate with 10% (v/v) amendment of PSM substrate. Ecowitt WH51 humidity monitoring probes were introduced into the glass containers to control humidity value of 60 % during the experiment (Figure 1). The experiment lasted for 9 months, during which the earthworms were counted once a month, weighed, measured, and feed dose (vegetable and fruit peelings, coffee grounds with an always constant composition) was added in equal amounts to both containers. At the end of the experimental period, the change in the morphology of earthworms was examined (dissection, preparation of microscopic slides) and then, after emptying the digestive tract, the earthworms were dried in a laboratory oven at 60 °C, homogenized and prepared representative sample was subjected to wet digestion using digestion vessels and concentrated HNO_3 as a mineralizing agent. Samples of the digests were filtered through a syringe filter with a polytetrafluoroethylene (PTFE) membrane (0.45 µm) and stored in a refrigerator at 5 °C for metal quantification by atomic absorption spectrometry (AAS) analysis (graphite furnace (GF)-AAS and flame (F)-AAS, Agilent 240FS AA (Agilent Technologies, USA)).

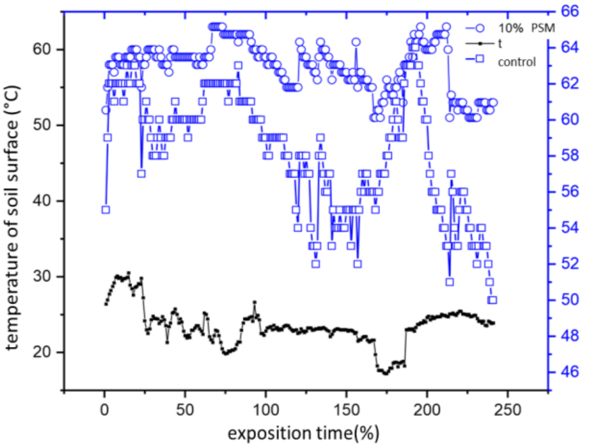

Figure 1. Experimental conditions (soil moisture and outdoor temperature) of exposure of earthworms *E. andrei* in control environment without amendment and environment with 10 % addition of PSM monitored by Ecowitt WH51 sensor system.

Statistical analysis

Generalized Linear Mixed Model (GLMM) was used to analyse the data. Earthworm occurrence in the experimental (1) or control (0) substrate was defined as the binomial dependent variable. Treatment (1 % vs. 5 %) was defined as a categorical predictor, and the individual body mass of each earthworm was a continuous predictor. The measures order (1 –5) was defined as a random effect. We also examined how the earthworm's preference for any of the two environments could affect its body mass. To test this, we employed the same GLMM, but earthworm body mass was defined as the dependent variable with a normal distribution (Kolmogorov-Smirnov test, p > 0.2) Treatment (1 % vs. 5 %) and the earthworm occurrence in the experimental (1) or control (0) substrate were defined as fixed predictors. The measures order (1 – 5) was defined as a random effect. For statistical assessment of data from long-term cultivation experiment the Student's t-test has been used. All analyses were performed with SPSS v. 26 (IBM Corp., 2019, USA) and OriginPro 2016 (OriginLab Corporation, Northampton, MA, USA).

Results and Discussion

The basic physicochemical characterization of PSM produced at 603 – 615 °C showed that the active pH of the material is in the neutral region (Table 1). Zielińska *et al*. (2015) in their work dealt with the pyrolysis of sewage sludge at temperatures of 500 °C, 600 °C and 700 °C and observed an increasing trend of active pH values with increasing pyrolysis temperature. They justified this process by the fact that during the thermal treatment of sludge, polymerisation/condensation reactions of aliphatic compounds occur, which are minimal at low temperatures, and at the same time the amount of acidic functional groups on the surface of the pyrolyzed sludge is reduced. The same trend was described by Wang *et al.* (2021), who investigated the pyrolysis of sewage sludge at temperatures of 350 °C, 550 °C and 750 °C and observed an increase in pH values towards the alkaline region. In addition, their work also investigated the co-pyrolysis of sewage sludge with additive materials such as bamboo and wood sawdust, rice husk, solid residues from leached tea and polyvinyl chloride, and they observed a significant decrease in the pH value in the case of co-pyrolysis with polyvinyl chloride (pH = 3.06). In the case of our PSM sample, the potential pH value was in the weakly acidic region (pH<7). Mierzwa-Hersztek *et al.* (2018) reported neutral values of potential pH of the material obtained by pyrolysis of sewage sludge from different regions of Poland (Krakow - 6.89; Krzeszowice - 7.06; Slomniki - 7.18) at 300 °C. In contrast, Souza *et al.* (2021) reported an increasing trend in potential pH due to pyrolysis temperature (300 °C < 500 °C < 700 °C), attributing this change, as mentioned above, to the loss of acidic functional groups (carboxyl, hydroxyl, or formyl) from the surface of the pyrolyzed sewage sludge. The increase in the alkaline character of the pyrolyzed sludge may be due to the separation of alkaline elements (Ca, Mg, K) from organic compounds during the pyrolysis process. The increase in the alkaline character of pyrolyzed sewage sludge can be justified based on the ongoing polymerisation/condensation reactions of aliphatic compounds; dehydration of the sludgeleading to a reduction in the amount of acidic functional groups; and finally the removal of alkali metal salts from the organic matrix with increasing pyrolysis temperature (Wang *et al.* 2020).

Table 1. Basic physicochemical characteristics of PSM.

pH_{H2O}	7.03 ± 0.03
pH_{KCl}	6.58 ± 0.04
EC (µS/cm)	1381.67 ± 109.56
ash content (%)	8.51 ± 0.07
Σ 18 PAHs (mg/kg)	1.05 ± 0.02
C (%)	35.52 ± 0.01
H (%)	0.95 ± 0.01
N (%)	4.21 ± 0.01
As (mg/kg)	<LOQ*
Cd (mg/kg)	<LOQ*
Cr (mg/kg)	107 ± 2.25
Cu (mg/kg)	582 ± 4.50
Ni (mg/kg)	67 ± 1.20
Pb (mg/kg)	87 ± 1.50
Zn (mg/kg)	2602 ± 10.50
Fe (g/kg)	138 ± 0.10
Hg (mg/kg)	<LOQ*

*LOQ As= 1 mg/kg, Cd = 1 mg/kg, Hg = 2 mg/kg.

The pH value of the pyrolyzed sludge is also significantly affected by its increased aromaticity (Zhang *et al.* 2022). The resulting electrical conductivity (EC) value of PSM is significantly influenced by the feedstock composition, pyrolysis conditions (residence time, maximum pyrolysis temperature reached) and the treatments of the feedstock precursor prior to pyrolysis. Compared to other works that dealt with the pyrolysis of sludge at approximately the same temperature (approximately 600 °C), the EC value is more than 3 times lower (Zoghlami *et al.* 2021). Zoghlami *et al.* (2021) also noted in their work that as the pyrolysis temperature increases (260 °C < 420 °C < 610 °C), the EC values of the pyrolyzed sludge decreased. The decrease in EC values is due to the accumulation of chemical compounds containing Na, K, Ca and Mg. During the sludge pyrolysis, the ash content increases while the solubility of salts and metals in water decreases, thus lowering the EC value of the material. Souza *et al.* (2021) attributed the decrease in solubility of salts and metal compounds at pyrolysis temperatures above 200 °C to the fixation of K^+, Ca^{2+}, Mg^{2+} and PO_4^{3-} ions in the mineral fractions, and the mineral content of the analysed material is directly related to the ash content. EC values are among the important parameters in the application of PSM to alkaline soils, which affect soil salinity and the excess of Na ions in the soil (Zoghlami *et al.* 2021).

Total polycyclic aromatic hydrocarbons in the pyrolysis product were present at 1.05 mg/kg (Table 1), which is a lower concentration compared to the EBC (2023) requirements that limit the concentration of PAHs to 12 mg/kg for conventional pyrolysis materials or to 4 mg/kg for premium pyrolysis materials. The predominant structures of PAHs present in the PSM were hydrocarbons with 4-ring structure followed by 3-ring structures. Due to the pyrolysis temperature, cracking of these structures to 2- and 3-ring hydrocarbons may occur, however, according to Tomczyk *et al.* (2021) the formation of multi-ring hydrocarbons occurs with increasing temperature, which was confirmed in their work. Additional analysis of methylated hydrocarbons revealed the presence of only 2-methylnaphthalene at a concentration of 0.129 mg/kg, which, according to Frišták *et al.* (2019) has a higher volatility than other PAH substances, making it more hazardous. The presence of PAHs in the BC sample indicates a potential risk of contamination and intoxication when applied to soil. However, the concentrations determined represent so-called total concentrations and are not readily mobile or mobilizable under normal environmental conditions.

For elemental analysis of PSM (Table 1), we focused on total heavy metals and semi-metals such as As, Cd, Cr, Cu, Ni, Pb, Zn, Fe and Hg. The total concentrations of the studied elements decreased in the order: Fe > Zn > Cu > Cr > Pb > Ni > Hg, As, Cd. The highest abundance in the PSM was observed for Fe, whose values exceeded those of the other elements studied by more than 52 times. In the work of Pedroza *et al.* (2014), authors reported an increase in Fe concentration in the pyrolysis product with increasing pyrolysis temperature (500 °C – 600 °C). This finding is supported by the fact of decomposition of organic compounds, increase in solids content in PSM and multiplication of non-volatile elements present. The values of Fe concentrations in PSM were almost 3-fold lower compared to the concentrations we found. Based on the literature review, we assumed the highest abundance of Zn among the group of metals investigated in the PSM sample. Zang *et al.* (2022) in their work reported an increasing trend of Zn concentration in PSM with increasing pyrolysis temperature, and the values reported in their work were more than 9-fold lower compared to the Zn concentration found by us. This trend was similarly noted in the work by Pedroza *et al.* (2014) and Mierzwa-Hersztek *et al.* (2018), who reported 2-fold lower Zn concentrations compared to our PSM sample. The higher concentrations of Fe and Zn in the pyrolyzed sludge are due to applied wastewater treatment technology and subsequently the higher concentrations of these elements in the input raw sewage sludge. The elevated concentrations of heavy metals in the PSM were probably due to the pyrolysis temperature, which affected the dissociation of organic compounds and some minerals such as carbonates. For example, chloride and sulfide compounds of heavy metals in sludge are easily released, but sulfide compounds of heavy metals are more resistant to this process (Mierzwa-Hersztek *et al.* 2018). On the contrary, the lowest abundance in PSM was observed for As, Cd and Hg, whose concentrations were below limits of quantification of analytical methods and techniques. We assumed very low Hg concentrations in PSM because Hg compounds are volatile even at pyrolysis temperatures below 600

°C. The results of Velli *et al.* (2021) observed a significant decrease in Cd concentrations in PSM at all temperatures studied (500 °C, 700 °C, 900 °C), whose concentrations did not exceed 8 mg/kg in the pyrolysis product and decreased with increasing pyrolysis temperature. The decrease in Cd concentrations can be attributed to the reduced oxide formation in the reducing atmosphere during the pyrolysis process, which contributes to the formation of volatile forms of Cd. The authors also report that the high release of Cd from the feedstock may be an advantage of pyrolysis compared to conventional combustion because more Cd oxides are present in the ash obtained from the combustion process than in the pyrolysis material. A decreasing trend in Cd concentration was also observed by Zoghlami *et al.* (2021) and Mierzwa-Hersztek *et al.* (2018). Wang *et al.* (2021) observed an increase in Cd concentration in pyrolyzed sludge up to 550°C, after exceeding this temperature Cd concentrations in product decreased rapidly. They attributed this decrease to the presence of Cd predominantly in the form of carbonates, which decompose thermally to volatile forms of Cd when the temperature increases above 600 °C. Several papers have reported decreasing concentrations of Cr, Cu, Pb, Cu, Ni in pyrolyzed sludges due to increasing pyrolysis temperature (Zheng *et al.* 2019; Alipour *et al.* 2021; Velli *et al.* 2021; Zoghlami *et al.* 2021; Zhang *et al.* 2022). Zheng *et al.* (2019) reported that in their investigation of pyrolyzed sewage sludge obtained from different areas of human activity, there are large differences in the total heavy metal content, which are closely related to the minerals contained in the sewage sludge, the type of pyrolysis, the pyrolysis temperature, and the process of pretreatments (drying, composting, and others). The authors (Zheng *et al.* 2019) also reported the total contents of Pb, Cd, Cu, Mn, Zn, Ni, Cr, and As in the investigated pyrolyzed sludges, which ranged from 44 – 506 (Pb), 2.6 – 10 (Cb), 148 – 2,361 (Cu), 403-1,543 (Mn), 542 – 3368 (Zn), 48 – 924 (Ni), 55 – 13,758 (Cr), and 3 – 51 (As) mg/kg, respectively. The total extractable concentrations of 17-PCDD/Fs (Table 2) in PSM were in all cases below than quantification limits used for the analysis. Pyrolysis reduced the amount of PCDD/Fs congeners. According Sørmo *et al.* (2024), the most persistent PCDD/F to thermal volatilization/degradation is 1,2,3,4,6,7,8- Hepta CDD which is found in all types of sewage sludge and also in pyrolyzed sludge. However, in our PSM, the concentration of this structure was <1.0 ng/kg. Sørmo *et al.* (2024) also addressed the effect of pyrolysis temperature on the ease of removal of HxCDFs, HxCDDs, PeCDFs and PeCDDs from the sludge matrix during pyrolysis. The concentration of 7-PCBs in PSM was below the limit of quantification for all structures studied (PCB28, PCB52, PCB101, PCB118, PCB138, PCB153, PCB180). Therefore, we can conclude that our PSM sample) was below EBC threshold for premium quality biochar (<0,2 mg/kg). Sørmo *et al.* (2024) and Schlederer *et al.* (2024) confirmed a similar effect of the pyrolysis process on the reduction of PCB congeners variety. The most abundant PCB in the sludge-derived biochar is PCB153, which can be found in 90 % of produced biochar (Sørmo *et al.* 2024). In the PSM material, neither this structure was confirmed at a concentration above the detection limit for the analytical method.

Table 3 compares the total concentration of selected inorganic and organic pollutants in the pyrolyzed sewage sludge against the IBI and EBC guidelines (International Biochar Initiative 2015; Schmidt *et al.* 2023). The selected parameters in the IBI guidelines represent a relatively wide range of values, as they are retrieved from regulation of several countries.

The EBC distinguishes between EBC-Agro and EBC-AgroOrganic biochar, which can be used for agricultural purposes. Both regulations consider the European fertilizer regulation (EU 2019/1009) and plus EBC-AgroOrganic covers the European Commission’s regulation for organic farming (EU 2019/2164). The PSM sample met all the requirements for organic and inorganic pollutant content according to the IBI (Table 3). Additionally, the PAH, PCB and PCDD/Fs concentrations also complied with the limits set according to EBC. In contrast, the concentrations of Cr, Cu, Ni, Zn exceed the levels for EBC-Agro and EBC-AgroOrganic as well.

Table 2. Total contents of 17-PCDD/Fs and 7-PCBs in PSM.

PCDD/Fs [ng/kg]		**PCBs [ng/kg]**	
2,3,7,8-Tetra CDD*	<0.20	PCB (28)	<100.0
1,2,3,7,8-Penta CDD*	<0.20	PCB (52)	<50.0
1,2,3,4,7,8-Hexa CDD*	<0.20	PCB (101)	<200.0
1,2,3,6,7,8-Hexa CDD*	<0.20	PCB (138)	<50.0
1,2,3,7,8,9-Hexa CDD*	<0.20	PCB (153)	<5.0
1,2,3,4,6,7,8 Hepta CDD*	<1.0	PCB (180)	<50.0
Octa CDD*	<2.0	PCB (118)	<50.0
2,3,7,8-Tetra CDF**	<0.20		
1,2,3,7,8-Penta CDF**	<0.20		
2,3,4,7,8-Penta CDF**	<0.20		
1,2,3,4,7,8-Hexa CDF**	<0.20		
1,2,3,6,7,8-Hexa CDF**	<0.20		
1,2,3,7,8,9-Hexa CDF**	<0.20		
2,3,4,6,7,8-Hexa CDF**	<0.20		
1,2,3,4,6,7,8-Hepta CDF**	<0.60		
1,2,3,4,7,8,9-Hepta CDF**	<0.60		
Octa CDF**	<2.00		

*****chlorodibenzodioxin, **chlorodibenzofuran

Table 3. Comparison of the inorganic and organic pollutant amount in PSM with IBI (version 2.1) (International Biochar Initiative 2015) and EBC (Version 10.3E (European Biochar Certification 2023) regulation.

	PSM	**IBI (2015)**	**EBC-Agro (2022)**	**EBC-AgroOrganic (2022)**
Σ 16-PAHs (mg/kg)*	0.88 ± 0.01	6-300	6 ± 2.4	6 ± 2.4
Σ 17-PCDD/Fs (ng/g)	<0.017	0.017	0.02	0.02
Σ 7- PCBs (ng/g)	<200	200-1000	200	200
As (mg/kg)	<1	13-100	13	13
Cd (mg/kg)	<1	1.4-39	1.5	0.7
Cr (mg/kg)	107 ± 2.25	93-1200	90	70
Cu (mg/kg)	582 ± 4.50	143-6000	100	70
Ni (mg/kg)	67 ± 1.20	47-420	50	25
Pb (mg/kg)	87 ± 1.50	121-300	120	45
Zn (mg/kg)	2602 ± 10.50	416-7400	400	200
Fe (g/kg)	138 ± 0.10	NL**	NL**	NL**
Hg (mg/kg)	<2	1-17	1	0.4

[*] 16-PAHs- represents concentration values of total 16 PAHs structures defined by IBI and EBC excluded to 1-methylnaphthalene and 2-methylnaphthalene;

[**] NL – no limit value

Figure 2. Cocoons and juveniles of earthworm *E. andrei* found in 10 % PSM-amended substrates after 5 months of exposure.

On the other hand, concentrations of As, Cd and Pb fulfilled the limits for EBC-Agro and concentration of Hg was under limit of quantification of selected analysis. Both EBC and IBI guidelines do not consider Fe concentration so far.

Earthworm substrate preferences and long-term effect of PSM

In 1 % and 5 % PSM enriched environments, 61.5 % and 57.5 % of earthworms preferred the experimental (amended) environment over the control environment (non-amended), respectively. However, earthworm's bias toward the experimental environment was not significant. There was no significant influence of earthworm's body mass on their preferences for the experimental environment (GLMM; $F_{(1,83)} = 0.11$; $p = 0.75$). The order of experiments showed no influence on the results reported above (estimate = 0.023, Z = 0.90, p = 0.37). There was neither effect of treatment, the earthworm occurrence in the experimental (1) or control (0) substrate, nor the interaction between variables on earthworm body mass (GLMM, $F_{(1,82)} = 0.0$, 0.12 and 0.15, p = 0.99, 0.73 and 0.70, respectively). The random effect did not influence these results (estimate = 0.003, Z = 0.73, p = 0.46). Based on the results found, there is no relevant evidence of a negative impact of pyrolyzed sewage sludge on the behaviour of soil earthworms. As is evident, volatiles that could be released from the material may affect behaviour and thus lead to escapes. However, this was not confirmed as no statistical difference was shown between the control and experimental sample. On the other hand, the short-term experiment (48 h) also did not lead to significant data to confirm that earthworms in PSM-enriched substrate would be stressed and thus stop taking food. The weight changes of the individuals did not show a statistical difference either.

To confirm the ecological safety of PSM material, earthworms were subjected to a long-term (9 months) test in the presence of 10 % amendment of PSM to test the chronic effect. Earthworms can bioaccumulate a wide range of contaminants, including heavy metals present in a waste or soil (Malińska *et al.* 2017). During the duration of the experiment -exposure period, we monitored earthworms every month. After 3 months of the experiment, we recorded the mortality of two of the individuals from the control group. After 5 months of the experiment, we observed the presence of 4 earthworm cocoons in the exposure with 10 % addition of PSM (Figure 2). Malińska *et al.* (2017) observed the presence of California earthworm cocoons in wood chips with pyrolyzed sewage sludge addition after only 4 weeks of exposure. The occurrence of earthworm cocoons in the control exposure was observed only after the 8th month of the experiment. After the end of the experiment, we observed 18 adults, 71 juveniles in the exposure with 10 % PSM addition and 8 original individuals and 3 juveniles of earthworm were present in the control exposure. After dissecting individuals from both exposures, we found that earthworms did not differ morphologically at all. We did not observe any anomaly in organ size or structure, nor visible changes in the slides of the two individuals, but we noted a difference in the pigmentation of the earthworms. Even there was no negative effect on biomass growth presented in case of conventional biochar amendments (Zhang *et al*. 2019; Huang *et al.* 2020) Earthworms originating from the exposure with 10% addition of PSM were pigmented slightly darker - to purple - than earthworms (brown) originating from the control sample.

Wet mineralization with subsequent spectral analysis was used to determine the total heavy metal concentrations (concretely Cd, Cr, Cu, Fe, Ni, Pb, Zn) in the earthworms from control sample and individuals from the 10 % PSM amended exposure (Figure 3). Bioaccumulated concentrations of As and Hg were not monitored because of low concentrations in the PSM. Based on the above result, we predicted a higher presence of Fe in the specimens that came from the exposure with 10 % PSM amendment. The value of Fe concentration was more than 3-fold higher compared to the value of Fe concentration in the control exposure. Higher concentrations in earthworm tissues cultured in PSM medium were also observed for Cr and Zn. Differences in concentrations of accumulated Cd, Cu, Ni, Pb in earthworms from PSM medium were not statistically significant compared to control (*p* = 0.05). The distribution of metals among the soil phases is important for the bioaccumulation by earthworms.

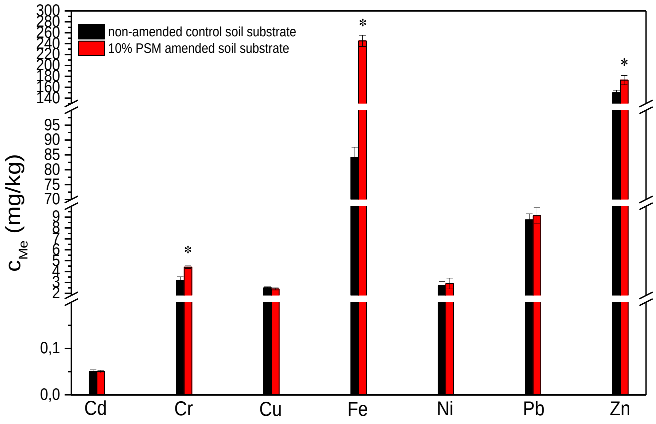

Figure 3. Total concentrations (c_{Me}) of Cd, Cr, Cu, Fe, Ni, Pb and Zn determined in tissues of earthworms *E. andrei* cultivated in non-amended control and 10 % PSM amended soil substrates at controlled moisture, temperature and exposition time 9 months. Error bars correspond to standard error (n = 3). Stars represent statistical significance of difference between control and amended environment (Student's t-test, *p* = 0.05).

Morera *et al.* (2001) showed for various soils (amended and non-amended) that the relative affinity of metals is consistent with the value of the first hydrolysis constant of the cations proposed by Basta and Tabatabai (1992). Shi *et al.* (2021) reported that bioaccumulation of heavy metals by earthworms depends on several factors such as physiological and morphological characteristics of these organisms, nutritional requirements, and dietary intake of elements. According to authors, heavy metals can enter the earthworm body by two mechanisms: chemical sorption of dissociated heavy metals through the skin and the digestion process through the earthworm gut. Liu *et al.* (2023) confirmed similar concentrations of bioaccumulated Cr in tissues of earthworms with response in unbalanced intestinal bacterial phyla. The concentrations of Cu and Pb in the earthworm tissues were of the same order than those reported by Kennette *et al.* (2002) and Dai *et al.* (2004). The PSM did not cause growth inhibition or reproduction inhibition of *E. andrei*. The material cannot be compared with conventional wood-based biochar in its pH effect, which precisely due to the change in the pH of the soil substrate causes a change in the activity of antioxidant enzymes (Huang *et al.* 2020). Based on our preliminary studies with PSM, it can be concluded that this soil amendment does not cause physiological or biochemical stress to earthworms even because of low concentrations of organic contaminants. On the other hand, higher concentrations of heavy metals in pyrolyzed sludge may lead to higher accumulation of inorganic contaminants over a longer exposure period than was investigated in our work. Therefore, it is necessary to carry out a longer-term test, as well as a test that reflects realistic environmental conditions where natural ageing of pyrolysis materials occurs.

Conclusion

Physicochemical characterization of PSM revealed the concentrations of heavy metals in PSM: Fe (138 g/kg), Zn (2602 mg/kg), Cu (582 mg/kg), Cr (107 mg/kg), Pb (87 mg/kg), Ni (67 mg/kg), As (<1 mg/kg), Hg (<2 mg/kg), Cd (<1 mg/kg). Total extractable concentrations of Σ 16-PAHs were 0.88 ± 0.01 mg/kg, Σ 17-PCDD/Fs were lower than quantification limits applied for analysis <0.017 ng/g. Concentration of Σ 7-PCBs in PSM were under limit of quantification (<200 ng/g). There was no relevant evidence of a negative impact of PSM on the behaviour of soil earthworms in avoidance test. PSM did not cause physiological or biochemical stress to earthworms in long-term cultivation test because of higher reproductive activity. 10% PSM amendment caused more than 3-fold higher concentration of bioaccumulated Fe in tissues of *E. andrei* compared to the value of Fe concentration in the control exposure. Higher concentrations in earthworm tissues cultured in PSM medium were also observed for Cr and Zn. Differences in concentrations of accumulated Cd, Cu, Ni, Pb in earthworms from PSM medium were not statistically significant compared to control. It is necessary to carry out a longer-term test, as well as a test that reflects realistic environmental conditions where natural ageing of pyrolysis materials occurs.

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