COMPOSTING AND VERMICOMPOSTING USED TO BREAK DOWN AND REMOVE POLLUTANTS FROM ORGANIC WASTE: A MINI REVIEW

ALENA GRASSEROVÁ[1,2], ALEŠ HANČ[3], PETRA INNEMANOVÁ[1,4], and TOMÁŠ CAJTHAML[1,2,*]

[1] Institute of Microbiology of the Czech Academy of Sciences, Vídeňská 1083, 14220, Prague 4, Czech Republic
[2] Institute for Environmental Studies, Faculty of Science, Charles University, Benátská 2, 12801, Prague 2, Czech Republic
[3] Department of Agro-Environmental Chemistry and Plant Nutrition, Czech University of Life Sciences Prague, Kamýcká 129, 16500, Prague 6, Czech Republic
[4] DEKONTA a.s., Dřetovice 109, 273 42 Stehelčeves, Czech Republic
* Corresponding author: cajthaml@biomed.cas.cz

ABSTRACT

The advantages of combining composting and vermicomposting to break down and remove pollutants from organic waste are reviewed. This mini-review aims to present the benefits of combining these methods and the outcome of specific cases of environmental remediation.

Keywords: composting; earthworms; heavy metals; organic pollutants; sewage sludge

Introduction

Organic substances occur in nature due to human activities and natural processes (Luo et al. 2014). These compounds occur throughout the environment at low trace concentrations; nevertheless, their negative effects on organisms including humans are well known. Such organic substances include pesticides, pharmaceuticals, personal care products, endocrine disruptors and industrial chemicals. Organic contaminants and heavy metals are not completely removed by wastewater treatment plants (WWTPs). The by-product, WWTP sludge, is rich in nutrients and therefore used as an agricultural fertilizer (Clarke and Smith 2011). In order to remove micropollutants from bio solids and at the same time maintain their valuable properties, green technologies called bioremediations are being developed (Hickman and Reid 2008). Typical biotechnologies for this purpose are composting or vermicomposting, which are environmentally friendly, low-maintenance and low-cost methods. When these two processes are combined, even better outcomes can be achieved in terms of breaking down organic matter and removal of pollutants from bio solids (Lim et al. 2016). The objective of this paper is to review the advantages of combining composting and vermicomposting in terms of both the properties of the end product and removal of pollutants.

Composting

During thermogenic composting, the organic matter is decomposed by microorganisms (Finstein and Morris 1975), so it is important to aerate the compost, in order to replenish the oxygen. Under optimal conditions, the thermal phase takes around one month (de Bertoldi et al. 1983). The main decomposers are bacteria, fungi, actinomycetes or protozoa. Over many years, composting has also been used for the bioremediation of polluted substrates (Cajthaml et al. 2002; Cai et al. 2007; Covino et al. 2016; Iranzo et al. 2018; Guo et al. 2020; Wei et al. 2020).

Vermicomposting

Vermicomposting is a process in which earthworms are used to break down organic matter (Domínguez 2004). The decomposition starts in the gizzards of the earthworms' after which the organic matter is digested by enzymes and microorganism in their guts. Product of vermicomposting is rich in nutrients and can be re-used as organic fertilizer (Yadav and Garg 2019). If a pollutant is removed from the soil via vermicomposting, the process is called vermiremediation (Rodriguez-Campos et al. 2014). Shi et al. (2019) recently defined vermiremediation as "an earthworm-based bioremediation technology that makes use of the earthworm's life cycle (i.e., feeding, burrowing, metabolism, secretion) or their interaction with other abiotic and biotic factors to accumulate and extract, transform, or degrade contaminants in the soil environment". Processes involved in vermiremediation are vermiaccumulation, vermiextraction, vermitransformation and drilodegradation (Shi et al. 2019). Earthworms can successfully remove organic micropollutants (Chachina et al. 2016; Chevillot et al. 2017; Havranek et al. 2017; Rorat et al. 2017; Lin et al. 2019; Owagboriaye et al. 2020) and heavy metals (Azizi et al. 2013a; Suthar et al. 2014; He et al. 2016).

Grasserová, A., Hanč, A., Innemanová, P., Cajthaml, T.: Composting and vermicomposting used to break down and remove pollutants from organic waste: a mini review
European Journal of Environmental Sciences, Vol. 10, No. 1, pp. 9–14
https://doi.org/10.14712/23361964.2020.2

Combination of composting and vermicomposting used to break down organic waste

Composting and vermicomposting alone are very successful methods for decomposing organic waste. However, each has its drawbacks, which can be overcome by combining the two techniques (Lim et al. 2016). Temperatures during self-heating of the compost can increase to 80 °C (Finstein and Morris 1975). These elevated temperatures (higher than 55 °C) are necessary to suppress pathogens in sludge (Grewal et al. 2006) but at the same time are lethal for earthworms (Domínguez 2004). It is therefore reasonable to start with composting, during which the pathogens are killed and the decomposition process begins. Ndegwa and Thompson (2001) confirm that by combining composting-vermicomposting eliminates pathogens. On the contrary, starting with vermicomposting, followed by composting, results in that the system does not reach temperatures high enough to kill the pathogens.

When the thermophilic phase is completed, earthworms are added to continue the decomposition and facilitate aeration of the material. The earthworms disturb the organic material and produce very small particles with favourable agrochemical properties resulting in high concentrations of available nitrogen and phosphorus (Hanc and Dreslova 2016). The size of the particles is crucial, as small particles have a large total surface area, which makes it easier for the microbes to access the material. Tognetti et al. (2005) report another benefit of the composting-vermicomposting method. As mentioned above, the thermophilic phase is detrimental to earthworms, therefore large areas are needed for spreading the material to prevent overheating. However, if the waste is subjected to the thermophilic phase prior to vermicomposting, the latter can be initiated in the surface layer, which reduces the demands on space. The same authors report a difference between compost and pre-composted vermicompost in terms of nutrient content. The pre-composted vermicompost has higher nutrient concentrations and an enhanced microbial activity resulting in a higher yield of ryegrass when applied as a fertiliser. However, these authors also point out that the quality of the product is not only dependent on the technology used, but also on the starting material, that is, the nature of the waste and bulking agent.

Table 1 gives examples of composting-vermicomposting using different kinds of organic waste. It is apparent that the incubation time for composting and vermicomposting is not the same. Composting usually takes around two to four weeks depending on the starting material and duration of the thermophilic phase. For instance, Nair et al. (2006) suggest for producing pathogen free compost from kitchen waste 9 days of pre-composting followed by 2.5 months of vermicomposting. Thermocomposting reduces both the time and area needed for vermicomposting by reducing the volume of material to be processed.

Composting-vermicomposting used to remove pollutants

Earthworms can accumulate heavy metals and organic pollutants (Sinha et al. 2008). Moreover, they increase their availability for microorganisms by grinding the waste into smaller particles. Earthworms generally improve soil microbial activity by stimulating the growth of bacteria and fungi both in their intestine and their faeces (Dendooven et al. 2011). Increase in activity of the detoxification enzymes cytochrome P450 and glutathione-S-transferase in earthworms are reported when they ingest different kinds of pollutants, which indicates earthworms are also able to degrade pollutants (Achazi et al. 1998; Zhang et al. 2009; Zhao et al. 2020). Earthworms are great accumulators of metals, especially zinc and cadmium, which are incorporated into their soft tissues. In that sense, earthworms can also act as indicators of metal pollution. Metals can also be transformed to a valent state inside earthworms, which makes them more available for plants.

Pollutants are not always completely removed from WWTP (Luo et al. 2014), in which case the WWTP sludge should not be used as a fertiliser, as it would contaminate field plants and the whole food chain. Composting and vermicomposting are both proven to be successful methods for removing pollutants (Poulsen and Bester 2010). However, vermicomposting is not suitable for the immediate remediation of WWTP sewage sludge due to the toxicity of NH_3 and CH_4 (Awiszus et al. 2018). Pre-composting with a nutrient-rich bulking agent, such as cow manure or green waste, stabilizes sewage sludge (Kaushik and Garg 2003; Hanc and Dreslova 2016). It not only reduces its toxicity to earthworms, but also adds nutrients to the final product.

Vermiremediation of sewage sludge or contaminated soil using pre-composting has been investigated (see Table 2). However, there is no detailed comparison of pre-composting-vermicomposting with composting and vermicomposting in terms of pollutant removal. Composting followed by vermiremediation is studied mainly for its efficiency in removing heavy metals and polycyclic aromatic hydrocarbons. Maňáková et al. (2014) report that combining these processes results in a greater reduction in the mobility and bioavailability of arsenic. The mobile arsenic pool is reduced to 4/9 of its initial value due to bioaccumulation. Soobhany et al. (2015) confirm the vermiaccumulation of other heavy metals with decrease in the bioaccumulation factors (BCFs) as follows: Cd > Ni > Cu > Co > Cr > Zn. In contrast, composting without earthworms results in a progressive increase in heavy metal concentrations due to the reduction in the volume of compost due to decomposition. Rorat et al.

Table 1 Summary of the results of composting-vermicomposting of organic wastes.

Organic waste	Bulking agent (amendment)	Composting duration (days)	Vermicomposting duration (days)	Earthworms used	Notes/Findings	Reference
Municipal sewage sludge digestate	Green waste, spent mushroom compost, wheat straw, biochar	43	90	*Eisenia fetida*	Similar outcomes as conventional composting, but kinetin concentration was two times higher.	Rékási et al. 2019
Vinasse	Bagasse, cow manure, zeolite	21	60	*Eisenia fetida*	Lower content of vinasse and higher content of zeolite resulted in better quality compost.	Alavi et al. 2017
Sewage sludge	Municipal solid waste, grass clippings, sawdust	30	45	*Eisenia andrei, Eisenia fetida, Dendrobaena veneta*	*Eisenia* species of earthworms exhibited stronger defence and higher ability to accumulate heavy metals.	Suleiman et al. 2017
Press mud	Cow dung, green manure plants	21	50	*Eudrilus eugeniae*	Ratio 2:1:1 (pressmud : cow dung : green manure plants) resulted in the high quality compost.	Balachandar et al. 2020
Garden waste	Cattle manure, spent mushroom substrate	21	70	*Eisenia fetida*	Ratio 2:1:1 (garden waste : cattle manure : spent mushroom substrate) resulted in high quality compost.	Gong et al. 2019
Pistachio waste	Cow dung	45	45	*Eisenia fetida*	Ratio 1:3 (pistachio waste : cow dung) resulted in high quality compost.	Esmaeili et al. 2020
Vegetable waste	Cow dung, saw dust, dried leaves	8	20	*Eisenia fetida, Eudrilus eugeniae*	Stabilized end product within a short period of time using rotary drum.	Varma and Kalamdhad 2016
Sugarcane press mud	Bagasse, sugarcane trash	30	40	*Drawida willsi*	Composting-vermicomposting method reduced the time required for composting.	Kumar et al. 2010
Rice straw, paper waste	Cow dung	21	105	*Eisenia fetida*	High fragmentation and homogeneity of vermicompost based on SEM pictures.	Sharma and Garg 2018
Press mud sludge	Cattle dung	15	135	*Eisenia fetida*	Ratio 1:3 (compressed sludge : cattle dung) resulted in good growth and fecundity of earthworms.	Bhat et al. 2016
Sewage sludge, vinasse	Rabbit manure	21	56	*Eisenia fetida*	Rabbit manure enhanced the reproduction and weight of earthworms.	Molina et al. 2013
Tomato crop residues	Almond shells	63	198	*Eisenia andrei, Eisenia fetida*	Vermicompost and pre-composted vermicompost had similar properties.	Fornes et al. 2012

(2017) also report vermiaccumulation of heavy metals, with decreases in BCFs as follows: Cd > Cu > Zn > Ni >Cr > Pb. Kharrazi et al. (2014), on the other hand, report increases in heavy metals concentrations in compost produced by the composting-vermicomposting process. These authors discuss possible reasons for this e.g. mineralization making metals more available or loss of the overall mass due to decomposition. They did not study the vermiaccumulation of heavy metals. Suleiman et al. (2017) report the accumulation of heavy metals by three species of earthworm, namely *Eisenia andrei, Eisenia fetida* and *Dendrobaena veneta*. BCFs were ranked as follows: Cd > Co > Cu > Zn > Ni > Pb > Cr. Of the earthworms studied, the *Eisenia* species exhibit the highest ability to vermiaccumulate heavy metals.

The fate of polycyclic aromatic hydrocarbons (PAHs) during vermicomposting is also reported. Rorat et al. (2017) report a significant reduction in 16 priority PAHs after 30 days of composting followed by 35 days of vermicomposting. Total amount of PAHs is reduced by up to 85.75%, with the reduction in naphthalene, acenaphtylene, phenanthrene and benzo(g,h,i)perylene the most marked. In addition to vermiaccumulation, degradation is reported, namely that of 5-rings PAHs to 3- and 4-rings PAHs. Composting alone is efficient when degrading PAHs (Cajthaml and Šašek 2005). However, degradation occurs in the final maturation phase, which can take up to 300 days. Composting-vermicomposting could therefore potentially decrease the time required to remove PAHs.

Table 2 Summary of the results of pre-composting followed by vermiremediation of pollutants.

Pollutant	Matrix	Bulking agent (amendment)	Composting duration (days)	Vermicomposting duration (days)	Earthworms used	Notes/Findings	Reference
Arsenic	Sewage sludge	Horse manure, sawdust, grass clippings	90	90	*Eisenia fetida*	Decrease in mobility to 4/9.	Maňáková et al. 2014
Heavy metals	Municipal solid waste	Food waste, paper waste, yard waste, cow dung	17	53	*Eudrilus eugeniae*	BCFs: Cd > Ni > Cu > Co > Cr > Zn.	Soobhany et al. 2015
Heavy metals	Sewage sludge	*Miscanthus* green waste, market waste, organic fraction of municipal solid waste	30	35	*Eisenia andrei*	BCFs: Cd > Cu > Zn > Ni > Cr > Pb.	Rorat et al. 2017
Heavy metals	Sewage sludge	Corn waste, cow dung, compost, paper	30	40	*Eisenia fetida*	Increase in heavy metal content due to the decrease in overall mass.	Kharrazi et al. 2014
Heavy metals	Pig manure	Rice straw	15	45	*Eisenia fetida*	Increase in the Cu and Zn availability after vermicomposting.	Zhu et al. 2014
Heavy metals	Sewage sludge	Spent mushroom compost	21	105	*Lumbricus rubellus*	90–98.7% removal of Cr, Cd and Pb.	Azizi et al. 2013a
Heavy metals	Sewage sludge	Municipal solid wastes, grass clippings, sawdust	30	45	*Eisenia andrei, Eisenia fetida, Dendrobaena veneta*	BCFs: Cd > Co > Cu > Zn > Ni > Pb > Cr.	Suleiman et al. 2017
Petroleum hydrocarbons	Soil	Compost	x (compost as amendment)	15	*Eisenia fetida*	Enrichment of microorganisms after adding compost as an amendment.	Ceccanti et al. 2006
16 priority PAHs	Sewage sludge	*Miscanthus* green waste, markets waste, organic fraction of municipal solid waste	30	35	*Eisenia andrei*	Degradation of 5-ring PAHs to 3- and 4-ring PAHs is reported.	Rorat et al. 2017
Anthracene, phenanthrene, benzo(α)pyrene	Soil, sewage sludge	x	21	60	*Lumbricus rubellus*	99.99% PAHs removed.	Azizi et al. 2013b
Asphaltenes	Heavy fuel oil	Cow bedding, rice husks, seaweed extracts, potato peelings	112	183	*Eisenia fetida*	Microorganisms obtained carbon and energy from asphaltenes.	Martín-Gil et al. 2008

Conclusion

Pre-composting is an important step when decomposing organic waste by vermicomposting. It facilitates its breakdown, suppresses pathogens and decomposes toxic compounds, which could harm the earthworms. Moreover, as it results in a reduction in mass, less time and space is needed for vermicomposting. Pre-composting can be also used prior to the vermiremediation of WWTPs sludge and contaminated soil. A combination of composting and vermicomposting has been successfully used for removing polycyclic aromatic hydrocarbons and heavy metals. However, no research has been done on using this method for removing other organic pollutants, such as pharmaceuticals or endocrine disruptors. This mini-review indicates that composting-vermicomposting is a promising low-cost and environmentally friendly way of treating contaminated WWTP sludge.

Acknowledgements

This work was supported by The Ministry of Agriculture of the Czech Republic (grant number QK1910095).

REFERENCES

Achazi RK, Flenner C, Livingstone DR, Peters LD, Schaub K, Scheiwe E (1998) Cytochrome P450 and dependent activities in unexposed and PAH-exposed terrestrial annelids. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 121: 339–350.

Alavi N, Daneshpajou M, Shirmardi M, Goudarzi G, Neisi A, Babaei AA (2017) Investigating the efficiency of co-composting and vermicomposting of vinasse with the mixture of cow manure wastes, bagasse, and natural zeolite. Waste Manag 69: 117–126.

Awiszus S, Meissner K, Reyer S, Müller J (2018) Ammonia and methane emissions during drying of dewatered biogas digestate in a two-belt conveyor dryer. Bioresour Technol 247: 419–425.

Azizi AB, Lim MPM, Noor ZM, Abdullah N (2013a) Vermiremoval of heavy metal in sewage sludge by utilising *Lumbricus rubellus*. Ecotoxicol Environ Saf 90: 13–20.

Azizi AB, Liew KY, Noor ZM, Abdullah N (2013b) Vermiremediation and mycoremediation of polycyclic aromatic hydrocarbons in soil and sewage sludge mixture: a comparative study. Int J Environ Sci Dev 4: 565–568.

Balachandar R, Baskaran L, Yuvaraj A, Thangaraj R, Subbaiya R, Ravindran B, Chang SW, Karmegam N (2020) Enriched pressmud vermicompost production with green manure plants using *Eudrilus eugeniae*. Bioresour Technol 299: 122578.

Bhat SA, Singh J, Vig AP (2016) Effect on growth of earthworm and chemical parameters during vermicomposting of pressmud sludge mixed with cattle dung mixture. Procedia Environ Sci 35: 425–434.

Cai Q-Y, Mo C-H, Wu Q-T, Zeng Q-Y, Katsoyiannis A, Ferard J-F (2007) Bioremediation of polycyclic aromatic hydrocarbons (PAHs)-contaminated sewage sludge by different composting processes. J Hazard Mater 142: 535–542.

Cajthaml T, Bhatt M, Šašek V, Matějů V (2002) Bioremediation of PAH-contaminated soil by composting: A case study. Folia Microbiol 47: 696–700.

Cajthaml T, Šašek V (2005) Application of supercritical fluid extraction (SFE) to predict bioremediation efficacy of long-term composting of PAH-contaminated soil. Environ Sci Technol 39: 8448–8452.

Ceccanti B, Masciandaro G, Garcia C, Macci C, Doni S (2006) Soil bioremediation: combination of earthworms and compost for the ecological remediation of a hydrocarbon polluted soil. Water Air Soil Pollut 177: 383–397.

Chachina SB, Voronkova NA, Baklanova ON (2016) Biological remediation of the petroleum and diesel contaminated soil with earthworms *Eisenia fetida*. Procedia Eng 152: 122–133.

Chevillot F, Convert Y, Desrosiers M, Cadoret N, Veilleux É, Cabana H, Bellenger JP (2017) Selective bioaccumulation of neonicotinoids and sub-lethal effects in the earthworm *Eisenia andrei* exposed to environmental concentrations in an artificial soil. Chemosphere 186: 839–847.

Clarke BO, Smith SR (2011) Review of 'emerging' organic contaminants in biosolids and assessment of international research priorities for the agricultural use of biosolids. Environ Int 37: 226–247.

Covino S, Fabianová T, Křesinová Z, Čvančarová M, Burianová E, Filipová A, Voříšková J, Baldrian P, Cajthaml T (2016) Polycyclic aromatic hydrocarbons degradation and microbial community shifts during co-composting of creosote-treated wood. J Hazard Mater 301: 17–26.

de Bertoldi M, Vallini G, Pera A (1983) The biology of composting: A review. Waste Manag Res 1: 157–176.

Dendooven L, Alvarez-Bernal D, Contreras-Ramos SM (2011) Earthworms, a means to accelerate removal of hydrocarbons (PAHs) from soil? A mini-review. Pedobiologia 54: 187–192.

Domínguez J (2004) State-of-the-art and new perspectives on vermicomposting research. In: Earthworm Ecology (2nd Edition). CRC Press, USA, pp 401–424.

Esmaeili A, Khoram MR, Gholami M, Eslami H (2020) Pistachio waste management using combined composting-vermicomposting technique: physico-chemical changes and worm growth analysis. J Clean Prod 242: 118523.

Finstein MS, Morris ML (1975) Microbiology of municipal solid waste composting. Adv Appl Microbiol 19: 113–151.

Fornes F, Mendoza-Hernández D, García-de-la-Fuente R, Abad M, Belda RM (2012) Composting versus vermicomposting: a comparative study of organic matter evolution through straight and combined processes. Bioresour Technol 118: 296–305.

Gong X, Li S, Carson MA, Chang SX, Wu Q, Wang L, An Z, Sun X (2019) Spent mushroom substrate and cattle manure amendments enhance the transformation of garden waste into vermicomposts using the earthworm *Eisenia fetida*. J Environ Manage 248: 109263.

Grewal SK, Rajeev S, Sreevatsan S, Michel FC (2006) Persistence of Mycobacterium avium subsp. *paratuberculosis* and other zoonotic pathogens during simulated composting, manure packing, and liquid storage of dairy manure. Appl Env Microbiol 72: 565–574.

Guo Y, Rene ER, Wang J, Ma W (2020) Biodegradation of polyaromatic hydrocarbons and the influence of environmental factors during the co-composting of sewage sludge and green forest waste. Bioresour Technol 297: 122434.

Hanc A, Dreslova M (2016) Effect of composting and vermicomposting on properties of particle size fractions. Bioresour Technol 217: 186–189.

Havranek I, Coutris C, Norli HR, Rivier PA, Joner EJ (2017) Uptake and elimination kinetics of the biocide triclosan and the synthetic musks galaxolide and tonalide in the earthworm *Dendrobaena veneta* when exposed to sewage sludge. Environ Toxicol Chem 36: 2068–2073.

He X, Zhang Y, Shen M, Zeng G, Zhou M, Li M (2016) Effect of vermicomposting on concentration and speciation of heavy metals in sewage sludge with additive materials. Bioresour Technol 218: 867–873.

Hickman ZA, Reid BJ (2008) Earthworm assisted bioremediation of organic contaminants. Environ Int 34: 1072–1081.

Iranzo M, Gamón M, Boluda R, Mormeneo S (2018) Analysis of pharmaceutical biodegradation of WWTP sludge using composting and identification of certain microorganisms involved in the process. Sci Total Environ 640–641: 840–848.

Kaushik P, Garg VK (2003) Vermicomposting of mixed solid textile mill sludge and cow dung with the epigeic earthworm *Eisenia foetida*. Bioresour Technol 90: 311–316.

Kharrazi SM, Younesi H, Abedini-Torghabeh J (2014) Microbial biodegradation of waste materials for nutrients enrichment and heavy metals removal: An integrated composting-vermicomposting process. Int Biodeterior Biodegrad 92: 41–48.

Kumar R, Verma D, Singh BL, Kumar U (2010) Composting of sugar-cane waste by-products through treatment with microorganisms and subsequent vermicomposting. Bioresour Technol 101: 6707–6711.

Lim SL, Lee LH, Wu TY (2016) Sustainability of using composting and vermicomposting technologies for organic solid waste biotransformation: recent overview, greenhouse gases emissions and economic analysis. J Clean Prod 111: 262–278.

Lin Z, Zhen Z, Liang Y, Li J, Yang J, Zhong L, Zhao L, Li Y, Luo C, Ren L (2019) Changes in atrazine speciation and the degradation pathway in red soil during the vermiremediation process. J Hazard Mater 364: 710–719.

Luo Y, Guo W, Ngo HH, Nghiem LD, Hai FI, Zhang J, Liang S, Wang XC (2014) A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. Sci Total Environ 473: 619–641.

Maňáková B, Kotyza J, Svobodová M, Hofman J (2014) Effects of combined composting and vermicomposting of waste sludge on arsenic fate and bioavailability. J Hazard Mater 280: 544–551.

Martín-Gil J, Navas-Gracia LM, Gómez-Sobrino E, Correa-Guimaraes A, Hernández-Navarro S, Sánchez-Báscones M, Ramos-Sánchez MdC (2008) Composting and vermicomposting experiences in the treatment and bioconversion of asphaltens from the Prestige oil spill. Bioresour Technol 99: 1821–1829.

Molina MJ, Soriano MD, Ingelmo F, Llinares J (2013) Stabilisation of sewage sludge and vinasse bio-wastes by vermicomposting with rabbit manure using *Eisenia fetida*. Bioresour Technol 137: 88–97.

Nair J, Sekiozoic V, Anda M (2006) Effect of pre-composting on vermicomposting of kitchen waste. Bioresour Technol 97: 2091–2095.

Ndegwa PM, Thompson SA (2001) Integrating composting and vermicomposting in the treatment and bioconversion of biosolids. Bioresour Technol 76: 107–112.

Owagboriaye F, Dedeke G, Bamidele J, Aladesida A, Isibor P, Feyisola R, Adeleke M (2020) Biochemical response and vermiremediation assessment of three earthworm species (Alma *millsoni*, *Eudrilus eugeniae* and *Libyodrilus violaceus*) in soil contaminated with a glyphosate-based herbicide. Ecol Indic 108: 105678.

Poulsen TG, Bester K (2010) Organic micropollutant degradation in sewage sludge during composting under thermophilic conditions. Environ Sci Technol 44: 5086–5091.

Rékási M, Mazsu N, Draskovits E, Bernhardt B, Szabó A, Rivier P-A, Farkas C, Borsányi B, Pirkó B, Molnár S (2019) Comparing the agrochemical properties of compost and vermicomposts produced from municipal sewage sludge digestate. Bioresour Technol 291: 121861.

Rodriguez-Campos J, Dendooven L, Alvarez-Bernal D, Contreras-Ramos SM (2014) Potential of earthworms to accelerate removal of organic contaminants from soil: A review. Appl Soil Ecol 79: 10–25.

Rorat A, Wloka D, Grobelak A, Grosser A, Sosnecka A, Milczarek M, Jelonek P, Vandenbulcke F, Kacprzak M (2017) Vermiremediation of polycyclic aromatic hydrocarbons and heavy metals in sewage sludge composting process. J Environ Manage 187: 347–353.

Sharma K, Garg VK (2018) Comparative analysis of vermicompost quality produced from rice straw and paper waste employing earthworm *Eisenia fetida* (Sav.). Bioresour Technol 250: 708–715.

Shi Z, Liu J, Tang Z, Zhao Y, Wang C (2019) Vermiremediation of organically contaminated soils: Concepts, current status, and future perspectives. Appl Soil Ecol 147: 103377.

Sinha RK, Bharambe G, Ryan D (2008) Converting wasteland into wonderland by earthworms – a low-cost nature's technology for soil remediation: a case study of vermiremediation of PAHs contaminated soil. Environmentalist 28: 466–475.

Soobhany N, Mohee R, Garg VK (2015) Comparative assessment of heavy metals content during the composting and vermicomposting of municipal solid waste employing *Eudrilus eugeniae*. Waste Manag 39: 130–145.

Suleiman H, Rorat A, Grobelak A, Grosser A, Milczarek M, Płytycz, B, Kacprzak M, Vandenbulcke F (2017) Determination of the performance of vermicomposting process applied to sewage sludge by monitoring of the compost quality and immune responses in three earthworm species: *Eisenia fetida*, *Eisenia andrei* and *Dendrobaena venet*a. Bioresour Technol 241: 103–112.

Suthar S, Sajwan P, Kumar K (2014) Vermiremediation of heavy metals in wastewater sludge from paper and pulp industry using earthworm *Eisenia fetida*. Ecotoxicol Environ Saf 109: 177–184.

Tognetti C, Laos F, Mazzarino MJ, Hernández MT (2005) Composting vs. vermicomposting: a comparison of end product quality. Compost Sci Util 13: 6–13.

Varma VS, Kalamdhad AS (2016) Efficiency of rotary drum composting for stabilizing vegetable waste during pre-composting and vermicomposting. Environ Process 3: 829–841.

Wei Y, Zhao Y, Zhao X, Gao X, Zheng Y, Zuo H, Wei Z (2020) Roles of different humin and heavy-metal resistant bacteria from composting on heavy metal removal. Bioresour Technol 296: 122375.

Yadav A, Garg VK (2019) Biotransformation of bakery industry sludge into valuable product using vermicomposting. Bioresour Technol 274: 512–517.

Zhang X, Lu Y, Shi Y, Chen C, Yang Z, Li Y, Feng, Y (2009) Antioxidant and metabolic responses induced by cadmium and pyrene in the earthworm *Eisenia fetida* in two different systems: contact and soil tests. Chem Ecol 25: 205–215.

Zhao S, Wang B, Zhong Z, Liu T, Liang T, Zhan J (2020) Contributions of enzymes and gut microbes to biotransformation of perfluorooctane sulfonamide in earthworms (*Eisenia fetida*). Chemosphere 238: 124619.

Zhu W, Yao W, Zhang Z, Wu Y (2014) Heavy metal behavior and dissolved organic matter (DOM) characterization of vermicomposted pig manure amended with rice straw. Environ Sci Pollut Res 21: 12684–12692.

nanomaterials

MDPI

Article

In Vitro Study of the Toxicity Mechanisms of Nanoscale Zero-Valent Iron (nZVI) and Released Iron Ions Using Earthworm Cells

Jaroslav Semerad [1,2], Natividad Isabel Navarro Pacheco [1,3], Alena Grasserova [1,2], Petra Prochazkova [1], Martin Pivokonsky [4], Lenka Pivokonska [4] and Tomas Cajthaml [1,2,*]

1 Institute of Microbiology of the Czech Academy of Sciences, Vídeňská 1083, CZ-142 20, Prague 4, Czech Republic; jaroslav.semerad@biomed.cas.cz (J.S.); natividad.pacheco@biomed.cas.cz (N.I.N.P.); alena.grasserova@biomed.cas.cz (A.G.); kohler@biomed.cas.cz (P.P.)
2 Institute for Environmental Studies, Faculty of Science, Charles University, Benátská 2, CZ-128 01, Prague 2, Czech Republic
3 First Faculty of Medicine, Charles University, Kateřinská 1660/32, CZ-121 08, Prague 2, Czech Republic
4 Institute of Hydrodynamics of the Czech Academy of Sciences, Pod Paťankou 30/5, CZ-166 12, Prague 6, Czech Republic; pivo@ih.cas.cz (M.P.); pivokonska@ih.cas.cz (L.P.)
* Correspondence: cajthaml@biomed.cas.cz

Received: 29 September 2020; Accepted: 28 October 2020; Published: 3 November 2020

Abstract: During the last two decades, nanomaterials based on nanoscale zero-valent iron (nZVI) have ranked among the most utilized remediation technologies for soil and groundwater cleanup. The high reduction capacity of elemental iron (Fe^0) allows for the rapid and cost-efficient degradation or transformation of many organic and inorganic pollutants. Although worldwide real and pilot applications show promising results, the effects of nZVI on exposed living organisms are still not well explored. The majority of the recent studies examined toxicity to microbes and to a lesser extent to other organisms that could also be exposed to nZVI via nanoremediation applications. In this work, a novel approach using amoebocytes, the immune effector cells of the earthworm *Eisenia andrei*, was applied to study the toxicity mechanisms of nZVI. The toxicity of the dissolved iron released during exposure was studied to evaluate the effect of nZVI aging with regard to toxicity and to assess the true environmental risks. The impact of nZVI and associated iron ions was studied in vitro on the subcellular level using different toxicological approaches, such as short-term immunological responses and oxidative stress. The results revealed an increase in reactive oxygen species production following nZVI exposure, as well as a dose-dependent increase in lipid peroxidation. Programmed cell death (apoptosis) and necrosis were detected upon exposure to ferric and ferrous ions, although no lethal effects were observed at environmentally relevant nZVI concentrations. The decreased phagocytic activity further confirmed sublethal adverse effects, even after short-term exposure to ferric and ferrous iron. Detection of sublethal effects, including changes in oxidative stress-related markers such as reactive oxygen species and malondialdehyde production revealed that nZVI had minimal impacts on exposed earthworm cells. In comparison to other works, this study provides more details regarding the effects of the individual iron forms associated with nZVI aging and the cell toxicity effects on the specific earthworms' immune cells that represent a suitable model for nanomaterial testing.

Keywords: nanoecotoxicology; earthworms; coelomocytes; reactive oxygen species; nanoscale zero-valent iron (nZVI); ferrous and ferric ions; phagocytosis; lipid peroxidation; apoptosis

1. Introduction

Nanoscale zero-valent iron (nZVI) and derived nanomaterials are widely applied for nanoremediation and have shown the potential to degrade inorganic and organic pollutants in many laboratory experiments and pilot field applications [1,2]. For remediation, nZVI-based materials in the form of highly concentrated suspensions (up to 10–30 g/L) are usually injected into contaminated soil or groundwater [1]. In addition to interacting with the targeted pollutant, nZVI can also interact with resident soil (micro)organisms [3]. It is noteworthy that due to the mobility of nZVI, highly concentrated suspension disperses in the groundwater aquifer after application and within a relatively short time period the local, environmentally relevant concentrations of this nanomaterial are in the range of mg/L [4,5]. Over the past decade, many authors have studied the negative effects of nZVI and materials derived from it on potentially exposed organisms [6]. The majority of these studies were mainly focused on toxicity and the mechanisms of adverse effects in exposed microorganisms [7]. Only a few authors have explored the effects of nZVI on earthworms and, due to the heterogeneity of nanomaterial types (size, crystallinity, and coating), results have varied greatly [8–14]. Using OECD tests, some of the authors were able to observe a decrease in the viability, body weight, cocoons production, growth, and reproduction rate of earthworms after exposure to nZVI synthesized by the borohydride method [8,9,11,12]. However, different types of nZVI at similar concentrations did not cause any changes in earthworm viability [13,14]. Intrinsically, nZVI is a reactive material with a high reduction capacity, which can result in reactive oxygen species (ROS) generation and oxidative stress induction [15]. Many authors have already described the oxidative stress and associated cellular damage that occurs in microorganisms after exposure to nZVI [16,17]. It is worth noting that only two studies employed oxidative stress determination in earthworms after exposure to nZVI [11,14]. Earthworm immune cells float freely in the coelomic fluid, where they encounter pollutants, bacteria, viruses and nanoparticles (NPs). These cells are divided into two main populations, eleocytes (free chloragogen cells with mainly nutritive function) and amoebocytes (hyaline and granular immune effector cells) and are frequently used for in vitro toxicity studies of pesticides, heavy metals or nanoparticles [18–22]. For example, Hayashi and Bigorgne described how Ag and TiO_2 nanoparticles induce oxidative stress and affect the immune function of these cells, demonstrating their potential for studying the fate and adverse effects of new nanomaterials [23,24].

When it is used, nZVI interacts with targeted pollutant molecules and untargeted compounds dissolved in the groundwater or naturally present in the soil [25,26]. This process of oxidation—the transformation of elemental iron to iron oxides and hydroxides—is called aging. Aggregation of nanoparticles is one of the most crucial factors affecting their toxicity, as an increase in their size results in decreased bioavailability and loss of their unique properties at the nano level. Therefore, direct characterization of nanomaterials in the exposure medium is necessary to understand the mechanisms of toxicity and avoid data misinterpretation. It was recently demonstrated that the aging process is accompanied by strong morphological nanomaterial changes, aggregate formation, soluble iron release, and a marked decrease in the toxicity of nZVI to bacterial species [25,27]. The effect of nZVI and its aggregates/aging products on earthworms is still not well described. Specifically, the toxicity of soluble forms of iron released from nZVI particles during the aging process to earthworms is still unknown. For this reason, the present work studies the sublethal effects of nZVI on the two subtypes of coelomocytes with a focus on the role of iron ions in oxidative stress and overall toxicity during the aging process.

2. Materials and Methods

2.1. Zero-Valent Iron Nanoparticles (nZVI NPs)

In the present study only one type of commercially available, air-stable modified form of nZVI was tested (NANOFER STAR; NANO IRON, Czech Republic). The surface-passivated nZVI particles were synthesized by solid–gas thermal reduction of an iron oxide precursor and partially oxidized to achieve stability in air. The synthesis and characterization of the particles used were recently described in detail

by Kaslik et al. (2018) [28]. Briefly, NANOFER STAR particles are core-shell spherical particles that reach a size of approximately 70 nm and are composed of a core of elemental iron (approximately 90%) and a 4-nm-thick shell predominantly made of magnetite (Fe_3O_4).

2.2. Characterization of nZVI and Quantification of Soluble Iron Species

The aggregation of nZVI NPs was analyzed by scanning electron microscopy (SEM). The aggregation experiments were performed under the same conditions as the exposure experiments to reflect the state of nanoparticles during the exposure (darkness, 20 °C, 0 and 24 h, distilled water and RPMI 1640 medium; Lonza, Walkersville, MD, USA). After the experiments, the samples of nZVI and RPMI 1640 medium + nZVI NPs were passed through filters to retain the particles. A glass vacuum filtration device (Advantec MFS, Inc., Tokyo, Japan) and a stainless-steel manifold (Speed Flow, Crami Group Srl, Milan, Italy) connected to a vacuum pump (Rocker 300, Rocker Scientific Co., Taiwan, ROC) were used. Separate filters were prepared for quantitative analysis of the particles by SEM. For SEM, polytetrafluoroethylene (PTFE) membrane filters (diameter of 13 mm; Merck Millipore, ME, USA) with a 0.2-µm pore size were used. Before analysis, the filters were dried in an oven (30 °C, 30 min) and stored in capped glass Petri dishes in a desiccator. A Vega high-resolution scanning electron microscope (Tescan, Brno, Czech Republic) was used to determine the number and size of the particles on the filters. Three cut-outs (3 × 8 mm) from each filter were prepared and analyzed. Prior to imaging, a conductive gold layer was sputtered onto the filter cut-outs. Images were then taken with an optimized acceleration voltage of 10 kV and detector working distance of approximately 9 mm. The number and size of the particles from each cut-out were determined by using SigmaScan 5 software (Systat Software, Inc., San Jose, CA, USA), and the results were then recalculated for the whole filter area. The SEM analyses were performed with 100 mg/L nZVI NPs dispersed in distilled water and RPMI 1640 cultivation medium at 2, 6, and 24 h. Fe concentrations were measured using inductively coupled plasma optical emission spectrometry (ICP-OES, 5110 Series, Agilent Technologies, Santa Clara, CA, USA). To separate undissolved NPs, the samples were centrifuged three times at 10,000 g for 10 min. Fe measurements were conducted in triplicate with errors at less than 2%. A calibration standard (Astasol) was purchased from ANALYTIKA, spol. s r. o. (Prague, Czech Republic). The range of calibration of Fe concentrations was 0.02–5 mg/L.

2.3. Earthworms and Coelomocytes Extrusion

The *Eisenia andrei* earthworms were collected from our laboratory vermicompost breeding. Adult earthworms with clitella were used for the extrusion of earthworm coelomocytes. Prior to coelomocytes extrusion, the earthworms were kept on wet filter paper for 48 h to clean their gut contents. Then, coelomocytes were extracted via a noninvasive method. Briefly, the earthworms were placed into a falcon tube andextrusion buffer (50.4 mM guaiacol glyceryl ether (GGE; Sigma-Aldrich; Steinheim, Germany) and 5.37 mM EDTA (Sigma-Aldrich, Steinheim, Germany) in PBS 3:2; 2 mL per earthworm) was applied to the earthworms for 2 min. Then, the earthworms were removed from the falcon tube and the collected cells were kept on ice before the following washing steps. The coelomocytes were washed twice with PBS (3:2, 176 mOsm, pH 7.3; 200× *g*, 4 °C, 10 min). RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum (FBS; Life technologies, Carlsbad, CA, USA), 1 M HEPES (Sigma-Aldrich; Gillingham, UK), 100 mM sodium pyruvate (Sigma-Aldrich, Steinheim, Germany), 100 mg/mL gentamycin (Corning, Manassas, VA, USA) and antibiotic-antimycotic solution (Sigma-Aldrich, Steinheim, Germany) was used for cell culture. The culture medium was diluted to 60% (*v*/*v*) with autoclaved milli-Q water (0.05 µS/cm) [29]. Later, nZVI NPs were freshly suspended in water and dispersed in an ultrasound bath (15 min, 300 W, 38 kHz). The nZVI NPs were then vortexed prior to being resuspended in the culture medium (1, 10, and 100 mg/L nZVI NPs). Coelomocytes were incubated in the dark at 20 °C for 2, 6 and 24 h in triplicate (96 well plates, total volume of 100 µL, phagocytosis and apoptosis: 2×10^5 cells/well, ROS quatification: 1×10^5 cells/well).

2.4. Quantification of Reactive Oxygen Species and Lipid Peroxidation

After incubation with 1, 10 and 100 mg/L nZVI NPs for 2, 6 and 24 h, the cells were washed with PBS (3:2; 200× *g*, 4 °C, 10 min); then, 2′,7′-dichlorofluorescein diacetate (DCF-DA; 1:1000 in PBS 3:2; Sigma-Aldrich; Steinheim, Germany) was applied to the cells for 15 min. Afterwards, the cells were washed twice with PBS (3:2; 200× *g*, 4 °C, 10 min). Then, the samples were stained with propidium iodide (PI, 1 mg/L; Sigma-Aldrich; Steinheim, Germany) and analyzed by flow cytometry. To quantify the lipid peroxidation induced by nZVI NPs, a previously optimized protocol for measuring malondialdehyde (MDA) levels in bacterial cultures was used with slight modification to examine earthworm cells [16]. The method is based on quantification of the MDA complex with thiobarbituric acid by HPLC-FLD. Earthworm coelomocytes (1 million cells/mL) were exposed to 3 different concentrations of nZVI NPs (1, 10, and 100 mg/L) and two different ionic forms of iron ($FeCl_2$ and $Fe_2(SO_4)_3$; 100 mg/L). Following exposure, all samples were frozen, stored at −20 °C and analyzed within 2 weeks by high-performance liquid chromatography with fluorescence detection (HPLC-FLD, Waters, Milford, MA, USA). Using external calibration, the final results were determined and are expressed as the production of MDA [nmol/L] per 1 million cells.

2.5. Phagocytosis

The phagocytic assay was performed with fluorescent beads (Fluoresbrite® Plain YG; 1 µm microspheres diameter; Polysciencies, Inc., Warrington, PA, UK). After incubation with 1, 10 and 100 mg/L nZVI NPs after 2, 6 and 24 h, the beads were added at a ratio of 1:100 (cells:beads) and incubated for 18 h at 17 °C. After incubation, the cells were washed twice with PBS (3:2; 200× *g*, 4 °C, 10 min), stained with PI (1 mg/L) and analyzed by flow cytometry. Finally, the phagocytosis is expressed as a percentage of engulfed fluorescence beads by the cells.

2.6. Viability, Apoptosis, and Necrosis Analyses

Viability and cell death stage assays were performed after incubation of the coelomocytes with 1, 10 and 100 mg/L nZVI NPs. The cell suspension was washed twice with Annexin V buffer (200× *g*, 4 °C, 10 min; 0.01M Hepes (pH 7.4), 0.14M NaCl, and 2.5 mM $CaCl_2$ solution) and stained with 30 µL of Annexin V (Thermo Fisher Scientific, Eugene, OR, USA) for 15 min in the dark at room temperature. Prior to flow cytometry analysis, the suspended cells were stained with PI (1 mg/L; Sigma-Aldrich; Steinheim, Germany).

2.7. Flow Cytometry

Cellular ROS production, viability, phagocytic activity, and the stages of cell death were analyzed with a flow cytometer (LSR II; BD biosciences, San Jose, CA, USA). To assess the reliability of these assays, 10 mM and 100 mM H_2O_2 (Lachner, Neratovice, Czech Republic) were used as positive controls for the endpoints measured by flow cytometry. The forward and side scatter and stain fluorescence settings were adjusted for each coelomocyte subtype and fluorescent probe (Figure S1). For analysis, 10,000 events were detected, and the obtained data were analyzed using FlowJo (9.9.4 version, BD Biosciences, San Jose, CA, USA). Each experiment was performed in triplicate for each component/substance, and the calculated standard deviation is plotted in the figures. For postprocessing the statistical analysis, two-way ANOVA and Bonferroni's post test were used (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) by GraphPad Prism (8.3.1 version, San Diego, CA, USA)

3. Results and Discussion

3.1. Particle Aggregation and Iron Release

The results of SEM examination revealed an increase in aggregate formation between fresh nZVI NPs and nZVI NPs aged for 24 h in distilled water (Figure 1a,b). However, the aggregation process was

much more intense upon RPMI 1640 medium exposure than in distilled water (Figure 1c,d). Analysis of particle size distribution confirmed an increase in aggregate formation over time and revealed a higher aggregation rate in the RPMI 1640 medium than in distilled water (Figure 2). The aggregate size reached values of 19.68 ± 2.77, 33.83 ± 4.11, 228.44 ± 11.30, and 359.06 ± 16.82 µm for fresh nZVI in distilled water, aged nZVI (24 h) in distilled water, fresh nZVI in RPMI 1640 medium, and aged nZVI (24 h) in RPMI 1640 medium, respectively. nZVI particles aggregated and formed larger clusters after exposure, suggesting that after the application of nZVI NPs in remediation practices, rapid aggregation can be expected depending on the complexity of the soil/groundwater [30–32]. On the other hand, the composition of the exposure media, especially the protein content, could also affect the behavior of NPs such as aggregation, bioavailability, and respective toxicity [33]. Many different proteins present in biological tissues/media could form a protein corona around NPs that affects NPs' behavior and its transport into the cells [34]. The RPMI 1640 medium has been supplemented with FBS which contains mainly bovine serum albumin and a great variety of other proteins. One of the possible explanations of the nZVI aggregation in RPMI 1640 medium was the presence of albumin (and other proteins) as it was documented in a study with TiO_2 NPs conducted by Márquez et al. (2017) [35].

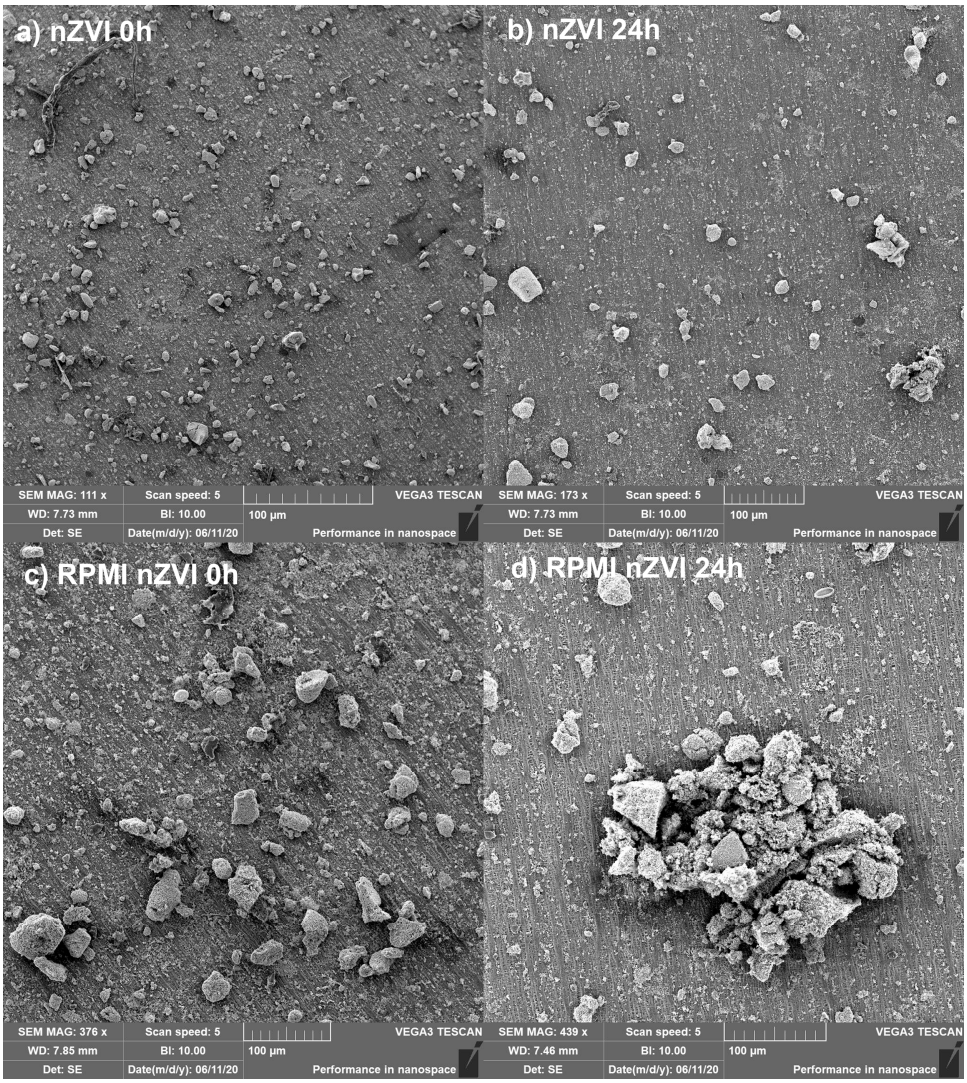


Figure 1. Scanning electron microscopy (SEM) images of fresh and aged nanoscale zero-valent iron (nZVI) in water and in the exposure medium (RPMI 1640): (**a**) Freshly prepared nZVI in distilled water; (**b**) nZVI aged for 24 h in distilled water; (**c**) Freshly prepared nZVI in RPMI 1640 medium; (**d**) nZVI aged for 24 h in RPMI 1640 medium.

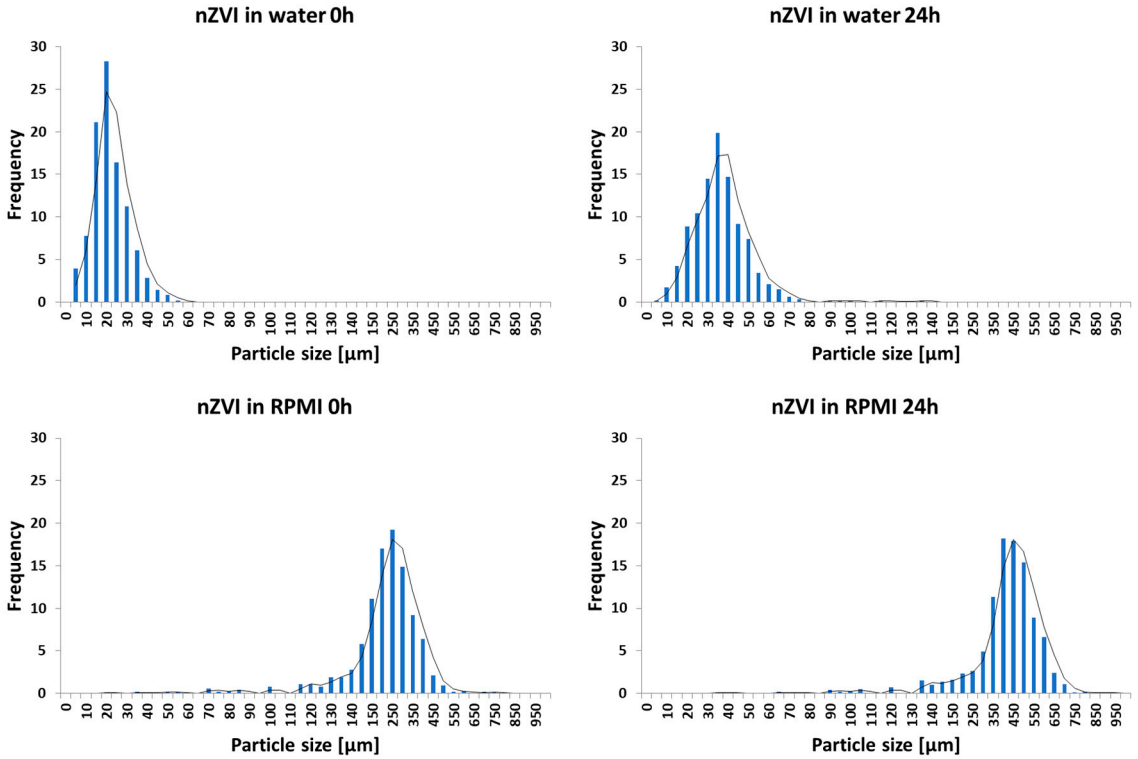


Figure 2. Particle size distribution of fresh and aged nZVI in distilled water and the exposure medium (RPMI 1640).

Moreover, ICP-OES analysis of dissolved iron demonstrated that ions were released from the nanomaterial (Figure 3). The dissolved concentrations corresponded to only approximately 0.1–0.2% of the original amount of nZVI NPs depending on the duration of exposure in RPMI 1640 medium. The analysis of the dissolved iron forms revealed that the concentration in case of nZVI reached maximally 0.15 mg/L in comparison with 7.72 to 9.56 and 3.82 to 4.46 mg/L detected from $FeCl_2$ (source of Fe^{2+} ions) and $Fe_2(SO_4)_3$ (source of Fe^{3+} ions). Notably, under specific conditions, nZVI can be oxidized/transformed into ferric and ferrous species that can be involved in ROS generation through Fenton-like processes [36,37].

3.2. Viability, Apoptosis, and Necrosis

Viability analysis did not show any significant effect following exposure of both granular and hyaline amoebocytes to either nZVI NPs at the tested concentrations or the tested ionic forms of iron (Figure 4; Figure 5; Figures S2–S8). The absence of a significant decrease in earthworm cell viability at concentrations of up to 100 mg/L is consistent with previous in vivo studies demonstrating no effect on whole organisms at these levels. Yirsaw et al. (2016) did not observe decreased survival in 3 different soils at concentrations of up to 3 g/kg [14]. However, it is worth noting that other authors have tested different types of nZVI NPs and exposure setups, which resulted in a significant decrease in the viability and body weight of exposed earthworms [8–10]. One possible explanation for the contradictory results of these published works is the formation of heterogeneous aggregates and, thus, different cellular (or animal) uptake due to differences in nZVI NPs aggregation rate [30,38]. The wide variety of modified forms of nZVI NPs being tested currently further complicates comparisons of the results; however, based on the results of previous studies, it is clear that nZVI NPs can be toxic to earthworms [8,9,11,12].

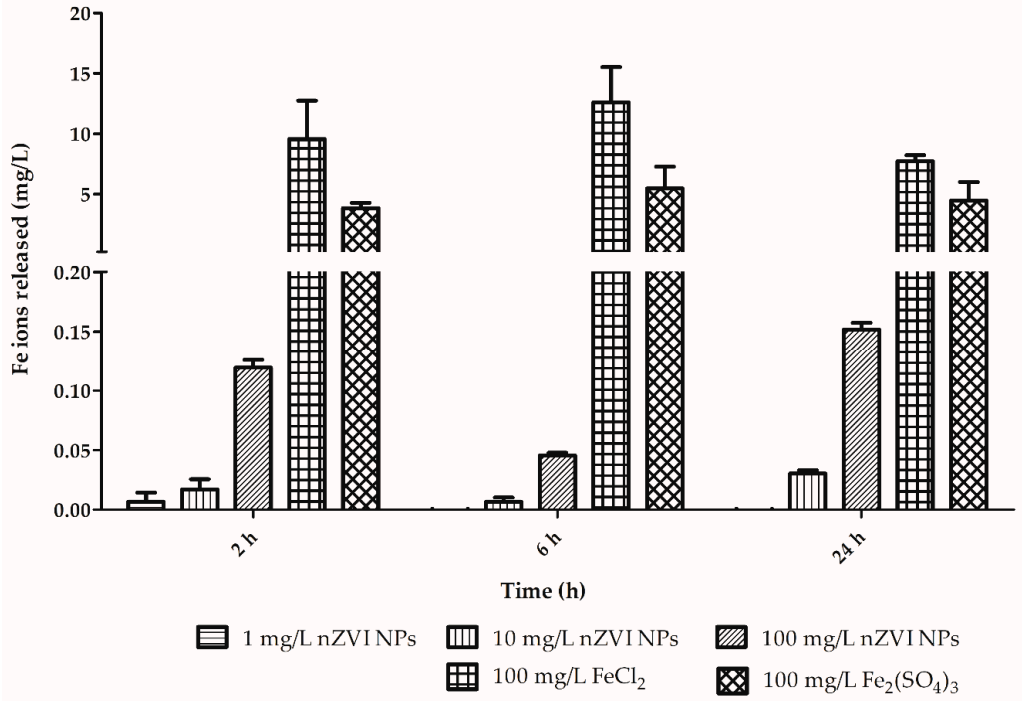


Figure 3. Soluble iron released from different sources during exposure in RPMI 1640 medium.

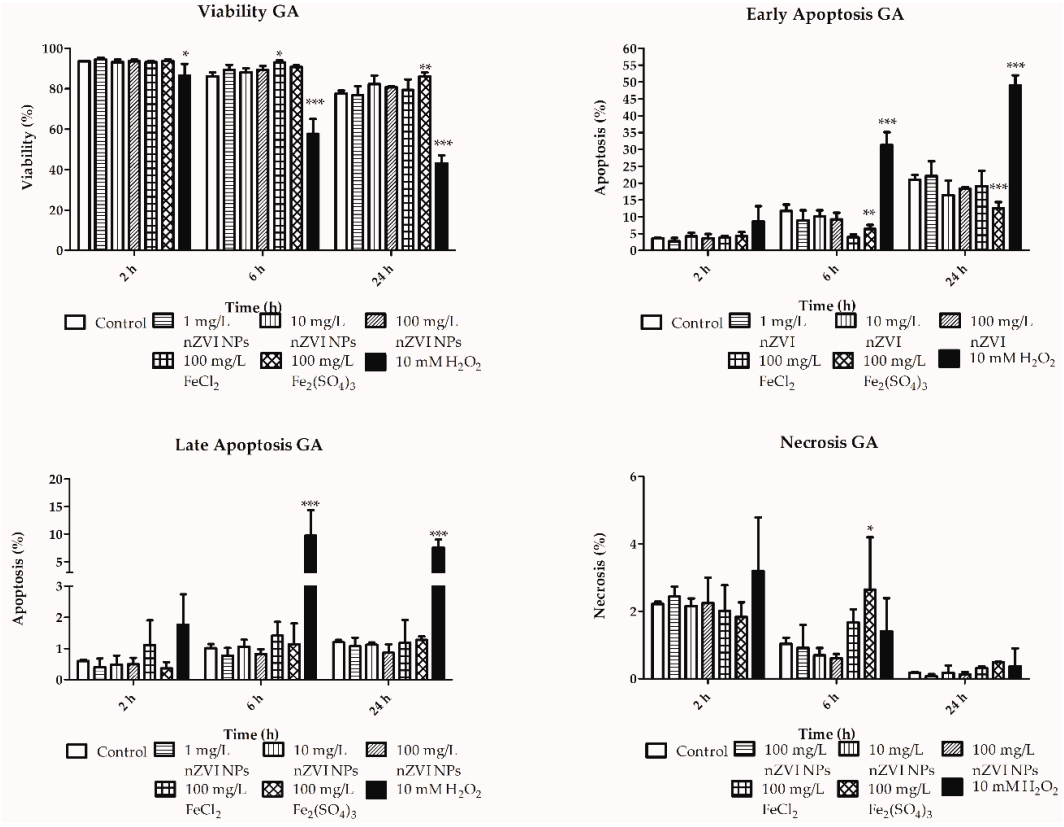


Figure 4. Viability, early and late apoptosis, and necrosis of granular amoebocytes (GA) after exposure to nZVI NPs, $FeCl_2$ and $Fe_2(SO_4)_3$ for 2, 6 and 24 h; 10 mM H_2O_2 was used as a positive control; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

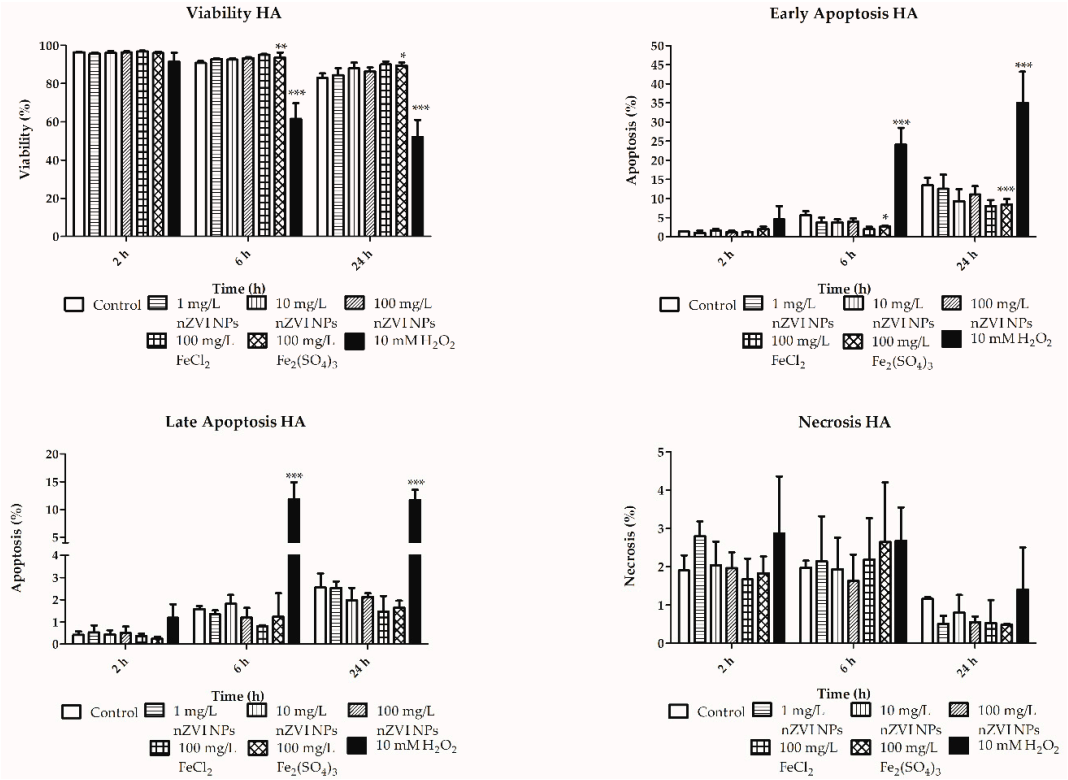


Figure 5. Viability, early and late apoptosis, and necrosis of hyaline amoebocytes (HA) after exposure to nZVI NPs, $FeCl_2$ and $Fe_2(SO_4)_3$ for 2, 6 and 24 h; 10 mM H_2O_2 was used as a positive control; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

In addition to viability studies, the use of flow cytometry allowed the monitoring of cell death stages, including apoptosis and necrosis, in coelomocytes. Moreover, based on the combination of different staining (PI and Annexin V) for the detection of membrane integrity and lipid phosphatidyl serine externalization (a typical marker of apoptosis), the early and late apoptosis were determined in the exposed cells. Only the ferric form of iron significantly decreased early apoptosis of granular amoebocytes (after 6 and 24 h) and increased their necrosis after 6 h (Figure 4). In contrast, no significant effect was observed at any of the tested concentrations of nZVI NPs or ferrous ions. Similar trends in early apoptosis were observed in hyaline amoebocytes, in which ferric iron also induced significant decreases after 6 and 24 h (Figure 5). Despite several changes in the cell death cycle induced by ionic forms of iron, no significant negative effects of nZVI NPs were observed in either type of amoebocytes at environmentally relevant concentrations. The absence of lethal effects is in accordance with in vivo studies focused on the toxicity of nZVI-based material in earthworms and other terrestrial organisms [13,14].

3.3. Sublethal Effects: Reactive Oxygen Species, Lipid Peroxidation, and Phagocytosis

Despite the fact that Yirsaw et al. (2016) did not observe a decrease in the survival of earthworms exposed to nZVI NPs, they were able to detect various concentration-dependent sublethal effects, such as lipid peroxidation and DNA damage [14]. In the current work, we performed a deeper exploration of the sublethal effects of nZVI NPs using the described in vitro approach. ROS level determination in coelomocytes after different treatments revealed an interesting trend that was similar for both types of earthworm immune effector cells (See Figure 6; Figures S8–S13). The highest level of ROS formation was detected in hyaline amoebocytes exposed to Fe^{2+} and Fe^{3+} for 2 h and in granular amoebocytes exposed to Fe^{3+} for the same amount of time. The differences in ROS production between GA and HA caused by Fe^{2+} and Fe^{3+} could be explained by different sensitivities of the two cell types

towards various redox forms of iron ions. Different antioxidative defense, uptake and biokinetics could result in different ROS production induced by Fe^{2+} and Fe^{3+}. Interestingly, after longer exposure (6 or 24 h), the production of ROS species decreased and did not differ significantly from that in the untreated controls. Several authors have found downward trends in ROS generation following longer exposure periods, and one possible explanation for this phenomenon is the activation of the antioxidative defense system [39,40]. Cellular metabolism involves many mechanisms for the detoxification of ROS that are triggered by oxidative stress. For example, coelomocytes express numerous antioxidative enzymes that protect the cells against the effects of oxidative stress. Metallothioneins are proteins produced by amoebocytes that are induced when metal-oxidative stress occurs [23,41]. The exposure of coelomocytes to nZVI NPs could induce the production of metallothioneins to protect the cells against the oxidative stress exerted by iron ions or a highly reactive nanomaterial. Moreover, there are other antioxidant enzymes (catalase and superoxide dismutase), that are activated in response to an imbalance in ROS metabolism. However, ROS formation in hyaline and granular amoebocytes after exposure to nZVI NPs showed a different trend. As observed with the DCF-DA test which measures the intracellular ROS, nZVI at 100 mg/L significantly increased nonspecific ROS generation after the longer exposure period of 24 h in both amoebocyte subpopulations. This opposite trend in ROS formation in cells after exposure to nZVI NPs could be explained by delayed cellular uptake of nanoparticles compared to iron ions or by dissolution/degradation of the oxidic shell [42]. These results emphasize the need for a further investigation of longer exposure times using an approach other than primary culture, which has a limited viability of approximately 24 h.

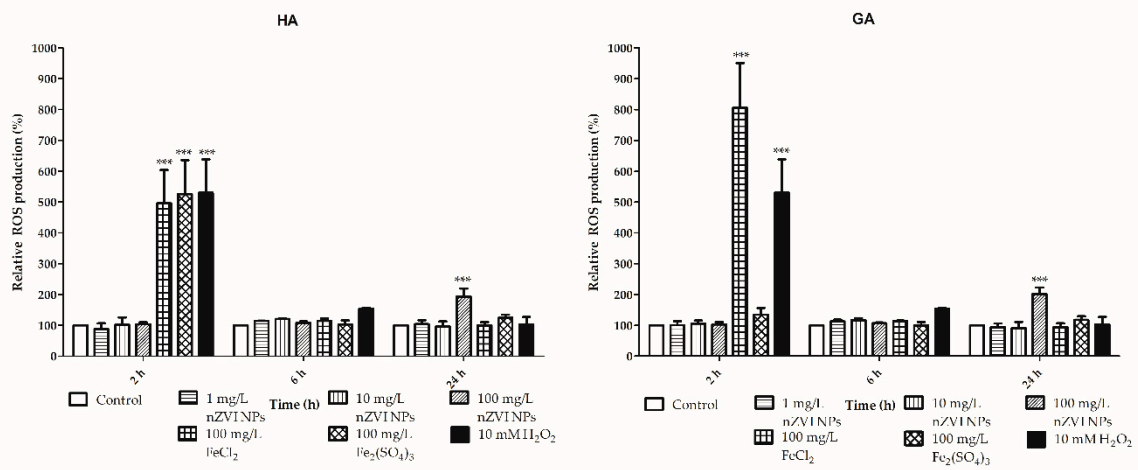


Figure 6. Reactive oxygen species (ROS) generation in granular (GA) and hyaline (HA) amoebocytes after exposure to nZVI NPs, $FeCl_2$ and $Fe_2(SO_4)_3$; 10 mM H_2O_2 was used as a positive control; *** $p < 0.001$.

Analysis of lipid peroxidation in coelomocytes after exposure to nZVI NPs showed a dose-dependent increase in MDA production (Figure 7). Even the tested concentration 10 mg/L of nZVI NPs induced a significant elevation of MDA levels compared to that in the control sample without the nanomaterial. Moreover, different forms of iron ions (Fe^{2+} and Fe^{3+}) also induced a significant increase in lipid peroxidation compared to that in the control sample. The same concentration of iron in the form of ferrous ions (Fe^{2+} dissolved from $FeCl_2$) produced significantly more MDA than iron in the form of ferric ions (Fe^{3+} dissolved from $Fe_2(SO_4)_3$). Determination of MDA levels was the most sensitive assay out of those used, as in a previous study focused on adverse effects in bacteria [27]. The applicability and sensitivity of the MDA determination assay has already been established using earthworms exposed to ZnO NPs, as well as to nZVI NPs [11,43]. The results of the MDA formation assay in amoebocytes exposed to nZVI NPs in the current study are consistent with an in vivo study by Liang et al. (2017) and show the potential of in vitro tests for NPs' toxicity testing [11].

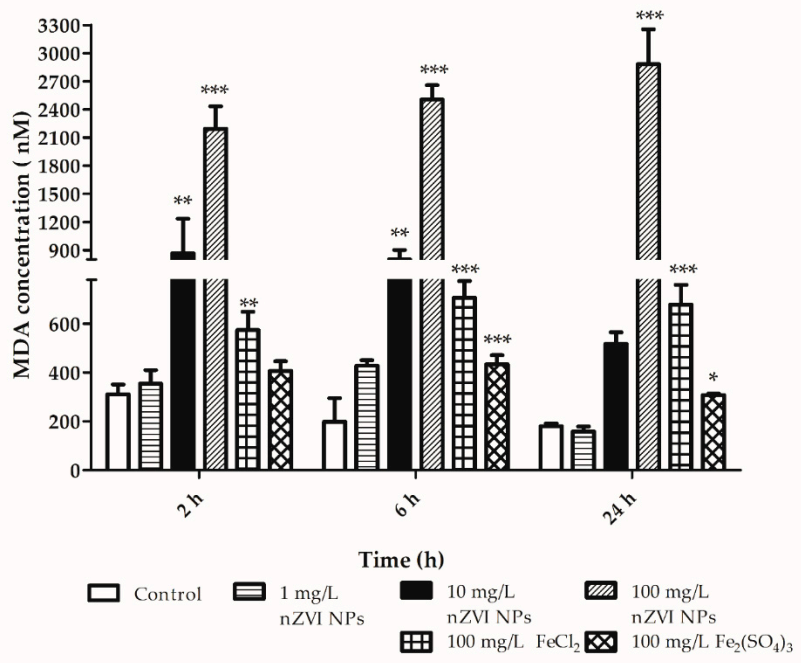


Figure 7. Malondialdehyde (MDA) formation in amoebocytes after exposure to nZVI NPs, $FeCl_2$ and $Fe_2(SO_4)_3$ for 2, 6 and 24 h; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The phagocytic activity of hyaline amoebocytes was not affected by the tested concentrations of nZVI NPs or iron ions (Figure 8; Figures S14–S19). The granular amoebocytes were more sensitive than the other subtype of coelomocytes. The tested concentrations of ferric iron significantly decreased the phagocytic activity of the exposed granular amoebocytes after 2, 6 and 24 h of exposure. The normal phagocytic activity of the untreated granular amoebocyte subpopulation was 52–58%, while that of granular amoebocytes treated with ferric iron was 37, 32, and 36% at 2, 6, and 24 h, respectively.

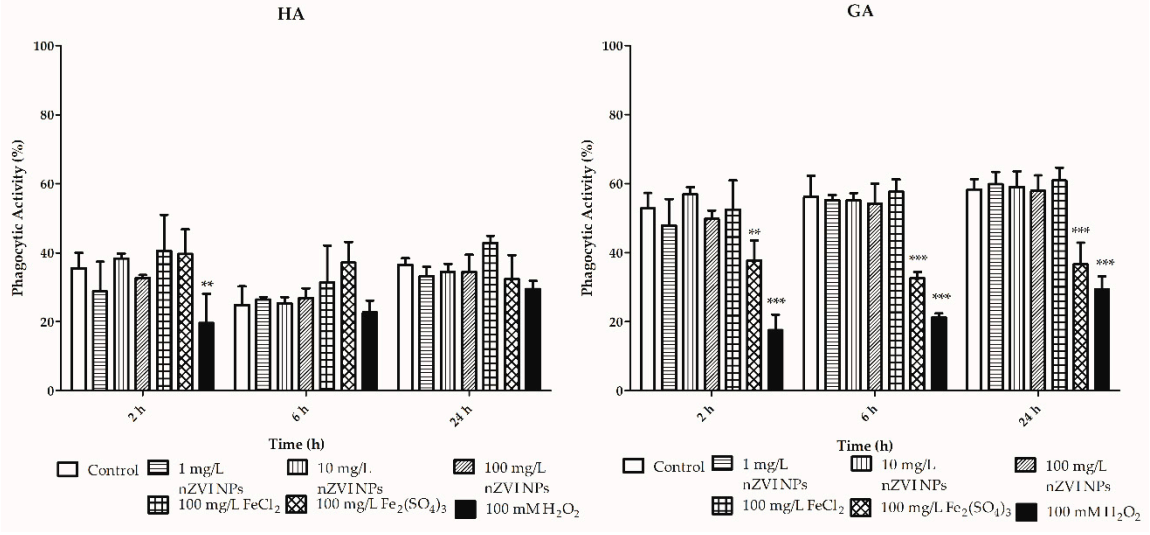


Figure 8. Phagocytic activity of hyaline (HA) and granular (GA) amoebocytes after exposure to nZVI NPs, $FeCl_2$ and $Fe_2(SO_4)_3$ for 2, 6 and 24 h; 100 mM H_2O_2 was used as a positive control; ** $p < 0.01$, *** $p < 0.001$.

3.4. The Effect of Dissolved Iron Species on Toxicity

As previously mentioned, many authors have studied the toxicity of nZVI NPs and elucidated the mechanisms of its adverse effects using microbial species. Despite the many different and contradictory

results, the majority of those studies concluded that oxidative stress is one of the main adverse effects induced by nZVI NPs and the materials derived from it [3]. The imbalance in ROS metabolism induced by increased ROS production or ineffective antioxidative defense mechanisms can result in oxidation of cellular biomolecules and subsequent cell death. Many reaction cascades have already been described in which nZVI NPs undergo oxidation/reduction with consequent ROS production [15]. Both ferrous and ferric ions are known to be able to induce intracellular ROS formation via Fenton reactions [44]. The redox reactions of ferrous and ferric ions with hydrogen peroxide result in the formation of hydroxyl and hydroperoxyl radicals, which can then degrade biomolecules such as nucleic acids, proteins, and lipids. Some recent studies performed on bacterial species and human cell lines have suggested that not only highly reactive nZVI NPs but also iron ions dissolved from nanomaterials could play a critical role in mediating the toxicity of nZVI NPs [45,46]. On the other hand, another study showed no significant relationship between toxicity/lipid peroxidation in bacteria and the concentration of dissolved iron from different nZVI-based materials during 2 months of aging [27]. Similarly, another study ascribed greater toxicity to *Escherichia coli* exposed to nZVI NPs than to *Escherichia coli* exposed to the same concentration of ferrous iron under both air-saturated and deaerated conditions [47]. The findings regarding early apoptosis and necrosis of granular amoebocytes exposed to ferric iron in this study are highly consistent with the decrease in phagocytic activity and demonstrate the potential negative effects of dissolved iron in this form (Figures 4 and 5). However, dissolved iron ions reached levels 0.1–0.2% of the amount reached with nZVI NPs in this experiment, which is negligible in comparison to dissolved iron species from $FeCl_2$ and $Fe_2(SO_4)_3$ that reached from 50 to 100 times higher concentrations (see Section 3.1 and Figure 3). It is noteworthy that approximately the same concentration levels of dissolved iron were detected in a previous 2-month aging experiment [27]. Nevertheless, even at such a high nZVI NPs concentration of 10 g/L, which is, from a practical perspective, irrelevant, as it is 100 times higher than the concentration used in our experiment or that is detected at sites of nZVI NPs applications, similar concentrations of ferric and ferrous ions could not theoretically be reached (considering that 0.1–0.2% of iron that is dissolved). This finding indicates that dissolved iron species do not play an important role in toxicity to earthworms in the context of nZVI NPs applications. It is worth noting that under different conditions in heterogenic matrices (e.g., real groundwater, different types of soil, etc.), the aging process is different, and dissolution occurs with different kinetics. Moreover, Fe^0 could be transformed by microbial species into various insoluble iron species or their oxidation products [25]. Despite the many aforementioned factors affecting the aging processes of nZVI NPs, the results of the current study show that nZVI NPs applications pose minimal risk to earthworms.

4. Conclusions

The results of the current study demonstrate the necessity of material characterization in the medium used for exposure conditions to achieve reliable results. While the fact that nZVI NPs aggregated in the RPMI 1640 medium makes the experimental setup inapplicable for determining the toxicity of fresh nZVI NPs, the formation of aggregates reflects the fate of nanoparticles after real application. Evaluation of the toxicity of nZVI NPs in the form of aggregates is equally or even more important than evaluation of freshly prepared particles, which are present in the environment only for several hours or days. Understanding the mechanisms of iron dissolution and ion release, as well as their impact on overall toxicity, can help in the assessment of the environmental safety of nZVI NPs. The toxicological data obtained in this study suggest that two sizes of nZVI NPs aggregates (after 0 and 24 h in RPMI 1640) and ferrous and ferric ions, as potentially released forms of iron, do not significantly affect the viability of coelomocytes and probably do not pose a risk to exposed earthworms. Determination of the sublethal effects including impacts on phagocytosis and oxidative stress-related markers showed that ferric and ferrous ions as well as nZVI NPs aggregates had a slight effect. The combination of the quantification of dissolved iron and the most sensitive assay (from the battery of assay used), malondialdehyde determination, showed that, even in the form of

aggregates, nZVI NPs cause higher lipid peroxidation than their potential transformation products (i.e., dissolved ferric and ferrous ions) in earthworm coelomocytes. Overall, the results of the present study confirm the environmental safety of nZVI NPs and suggest that the toxicity of nZVI NPs is potentially reduced during aging. Further studies incorporating the nZVI NPs characterization during the aging process and iron dissolution under different environmental conditions are needed to better understand material behavior and toxicity in real-world nanoremediation applications. Our results also show that the methodological concept of testing isolated immune earthworm cells is a suitable approach to testing for nanomaterials toxicity and to elucidate mechanistical aspects of the nanomaterials with respect to possible different modes of actions.

Supplementary Materials: The following are available online at http://www.mdpi.com/2079-4991/10/11/2189/s1. Figure S1. Detection of coelomocytes subpopulations through flow cytometry. Coelomocytes populations were detected and divided into eleocytes (E), hyaline (HA) and granular amoebocytes (GA). (A) coelomocytes non-treated (control) and coelomocytes exposed to 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$; (B) RPM 1640 medium and RPMI 1640 medium with 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ without coelomocytes after 6 h. Figure S2. Apoptosis activity of hyaline amoebocytes (HA) without annexin V, without treatment (control) and HA exposed to 10 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 2 h. Figure S3. Apoptosis activity of granular amoebocytes (GA) without annexin V, without treatment (control) and GA exposed to 10 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 2 h. Figure S4. Apoptosis activity of hyaline amoebocytes (HA) without annexin V, without treatment (control) and HA exposed to 10 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 6 h. Figure S5. Apoptosis activity of granular amoebocytes (GA) without annexin V, without treatment (control) and GA exposed to 10 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 6 h. Figure S6. Apoptosis activity of hyaline amoebocytes (HA) without annexin V, without treatment (control) and HA exposed to 10 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 24 h. Figure S7. Apoptosis activity of granular amoebocytes (GA) without annexin V, without treatment (control) and GA exposed to 10 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 24 h. Figure S8. Reactive Oxygen Species production of hyaline amoebocytes (HA) without fluorescence, without treatment (control) and HA exposed to 10 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 2 h. Figure S9. Reactive Oxygen Species production of granular amoebocytes (GA) without fluorescence, without treatment (control) and GA exposed to 10 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 2 h. Figure S10. Reactive Oxygen Species production of hyaline amoebocytes (HA) without fluorescence, without treatment (control) and HA exposed to 10 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 6 h. Figure S11. Reactive Oxygen Species production of granular amoebocytes (GA) without fluorescence, without treatment (control) and GA exposed to 10 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 6 h. Figure S12. Reactive Oxygen Species production of hyaline amoebocytes (HA) without fluorescence, without treatment (control) and HA exposed to 10 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 24 h. Figure S13. Reactive Oxygen Species production of granular amoebocytes (GA) without fluorescence, without treatment (control) and GA exposed to 10 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 24 h. Figure S14. Phagocytic activity of hyaline amoebocytes (HA) without Fluoresbrite, without treatment (control) and HA exposed to 100 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 2 h. Figure S15. Phagocytic activity of granular amoebocytes (GA) without annexin V, without treatment (control) and GA exposed to 100 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 2 h. Figure S16. Phagocytic activity of hyaline amoebocytes (HA) without Fluoresbrite, without treatment (control) and HA exposed to 100 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 6 h. Figure S17. Phagocytic activity of granular amoebocytes (GA) without Fluoresbrite, without treatment (control) and HA exposed to 100 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 6 h. Figure S18. Phagocytic activity of hyaline amoebocytes (HA) without Fluoresbrite, without treatment (control) and HA exposed to 100 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 24 h. Figure S19. Phagocytic activity of granular amoebocytes (GA) without Fluoresbrite, without treatment (control) and HA exposed to 100 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 24 h.

Author Contributions: Conceptualization, J.S. and T.C.; Data curation, A.G.; Formal analysis, J.S., N.I.N.P. and A.G.; Funding acquisition, T.C.; Methodology, J.S., N.I.N.P., M.P. and L.P.; Supervision, P.P. and T.C.; Writing—original draft, J.S., N.I.N.P. and T.C.; Writing—review & editing, P.P. and T.C. All authors have read and agreed to the published version of the manuscript.

Funding: The work was supported by the Ministry of Agriculture of the Czech Republic [grant number QK1910095] and by the Center for Geosphere Dynamics (UNCE/SCI/006). This project received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 67188. This work was supported by the Czech Academy of Sciences, Czech Republic [RVO 67985874 and RVO 61388971]; the authors acknowledge the financial assistance on this project.

Acknowledgments: We acknowledge the Cytometry and Microscopy Facility at the Institute of Microbiology of the ASCR, v.v.i, Vídeňská 1083, Prague, CZ for the use of cytometry equipment and the support from the staff.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Mueller, N.C.; Braun, J.; Bruns, J.; Cernik, M.; Rissing, P.; Rickerby, D.; Nowack, B. Application of nanoscale zero valent iron (nZVI) for groundwater remediation in Europe. *Environ. Sci. Pollut. Res.* **2012**, *19*, 550–558. [CrossRef] [PubMed]
2. Pasinszki, T.; Krebsz, M. Synthesis and application of zero-valent iron nanoparticles in water treatment, environmental remediation, catalysis, and their biological effects. *Nanomaterials* **2020**, *10*, 917. [CrossRef]
3. Semerad, J.; Cajthaml, T. Ecotoxicity and environmental safety related to nano-scale zerovalent iron remediation applications. *Appl. Microbiol. Biotechnol.* **2016**, *100*, 9809–9819. [CrossRef]
4. Kocur, C.M.; Chowdhury, A.I.; Sakulchaicharoen, N.; Boparai, H.K.; Weber, K.P.; Sharma, P.; Krol, M.M.; Austrins, L.; Peace, C.; Sleep, B.E.; et al. Characterization of nZVI mobility in a field scale test. *Environ. Sci. Technol.* **2014**, *48*, 2862–2869. [CrossRef] [PubMed]
5. Johnson, R.L.; Nurmi, J.T.; O'Brien Johnson, G.S.; Fan, D.; O'Brien Johnson, R.L.; Shi, Z.; Salter-Blanc, A.J.; Tratnyek, P.G.; Lowry, G.V. Field-Scale Transport and Transformation of Carboxymethylcellulose-Stabilized Nano Zero-Valent Iron. *Environ. Sci. Technol.* **2013**, *47*, 1573–1580. [CrossRef]
6. Jang, M.-H.; Lim, M.; Hwang, Y.S. Potential environmental implications of nanoscale zero-valent iron particles for environmental remediation. *Environ. Health Toxicol.* **2014**, *29*, e2014022. [CrossRef]
7. Lefevre, E.; Bossa, N.; Wiesner, M.R.; Gunsch, C.K. A review of the environmental implications of in situ remediation by nanoscale zero valent iron (nZVI). *Sci. Total Environ.* **2016**, *565*, 889–901. [CrossRef]
8. Sevcu, A.; El-Temsah, Y.S.; Filip, J.; Joner, E.J.; Bobcikova, K.; Cernik, M. Zero-valent iron particles for PCB degradation and an evaluation of their effects on bacteria, plants, and soil organisms. *Environ. Sci. Pollut. Res.* **2017**, *24*, 21191–21202. [CrossRef]
9. El-Temsah, Y.S.; Sevcu, A.; Bobcikova, K.; Cernik, M.; Joner, E.J. DDT degradation efficiency and ecotoxicological effects of two types of nano-sized zero-valent iron (nZVI) in water and soil. *Chemosphere* **2016**, *144*, 2221–2228. [CrossRef]
10. El-Temsah, Y.S.; Joner, E.J. Ecotoxicological effects on earthworms of fresh and aged nano-sized zero-valent iron (nZVI) in soil. *Chemosphere* **2012**, *89*, 76–82. [CrossRef]
11. Liang, J.; Xia, X.Q.; Zhang, W.; Zaman, W.Q.; Lin, K.F.; Hu, S.Q.; Lin, Z.F. The biochemical and toxicological responses of earthworm (*Eisenia fetida*) following exposure to nanoscale zerovalent iron in a soil system. *Environ. Sci. Pollut. Res.* **2017**, *24*, 2507–2514. [CrossRef] [PubMed]
12. Liang, J.; Xia, X.Q.; Yuan, L.; Zhang, W.; Lin, K.F.; Zhou, B.S.; Hu, S.Q. The reproductive responses of earthworms (*Eisenia fetida*) exposed to nanoscale zero-valent iron (nZVI) in the presence of decabromodiphenyl ether (BDE209). *Environ. Pollut.* **2018**, *237*, 784–791. [CrossRef] [PubMed]
13. Yoon, H.; Pangging, M.; Jang, M.H.; Hwang, Y.S.; Chang, Y.S. Impact of surface modification on the toxicity of zerovalent iron nanoparticles in aquatic and terrestrial organisms. *Ecotoxicol. Environ. Saf.* **2018**, *163*, 436–443. [CrossRef] [PubMed]
14. Yirsaw, B.D.; Mayilswami, S.; Megharaj, M.; Chen, Z.L.; Naidu, R. Effect of zero valent iron nanoparticles to *Eisenia fetida* in three soil types. *Environ. Sci. Pollut. Res.* **2016**, *23*, 9822–9831. [CrossRef]
15. Sevcu, A.; El-Temsah, Y.S.; Joner, E.J.; Cernik, M. Oxidative Stress Induced in Microorganisms by Zero-valent Iron Nanoparticles. *Microbes Environ.* **2011**, *26*, 271–281. [CrossRef]
16. Semerad, J.; Cvancarova, M.; Filip, J.; Kaslik, J.; Zlota, J.; Soukupova, J.; Cajthaml, T. Novel assay for the toxicity evaluation of nanoscale zero-valent iron and derived nanomaterials based on lipid peroxidation in bacterial species. *Chemosphere* **2018**, *213*, 568–577. [CrossRef]
17. Semerad, J.; Moeder, M.; Filip, J.; Pivokonsky, M.; Filipova, A.; Cajthaml, T. Oxidative stress in microbes after exposure to iron nanoparticles: Analysis of aldehydes as oxidative damage products of lipids and proteins. *Environ. Sci. Pollut. Res.* **2019**, *26*, 33670–33682. [CrossRef]
18. Manerikar, R.S.; Apte, A.A.; Ghole, V.S. In vitro and in vivo genotoxicity assessment of Cr(VI) using comet assay in earthworm coelomocytes. *Environ. Toxicol. Pharmacol.* **2008**, *25*, 63–68. [CrossRef]
19. Hayashi, Y.; Engelmann, P. Earthworm's immunity in the nanomaterial world: New room, future challenges. *Invertebr. Surviv. J.* **2013**, *10*, 69–76.

20. Muangphra, P.; Kwankua, W.; Gooneratne, R. Genotoxic effects of glyphosate or paraquat on earthworm coelomocytes. *Environ. Toxicol.* **2014**, *29*, 612–620. [CrossRef]
21. Bunn, K.E.; Thompson, H.M.; Tarrant, K.A. Effects of agrochemicals on the immune systems of earthworms. *Bull. Environ. Contam. Toxicol.* **1996**, *57*, 632–639. [CrossRef]
22. Yang, Y.; Xiao, Y.; Li, M.; Ji, F.; Hu, C.; Cui, Y. Evaluation of Complex Toxicity of Canbon Nanotubes and Sodium Pentachlorophenol Based on Earthworm Coelomocytes Test. *PLoS ONE* **2017**, *12*, e0170092. [CrossRef]
23. Hayashi, Y.; Engelmann, P.; Foldbjerg, R.; Szabo, M.; Somogyi, I.; Pollak, E.; Molnar, L.; Autrup, H.; Sutherland, D.S.; Scott-Fordsmand, J.; et al. Earthworms and Humans In Vitro: Characterizing Evolutionarily Conserved Stress and Immune Responses to Silver Nanoparticles. *Environ. Sci. Technol.* **2012**, *46*, 4166–4173. [CrossRef]
24. Bigorgne, E.; Foucaud, L.; Caillet, C.; Giamberini, L.; Nahmani, J.; Thomas, F.; Rodius, F. Cellular and molecular responses of *E. fetida* coelomocytes exposed to TiO_2 nanoparticles. *J. Nanopart. Res.* **2012**, *14*, 959. [CrossRef]
25. Wu, S.; Cajthaml, T.; Semerad, J.; Filipova, A.; Klementova, M.; Skala, R.; Vitkova, M.; Michalkova, Z.; Teodoro, M.; Wu, Z.; et al. Nano zero-valent iron aging interacts with the soil microbial community: A microcosm study. *Environ. Sci. Nano* **2019**, *6*, 1189–1206. [CrossRef]
26. Mangayayam, M.C.; Alonso-de-Linaje, V.; Dideriksen, K.; Tobler, D.J. Effects of common groundwater ions on the transformation and reactivity of sulfidized nanoscale zerovalent iron. *Chemosphere* **2020**, *249*, 126137. [CrossRef]
27. Semerad, J.; Filip, J.; Sevcu, A.; Brumovsky, M.; Nguyen, N.H.A.; Miksicek, J.; Lederer, T.; Filipova, A.; Bohackova, J.; Cajthaml, T. Environmental fate of sulfidated nZVI particles: The interplay of nanoparticle corrosion and toxicity during aging. *Environ. Sci. Nano* **2020**, *7*, 1794–1806. [CrossRef]
28. Kaslik, J.; Kolarik, J.; Filip, J.; Medrik, I.; Tomanec, O.; Petr, M.; Malina, O.; Zboril, R.; Tratnyek, P.G. Nanoarchitecture of advanced core-shell zero-valent iron particles with controlled reactivity for contaminant removal. *Chem. Eng. J.* **2018**, *354*, 335–345. [CrossRef]
29. Hayashi, Y.; Miclaus, T.; Scavenius, C.; Kwiatkowska, K.; Sobota, A.; Engelmann, P.; Scott-Fordsmand, J.J.; Enghild, J.J.; Sutherland, D.S. Species Differences Take Shape at Nanoparticles: Protein Corona Made of the Native Repertoire Assists Cellular Interaction. *Environ. Sci. Technol.* **2013**, *47*, 14367–14375. [CrossRef]
30. Keller, A.A.; Garner, K.; Miller, R.J.; Lenihan, H.S. Toxicity of Nano-Zero Valent Iron to Freshwater and Marine Organisms. *PLoS ONE* **2012**, *7*, e43983. [CrossRef] [PubMed]
31. Kocur, C.M.; O'Carroll, D.M.; Sleep, B.E. Impact of nZVI stability on mobility in porous media. *J. Contam. Hydrol.* **2013**, *145*, 17–25. [CrossRef] [PubMed]
32. O'Carroll, D.; Sleep, B.; Krol, M.; Boparai, H.; Kocur, C. Nanoscale zero valent iron and bimetallic particles for contaminated site remediation. *Adv. Water Resour.* **2013**, *51*, 104–122. [CrossRef]
33. Vidic, J.; Haque, F.; Guigner, J.M.; Vidy, A.; Chevalier, C.; Stankic, S. Effects of Water and Cell Culture Media on the Physicochemical Properties of ZnMgO Nanoparticles and Their Toxicity toward Mammalian Cells. *Langmuir* **2014**, *30*, 11366–11374. [CrossRef]
34. Uskokovic, V.; Huynh, E.; Wu, V.M. Mimicking the transit of nanoparticles through the body: When the path determines properties at the destination. *J. Nanopart. Res.* **2020**, *22*, 184. [CrossRef]
35. Marquez, A.; Berger, T.; Feinle, A.; Husing, N.; Himly, M.; Duschl, A.; Diwald, O. Bovine Serum Albumin Adsorption on TiO2 Colloids: The Effect of Particle Agglomeration and Surface Composition. *Langmuir* **2017**, *33*, 2551–2558. [CrossRef]
36. Nurmi, J.T.; Tratnyek, P.G.; Sarathy, V.; Baer, D.R.; Amonette, J.E.; Pecher, K.; Wang, C.M.; Linehan, J.C.; Matson, D.W.; Penn, R.L.; et al. Characterization and properties of metallic iron nanoparticles: Spectroscopy, electrochemistry, and kinetics. *Environ. Sci. Technol.* **2005**, *39*, 1221–1230. [CrossRef]
37. He, D.; Ma, J.X.; Collins, R.N.; Waite, T.D. Effect of structural transformation of nanoparticulate zero-valent iron on generation of reactive oxygen species. *Environ. Sci. Technol.* **2016**, *50*, 3820–3828. [CrossRef]
38. Albanese, A.; Chan, W.C.W. Effect of Gold Nanoparticle Aggregation on Cell Uptake and Toxicity. *ACS Nano* **2011**, *5*, 5478–5489. [CrossRef] [PubMed]
39. Katsumiti, A.; Thorley, A.J.; Arostegui, I.; Reip, P.; Valsami-Jones, E.; Tetley, T.D.; Cajaraville, M.P. Cytotoxicity and cellular mechanisms of toxicity of CuO NPs in mussel cells in vitro and comparative sensitivity with human cells. *Toxicol. In Vitro* **2018**, *48*, 146–158. [CrossRef] [PubMed]

40. Zhang, L.; Wu, L.L.; Si, Y.B.; Shu, K.H. Size-dependent cytotoxicity of silver nanoparticles to *Azotobacter vinelandii*: Growth inhibition, cell injury, oxidative stress and internalization. *PLoS ONE* **2018**, *13*, e0209020. [CrossRef]
41. Bodo, K.; Ernszt, D.; Nemeth, P.; Engelmann, P. Distinct immune- and defense-related molecular fingerprints in sepatated coelomocyte subsets of *Eisenia andrei* earthworms. *Invertebr. Surviv. J.* **2018**, *15*, 338–345.
42. Mosquera, J.; Garcia, I.; Liz-Marzan, L.M. Cellular Uptake of Nanoparticles versus Small Molecules: A Matter of Size. *Acc. Chem. Res.* **2018**, *51*, 2305–2313. [CrossRef]
43. Li, M.; Yang, Y.; Xie, J.W.; Xu, G.H.; Yu, Y. *In-vivo* and *in-vitro* tests to assess toxic mechanisms of nano ZnO to earthworms. *Sci. Total Environ.* **2019**, *687*, 71–76. [CrossRef] [PubMed]
44. Oh, E.; Andrews, K.J.; Jeon, B. Enhanced biofilm formation by ferrous and ferric iron through oxidative stress in *Campylobacter jejuni*. *Front. Microbiol.* **2018**, *9*, 1204. [CrossRef]
45. Chen, Q.; Li, J.; Wu, Y.; Shen, F.; Yao, M. Biological responses of Gram-positive and Gram-negative bacteria to nZVI (Fe^0), Fe^{2+} and Fe^{3+}. *RSC Adv.* **2013**, *3*, 13835–13842. [CrossRef]
46. Keenan, C.R.; Goth-Goldstein, R.; Lucas, D.; Sedlak, D.L. Oxidative Stress Induced by Zero-Valent Iron Nanoparticles and Fe(II) in Human Bronchial Epithelial Cells. *Environ. Sci. Technol.* **2009**, *43*, 4555–4560. [CrossRef]
47. Lee, C.; Kim, J.Y.; Lee, W.I.; Nelson, K.L.; Yoon, J.; Sedlak, D.L. Bactericidal effect of zero-valent iron nanoparticles on *Escherichia coli*. *Environ. Sci. Technol.* **2008**, *42*, 4927–4933. [CrossRef]

nanomaterials

MDPI

Supplementary Materials

In Vitro Study of the Toxicity Mechanisms of Nanoscale Zero-Valent Iron (nZVI) and Released Iron Ions Using Earthworm Cells

Jaroslav Semerad[1,2], Natividad Isabel Navarro Pacheco[1,3], Alena Grasserova[1,2], Petra Prochazkova[1], Martin Pivokonsky[4], Lenka Pivokonska[4] and Tomas Cajthaml[1,2,*]

[1] Institute of Microbiology of the Czech Academy of Sciences, Vídeňská 1083, CZ-142 20, Prague 4, Czech Republic
[2] Institute for Environmental Studies, Faculty of Science, Charles University, Benátská 2, CZ-128 01, Prague 2, Czech Republic
[3] First Faculty of Medicine, Charles University, Kateřinská 1660/32, CZ-121 08, Prague 2, Czech Republic
[4] Institute of Hydrodynamics of the Czech Academy of Sciences, Pod Paťankou 30/5, CZ-166 12, Prague 6, Czech Republic
* Correspondence: cajthaml@biomed.cas.cz

Electronic Supplementary Information

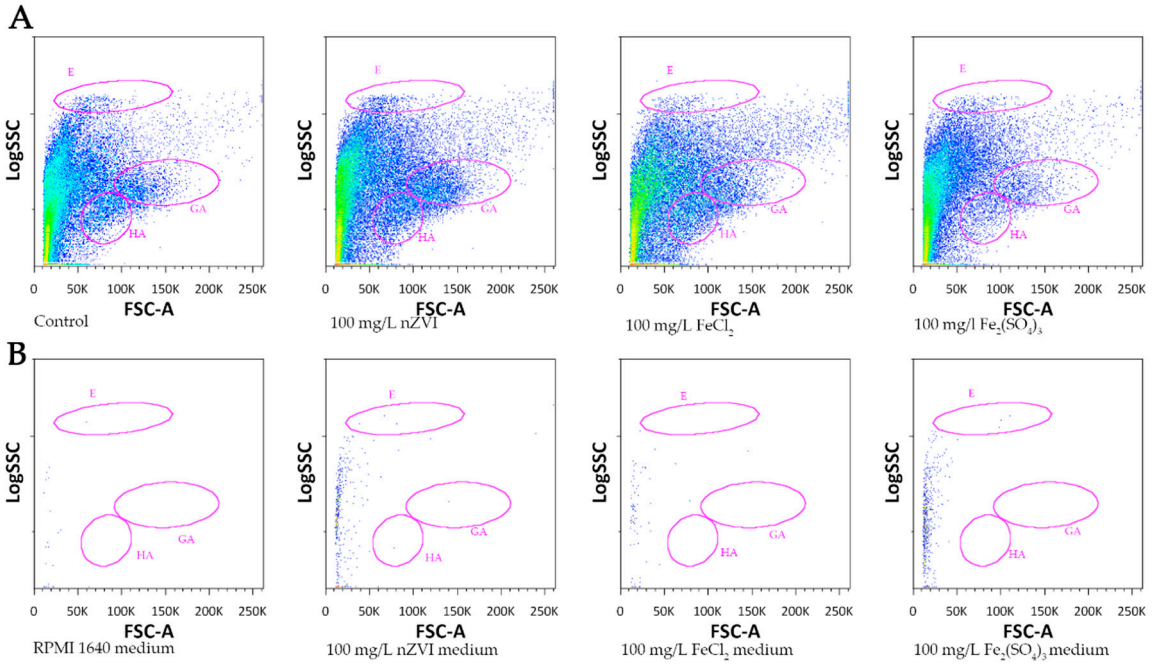


Figure S1. Detection of coelomocytes subpopulations through flow cytometry. Coelomocytes populations were detected and divided into eleocytes (E), hyaline (HA) and granular amoebocytes (GA). (**A**) coelomocytes non-treated (control) and coelomocytes exposed to 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$; (B) RPM1640 medium and medium with 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ without coelomocytes after 6 hours.

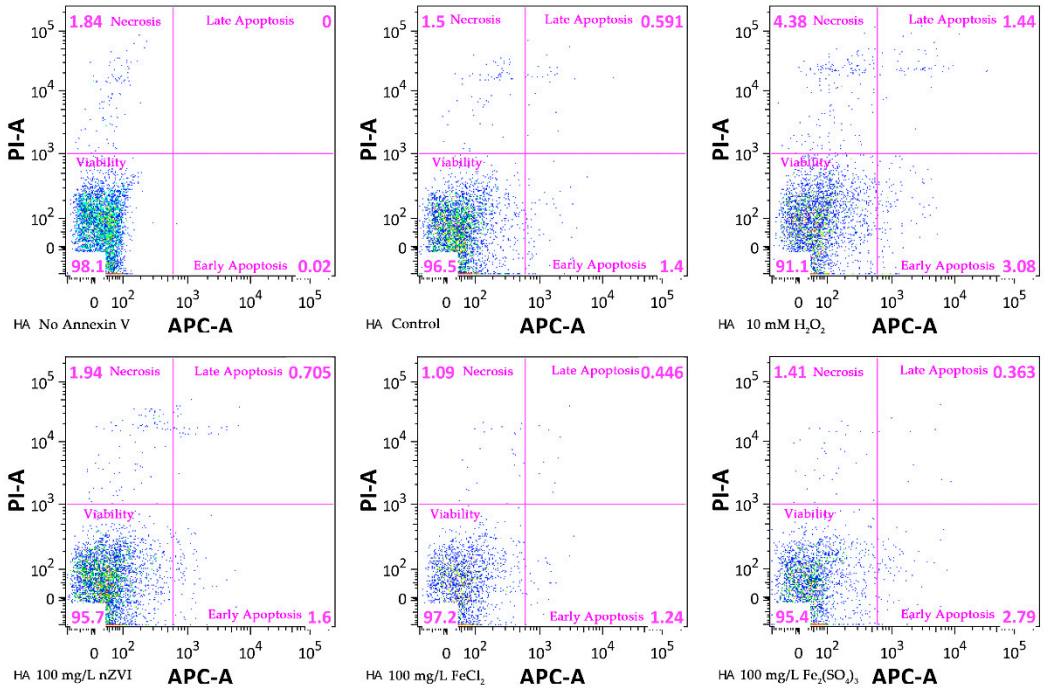


Figure S2. Apoptosis activity of hyaline amoebocytes (HA) without annexin V, without treatment (control) and HA exposed to 10 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 2 hours.

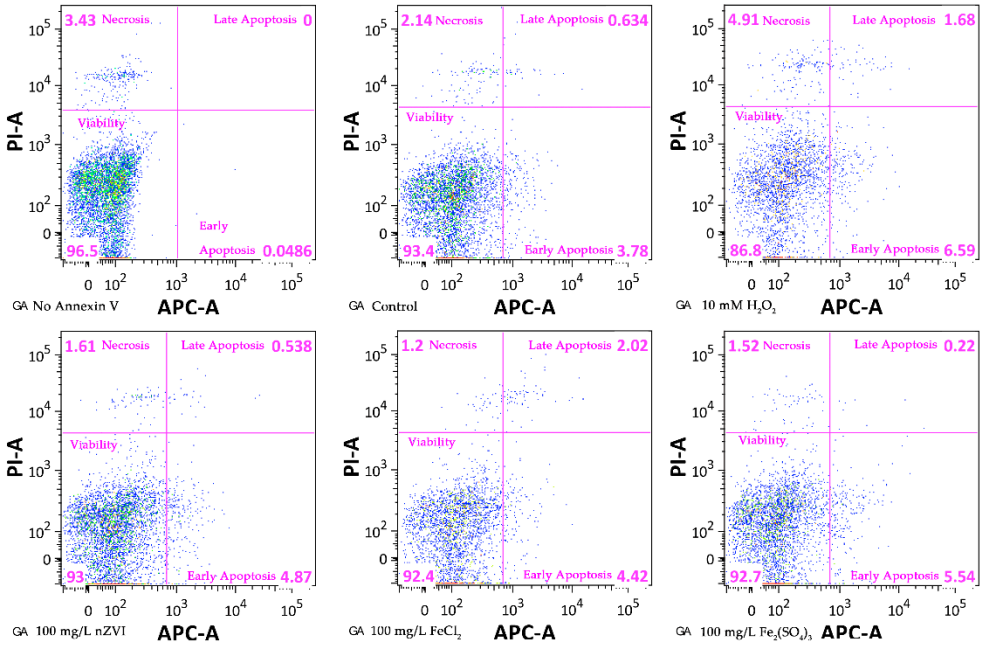


Figure S3. Apoptosis activity of granular amoebocytes (GA) without annexin V, without treatment (control) and GA exposed to 10 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 2 hours.

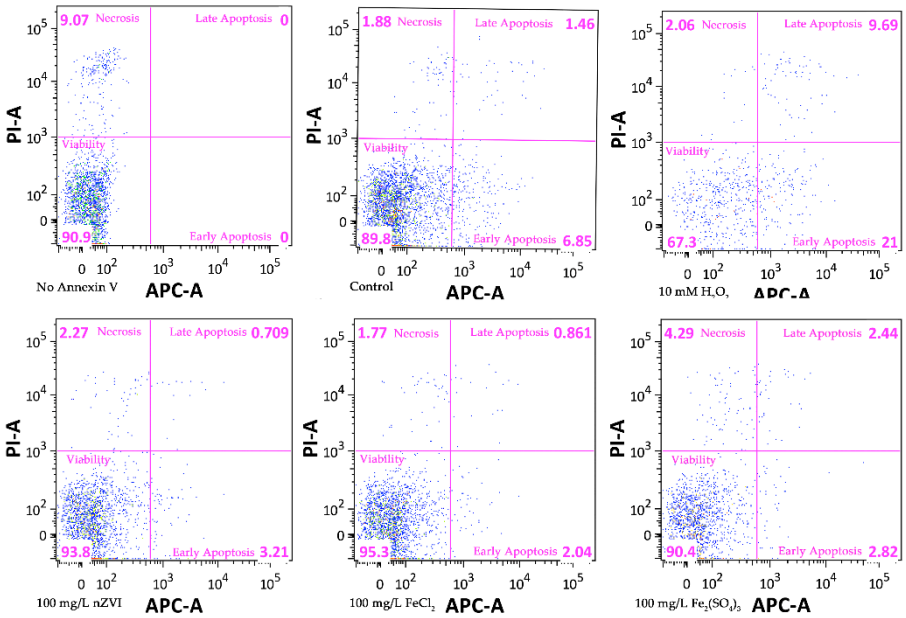


Figure S4. Apoptosis activity of hyaline amoebocytes (HA) without annexin V, without treatment (control) and HA exposed to 10 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 6 hours.

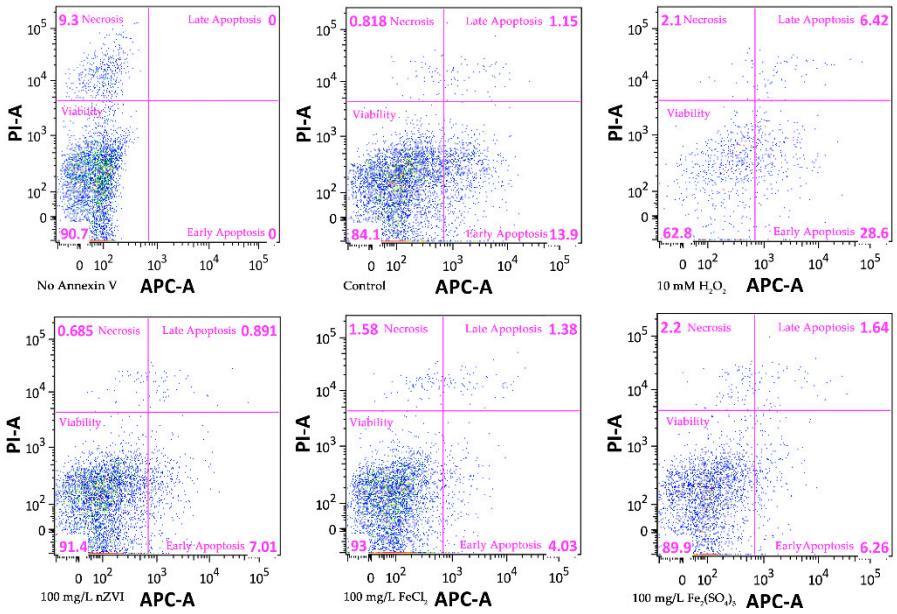


Figure S5. Apoptosis activity of granular amoebocytes (GA) without annexin V, without treatment (control) and GA exposed to 10 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 6 hours.

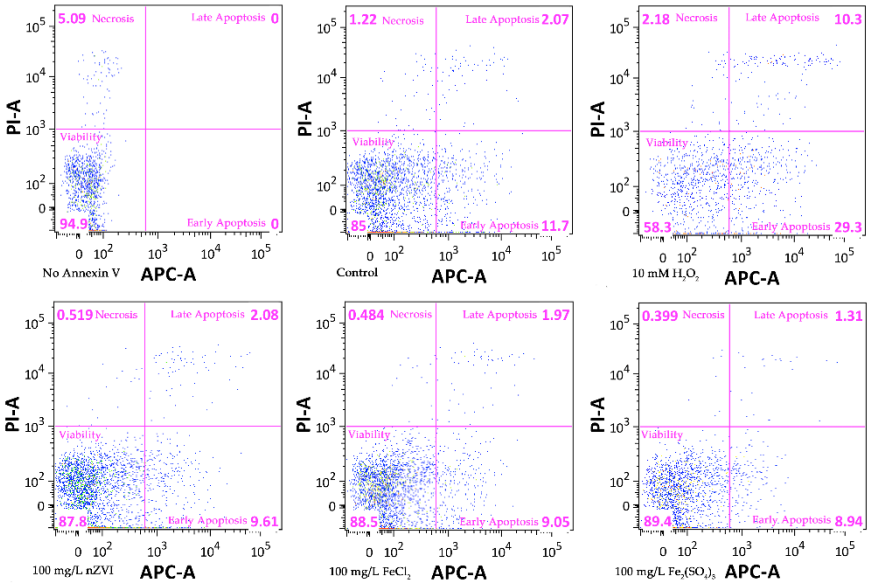


Figure S6. Apoptosis activity of hyaline amoebocytes (HA) without annexin V, without treatment (control) and HA exposed to 10 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 24 hours.

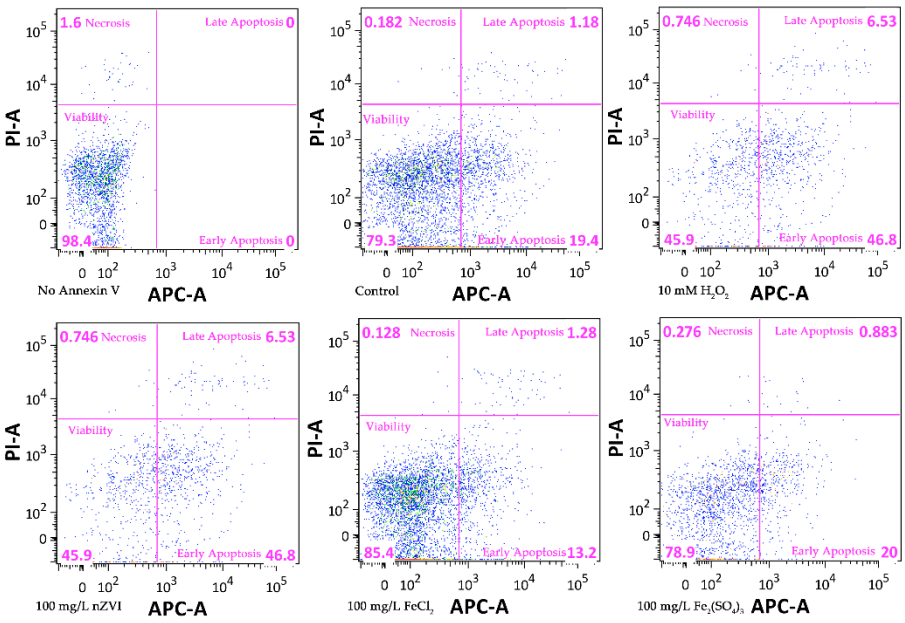


Figure S7. Apoptosis activity of granular amoebocytes (GA) without annexin V, without treatment (control) and GA exposed to 10 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 24 hours.

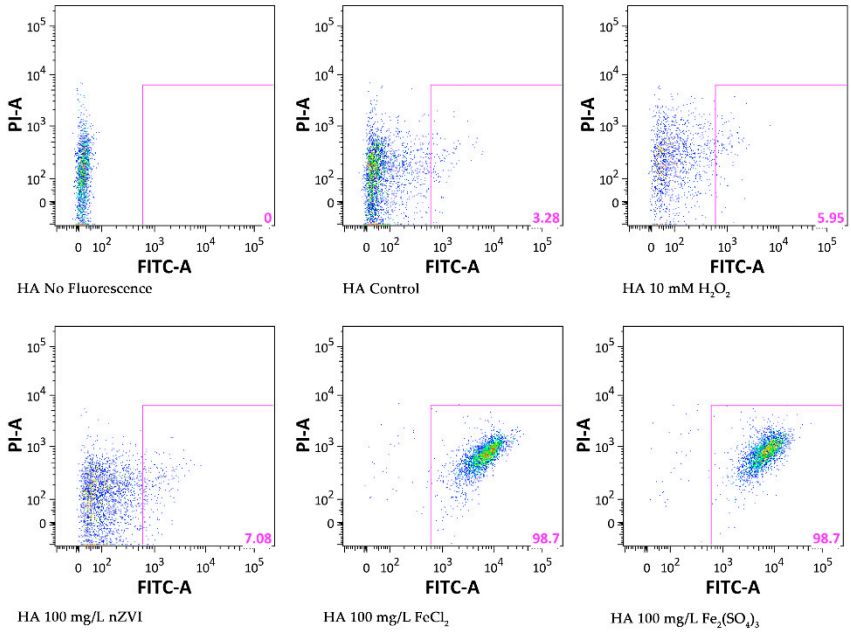


Figure S8. Reactive Oxygen Species production of hyaline amoebocytes (HA) without fluorescence, without treatment (control) and HA exposed to 10 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 2 hours.

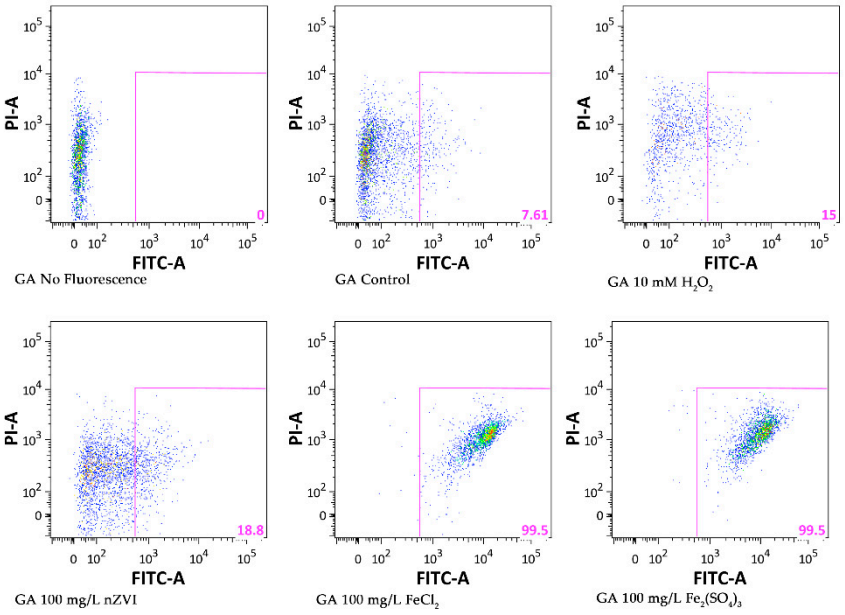


Figure S9. Reactive Oxygen Species production of granular amoebocytes (GA) without fluorescence, without treatment (control) and GA exposed to 10 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 2 hours.

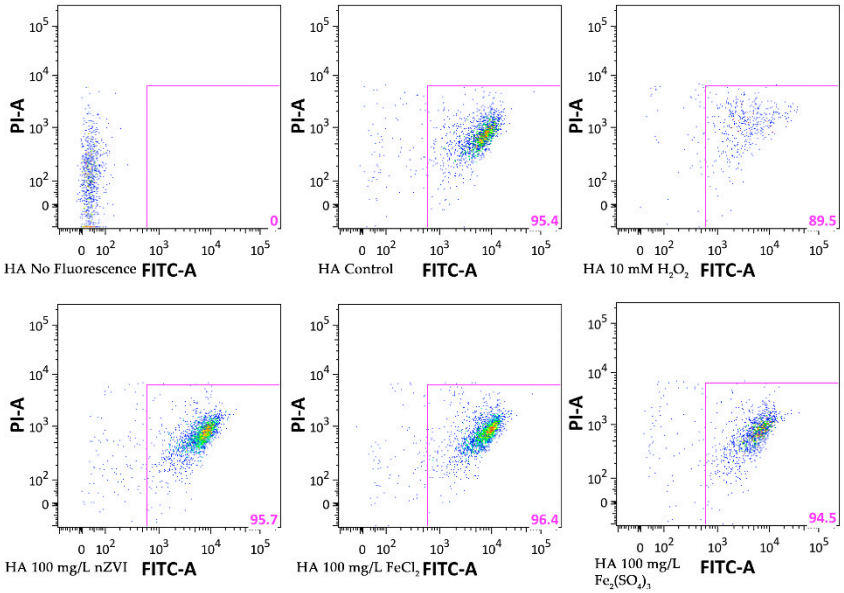


Figure S10. Reactive Oxygen Species production of hyaline amoebocytes (HA) without fluorescence, without treatment (control) and HA exposed to 10 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 6 hours.

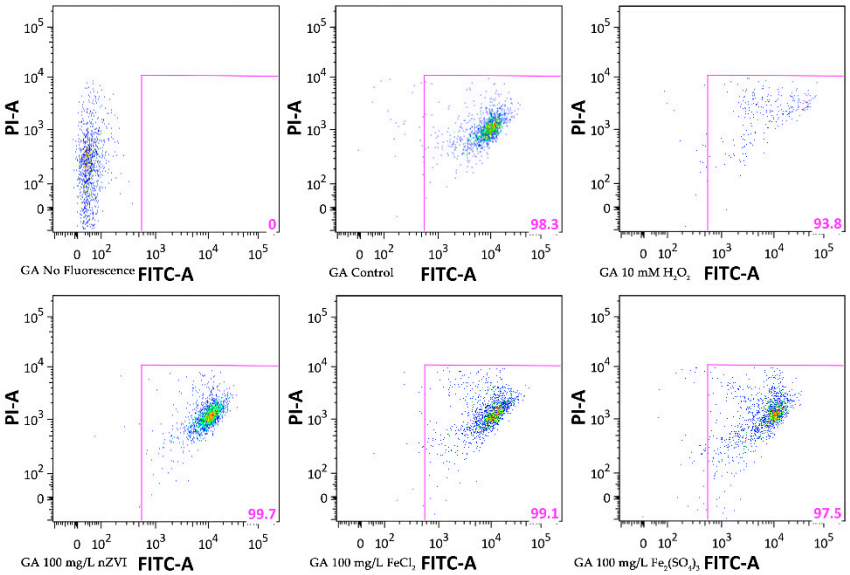


Figure S11. Reactive Oxygen Species production of granular amoebocytes (GA) without fluorescence, without treatment (control) and GA exposed to 10 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 6 hours.

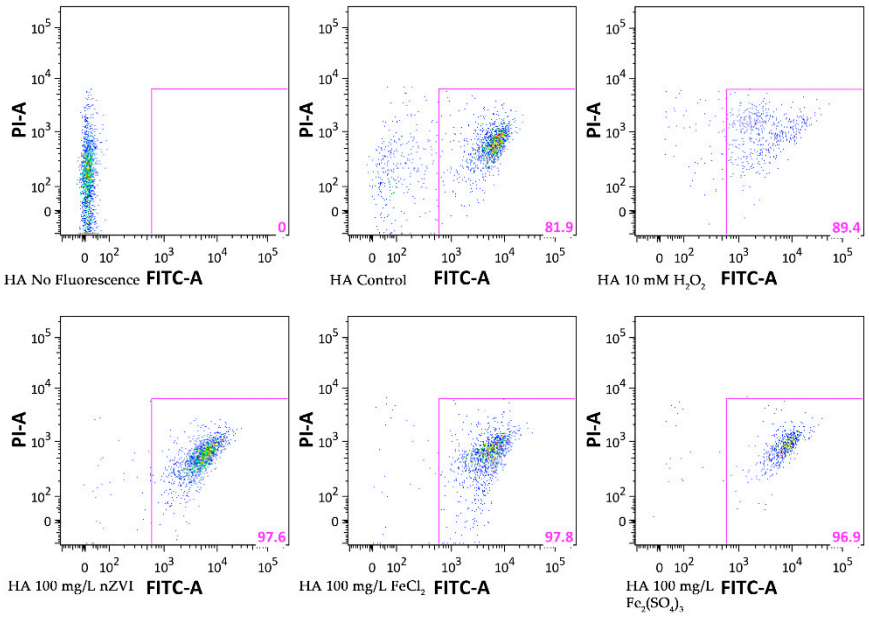


Figure S12. Reactive Oxygen Species production of hyaline amoebocytes (HA) without fluorescence, without treatment (control) and HA exposed to 10 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 24 hours.

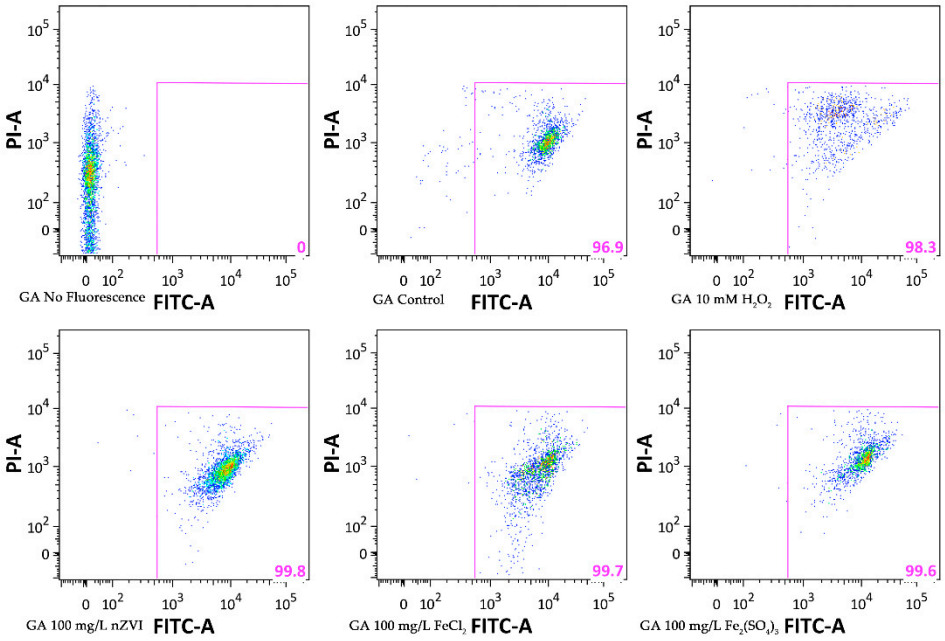


Figure S13. Reactive Oxygen Species production of granular amoebocytes (GA) without fluorescence, without treatment (control) and GA exposed to 10 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 24 hours.

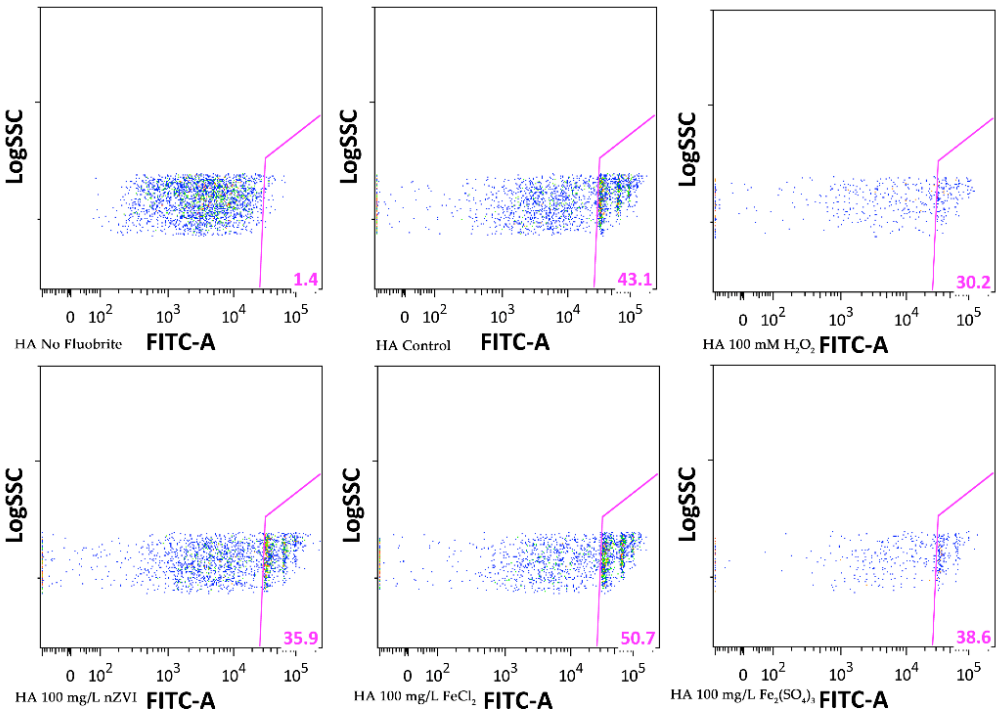


Figure S14. Phagocytic activity of hyaline amoebocytes (HA) without Fluoresbrite, without treatment (control) and HA exposed to 100 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 2 hours.

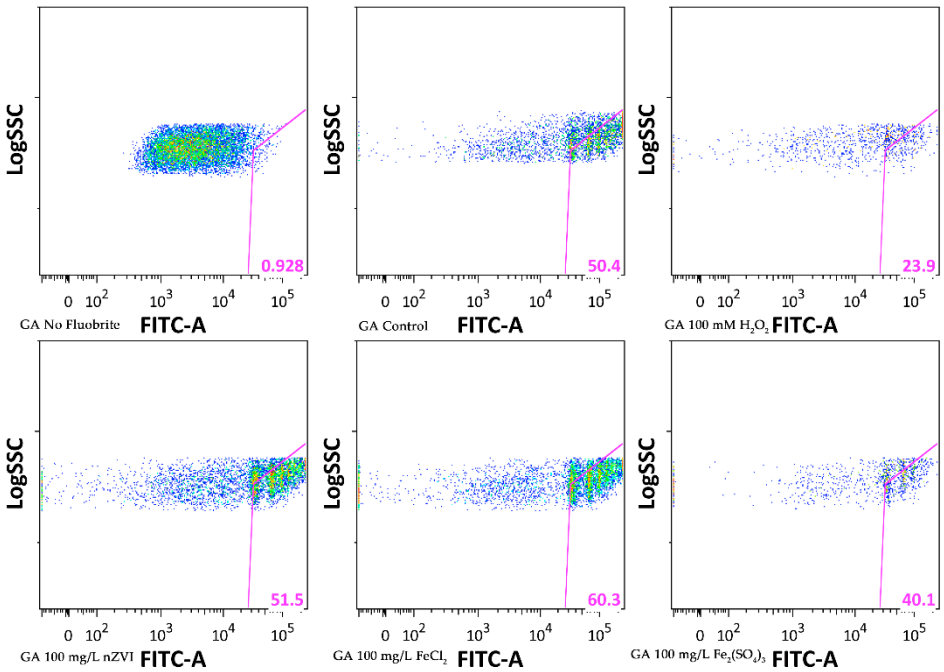


Figure S15. Phagocytic activity of granular amoebocytes (GA) without Fluoresbrite, without treatment (control) and GA exposed to 100 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 2 hours.

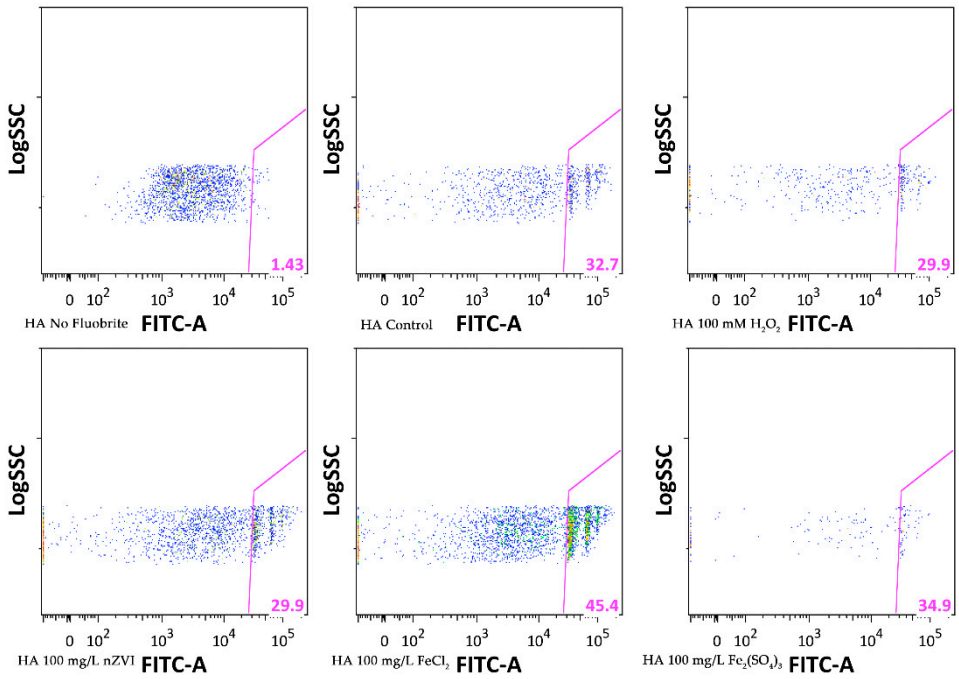


Figure S16. Phagocytic activity of hyaline amoebocytes (HA) without Fluoresbrite, without treatment (control) and HA exposed to 100 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 6 hours.

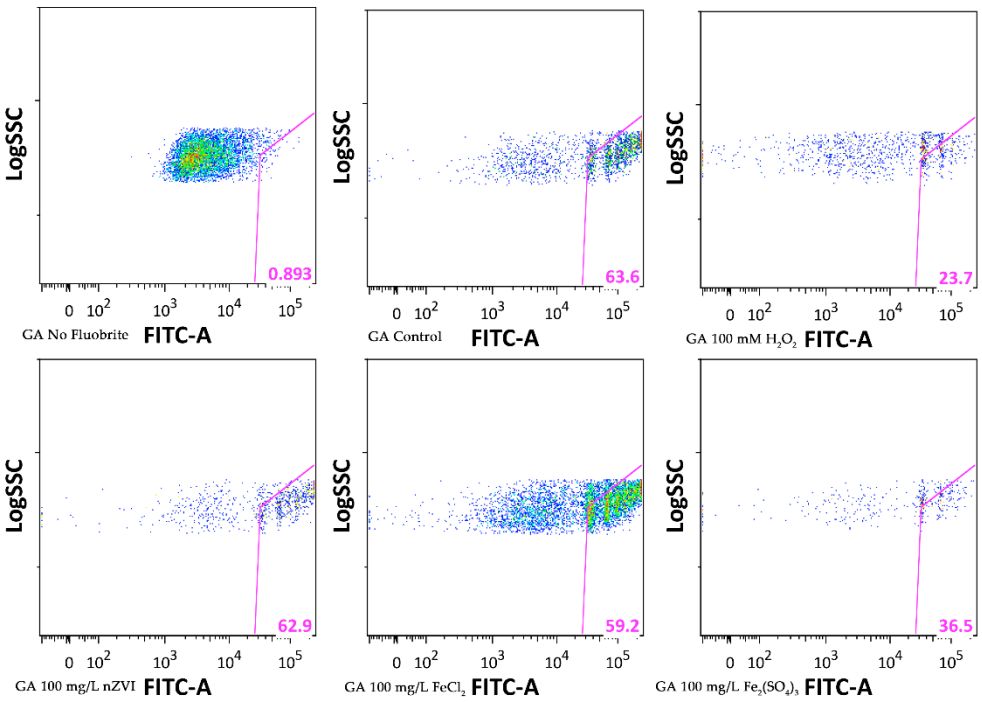


Figure S17. Phagocytic activity of granular amoebocytes (GA) without Fluoresbrite, without treatment (control) and GA exposed to 100 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 6 hours.

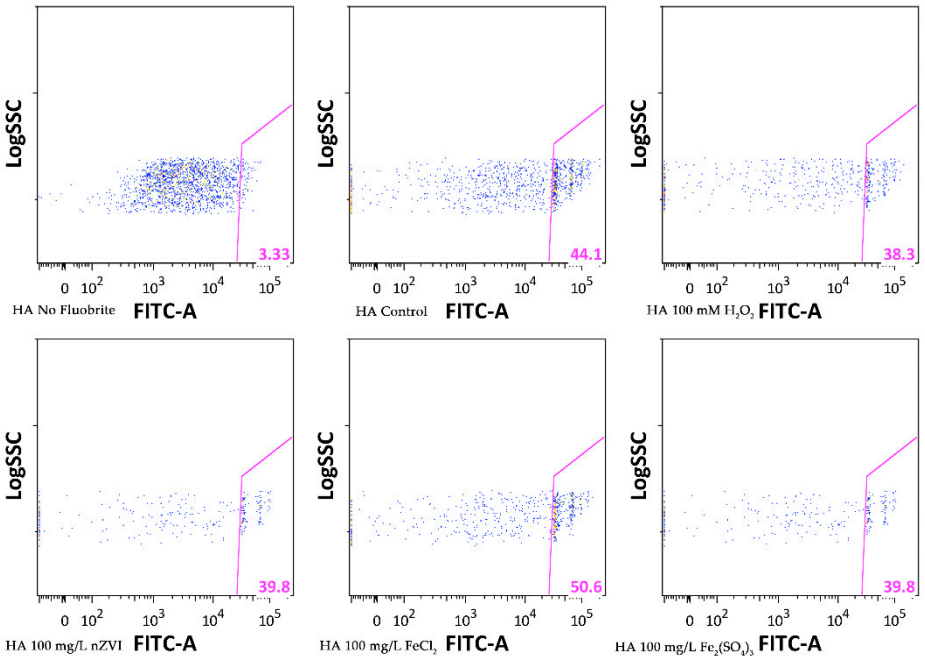


Figure S18. Phagocytic activity of hyaline amoebocytes (HA) without Fluoresbrite, without treatment (control) and HA exposed to 100 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 24 hours.

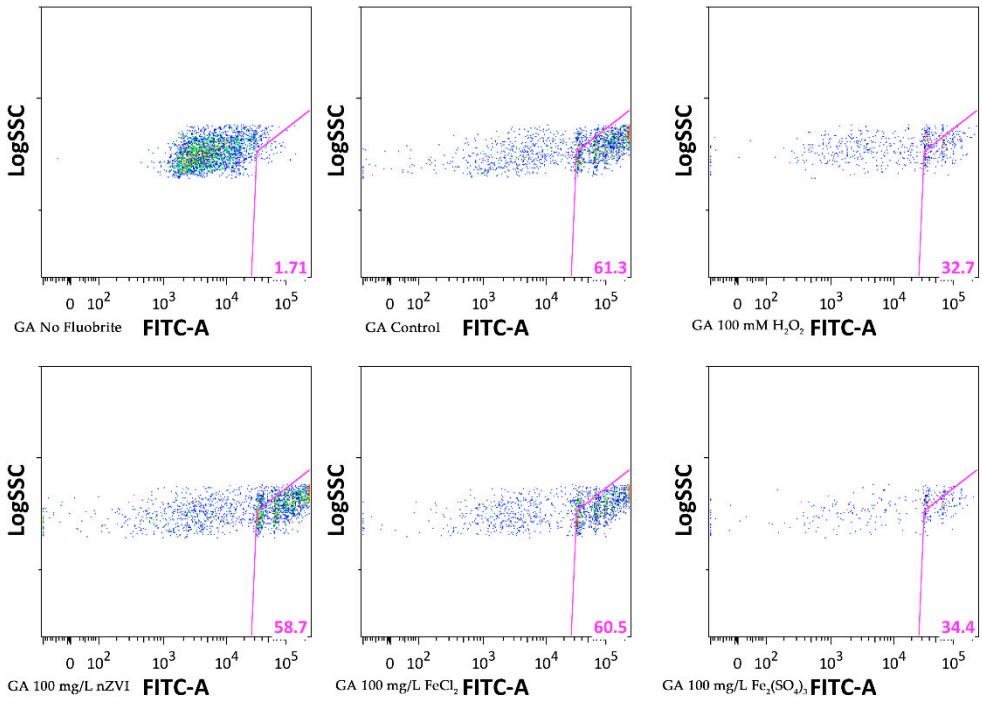


Figure S19. Phagocytic activity of granular amoebocytes (GA) without Fluoresbrite, without treatment (control) and GA exposed to 100 mM H_2O_2 (positive control), 100 mg/L of nZVI, $FeCl_2$ and $Fe_2(SO_4)_3$ after 24 hours.

Bioresource Technology 341 (2021) 125925

Contents lists available at ScienceDirect

Bioresource Technology

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

Conversion of spent coffee grounds into vermicompost

Ales Hanc [a,*], Tereza Hrebeckova [a], Alena Grasserova [b,c], Tomas Cajthaml [b,c]

[a] Department of Agro-Environmental Chemistry and Plant Nutrition, Czech University of Life Sciences Prague, Czech Republic
[b] Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic
[c] Institute for Environmental Studies, Faculty of Science, Charles University in Prague, Czech Republic

HIGHLIGHTS

- The addition of straw to spent coffee grounds supported earthworm development.
- The contents of fungi and enzymes decreased with the age of vermicompost.
- Earthworms were able to substantially reduce the caffeine stimulant content.
- Vermicompost contained more P, K and Mg than the variant without earthworms.
- Biowaste with earthworms was stabilized faster than without earthworms.

ARTICLE INFO

Keywords:
Spent coffee grounds
Vermicomposting
Straw pellets
Caffeine
Eisenia andrei

ABSTRACT

The present study was focused on vermicomposting of spent coffee grounds (SCG) and its mixtures with straw pellets. The process was evaluated in terms of biological and physico-chemical properties. The greatest number and biomass of earthworms was found in the treatment with 25% vol. SCG + 75% vol. straw pellets. In this treatment, the upper youngest layer exhibited 1.6-fold and 4.5-fold greater earthworm number and biomass, respectively, than the bottom oldest layer. Earthworm weight decreased in direct proportion to the layer age. The oldest treatment layer was characterized by lesser contents of fungi and six hydrolytic enzymes, compared to the younger layers. Further, the oldest treatment layer had suitable agrochemical properties. Earthworms were able to substantially reduce the caffeine stimulant content, which is considered the most representative pharmaceutically active compound.

1. Introduction

Worldwide, the coffee processing industry produces almost 33 million tons of solid coffee waste (coffee pulps, mucilages, and hulls) per year. The coffee processing industry utilizes almost 15 L of water per kilogram of freshly harvested green coffee during the various stages. The process discharges effluent which can pollute a receiving water system and soil (Alemayehu et al., 2020). In 2019, annual coffee bean production exceeded 10 million tons (International Coffee Organization, 2020). The residue after preparing the coffee drink are spent coffee grounds (SCG). Since coffee brewing involves the extraction of selected compounds from coffee beans, a large amount of unused waste is generated (about 90 % by weight) (McNutt and He, 2019; Blinová et al., 2017). Especially, in cafes, SCG can account for a substantial proportion of food waste. SCG can be defined as an organic residue with high humidity and small particle size (Esquivel and Jiménez, 2012). SCG contain large amounts of organic matter, including polysaccharides, especially cellulose (with glucose as a main component), and hemicellulose (with mannose, galactose, and arabinose as main components), which together make up half of the SCG dry mass. Lignin (25% wt. on a dry mass basis), protein (almost 20% wt. on a dry mass basis), and oil (with over 15% wt. on a dry mass basis) are also significant in SCG (Ballesteros et al., 2014; Mussatto et al., 2011; Yordanov et al., 2016; Kovalcik et al., 2018). Most SCG end up in landfills or in the sewage system, which is a serious environmental problem related to biodegradable organic matter decomposition and the release of potentially toxic compounds, for example, polyphenols, tannins, and caffeine (Low et al., 2015; Murthy and Madhava Naidu, 2012). SCG are characterized

* Corresponding authors at: Ales Hanc, Department of Agro-Environmental Chemistry and Plant Nutrition, Czech University of Life Sciences Prague, Kamycka 129, 165 00 Prague 6, Czech Republic.
E-mail address: hanc@af.czu.cz (A. Hanc).

https://doi.org/10.1016/j.biortech.2021.125925
Received 31 July 2021; Received in revised form 6 September 2021; Accepted 8 September 2021
Available online 13 September 2021

by a great caffeine content, but the specific value depends on the coffee sort and processing (Peshev et al., 2018). Caffeine is considered as pharmaceutically active compound (PhAC) pollutant. Due to its large amount in the environment, it is suitable as an indicator of anthropogenic inputs of PhACs in unregulated water bodies. Caffeine residues are very stable in the water environment (Li et al., 2020).

In practice, only a small part of SCG production is used. According McNutt and He (2019), possible applications for SCG include:

- biodiesel, bioethanol, biochar, and biogas production (Obruca et al., 2014; Tongcumpou et al., 2019)
- direct fuel source
- extraction of phenolic compounds, antioxidants, and phytosterols (Nzekoue et al., 2020)
- use as subgrade filler material, effective adsorbent for a wide range of contaminants, composites, and bricks incorporating SCG
- direct application into soil – there is frequent anecdotal recommendation for the use of locally produced SCG as fertilizer into the soil. However, this is against the conclusions of scientific experiments. For example, in an experiment with five horticultural plants in different soil types with various SCG amendment rates, detrimental plant growth effects were found, regardless of soil type and fertiliser addition. Growth suppression was not explained by a change in any of the major parameters, such as pH or soil nitrogen availability. The more likely reason was the phytotoxic effects of SCG caused by high levels of caffeine, tannins and polyphenols (Hardgrove and Livesley, 2016)
- composting is a viable way of valorising this residue (Santos et al., 2017)

Composting using earthworms (vermicomposting) is considered the most advanced composting method (Lim et al., 2016). Vermicomposting is a biooxidative and stabilizing process of organic materials conversion, which, unlike classical composting, uses interactions between earthworms and microorganisms, and does not involve the decomposition thermophilic phase (Domínguez and Edwards, 2011). It is evident that earthworms and endosymbiotic microbes during vermicomposting tend to eliminate pathogens by enhancing enzymatic activities in both gut- and cast-associated processes. Pathogen reduction during vermicomposting can be plausibly attributed to direct actions like microbial inhibition due to intestinal enzymatic action, and secretion of coelomic fluids with antibacterial properties, as well as indirect actions like stimulation of endemic microbes leading to competition and antagonism. Further, the pathogen reduction during vermicomposting is largely selective, and earthworms exert a differential effect according to the earthworm species and whether the pathogen considered is Gram-positive or -negative, owing to its cell wall composition (Swati and Hait, 2018). Digging, fragmentation, and aeration are provided to a greater extent by earthworms, making vermicomposting one of the low-cost waste treatment systems (Baghel et al., 2018). The technology is fully environmentally friendly (Abbasi et al., 2015).

Vermicomposts are characterized by very good maturity and stability, containing high-quality humic substances, enzymes, and plant growth hormones (Hanc et al., 2019a; Hřebečková et al., 2019a; Ravindran et al., 2016).

There are many reports on different valorization of SCG. Some of them are on classical composting. Only a few studies have been published on vermicomposting of this specific waste. Adi and Noor (2009) used *Lumbricus rubellus* for 49-day vermicomposting that was conducted after 21 days of pre-composting. Three different combination of treatments were prepared combined cow dung, kitchen waste and coffee grounds. The presence of coffee grounds showed higher percentage of nutrients in vermicompost produced. The data reveal that coffee grounds can be decomposed through vermicomposting and help to enhance the quality of vermicompost produced rather than sole use of kitchen waste in vermicomposting. Sanchez-Hernandez and Domínguez (2017) concluded that vermicomposting reduced substantially the residue mass of SCG in the very short term, yielding a nutrient-rich and enzymatically active vermicompost. González-Moreno et al. (2020) conducted 60-day laboratory vermicomposting experiment with 9 treatments differed with proportions of horse manure, spent coffee grounds (SCG) and coffee silver skin (CS). Best options were treatments with a medium–low amount of residue (25% for SCG and 25% or 50% for CS) due to the specific characteristics of these wastes and possible toxicity. They recommended that SCG is used with other amendments or alkaline residues in order to increase the pH level..

In comparison with the above studies, the main novelties of our work include using of straw pellets as amendment material, system with continuous feeding of earthworms where layers of different ages can be evaluated and effect of earthworms on reduction of caffeine.

The aim of the study was to investigate the feasibility of the vermicomposting SCG and its mixtures with straw pellets on the basis of: i) survival and development of earthworms, ii) occurrence of the main groups of microorganisms, iii) enzyme activity, iiii) physico-chemical parameters, and iiiii) caffeine content.

Our research results are useful for food waste producers, small-scale processors and large-scale vermicomposting plants, and vermicompost users, especially growers.

2. Materials and methods

2.1. Raw material and earthworms

A SCG mixture of coffee varieties from the La Divina Providencia from Nicaragua, Karuhiu Uhteri from Kenya, and Nensebo from Ethiopia was used for the vermicomposting experiment. It was obtained from Misto Café in Prague (N 50°5.94017′, E 14°24.26082′). After receiving this material, the SCG were refrigerated in dark sealed bags. Due to the lesser pH and C:N ratio in SCG, moistened straw pellets with 34% dry matter content was used in the experiment. The pellets exhibited an alkaline pH (7.9). The EC was 2 times greater, and the C:N 4 times greater compared with the SCG (Table 1). *Eisenia andrei* earthworms were used in the study. It belongs to the group of epigeic earthworms. It is one of the most commonly used earthworms for vermicomposting in a mild climate.

2.2. Experimental design

The experiment was set up under laboratory conditions in plastic vermicomposters Worm Factory with four perforated trays of individual size 40 × 40 × 18 cm, marked from oldest (I) to youngest layer (IV). They were gradually filled with biowaste every 6 weeks during 6 months. Five treatments were established:

1: SCG 100% vol. with *Eisenia andrei* (layers I to IV)
2: SCG 75% vol. + straw pellets 25% vol. with *Eisenia andrei* (layers I to IV)
3: SCG 50% vol. + straw pellets 50% vol. with *Eisenia andrei* (layers I to IV)
4: SCG 25% vol. + straw pellets 75% vol. with *Eisenia andrei* (layers I to IV)
5: SCG 50% vol. + straw pellets 50% vol. without earthworms (layers I to IV)

For the four first treatments, 10*L* of bedding layer containing grape marc with earthworms *Eisenia andrei* (50 earthworms per liter) was put down. After that, new layer – 15 L of feedstocks was placed into the new tray above. The top of the vermicomposter was covered with a composting fabric and a plastic lid. To prevent the earthworms from escaping or crawling among vermicomposters, the experiment was conducted in constant light. The room air temperature was maintained at 22 °C. Every 12 h the room air was replaced with outdoor air.

Three 1 kg samples were taken from each layer and weighed. All potential earthworms were separated manually, counted, and weighed from each sample taken. About 50 g of the remaining sample was stored in a refrigerator (temperature 4 °C). Another 500 g portion was placed in a drying room and dried for about 14 days at a constant temperature of

Table 1
Physico-chemical parameters of SCG and wet straw pellets used in the study.

	Dry matter[%]	pH/H_2O	EC[μS/cm]	ORP[mV]	C/N	$P_{tot.}$[mg/kg]	$K_{tot.}$[mg/kg]	$Mg_{tot.}$[mg/kg]	Caffeine[mg/g]
SCG	34.5 ± 1.0	6.0 ± 0.1	723 ± 47	45.5 ± 6.6	17.7 ± 1.3	1301.7 ± 131.4	9904.3 ± 917.4	1586.0 ± 238.6	3.27 ± 0.14
Straw pellets	34.2 ± 2.4	7.9 ± 0.1	1345 ± 59	−30.5 ± 3.1	65.7 ± 7.0	1100 ± 100	9300 ± 600	900 ± 100	n.d.

Values are means ± SD; n.d. – no data.
pH, EC, and ORP were determined in wet matter; other parameters in dry matter
SCG = spent coffee grounds

35 °C, and then ground. The remaining sample was frozen at −25 °C and lyophilized.

2.3. Physico-chemical and biological analyses

2.3.1. Analyses from refrigerated samples

To determine the pH, electrical conductivity (EC) and oxidation–reduction potential (ORP), a 10 g sample was weighed into a sealable flask, and then 50 ml of demineralized water was added. The mixture was then shaken for 10 min. Values of pH and ORP were measured with a calibrated meter (WTW 340i). Then, the sample was filtered and the EC was measured with the WTW cond 730 conductometer.

2.3.2. Analyses from dried samples

For total carbon (C_{tot}) and nitrogen (N_{tot}) determination, the CHNS Vario MACRO cube analyzer (Elementar Analysensysteme GmbH, Germany), was used. The total contents of macronutrients (P, K, and Mg) were determined by decomposition obtained by pressurized wet-ashing (HNO_3 + H_2O_2) of dried samples in a closed system of Ethos 1 (MLS GmbH, Germany). The contents of ammonium and nitrate nitrogen (N-NH_4^+, N-NO_3^-), dissolved organic carbon (DOC) and the available nutrients (P, K, and Mg) were determined in CAT solution, which is a mixture of 0.01 mol/L $CaCl_2$ and 0.002 mol/L diethylene triamine pentaacetic acid (DTPA) (1:10 w/v), according to the international BSI EN 13651, 2001. The N-NH_4^+, N-NO_3^-, and DOC contents in the extracts were measured using the SKALAR SANPLUS SYSTEM® (the Netherlands). The total and available element concentrations were determined using ICP-OES (VARIAN VistaPro, Australia).

2.3.3. Analyses from lyophilized samples

Phospholipid fatty acid (PLFA) analysis were determined in the samples (in triplicates) according to Stella et al., 2015. The samples were extracted using phosphate buffer, chloroform, and methanol (0.8:1:2; v/v/v) according to Bligh and Dyer, 1959. Gas chromatography-mass spectrometry (GC–MS; 450-GC, 240-MS Varian, Walnut Creek, CA, USA) was employed for determination of methylated esters of fatty acids. The authentic chemical standards were obtained from Sigma-Aldrich, Prague, Czech Republic and Matreya LLC, USA. Actinobacterial biomass was estimated as the sum of 10Me-16:0, 10Me-18:0, 10Me-17:0, biomass of gram positive bacteria (G +): a17:0, i17:0, i16:0, i15:0, a15:0, and i14:0; biomass of gram negative bacteria (G −): 16:1ω5,16:1ω7, cy17:0, 18:1ω7, and cy19:0. The total bacterial biomass was determined on the basis of 15:0, 17:0, 16:1ω7, and 16:1ω9, together with the other above mentioned bacterial PLFA. Fungi were estimated according to 18:2ω6,9.

The enzymatic activities of hydrolytic enzymes were measured in 96-well microplates. The mixture of lyophilized sample (0.2 g) and acetate buffer (20 ml; pH 5.0; c = 50 mmol/L) was homogenized using the Ultra-Turrax (IKA Labortechnik, Germany) according to Štursová and Baldrian (2011). The substrates for these hydrolytic enzymes were as follows: for β–D–glucosidase it was MUFG (c = 2.75 mmol/L), which is a mixture of 4-methylumbellyferyl-β-D-glucopyranoside and dimethyl sulfoxide, for acid phosphatase it was MUFP(c = 2.75 mmol/L), which is a mixture of 4-methylumbellyferyl-phosphate and dimethyl sulfoxide, for arylsulphatase it was MUFS (c = 2.50 mmol/L), which is a mixture of 4-methylumbellyferyl sulphate potassium salt and dimethyl sulfoxide, for lipase it was MUFY (c = 2.50 mmol/L), which is a mixture of 4-methylumbellyferyl-caprylate and dimethyl sulfoxide, for chitinase it was MUFN (c = 1.00 mmol/L), which is a mixture of 4-methylumbellyferyl-N-acetylglucosaminide and dimethyl sulfoxide, for cellobiohydrolase it was MUFC (c = 2.50 mmol/L), which is a mixture of 4-methylumbellyferyl-N-cellobiopyranoside and dimethyl sulfoxide, for alanine aminopeptidase it was AMCA (c = 2.50 mmol/L), which is a mixture of L-alanine-7-amido-4-methylcoumarin and dimethyl sulfoxide, for leucine aminopeptidase it was AMCL (c = 2.50 mmol/L), which is a mixture of L-leucine-7-amido-4-methylcoumarin and dimethyl sulfoxide. Enzymes were measured as a fluorescence change using the Tecan Infinite® M200 (Austria) after 5 min and 125 min of incubation (40 °C) according to Baldrian (2009).

Caffeine was analyzed in the lyophilized samples using liquid chromatography-tandem mass spectrometry (LC-MS/MS). At first, the lyophilised samples were extracted using Accelerated Solvent Extractor ASE 200 (Dionex; Palaiseau, France) with heated methanol as an extraction solvent (temperature 80 °C, pressure 10.3 MPa, 3 extraction cycles and 5-min static periods in between the cycles). Methanol extracts were appropriately diluted with 50% methanol and analysed with liquid LC-MS/MS (Cimetiere et al., 2013). The system consisted of Agilent 1260 Infinity II liquid chromatograph coupled with Agilent 6470 LC/TQ mass spectrometer equipped with Agilent Jet Stream electrospray ion source (Agilent Technologies, Santa Clara, CA, USA). The caffeine analyses was performed using chromatographic column Poroshell 120 2.7 μm, 3 mm × 100 mm (Agilent Technologies, Santa Clara, CA, USA). Injection volume was 2 μl, mobile phase consisted of 0.5 mM NH_4F in Milli-Q water (0.01% formic acid; Honeywell) (A) and methanol (Honeywell) (B); the flow rate was 0.4 ml min^{-1}. Column temperature throughout the analysis was maintained at 40 °C. Gradient elution was as follows (min/%B): 0/5; 1/20; 8 – 9/100; 9.1 – 12/5. Caffeine was monitored in positive ion mode, specific ion transitions (m/z) were: 195.1 → 83; 195.1 → 110 and 195.1 → 138 (fragmentor voltage: 105 V, collision energy: 20 eV). Following electrospray conditions were applied: drying gas temperature: 200 °C, drying gas flow 8 L/min, nebulizer pressure: 45 psi, sheath gas temperature: 400 °C, sheath gas flow: 12 L/min, capillary voltage: 2500 V, nozzle voltage: 0 V.

2.4. Statistical analysis

All the results are the means of three replicates. The tests of normality and homogeneity were performed. Since some data did not have normal and homogeneous distribution, a strict nonparametric Kruskal-Wallis ANOVA test ($P \leq 0.05$) was used with the help of STATISTICA 12 software (StatSoft, Tulsa, USA). Spearman's correlations were explored at the 0.05 probability levels.

3. Results and discussion

3.1. Earthworms

The youngest upper layers contained the greatest biomass and also a number of earthworms (Table 2). The increasing addition of straw pellets reduced the proportion of number and biomass of earthworms in the upper layer (number of earthworms: 88%, 64%, and 37%; earthworm

Table 2
Number and biomass of earthworms in individual treatments and layers.

Layer (age)	100% SCG(Treatment 1)		75% SCG + 25% straw pellets (Treatment 2)		50% SCG + 50% straw pellets (Treatment 3)		25% SCG + 75% straw pellets (Treatment 4)	
	Number [pcs/kg]	E. biomass [g/kg]	Number [pcs/kg]	E. biomass [g/kg]	Number [pcs/kg]	E. biomass [g/kg]	Number [pcs/kg]	E. biomass [g/kg]
IV(45 days)	42 ± 5	12.0 ± 1.7	99 ± 24	38.7 ± 6.5	70 ± 13	27.5 ± 3.4	629 ± 83	71.5 ± 7.1
III(90 days)	41 ± 18	13.0 ± 5.6	14 ± 3	4.2 ± 1.2	31 ± 8	13.6 ± 3.9	354 ± 66	28.4 ± 5.5
II(135 days)	6 ± 4	1.2 ± 0.8	0	0	8 ± 1	2.8 ± 1.4	320 ± 6	15.8 ± 0.6
I(180 days)	0	0	0	0	1 ± 1	0.2 ± 0.2	400 ± 51	16.0 ± 0.2

Values are means ± SD.
SCG = spent coffee grounds
E. biomass = Earthworm biomass
days in brackets = age of layers at the time of sampling

biomass: 90%, 62%, and 54% in treatments 2, 3, and 4, respectively), and thus increased their presence in the bottom and the middle layers. The biomass for layer I (age 180 days) was less for treatment 3 (0.2 g) than for treatment 4 (16 g). Their long presence in treatment 4 was due to the longer biodegradability because of the greater C/N ratio. Significant proportion of straw and, conversely, a lesser proportion of SCG created suitable conditions for earthworm survival (greater aeration and less bulk density of the mixture), and encouraged earthworms to multiply. Earthworm weight was calculated from Table 2 as ratio of earthworm biomass and number of earthworms, and decreased in direct proportion to the layer age. Compared to the other treatments, the earthworm weight was much lesser for treatment 4 (0,07 g). The fewest number and least biomass of earthworms were found in the first vermicomposter with 100% coffee grounds. They accounted for 4% of the total number and 10% of total earthworm biomass of all vermicomposters. The lesser earthworm biomass in SCG itself could be caused by the lesser carbon compound content as a source of energy for earthworms, and/or the relatively great density and thus lesser air content in SCG and pressure of the above layers. The toxicity of the SCG itself may be important here (Cervera-Mata et al., 2020). In our experiment, the greatest earthworm number, which exceeded all other treatments by about 15 times, was found in the fourth treatment with 75% straw pellets. Therefore, the description of further biological and chemical parameters will be directed to this treatment and treatment 3, where earthworms occurred in all layers.

3.2. Microorganisms

The oldest layers of treatment 3 and 4 contained by 23% and 11%, respectively, more total microbial biomass expressed as phospholipid

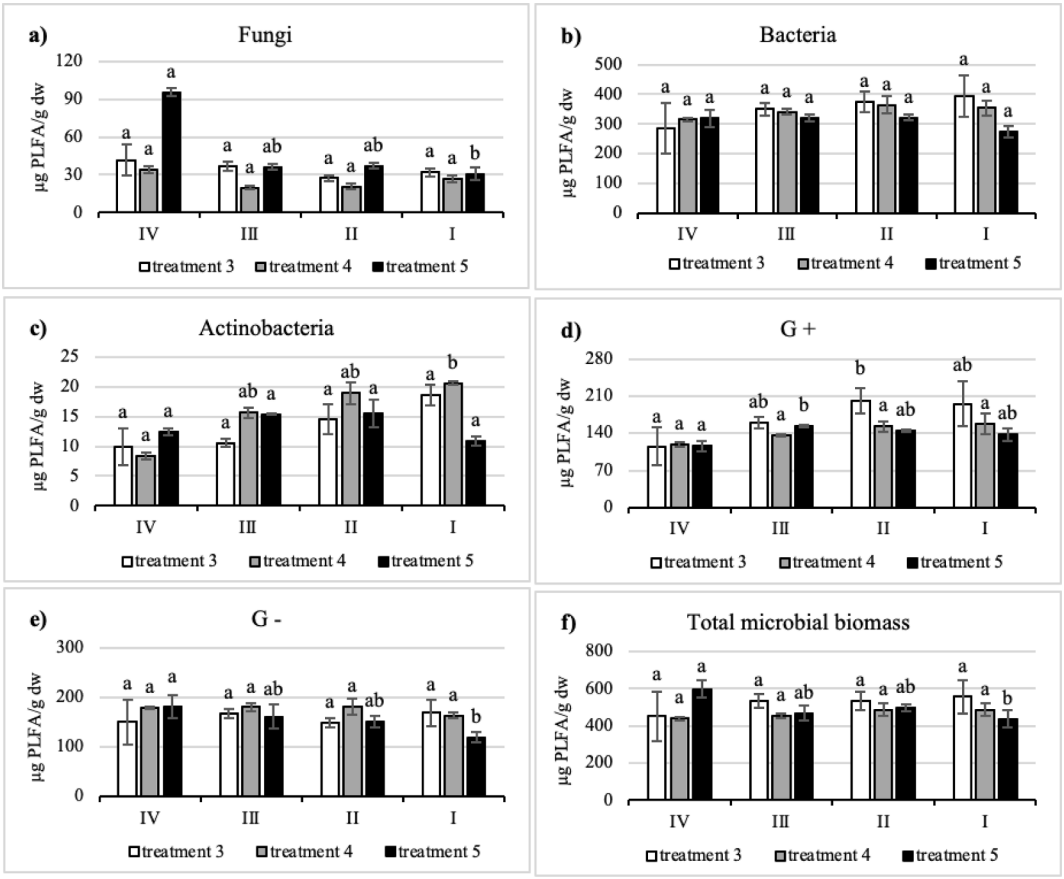


Fig. 1. Changes in the microbial biomass expressed as content of phospholipid fatty acids (PLFA) (a-f) in layers IV, III, II, and I of the treatments 3, 4, and 5. Values are the means ± SD. Letters indicate significant differences (Kruskal-Wallis test, $P \leq 0.05$) among layers within a treatment. dw – dry weight.

fatty acids (PLFA) than the youngest layer, in which the microorganisms were probably much represented in the earthworm digestive tracts (Fig. 1). This is confirmed by a strong indirect correlation between the total microbial biomass and earthworm number ($R = -0.94$, $p < 0.05$ for treatment 3; $R = -0.71$, $p < 0.05$ for treatment 4), and the total microbial biomass and earthworm biomass ($R = -0.92$, $p < 0.05$ for treatment 3; $R = -0.88$, $p < 0.05$ for treatment 4). Conversely, in treatment 5 without earthworms, which is identical to treatment 3 (with respect to composition), biomass decreased directly proportionally with the layer age. Fungi are a valuable food source for earthworms. Epigeic earthworms have enzymes in their digestive tract that allow them to digest fungi (Zhang et al., 2000). Likewise, treatments 3 and 4 contained 32% and 50%, respectively, less fungi than treatment 5 without earthworms. Conversely, the presence of bacteria was slightly greater in treatments 3 and 4, specifically by 14% and 11%, respectively, in comparison with treatment 5 without earthworms. A strong indirect correlation was found between bacteria and earthworm number ($R = -0.99$, $p < 0.05$ for treatment 3; $R = -0.91$, $p < 0.05$ for treatment 4), and between bacteria and earthworm biomass ($R = -0.99$, $p < 0.05$ for treatment 3; $R = -0.96$, $p < 0.05$ for treatment 4).

3.3. Enzyme activities

The activity of all enzymes varied in the individual treatments and layers depending on the specific enzyme (Fig. 2). The greatest enzymatic activity values were found in the non-earthworm treatment 5. At the beginning of the process in the youngest treatment layer, the activity of the 8 enzymes was 1.75 and 2.96 times greater compared to treatments 3 and 4, respectively. In the oldest layer, it was only 1.52 and 2.21-fold. This was probably due to the greater number and biomass of earthworms, where decomposition took place mainly in the earthworm bodies. Fifty-seven bacterial 16S rDNA clones, including enzyme-producing microorganisms, were identified in the intestines of *Eisenia fetida* by using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis (Hong et al., 2011). In our experiment, as the earthworm representation in layers III and II decreases, the enzymatic activity increases. In the oldest layer, in which the presence of earthworms slightly increased, enzymatic activity was reduced again. Activity of lipaze was the highest among 8 determined hydrolytic enzymes due to the high content of lipids in coffee, and in connection with caffeine, which promotes the breakdown of lipids.

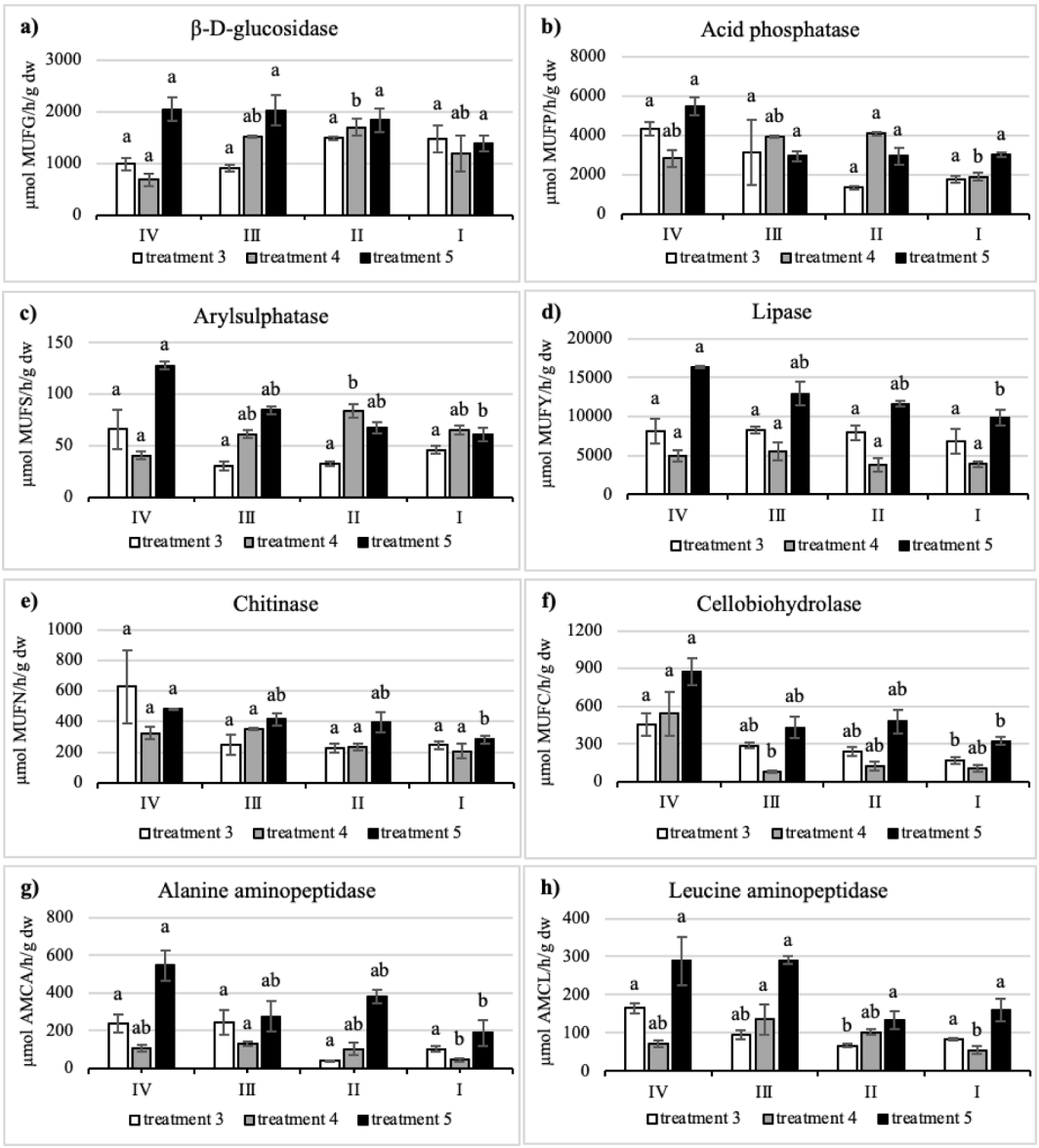


Fig. 2. Changes in enzymatic activity of hydrolytic enzymes (a-h) in layers IV, III, II, and I of the treatments 3, 4, and 5. Values are the means ± SD. Letters indicate significant differences (Kruskal-Wallis test, $P \leq 0.05$) among layers within a treatment. dw – dry weight.

Caffeine inhibits phosphodiesterase (an enzyme that catalyzes the hydrolysis of cyclic adenosinemonophosphate (cAMP)), leading to an increase in cAMP concentration. Thus, caffeine indirectly affects the regulation of cAMP-dependent protein kinases responsible for the regulation of glycogen, sugars and lipid metabolism. Activation of hormone-sensitive lipases leads to increased lipolysis, which causes increased plasma levels of free fatty acids and glycerol. There is also an increased release of catecholamines (Wahlang et al., 2018).

3.4. Physico-chemical parameters

The dry matter decreased from the youngest to the oldest layer (Table 3). The differences between layers IV and I were statistically significant. Among monitored treatments, the greatest increase was recorded in treatment 4 (by 47% between layers I and IV), where greater earthworm activity and proportion of straw pellets were important. Increasing moisture with layer age showed that the humified material (vermicompost) has a greater water absorption capacity than feedstock (Munnoli and Bhosle, 2011). The pH ranged from 7.1 to 8.1. In treatment 3 with earthworms, there was a significant pH increase over time, unlike the composition of an identical earthworm-free treatment 5, in which the pH was almost unchanged. There was a statistically significant difference between treatments 3 and 5 in the oldest layer I. There was no pH change in treatment 4, which was due to the greater proportion of straw pellets with their greater pH value (7.9). This probably did not give the possibility to increase the pH further. The EC decreased significantly (by 23%) in treatment 3 between layers IV and I. The layer I EC value was much less for treatment 3 (626 μS/cm) than for the earthworm-free treatment 5 (785 μS/cm). This can be explained by the fact that some of the salts were found in earthworm bodies removed from the sample prior to vermicompost analysis. In another 10-week SCG vermicomposting study, EC was maintained within the range of 0.64–0.70 ds m^{-1}, and treatments receiving cardboard had a lesser EC compared with the non-cardboard treatments (Liu and Price, 2011). Similar values and findings were obtained in our experiment. Redox potential gradually decreased with vermicompost age, which indicates gradual reduction of aerobic conditions. One of the important factors that reduced ORP in older layers was the pressure of the layers located above them. In control treatment 5, the ORP was by 35% and by 74% less compared to variants 3 and 4, respectively. This indicates that treatment 5 tended more to anaerobic conditions. On the contrary, earthworms have ability to intensively aerate material (Hanc and Dreslova, 2016) and thus ORP can increased. Earthworm number and earthworm biomass showed the same correlation with ORP in treatment 3 ($R = 0.91$, $p < 0.05$). In treatment 4, the ORP correlated more with earthworm biomass ($R = 0.70$, $p < 0.05$) than with earthworm number ($R = 0.42$, $p < 0.05$). The greater ORP in treatment 4 compared to treatments 3 and 5 was also due to the greater proportion of straw pellets having lesser bulk density and greater aeration ability. The C:N ratio decreased directly proportionally with increasing layer age. Treatment 4 exhibited the greatest C/N ratio, but there was the greatest decrease between layer IV and I (by 27%). For treatments 3 and 5, this decrease was only 8% and 20% respectively. The lesser C/N ratio in treatment 3 compared with treatment 5 (especially in the youngest layer) showed that earthworms accelerate biowaste decomposition. Wastes in which N-NH_4^+ values exceed 200 mg/kg of dry matter are unsuitable for vermicomposting, especially due to the volatilization of NH_3, which is lethal to earthworms (Míchal et al., 2019). N-NH_4^+ ranged between 40 and 50 mg/kg, and decreased during vermicomposting (Table 3). Lesser values were found in treatment 4 with 25% SCG compared with treatments 3 and 5, which suggests that the N-NH_4^+ level decreased with straw. Similarly, the N-NO_3^- values observed in treatment 4 were 6.5-fold less than in treatments 3 and 5, and accounted for only 0.03% of the total N content. With increasing composting time, the N-NO_3^- content increases (Hanc et al., 2017). This was confirmed in treatment 5 without earthworms, where after 180 days the N-NO_3^- value was 16

Table 3
Physico-chemical parameters in layers IV, III, II, and I of treatments 3, 4, and 5.

	50% SCG + 50% straw pelletswith earthworms (Treatment 3)	25% SCG + 75% straw pelletswith earthworms (Treatment 4)	50% SCG + 50% straw pelletswithout earthworms(Treatment 5)
Dry matter [%]			
IV (45 days)	19.9 ± 0.8 aA	22.6 ± 0.7 aA	24.6 ± 1.9 aA
III (90 days)	19.1 ± 0.8 abA	19.0 ± 0.7 abA	19.4 ± 0.6 abA
II (135 days)	17.4 ± 0.6 abAB	16.4 ± 0.3 abA	20.1 ± 1.1 abB
I (180 days)	16.5 ± 0.2 bAB	15.4 ± 0.5 bA	18.2 ± 0.2 bB
pH/H_2O			
IV (45 days)	7.2 ± 0 abA	7.8 ± 0.1 aB	7.3 ± 0 aAB
III (90 days)	7.1 ± 0 aA	7.9 ± 0 aB	7.2 ± 0.1 aAB
II (135 days)	7.6 ± 0.1 abAB	7.8 ± 0.1 aA	7.1 ± 0.1 aB
I (180 days)	8.1 ± 0.1 bA	7.8 ± 0 aAB	7.2 ± 0.1 aB
EC [μS/cm]			
IV (45 days)	808.7 ± 55.9 aA	664.7 ± 70.5 aA	767.0 ± 58.1 aA
III (90 days)	711.3 ± 19.7 abA	691.3 ± 37.0 aA	758.7 ± 166.7 aA
II (135 days)	766.7 ± 44.3 abA	742.0 ± 28.9 aA	805.7 ± 57.8 aA
I (180 days)	626.3 ± 33.9 bA	709.3 ± 46.7 aA	785.0 ± 127.5 aA
ORP [mV]			
IV (45 days)	36.8 ± 2.3 aAB	52.8 ± 7.1 aA	18.6 ± 2.3 aB
III (90 days)	25.0 ± 1.7 abA	51.5 ± 4.1 aB	13.4 ± 1.0 abA
II (135 days)	22.6 ± 2.2 abA	49.4 ± 7.6 aB	12.7 ± 2.6 abA
I (180 days)	8.3 ± 1.8 bA	44.1 ± 6.0 aB	8.2 ± 1.0 bA
C/N			
IV (45 days)	11.2 ± 0.3 abA	18.7 ± 0.7 aAB	13.6 ± 0.6 aB
III (90 days)	11.8 ± 0.1 aA	14.5 ± 0.1 abB	12.0 ± 0.2 abA
II (135 days)	11.1 ± 0.1 abAB	14.0 ± 0.4 abA	10.9 ± 0.4 abB
I (180 days)	10.3 ± 0.2 bA	13.6 ± 0.4 bB	10.3 ± 0.2 bA
N-NH_4^+ [mg N/kg]			
IV (45 days)	49.5 ± 0.2 aA	46.2 ± 2.9 aA	49.4 ± 0.7 aA
III (90 days)	47.2 ± 1.2 abA	46.7 ± 1.1 aA	45.3 ± 0.5 bA
II (135 days)	47.2 ± 0.4 abA	45.3 ± 1.6 aA	47.4 ± 0.7 abA
I (180 days)	45.4 ± 0.7 bAB	42.4 ± 1.2 aA	46.9 ± 1.2 abB
N-NO_3^- [mg N/kg]			
IV (45 days)	65.2 ± 3.1 aA	6.7 ± 2.4 abA	4.9 ± 0.3 aA
III (90 days)	65.2 ± 1.4 aAB	13.8 ± 0.7 aA	85.7 ± 0.7 bB
II (135 days)	48.1 ± 7.1 aAB	5.3 ± 0.8 bA	58.4 ± 0.5 abB
I (180 days)	33.6 ± 1.5 aAB	8.0 ± 0.8 abA	80.3 ± 1.1 abB

(continued on next page)

Table 3 (*continued*)

	50% SCG + 50% straw pelletswith earthworms (Treatment 3)	25% SCG + 75% straw pelletswith earthworms (Treatment 4)	50% SCG + 50% straw pelletswithout earthworms(Treatment 5)
$N\text{-}NH_4^+/N\text{-}NO_3^-$			
IV (45 days)	0.76 ± 0.04 abA	7.38 ± 2.08 abAB	10.15 ± 0.55 aB
III (90 days)	0.72 ± 0.01 aAB	3.39 ± 0.25 aA	0.53 ± 0.00 bB
II (135 days)	1.00 ± 0.14 abAB	8.71 ± 1.55 bA	0.81 ± 0.01 abB
I (180 days)	1.35 ± 0.08 bAB	5.33 ± 0.62 abA	0.58 ± 0.01 abB
DOC [mg C/kg]			
IV (45 days)	18808 ± 2440 aA	10084 ± 823 aA	16101 ± 2041 aA
III (90 days)	11875 ± 2468 abA	10560 ± 1706 aA	12910 ± 848 aA
II (135 days)	15025 ± 908 abA	11954 ± 1077 aA	12993 ± 1111 aA
I (180 days)	10245 ± 602 bA	10726 ± 651 aA	14898 ± 926 aA

Values are the means ± SD. Different lowercase letters in a column indicate significant differences among layers, capital letters indicate significant differences among treatments (Kruskal-Wallis test, $P \leq 0.05$).
SCG = spent coffee grounds; days in brackets = age of layers at the time of sampling

times greater. The nitrification index ($N\text{-}NH_4^+/N\text{-}NO_3^-$) is considered a parameter of compost and vermicompost maturity (Karak et al., 2017). There was a significant decrease in the $N\text{-}NH_4^+/N\text{-}NO_3^-$ ratio in treatment 5 between layers IV and III (45 and 90 days). In treatment 3 containing earthworms the $N\text{-}NH_4^+/N\text{-}NO_3^-$ ratio was on average 3 times less than in the identical treatment 5 without earthworms. Zhang and Sun (2017) found an indirect relationship between the increasing proportion of SCG and the nitrification index during green waste composting. Treatment 4 exhibited a much greater $N\text{-}NH_4^+/N\text{-}NO_3^-$ ratio than treatment 3 (Table 4). This result was probably due to the greater proportion of straw pellets, which increased the C/N ratio and thus provided more suitable conditions for earthworm activity, as seen previously (Biruntha et al., 2020). This is confirmed by the fairly even earthworm distribution in the whole profile of treatment 4. Earthworms are able to stabilize biowaste faster, as evidenced by the fact that after only 45 days the $N\text{-}NH_4^+/N\text{-}NO_3^-$ ratio was much less for treatment 3 (0.76), as opposed to treatment 5 (10.15). The proportion of DOC in the total carbon content was less in treatment 4 (2.6%) than in treatment 3 (3.2%). There was a decrease in DOC in treatments 3 and 5 between the youngest and the oldest layer by 45% and 7%, respectively. Although treatment 4 exhibited the least DOC, the values practically did not change, because the vermicomposting process was still occurring in all layers.

Table 4
Caffeine content in layers IV, III, II, and I of all studied treatments (ng/g of dry matter).

	IV (45 days)	III (90 days)	II (135 days)	I (180 days)
100% SCGwith earthworms (Treatment 1)	2447 ± 96.6 Aab	142.4 ± 21.6 bAB	2301 ± 89.1 abA	1748 ± 138.0 abAB
75% SCG + 25% straw pelletswith earthworms (Treatment 2)	95.4 ± 6.7 aAB	2791 ± 780.8 abAB	139.5 ± 51.3 abAB	7330 ± 218.9 bB
50% SCG + 50% straw pelletswith earthworms (Treatment 3)	42.0 ± 6.3 abAB	42.1 ± 4.6 abAB	73.9 ± 19.4 aAB	33.4 ± 2.3 bAB
25% SCG + 75% straw pelletswith earthworms (Treatment 4)	26.8 ± 1.9 aA	20.7 ± 0.6 abA	17.7 ± 0.8 bB	20.6 ± 0.5 abB
50% SCG + 50% straw pelletswithout earthworms (Treatment 5)	2174 ± 620.3 aB	4809 ± 48.3 abB	283.2 ± 43.6 abAB	136.8 ± 18.9 bAB

Values are the means ± SD. Different lowercase letters indicate significant differences among layers, capital letters indicate significant differences among treatments (Kruskal-Wallis test, $P \leq 0.05$).
SCG = spent coffee grounds
days in brackets = age of layers at the time of sampling

Total and available contents of basic macroelements are shown in Fig. 3. The contents were influenced by earthworm movement from the bedding layer and between the layers, the element contents in living earthworms, the decomposition of dead earthworm bodies, and conversely, the birth of new earthworms. The P_{tot} content ranged from 0.16 to 0.29%. There was an increase in P_{tot} content towards the bottom older layers, which was probably caused by organic matter mineralization. The greatest increase was recorded in treatment 4 (1.49-fold). The K_{tot} content was the greatest within the monitored elements (1.5 to 2.2%). The greatest increase of 1.25-fold was again seen in treatment 4. The average Mg_{tot} content was 0.24%, with values varying irregularly between layers. With the exception of Mg, the content of available macroelements increased over time. The percentage of the available contents of macronutrients (P, K, and Mg) on average in all of the layers and chosen treatments accounted for 47%, 45%, and 14%, respectively, on the total content. For vermicomposting of distillery residues, the proportion P, K, and Mg constituted 11%, 64%, and 10%, and in the case of kitchen waste 16%, 39%, and 2%, respectively (Hanc et al., 2019b; Hřebečková et al., 2019b). The greatest correlation between the total and available content in the layers was shown in treatment 5 (for P: $R = 0.94$, $p < 0.05$; for K: $R = 0.68$, $p < 0.05$; for Mg: $R = 0.95$, $p < 0.05$). In other treatments, the correlation was strongly influenced by earthworm movement and activity.

3.5. Caffeine

Table 4 shows the caffeine content in all experimental treatments. *Eisenia andrei* were able to decrease the caffeine content, as evidenced by the lesser caffeine content in treatment 3 (average of the layers 48 ng/g) compared to the control treatment 5 of the same composition without earthworms (average of the layers 1851 ng/g) which is 38 times less. The greater content in the control treatment 5 layer III could be caused by the natural movement of the extract from the upper layer. In treatments with a predominance of SCG, lesser caffeine values were found in the younger layers. In treatments 3 to 5, on the contrary, lesser caffeine values were found in the older layers.

Although caffeine has a negative effect on the environment, it is a recognized stimulator of the central nervous system and beneficial health effects have also been described. By using low-pressure or supercritical CO_2 extraction within the biorefinery of SCG extract it is possible to obtain between 0.734 and 41.3 μg/mg of caffeine which corresponded to 18–48% of extracted compounds from coffee beans, and 8–31% from roasted coffee (Santos et al., 2021).

4. Conclusions

Due to the content of toxic substances, SCG itself are not suitable for vermicomposting. The addition of straw pellets to the SCG (up to 75% vol.) improved aeration and reduced bulk density, resulting in the development of earthworms. Strong indirect correlation between the total microbial biomass and earthworm number was found caused by the presence of microorganisms in digestive tract of earthworms. Vermicomposting of SCG was characterized by very strong activity of lipaze due to the high content of lipids and caffeine in coffee. Vermicomposting increased the content of P, K and Mg and decreased the content of caffeine.

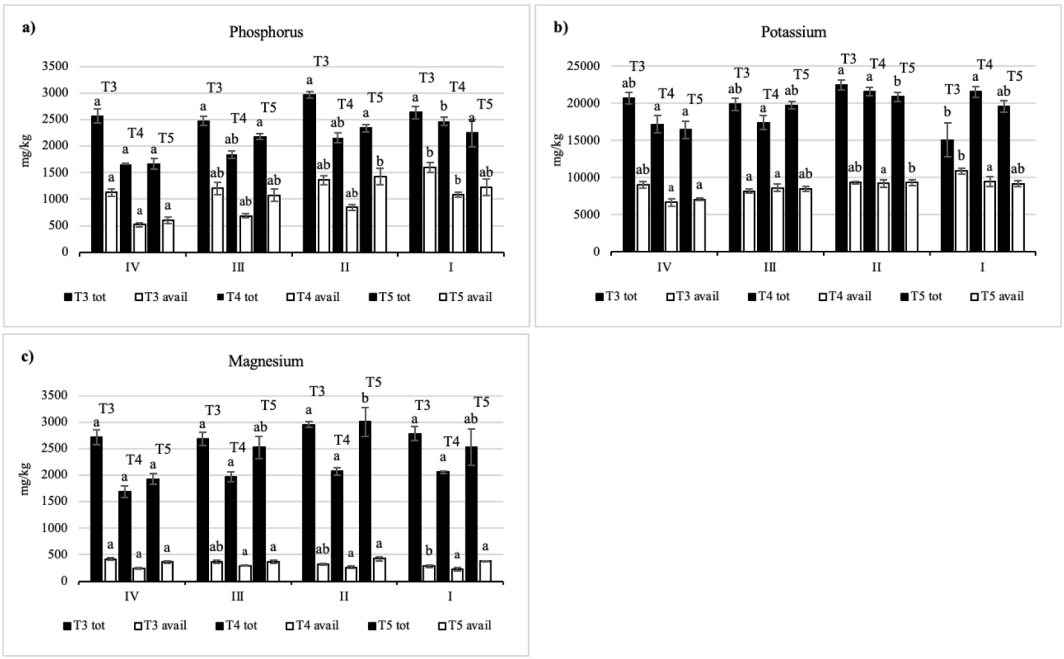


Fig. 3. Total and available content of phosphorus, potassium, and magnesium (a-c) in layers IV, III, II, and I of the treatments 3, 4, and 5. Values are the means ± SD. Letters indicate significant differences (Kruskal-Wallis test, $P \leq 0.05$) among layers within a treatment. T3 – treatment 3 (50% SCG + 50% straw pellets with earthworms), T4 – treatment 4 (25% SCG + 75% straw pellets with earthworms), T5 – treatment 5 (50% SCG + 50% straw pellets without earthworms), tot – total content, avail – available content.

CRediT authorship contribution statement

Ales Hanc: Conceptualization, Methodology, Investigation, Visualization, Funding acquisition, Project administration. **Tereza Hrebeckova:** Resources, Formal analysis, Data curation. **Alena Grasserova:** Formal analysis, Methodology. **Tomas Cajthaml:** Conceptualization, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the Ministry of Agriculture of the Czech Republic under NAZV project No. QK1910095. The authors would like to thank Sona Houzvova from KOKOZA, o.p.s. for consultations on SCG issue and Christina Baker Starrman for revision of the English text.

References

Abbasi, S.A., Nayeem-Shah, M., Abbasi, T., 2015. Vermicomposting of phytomass: limitations of the past approaches and the emerging directions. J. Clean. Prod. 93, 103–114.
Adi, A.J., Noor, Z.M., 2009. Waste recycling: Utilization of coffee grounds and kitchen waste in vermicomposting. Bioresour. Technol. 100 (2), 1027–1030.
Alemayehu, Y.A., Asfaw, S.L., Tirfie, T.A., 2020. Management options for coffee processing wastewater. A review. J. Mater. Cycles Waste 22 (2), 454–469.
Baghel, B., Sahu, R., Pandey, D., 2018. Vermicomposting an economical enterprise for nutrient and waste management for rural agriculture. Int. J. Curr. Microbiol. Appl. Sci. 7 (2), 3754–3758.
Baldrian, P., 2009. Microbial enzyme-catalyzed processes in soils and their analysis. Plant Soil Environ. 55 (No. 9), 370–378.
Ballesteros, L.F., Teixeira, J.A., Mussatto, S.I., 2014. Chemical, functional, and structural properties of spent coffee grounds and coffee silverskin. Food Bioprocess. Technol. 7 (12), 3493–3503.
Biruntha, M., Karmegam, N., Archana, J., Karunai Selvi, B., John Paul, J.A., Balamuralikrishnan, B., Chang, S.W., Ravindran, B., 2020. Vermiconversion of biowastes with low-to-high C/N ratio into value added vermicompost. Bioresour. Technol. 297, 122398. https://doi.org/10.1016/j.biortech.2019.122398.
Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Phys. 37 (8), 911–917.
Blinová, L., Bartošová, A., Sirotiak, M., 2017. Biodiesel production from spent coffee grounds. Research Papers Faculty of Materials Science and Technology Slovak University of Technology, 25, 113–121.
BSI EN 13651, 2001. Soil Improvers and Growing Media - Extraction of Calcium Chloride/DTPA (CAT) Soluble Nutrients.
Cervera-Mata, A., Navarro-Alarcón, M., Rufián-Henares, J.Á., Pastoriza, S., Montilla-Gómez, J., Delgado, G., 2020. Phytotoxicity and chelating capacity of spent coffee grounds: Two contrasting faces in its use as soil organic amendment. Sci. Total Environ. 717, 137247. https://doi.org/10.1016/j.scitotenv.2020.137247.
Cimetiere, N., Soutrel, I., Lemasle, M., Laplanche, A., Crocq, A., 2013. Standard addition method for the determination of pharmaceutical residues in drinking water by SPE–LC–MS/MS. Environ. Technol. 34 (22), 3031–3041.
Domínguez, J., Edwards, C.A., 2011. Relationships between composting and vermicomposting. in: Edwards, C.A., Arancon, N.Q., Sherman, R. (Eds.), Vermiculture Technology. CRC Press, Taylor & Francis Group, Boca Raton, pp. 11–25.
Esquivel, P., Jiménez, V.M., 2012. Functional properties of coffee and coffee by-products. Food Res. Int. 46 (2), 488–495.
González-Moreno, M.A., García Gracianteparaluceta, B., Marcelino Sádaba, S., Zaratiegui Urdin, J., Robles Domínguez, E., Pérez Ezcurdia, M.A., Seco Meneses, A., 2020. Feasibility of vermicomposting of spent coffee grounds and silverskin from coffee industries: A laboratory study. Agronomy 10 (8), 1125. https://doi.org/10.3390/agronomy10081125.
Hanc, A., Dreslova, M., 2016. Effect of composting and vermicomposting on properties of particle size fractions. Bioresour. Technol. 217, 186–189.
Hanc, A., Enev, V., Hrebeckova, T., Klucakova, M., Pekar, M., 2019a. Characterization of humic acids in a continuous-feeding vermicomposting system with horse manure. Waste Manage. 99, 1–11.
Hanc, A., Hrebeckova, T., Kuzel, S., 2019b. Vermicomposting of distillery residues in a vertical-flow windrow system. Waste Biomass Valori. 10 (12), 3647–3657.
Hanc, A., Ochecova, P., Vasak, F., 2017. Changes of parameters during composting of bio-waste collected over four seasons. Environ. Technol. 38 (13-14), 1751–1764.
Hardgrove, S.J., Livesley, S.J., 2016. Applying spent coffee grounds directly to urban agriculture soils greatly reduces plant growth. Urban for. urban gree. 18, 1–8.
Hong, S.W., Lee, J.S., Chung, K.S., 2011. Effect of enzyme producing microorganisms on the biomass of epigeic earthworms *(eisenia fetida)* in vermicompost. Bioresour. Technol. 102 (10), 6344–6347.
Hřebečková, T., Hanč, A., Roy, A., 2019a. Changes of chemical and biological parameters during vermicomposting of kitchen biowaste with an emphasis on pathogens. Waste Forum 2019, 187–196.
Hřebečková, T., Wiesnerová, L., Hanč, A., 2019b. Changes of enzymatic activity during a large-scale vermicomposting process with continuous feeding. J. Clean. Prod. 239, 118127. https://doi.org/10.1016/j.jclepro.2019.118127.

International Coffee Organization, 2020. http://www.ico.org/documents/cy2019-20/cmr-1219-e.pdf. Accessed 28 January 2020.
Karak, T., Kutu, F.R., Paul, R.K., Bora, K., Das, D.K., Khare, P., Das, K., Dutta, A.K., Boruah, R.K., 2017. Co-composting of cow dung, municipal solid waste, roadside pond sediment and tannery sludge: role of human hair. Int. J. Environ. Sci. Technol. 14 (3), 577–594.
Kovalcik, A., Obruca, S., Marova, I., 2018. Valorization of spent coffee grounds: A review. Food Bioprod. Process 110, 104–119.
Li, S., Wen, J., He, B., Wang, J., Hu, X., Liu, J., 2020. Occurrence of caffeine in the freshwater environment: Implications for ecopharmacovigilance. Environ. Pollut. 263, 114371. https://doi.org/10.1016/j.envpol.2020.114371.
Lim, S.L., Lee, L.H., Wu, T.Y., 2016. Sustainability of using composting and vermicomposting technologies for organic solid waste biotransformation: recent overview, greenhouse gases emissions and economic analysis. J. Clean. Prod. 111, 262–278.
Liu, K., Price, G.W., 2011. Evaluation of free composting systems for the management of spent coffee grounds. Bioresour. Technol. 102, 7966–7974.
Low, J.H., Rahman, W.A.W.A., Jamaluddin, J., 2015. The influence of extraction parameters on spent coffee grounds as a renewable tannin resource. J. Clean. Prod. 101, 222–228.
McNutt, J., He, Q.(., 2019. Spent coffee grounds: A review on current utilization. J. Ind. Eng. Chem. 71, 78–88.
Míchal, P., Hanč, A., Švehla, P., 2019. Inhibiting effect of ammonia during vermicomposting of sewage sludge and the possibilities of its elimination. Waste Forum 2019, 144–152.
Munnoli, P.M., Bhosle, S., 2011. Water-holding capacity of earthworms' vermicompost made of sugar industry waste (press mud) in mono- and polyculture vermireactors. Environmentalist 31 (4), 394–400.
Murthy, P.S., Madhava Naidu, M., 2012. Sustainable management of coffee industry by-products and value addition: A review. Resour. Conserv. Recy. 66, 45–58.
Mussatto, S.I., Machado, E.M.S., Martins, S., Teixeira, J.A., 2011. Production, composition, and application of coffee and its industrial residues. Food Bioprocess. Technol. 4 (5), 661–672.
Nzekoue, F.K., Khamitova, G., Angeloni, S., Sempere, A.N., Tao, J., Maggi, F., Xiao, J., Sagratini, G., Vittori, S., Caprioli, G., 2020. Spent coffee grounds: A potential commercial source of phytosterols. Food Chem. 325, 126836. https://doi.org/10.1016/j.foodchem.2020.126836.
Obruca, S., Benesova, P., Petrik, S., Oborna, J., Prikryl, R., Marova, I., 2014. Production of polyhydroxyalkanoates using hydrolysate of spent coffee grounds. Process Biochem. 49 (9), 1409–1414.
Peshev, D., Mitev, D., Peeva, L., Peev, G., 2018. Valorization of spent coffee grounds – A new approach. Sep. Purif. Technol. 192, 271–277.
Ravindran, B., Wong, J.W.C., Selvam, A., Sekaran, G., 2016. Influence of microbial diversity and plant growth hormones in compost and vermicompost from fermented tannery waste. Bioresour. Technol. 217, 200–204.
Sanchez-Hernandez, J.C., Domínguez, J., 2017. Vermicompost derived from spent coffee grounds: assessing the potential for enzymatic bioremediation. In: Galanakis, C. (Ed.), Handbook of Coffee Processing By–product. Academic Press, Elsevier, London, pp. 369–398.
Santos, C., Fonseca, J., Aires, A., Coutinho, J., Trindade, H., 2017. Effect of different rates of spent coffee grounds (SCG) on composting process, gaseous emissions and quality of end-product. Waste Manage. 59, 37–47.
Santos, É.M.D., Macedo, L.M.d., Tundisi, L.L., Ataide, J.A., Camargo, G.A., Alves, R.C., Oliveira, M.B.P.P., Mazzola, P.G., 2021. Coffee by-products in topical formulations: A review. Trends Food Sci. Technol. 111, 280–291.
Stella, T., Covino, S., Burianová, E., Filipová, A., Křesinová, Z., Voříšková, J., Větrovský, T., Baldrian, P., Cajthaml, T., 2015. Chemical and microbiological characterization of an aged PCB-contaminated soil. Sci. Total Environ. 533, 177–186.
Swati, A., Hait, S., 2018. A comprehensive review of the fate of pathogens during vermicomposting of organic wastes. J. Environ. Qual. 47 (1), 16–29.
Štursová, M., Baldrian, P., 2011. Effects of soil properties and management on the aktivity of soil organic matter transforming enzymes and the quantification of soil-bound and free activity. Plant Soil 338, 99–110.
Tongcumpou, C., Usapein, P., Tuntiwiwattanapun, N., 2019. Complete utilization of wet spent coffee grounds waste as a novel feedstock for antioxidant, biodiesel, and biochar production. Ind. Crop Prod. 138, 111484. https://doi.org/10.1016/j.indcrop.2019.111484.
Wahlang, B., McClain, C., Barve, S., Gobejishvili, L., 2018. Role of cAMP and phosphodiesterase signaling in liver health and disease. Cell. Signal. 49, 105–115.
Yordanov, D., Mustafa, Z., Milina, R., Tsonev, Z., 2016. Multi-criteria optimisation process of the oil extraction from spent coffee ground by various solvents. Oxid. Commun. 39, 1478–1487.
Zhang, B., Li, G., Shen, T., Wang, J., Sun, Z., 2000. Changes of microbial biomass C, N, and P and enzyme activities in soil incubated with the earthworms *Metaphire guillelmi* or *Eisenia fetida*. Soil Biol. Biochem. 32, 2055–2062.
Zhang, L.u., Sun, X., 2017. Using cow dung and spent coffee grounds to enhance the two-stage co-composting of green waste. Bioresour. Technol. 245, 152–161.

detritus | Multidisciplinary Journal for Waste Resources & Residues

CISA

PILOT-SCALE VERMICOMPOSTING OF DEWATERED SEWAGE SLUDGE FROM MEDIUM-SIZED WASTEWATER TREATMENT PLANT (WWTP)

Petra Innemanová [1,2,*], Alena Grasserová [1,3] and Tomáš Cajthaml [1,3]

[1] *Institute for Environmental Studies, Faculty of Science, Charles University, Benátská 2, 128 01 Prague 2, Czech Republic*
[2] *DEKONTA a.s., Dřetovice 109, 273 42 Stehelčeves, Czech Republic*
[3] *Institute of Microbiology Czech Academy of Sciences, Vídeňská 1083, 14220, Prague 4, Czech Republic*

Article Info:
Received:
15 December 2021
Revised:
17 February 2022
Accepted:
9 March 2022
Available online:
31 March 2022

Keywords:
Sewage sludge
Vermicomposting
Organic micropollutants
PPCPs

ABSTRACT

The transformation of dewatered sewage sludge into vermicompost provides an advantageous solution in cases where the sludge is not too contaminated with inorganic pollutants, especially heavy metals. In addition to the conversion of the sludge to a product with a higher-added value, undesirable organic pollutants and micropollutants are partially eliminated. Anaerobically stabilized dewatered sewage sludge from a medium-sized Wastewater Treatment Plant (WWTP) was subjected to the vermicomposting process under field conditions. Straw was used as the bedding material in the form of two mixing ratios. The almost 1 year of the monitoring of the process focused on the hazardous substances present, the concentrations of which are regulated by legislation on the use of sludge on agricultural land. In addition, the contents of macro- and micro-nutrients such as N, P, K, Mo, Ca, Mg, and the wintering of the earthworm inocula were monitored. The potential of the vermicomposting process to reduce the content of emergent pollutants from the PPCP group was described with respect to 35 detected substances, including five endocrine disruptors. The study suggested that the bio-stabilization of dewatered sewage sludge using earthworms provides an effective technology for converting noxious wastewater treatment products into nutrient-rich bio-fertilizers.

1. INTRODUCTION

Sewage sludge contains nutrients and other substances that are able to positively contribute to the enhancement of the properties of soil and overall fertility (Latare et al., 2014; Shanta Mendis et al., 2020). Its reuse, where suitable, is encouraged by European Council Directive 91/271/EEC. Treated sludge in the Czech Republic must fulfil the quality criteria set for toxic metals (As, Cd, Cr, Cu, Hg, Ni, Pb and Zn), adsorbable organic halogens (AOX), polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs) and the microbial pathogens *Salmonella sp.* and *Escherichia coli* (Ministry of the Environment of the Czech Republic, 2021). National legislation has incorporated the relevant regulations of the European Union, including Directive 86/278/EEC. Apart from those pollutants whose concentrations are regulated, a broad spectrum of so-called 'emerging' organic chemicals, including pharmaceuticals and other personal care products (PPCPs), may be transferred to residual solids during the treatment of wastewater. Thus, a reliable assessment is required of their significance and implications for the beneficial recycling of treated sewage sludge (Khakbaz et al., 2020).

Vermicomposting is a process via which earthworms act to convert organic materials (usually waste) into a humus-like material known as vermicompost. It comprises a bio-oxidative and stabilizing process for the conversion of organic material which, unlike classical composting, uses the interaction between the intensive activity of earthworms and microorganisms, and does not involve the thermophilic decomposition phase (Domínguez and Edwards, 2011; Champar Ngam et al., 2010). Vermicompost generally appears to be superior to conventionally-produced compost in terms of a number of important parameters including a higher content of available nutrients associated with the enhanced hydrolytic activity and microbial population size (Tognetti et al., 2005; Sinha et al., 2010).

Our study concerns the long-term field testing of sludge vermicomposting in two separate pits, each with a working volume of 3m^3. Straw was used as the bulking material in two mixing ratios. The research covered the testing phase of a pilot vermicomposter conducted for the purpose of

* Corresponding author:
Petra Innemanová
email: petra.innemanova@dekonta.cz

Detritus / Volume 18 - 2022 / pages 35-41
https://doi.org/10.31025/2611-4135/2022.15166

follow-up experiments focusing on the reprocessing and sanitation of sewage sludge. The aim was to ensure a sufficient inoculum density and to test the overwintering of the system under outdoor conditions. However, even during this start-up phase, all the parameters required to be monitored by Czech legislation, as well as the contents of macro- and micro-nutrients such as N, P, K, Mo, Ca and Mg were monitored, as was the development of the concentration of selected PPCP micropollutants.

2. MATERIAL AND METHODS

2.1 Material and the design of the field experiment

The dewatered anaerobically stabilized sewage sludge with an initial dry matter content (DMC) of 24.9 ± 0.7% was taken from WWTPs of a 33 thousand population-equivalent (p.e.) located in South Bohemia. The straw was supplied by a local farmer. The earthworms (*Eisenia andrei*) were supplied by the FLORIUM s.r.o. vermicomposting plant.

The pilot-scale vermicomposting experiment is being conducted in segments A and B of a field vermicomposter (see Figure 1). The working volume of each segment (A and B) is 3 m^3. The working volume of the backup segment (C) is 3.5 m^3; this part of the vermicomposter serves as the earthworm inoculum for subsequent experiments. The drainage system of the field vermicomposter allows for the leachate sampling of each segment. The excess leachate is collected in an underground tank with a volume of 1 m^3 and subsequently disposed of at the nearest WWTP. This experiment does not include the monitoring of the leachate (Figure 2).

A perforated drainage pipe made of polyvinyl chloride was positioned at the bottom of each segment and covered with a layer of straw (36 kg for each of segment A and B). After separating this drainage layer with a geotextile material, each of the segments was filled with the test material according to the following arrangements:

Segment A: 4 layers of straw (40 kg in total) and 3 layers of dewatered sewage sludge (608 kg in total, representing 159 kg of dry matter). Straw formed the bottom and upper layers. The weight ratio of the straw to the dry sludge was 1:4.

FIGURE 2: Field experiment.

Segment B: 3 layers of straw (30 kg in total) and 2 layers of dewatered sewage sludge (the same amounts as in segment A). Straw formed the bottom and upper layers. The weight ratio of the straw to the dry sludge was 1:5.3.

After filling the vermicomposter with a substrate, two perforated polypropylene boxes containing the earthworm hybrid *Eisenia andrei* were placed in each segment. The total weight of the earthworm inoculum was 7 kg for each segment.

The layers of straw created air pockets that improved the level of comfort for the earthworms. Sludge samples

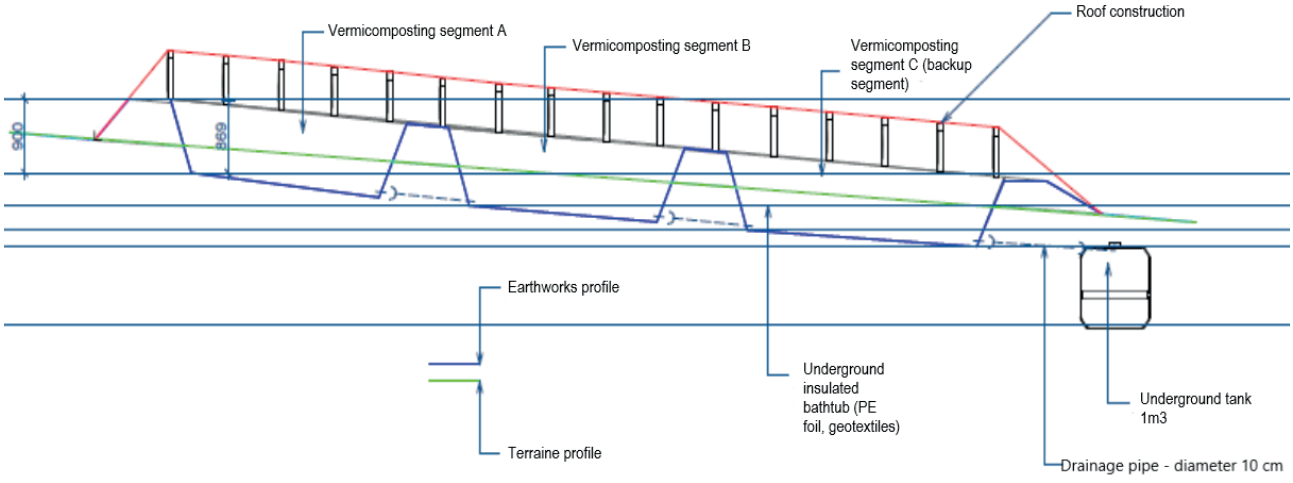


FIGURE 1: Pilot-scale vermicomposter scheme (if not directly specified, the dimensions are given in cm).

for subsequent analysis purposes were taken from the layers without straw.

The vermicomposting process commenced on 4 June 2020 and is ongoing. Both segments were sprinkled twice with the same amount of water during the dry summer of 2020. Otherwise, a perforated cover was sufficient to provide the necessary irrigation.

In November 2020, compact piles were formed from the vermicompost layers of the two segments, in the lower one-third of the segment in both cases. This arrangement allowed the earthworms to overwinter comfortably via the creation of non-freezing zones. In addition, this form of vermicompost will serve as the inoculum for the next batch of sludge in the so-called wedge system (currently in progress).

2.2 Sample analysis

The earthworm biomass was determined on the basis of the manual counting of individual worms (adults and juveniles) in a 1 l sample of vermicompost. 5 parallel samples were taken from the two segments A and B. The dry matter content (DMC) was measured gravimetrically after the drying of the samples at 120^{0}C. DMC was expressed as a percentage of the dry weight of the respective sample.

E. coli was determined according to the Czech ČSN EN ISO 9308-1 national standard. The *Salmonella sp.* was determined according to ČSN EN ISO 6579.

The determination of heavy metals, the Ca, Mg, K, P and N contents, the pH, the DMC and the content of TOC, PCBs (the sum of 7 congeners 28+52+101+118+138+153+180), PAHs (the sum of anthracene, benzo(a) anthracene, benzo(b) fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, benzo(ghi)perylene, phenanthrene, fluoranthene, chrysene, indeno(1,2,3-cd)-pyrene, naphthalene and pyrene) and AOX was conducted by an accredited analytical laboratory (Dekonta, a.s., Ústí nad Labem, Czech Republic). All the measurements were taken in triplicate.

The samples intended for the PPCP and endocrine disruptor analysis using LC-MS/MS were freeze-dried and homogenized. Each sample (weights of 1-2 g) was then transferred to an extraction cell and positioned in an Accelerated solvent extractor (ASE, Dionex). The extraction method was as follows: the preheating of the methanol solvent and the cell to 80°C with a pressure of 1500 psi; 3 extraction cycles and 5-minute static periods between the cycles. The extracts were then evaporated to 5 mL and centrifuged (6000g, 10 min), whereupon the supernatants were transferred to 2mL vials for subsequent analysis purposes. The extracts were analyzed using the LC system (Agilent 1260 Infinity) coupled with a triple quadrupole mass detector (Agilent 6470 LC/TQ). Separation was performed using a Poroshell 120 EC-C18 column (2.7 µm, 3 mm x 100 mm, Agilent) equipped with a Poroshell 120 EC-C18 precolumn (2.7 µm, 3 mm x 5 mm, Agilent); both were heated to 40°C. The mobile phase consisted of phase A (0.5mM ammonium fluoride in MQ water + 0.01% formic acid, LC-MS grade) and phase B (100% methanol, LC-MS grade). The gradient elution program was as follows (time [min], % phase B): 0, 5; 4, 50; 6, 50; 18, 100; 21, 100; 22, 5 and 23, 5. The mobile phase flow was 0.4 mL/min; one run lasted 23.50 min and the injection volume was 2 µL. In order to suppress the matrix effect, the samples were measured with automatic standard additions of 1, 5 and 25 ng/mL. The mass spectrometric parameters were optimized using MassHunter Workstation Optimizer and Source Optimizer (both Version 10.0, SR1, Agilent).

The output values of the monitored chemical parameters represented the results of the field sampling after 9 months (which included the winter season). With respect to the microbiological parameters, the sanitation efficiency of the process was evaluated after approximately 5 and 11 months of the duration of the process.

3. RESULTS AND DISCUSSON

3.1 Dry matter content and earthworm biomass

The dry matter contents of segments A and B after 11 months of processing were 28.6 ± 0.4% and 26.8 ± 0.9%, respectively. The small (but statistically significant difference, $p < 0.01$) did not lead to a differing worm density, i.e. 54.8 ± 15.2 individuals per liter in segment A and 46.2 ± 20.2 individuals per liter in segment B (mean and standard deviation of 5 measurements) 11 months after the start of the experiment. Nevertheless, the observed average weight of the adults of 0.86 ± 0.27 g in segment B was significantly higher than the value of 0.47 ± 0.18 g determined for segment A ($p < 0.01$, mean and standard deviation of 30 measurements). The slight difference in the moisture contents of segments A and B was the most likely reason for the observed differences in the body weights of the earthworms. Similar observations were described in a study by Domínguez and Edwards (1997), which described that beneath an 85% moisture level, higher moisture conditions clearly facilitated growth as measured by an increase in the individual biomass of *Eisenia andrei*.

3.2 Chemical and microbiological parameters

Adequate sanitation had not been achieved after 5 months of the process. *Salmonella sp.* was not detected in the sludge used in the experiment but concerning the *E. coli* parameter, the required limits were met after approximately 11 months of the duration of the experiment (see Table 1), thus indicating that sludge sanitation is possible in the absence of a pre-composting step with the thermal phase of the process; however, it requires a longer time period. These results are at variance with trends reported in the literature. For example, Procházková et al. (2018) observed a decrease in *E. coli* to an undetectable level after 8 weeks of the vermicomposting of apple pomace waste with an artificial bacterial load. In addition, a study by Parseh et al. (2021) described the extensive ability of *E. fetida* to reduce pathogens within 8 weeks in dewatered sludge without the need for an increase in temperature. However, the results of these studies are difficult to compare since they are usually recorded under optimal laboratory conditions. It is necessary to take into account that a longer period of time is required for complete sanitation under real conditions. This is due not only to temperature and moisture fluctuations; it was observed during the experiment that due to the inhomogeneity of the mixture, random layers without

TABLE 1: Concentrations of *E. coli* in segments A and B at the commencement and after 140 and 340 days of the process.

	E. coli in parallel samples (CFU/g)					Czech legislation limit
Input	7.8×10^4	9.2×10^4	1×10^5	2.2×10^5	2.8×10^5	Max. 10^3CFU/g for 4 samples and 5×10^3CFU/g for one sample from 5 parallel samples
A (day 140)	3.1×10^4	3.5×10^4	3.5×10^4	3.6×10^4	4.2×10^4	
B (day 140)	2×10^4	2.5×10^4	2.9×10^4	3.4×10^4	3.5×10^4	
A (day 340)	Negative	Negative	Negative	Negative	Negative	
B (day 340)	Negative	Negative	Negative	4×10^2	2.4×10^3	

the presence of earthworm settlements occurred over relatively longer time period.

As can be seen in Table 2, the treated sludge complied with the limits for hazardous substances set by Czech legislation (Ministry of the environment of the Czech Republic, 2021) for the application of treated sludge to agricultural land even before the start of the vermicomposting process. The relative stabile concentration at the most of monitored heavy metals can be explained by the combination of two conflicting phenomena: the concentration through the decomposition of the organic matter and elimination due to ingestion by the earthworms and following bioaccumulation. The predominant effect of bioaccumulation may provide an explanation for the decrease in the content of Cu and As. According to Rorat et al. (2017), *Eisenia andrei* accumulated heavy metals as follows: Cd>Cu>Zn>Ni>Cr>Pb. Kilpi-Koski et al. (2019) observed a high bioaccumulation factor (BAF) for As, but a low BAF for Cu. Moreover, other studies have provided differing information on heavy metal bioaccumulation factors (Suleiman et al., 2017; Wang et al., 2018), and further research is required in this regard. In any case, bioaccumulation cannot be considered to provide a tool for the removal of heavy metals from vermicomposted material since the continuous earthworm mortality and their subsequent decomposition during a full-scale application leads to the re-supply of accumulated metals back into the final vermicompost. Therefore, only the initial concentration of heavy metals in the sludge is a key factor in the design of the appropriate technology. The limits set for selected organic substances from the persistent organic pollutants (POPs) category were fulfilled. The AOX concentration dropped to below the detection limit for both treatments. Some studies (for example Khakbaz et al., 2020) have used sludge parameter extractable organic halogens (EOX) for the quantification of organic halogens in sewage because of the suitability of this parameter to characterize complex two-phase matrices as a sludge (Rizzardini and Goi, 2014). Our study followed the requirements of the Ministry of the environment of the Czech Republic (2021) according to which, in addition to the AOX, the monitoring of PCBs (the sum of 7 congeners: 28+52+101+118+138+153+180) and PAHs (the sum of anthracene, benzo(a)anthracene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, benzo(ghi)perylene, phenanthrene, fluoranthene, chrysene, indeno(1,2,3-cd)-pyrene, naphthalene and pyrene) is mandatory. While PCBs were under the detection limit even in the initial samples, there was no tendency for the sum of 7 selected PAHs to decrease from the initial value of 2.8 ± 1.1 ppm. The opposite tendency, i. e. concentration over time, indicates the presence of highly-persistent substances. The bioavailability of PAHs has been found to be strongly related to the number of their aromatic rings, their molecular weight and their structure (Amir et al., 2005). In this specific case, more persistent PAH representatives were probably present in the sludge while, for example, a study by Rorat et al. (2017) reported that of the PAHs regulated, indeno(1,2,3-c,d)-pyrene was not detected and the most efficient rate of removal was recorded for the two- and three-ring substances naphtalene and phenanthrene. When designing the appropriate technology, it is, therefore, necessary to take into account the relative concentrations of individual PAHs in the sum of those that are subject to regulation.

In addition to hazardous substances, the monitoring of which is required by legislation, the content of selected biogenic elements was also monitored. As can be seen from Table 2, an obvious increase was observed especially in the case of phosphorus. An elevated level was also observed in potassium concentrations. Conversely, the total nitrogen content dropped and, at the same time, a marked shift was observed in the ratio of the NH^{4+}/NO^{3-} form of nitrogen. It decreased by 4 orders of magnitude from the original value of approximately 10^4. The levels of calcium and magnesium remained at similar levels for 9 months. These trends have also been observed under laboratory conditions (Zhang et al., 2020) and even without sludge blending (Khwairakpam and Bhargawa, 2009), which indicates that sewage sludge can be recycled as a good quality fertilizer. The observed loss of organic carbon can be attributed to the loss of organic matter from feed mixtures as carbon dioxide through earthworms and microbial respiration (Garg et al., 2008). The pH dropped from the original 7.6 ± 0.1 to 5.2 ± 0.3 and 5.4 ± 0,1 which is consistent with the results of other authors (Gupta and Garg, 2008; Bhat et al., 2016). A gradual return to neutral values was observed after longer processing times (data not shown).

3.3 Development of the micropollutant concentration

Although samples were taken for analysis from the sludge layer, it cannot be ruled out that the action of the earthworms and mechanical manipulation did not result in the mixing of the substrates and, thus, a reduction in the content of the monitored substances via dilution. On the other hand, during the vermicomposting process, the mass of the mixture generally declines due to the partial mineralization of the organic matter (Suleiman at al., 2017). Thus, recalcitrant substances may become concentrated as a result. The calculation of the actual loss of pollutants is subsequently complicated. Similar laboratory studies have applied an internal standard for the recalculation of the ef-

TABLE 2: Concentration of hazardous substances, nutrients and TOC in segments A and B at the commencement and after 9 months of the process (mean and standard deviation of 3 paralel samples).

Hazardous substances (mg/kg)	Input	A – output	B- output	Czech legislation limit
As	8.0 ± 0.2	5.2 ± 1.1	6.1 ± 0.6	30
Cd	1.3 ± 0.03	1.2 ± 0.1	1.2 ± 0.1	5
Cr	42.8 ± 4.2	42.7 ± 8.2	40.9.0 ± 0.7	200
Cu	249.0 ± 11.0	201.0 ± 21.0	202.0 ± 16	500
Hg	1.7 ± 0.5	1.3 ±0.4	1.2 ±0.1	4
Ni	29.5 ± 1.9	28.5 ± 2.8	31.1 ± 5.4	100
Pb	37.6 ± 2.6	34.6 ± 4.5	34.4 ± 2.8	200
Zn	804.0 ± 45.0	754.0 ± 80.0	714.0 ± 98.0	2500
Mo	6.0 ± 0.65	5.0 ± 0.65	4.7 ± 0.6	
AOX	96.0 ± 57.0	n.d.	n.d.	500
PCB	n.d.	n.d.	n.d.	0.6
PAH	2.8 ± 1.1	3.5 ± 0.4	6.8 ± 1.4	10
P	(26.0 ± 0.5) x10^3	(39.3 ± 4.1) x10^3	(39.4 ± 4.7) x10^3	
K	(2.7 ± 0.4) x 10^3	(4.5 ± 0.3) x10^3	(3.9 ± 0.2) x10^3	
N_{total}	(8.2 ± 2.5) x10^3	(3.7 ± 0.8) x10^3	(3.9 ± 0.3) x10^3	
Ca	(23.8 ± 0.4) x10^3	(22.6 ± 1.7) x10^3	(19.5 ± 4.6) x10^3	
Mg	(5.0 ± 0.2) x10^3	(5.0 ± 0.4) x10^3	(4.8 ± 0.7) x10^3	
TOC	(308 ± 44) x10^3	(227 ± 14) x 10^3	(235 ± 10) x10^3	

n.d.: not detected

ficacy. For example, Covino et al. (2016) used a selected heavy metal, which was present in only one part of the composting mixture (wooden chips). The removal efficiency of micropollutants and hazardous substances indicated as a percentage of reduction on the basis of the initial and final concentrations without the calculation of the actual loss may thus be misleading. The declared loss of monitored substances should be considered as a combination of the processes described above. However, with regard to the subsequent applicability of the sludge, we were primarily interested in the final quality of the product. For our purposes, the final removal of micropollutants might be referred to provisionally as the "operating removal efficiency".

The initial concentrations of the monitored pharmaceuticals ranged from 0.5 ± 0.1 ppb (Sulfamethazine) to 8.0 ± 0.4 ppm (Telmisartan). The initial and output concentrations of 35 substances detected from the PPCP group are summarized in Table 3.

The operating removal efficiency of the vermicomposting process differed between 0% and 100%, with the highest values (above 90%) determined for Acesulfame, Equilin, Equol, Furosemide, Hydrochlorothiazide, Ibuprofen, Saccharine and Sulfamethazine and endocrine disruptor 17beta-estradiol. With respect to this case study, 28.7% (segment A) and 29.2% (segment B) of the most abundant micropollutant, Telmisartan, were removed. The only increased level after 9 months of processing was observed for Bisphenol S, which was probably related to the composter insulation material used. The total operating removal efficiency of all the detected micropollutants was 35.3% and 34%. To date, only a small number of similar studies have been published, a review of which has recently been provided by Chowdhury et al. (2022). The cited studies differ in terms of the specific observed substances included in the groups of pharmaceuticals and PPCPs and are, therefore, difficult to compare. It is clear that further research is essential in the field, especially concerning the overall effects on the environment, e. g. endocrine disruptivity and ecotoxicity.

4. CONCLUSIONS

During the first year of the operation of the field vermicomposter, the earthworm inoculum in the mixture of sewage sludge and straw multiplied to a sufficient extent and the culture overwintered successfully, even though frosts reached temperatures of below -20°C in the winter of 2020/2021.

The different mixing ratio of the sludge/straw exerted a slight effect on the output dry matter content, which led to a minimal difference in the density of the earthworm populations and a significant difference in the biomass of the *Eisenia andrei*. No significant difference was observed between segments A and B with respect to the monitored parameters.

The sludge used in the experiment met the respective legislative requirements for agricultural land application in terms of the content of heavy metals and that of the monitored organic substances and *Salmonella sp.*, the content of which met legislative requirements even at the outset of the process. The *E. coli* content met the criteria in the 11th month.

TABLE 3: Concentration of selected PPCPs in segments A and B at the commencement and after 9 months of the process, mean and standard deviation of 18 input samples and 6 output samples from each of segment A and B; the variance associated with the compound content via the analysis of variance (ANOVA) and its significance, *p <0.05; **p <0.01.

Pollutant	Input (ng/g)	Segment A (ng/g)	Segment B (ng/g)
Acesulfame	47.5 ± 9.0	4.1 ± 1**	3.9 ± 0.7**
Acetaminophen (Paracetamol)	10.8 ± 2.2	4.8 ± 1.5**	3.9 ± 0.4**
Amitriptyline	64.4 ± 11.5	52.2 ± 11.6	51.7 ± 7.3*
Atorvastatin	13.1 ± 4.2	10.3 ± 4	13.5 ± 5
Azithromycin	41.4 ± 11.7	59.8 ± 18.1	45.7 ± 25.4
Bisphenol A	615.5 ± 63.4	93.4 ± 23.6**	158.5 ± 24.6**
Bisphenol F	29.6 ± 4.3	21.5 ± 8.4**	17.8 ± 2.2**
Bisphenol S	27.3 ± 2.6	95.0 ± 31.5*	45.6 ± 29.1
Caffeine	50.0 ± 4.0	40.8 ± 5.2**	42.9 ± 2.3**
Carbamazepine	132.5 ± 38.4	75.8 ± 11.4**	87.9 ± 12.3*
Carbamazepine 10,11-epoxide	4.9 ± 0.4	3.2 ± 0.6**	3.7 ± 0.6**
Cetirizine	152.8 ± 9.6	84 ± 15.7**	91.5 ± 12.5**
Citalopram	421.1 ± 32.1	320.1 ± 62.8**	287.3 ± 61.8**
Daidzein	7.4 ± 1.1	2.6 ± 0.2**	2.5 ± 0.1**
Equilin	1.2 ± 1.4	n.d.	n.d.
Equol	39.7 ± 8.6	2.6 ± 0.8**	4.9 ± 4.2**
Estrone	3.5 ± 2.7	0.4 ± 1.0*	0.9 ± 1.3
Fluoconazole	1.3 ± 0.2	1.0 ± 0.2**	1.1 ± 0.1*
Furosemide	19.2 ± 2.8	n.d.**	n.d.**
Gabapentin	38.5 ± 9.8	7.0 ± 2.8**	9.8 ± 1.9**
Genistein	3.5 ± 2.7	2 ± 0.4	2.0 ± 0.2
Hydrochlorothiazide	2.6 ± 0.5	n.d.**	n.d.**
Ibuprofen	129.7 ± 79.2	7.7 ± 7.2**	n.d.**
Lamotrigine	94.7 ± 12.6	21.4 ± 7.8**	27.6 ± 5.6**
Metoprolol	135.8 ± 8.2	45.2 ± 7.5**	48.0 ± 13.1**
Mirtazapine	74.2 ± 5.7	33.8 ± 10.2**	36.3 ± 5**
Saccharine	28.4 ± 13.2	n.d.**	n.d.**
Sulfamethazine	0.5 ± 0.1	n.d.**	n.d.**
Sulfanilamide	9.4 ± 3.1	2.2 ± 0.4**	2.9 ± 0.5**
Sulfapyridine	8.1 ± 1.9	1.9 ± 0.2**	2.6 ± 0.8**
Telmisartan	(8.0 ± 0.4) x10^3	(5.7 ± 0.8) x10^3 **	(5.7 ± 0.8) x10^3 **
Tramadol	58.4 ± 4.3	28.8 ± 4.3**	31.3 ± 5.9**
Trimethoprim	10.2 ± 1.6	1.6 ± 0.1**	1.3 ± 0.2**
Venlafaxine	128.6 ± 9.6	91.2 ± 15.1**	90.5 ± 11.3**
17beta-estradiol	24.8 ± 20	n.d.**	n.d.**

n.d.: not detected

The degradation potential of selected micropollutants from the PPCP group differed. A total of 35.3% degradation of the monitored substances was observed in segment A and 34% in segment B.

Vermicomposting led to a significant decrease in the concentration of the 4 detected endocrine disruptors (Bisphenol A, Bisphenol F, Estrone and 17beta-estradiol). Conversely, an increase was observed in the content of Bisphenol S, which was probably due to the film material that was used for the insulation of the vermicomposter.

Thus, vermicomposting appears to be a useful method for processing sewage sludge from at least smaller WWTPs. It is recommended that the further potential of this process be explored in subsequent research.

AKNOWLEDGEMENTS

The "Use of Vermicomposting to Eliminate Micropollutants for the Safe Application of Sewage Sludge on Agricul-

tural Land" project (No. QK1910095) was financially supported by the Ministry of Agriculture of the Czech Republic as part of the "ZEMĚ" program.

REFERENCES

Amir, S., Hafidi, M., Merlina, G., Hamdi, H., Revel, J.C. 2005. Fate of polycyclic aromatic hydrocarbons during composting of lagooning sewage sludge. Chemosphere 58(4). 449-458. doi.org/10.1016/j.chemosphere.2004.09.039

Bhat, S. A., Singh, J., Vig, A.P. 2016. Effect on growth of earthworm and chemical parameters during vermicomposting of pressumd sludge mixed with cattle dung mixture. Procedia Environmental Sciences 35. 425-434. doi.org/10.1016/j.proenv.2016.07.025

Chowdhury, S.D., Surampalli, R.Y., Bhunia, P. 2022. Potential of the constructed wetlands and the earthworm-based treatment technologies to remove the emerging contaminants: A review. J hazard Toxic radioact Waste 26(2). 04021066. doi.10.1061/(ASCE)HZ.2153-5515.0000668

Covino, S., Fabianová, T., Křesinová, Z., Čvančarová, M., Burianová, E., Filipová, A., Voříšková, J., Baldrian, P., Cajthaml, T. 2016. Polycyclic aromatic hydrocarbons degradation and microbial community shifts during co-composting of creosote-treated wood. J Hazard Mater. 301(15). 17-26. doi.org/10.1016/j.jhazmat.2015.08.023

Domínguez, J., Edwards, C., A. 1997. Effects of stocking rate and moisture content on the growth and maturation of *Eisenia andrei* (Oligochaeta) in pig manure. Soil Biol Biochem. 29(3/4). 743-746. doi.org/10.1016/S0038-0717(96)00276-3

Domínguez, J., Edwards, C., A. 2011. Relationships between composting and vermicomposting. In: Vermiculture Technology (Eds Edwards C.A., Arancon N.Q., Sherman R.), Boca Raton: CRC Press, Taylor & Francis Group, 11-25.

Garg, V. K., Gupta, R., Yadav, A. 2008. Potential of vermicomposting technology in solid waste management. In: Pandey A et al (ed) Current developments in solid state fermentation. Asia-Tech. Publishers Inc. New Delhi. 468-511. doi.org/10.1007/978-0-387-75213-6_20

Gupta, R., Garg, V.K. 2008. Stabilization of primary sewage sludge during vermicomposting. J Hazard Mater. 153(3). 1023-1030. doi.org/10.1016/j.jhazmat.2007.09.055

Champar-Ngam, N., Iwai, C.B., Ta-oun, M. 2010. Vermicompost: tool for agro-industrial waste management and sustainable agriculture. IJERD. 1-2. 38-43

Khakbaz, A., De Nobili, M., Mainardis, M., Contin, M., Aneggi, E., Mattiussi, M., Cabras, I., Busut M., Goi, D. 2020. Monitoring of heavy metals, EOX and LAS in sewage sludge for agricultural use: a case study. Detritus Journal, 12-2020.160-168. doi.org/10.31025/2611-4135/2020.13993

Khwairakpam, M., Bhargava, R. 2009. Vermitechnology for sewage sludge recycling. J Hazard Mater 161. 948-954. doi.org/10.1016/j.jhazmat.2008.04.088

Kilpi-Koski, J., Penttinen, OP., Väisänen, A.O., van Gestel, C.A.M. 2019. An uptake and elimination kinetics approach to assess the bioavailability of chromium, copper, and arsenic to earthworms (*Eisenia andrei*) in contaminated field soils. Environ Sci Pollut Res 26. 15095–15104. doi.org/10.1007/s11356-019-04908-6

Latare, A.M., Kumar, O., Singh, S.K., Gupta, A. 2014. Direct and residual effect of sewage sludge on yield, heavy metals content and soil fertility under rice-wheat system. Ecol. Eng. 69. 17-24. doi.org/10.1016/j.ecoleng.2014.03.066

Ministry of the environment of the Czech Republic. 2021. Vyhláška č. 273/2021 Sb., vyhláška o podrobnostech nakládání s odpady. In: Sbírka zákonů ČR, volume 2021, Number 273. https://www.zakonyprolidi.cz/cs/2021-273

Parseh, I., Mousavi, K., Badieenejad, A., Golbini Mofrad, M.M., Hashemi, M., Azadbakht, O., Karimi, H. 2021. Microbial and composition changes during vermicomposting process resulting from decomposable domestic waste, cow manure and dewatered sludge. Int J Env Health Eng 10:3. doi.10.4103/ijehe.ijehe_56_20

Procházková, P., Hanč, A., Dvořák, J., Roubalová, R., Drešlová, M., Částková, T., Šustr, V., Škanta, F., Navarro Pacheco, N.I., Bilej, M. 2018. Contribution of *Eisenia andrei* earthworms in pathogen reduction during vermicomposting. Environ Sci Pollut R 25. 26267-26278

Rizzardini, C.B., Goi, D. 2014. Sustainability of domestic sewage sludge disposal. Sustainability 2014. 6(5). 2424-2434. doi.org/10.3390/su6052424

Rorat, A., Wloka, D., Grobelak, A., Grosser, A.S., Milczarek, M., Jelonek, P., Vandenbulcke, F., Kacprzak, M. 2017. Vermiremediation of polycyclic aromatic hydrocarbons and heavy metals in sewage sludge composting process. J Environ Manage 187. 347-353. doi.org/10.1016/j.jenvman.2016.10.062

Sinha, R.K., Herat, S., Bharambe, G., Brahambhatt, A. 2010. Vermistabilization of sewage sludge (biosolids) by earthworms: converting a potential biohazard destined for landfill disposal into a pathogen-free, nutritive and safe biofertilizer for farms. Waste Manage Res 2010:28. 872-881

Shanta Mendis, A. S., Dunuweera, S.P., Walpolage, S. & Gamini Rajapakse, R. M. (2020). Conversion of biological treatment plant sludge to organic fertilizer for applications in organic farming. Detritus Journal, 9-2020. 83-93. doi.org/10.31025/2611-4135/2020.13899

Suleiman, H., Rorat, A., Grobelak, A., Grosser, A., Milczarek, M., Plytycz, B., Kacprzak, M., Vandebulcke, F. 2017. Determination of the performance of vermicomposting process applied to sewage sludge by monitoring of the compost quality and immune responses in three earthworm species: *Eisenia fetida*, *Eisenia andrei* and *Dendrobaena veneta*. Bioresource Technol 241. 103-112. doi.org/10.1016/j.biortech.2017.05.104

Tognetti, C., Laos, F., Mazzarino, M.J., Hernández, M.T. 2005. Composting vs. Vermicomposting: A Comparison of End Product Quality, Compost Sci Util, 13:1. 6-13. doi: 10.1080/1065657X.2005.10702212

Wang, K., Qiao, Y., Zhang, H., Yue, S., Li, H., Ji, X., Liu, L. 2018. Bioaccumulation of heavy metals in earthworms from firld contaminated soil in a subtropical aera of China. Ecotox Environ Safe 148. 876-883. doi.org/10.1016/j.ecoenv.2017.11.058

Zhang, H., Li, J., Zhang, Y., Huang, K. 2020. Quality of vermicompost and microbial community deversity affected by the contrasting temperature during vermicomposting of dewatered sludge. Int J Environ Res Public Health 17(5). 1748. doi.org/10.3390/ijerph17051748

Journal of Cleaner Production 416 (2023) 137869

Contents lists available at ScienceDirect

Journal of Cleaner Production

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

Influence of earthworms on the behaviour of organic micropollutants in sewage sludge

Bayu Dume [a,*], Aleš Hanč [a], Pavel Švehla [a], Pavel Michal [a], Vojtěch Pospíšil [a], Alena Grasserová [b,c], Tomáš Cajthaml [b,c], Abraham Demelash Chane [a], Abebe Nigussie [d]

[a] Czech University of Life Sciences, Faculty of Agrobiology, Food, and Natural Resources, Department of Agro-Environmental Chemistry and Plant Nutrition, Kamycka 129, Prague, 16500, Czech Republic
[b] Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic
[c] Institute for Environmental Studies, Faculty of Science, Charles University in Prague, Czech Republic
[d] Jimma University, College of Agriculture, 307, Jimma, Ethiopia

ARTICLE INFO

Handling Editor: Cecilia Maria Villas Bôas de Almeida

Keywords:
Bio-waste
Vermidegradation
Eisenia andrei
Vermiaccumulation
Vermicompost

ABSTRACT

The objective of this study was to evaluate the concentration of pharmaceuticals and personal care products (PPCPs) and endocrine disrupting chemicals during vermicomposting of sewage sludge using *Eisenia andrei* and in earthworm tissues with the aim of evaluating the effectiveness of earthworms to remove these substances. The experiment was carried out for 120 days with and without earthworms in varying proportions of sewage sludge in a mixture with dried straw pellets at 100, 75, 50, and 25% (w/w) of sludge. The results revealed that the earthworms had the most significant removal efficiencies on triclosan (37%) and mirtazapine (14%). Venlafaxine (193%), triclosan (43%), and citalopram (37%), had the most earthworm influence efficiency of degradation. The maximum vermiaccumulation of caffeine (72%), carbamazepine (65%), cetirizine (32%), citalopram (16%), diclofenac (183%), and triclosan (118%) was obtained. Based on these findings, earthworms show great promise in removing monitored compounds from sewage sludge during vermicomposting. However, further research is needed to optimize the process for maximum removal efficiency and confirm this approach's effectiveness.

1. Introduction

Organic micropollutants, including pharmaceuticals and personal care products (PPCPs) and endocrine-disrupting chemicals (EDCs), pose significant threats to ecosystems and human health (Thomas et al., 2020). PPCPs comprehend a wide range of substances, such as antibiotics, hormones, fungicides, disinfectants, antidepressants, and non-steroidal anti-inflammatory drugs (Jiang et al., 2023). EDCs include detergents, plasticizers, personal care products, and biocides, which can potentially interfere with hormonal systems, causing various developmental, reproductive, and behavioural disturbances (Schug et al., 2016). PPCPs and EDCs have become widespread in the aquatic environment, including surface water, sediments, and soils, where the most important primary source of these compounds usually represents wastewater (Nunes et al., 2021). PPCPs and EDCs represent bioactive substances that provide additional concern due to their hazardous bioactivity, even at very low concentrations (Hu et al., 2021).

In a wastewater treatment system, organic micropollutants are typically removed from wastewater through microbial degradation and sorption on sludge (Menon et al., 2020). For this reason, the content of PPCPs and EDCs in sewage sludge could be significant (Nunes et al., 2021). Considering the significant production of sewage sludge and its potential use as a fertilizer or soil amendment, addressing the issue of organic micropollutants in this waste material is crucial (Mazzeo et al., 2023). In the EU27, nearly 10 million tonnes of dry sludge are produced annually, with more than half of this amount applied to farmland for agricultural uses (Samaras et al., 2014). However, caution is necessary for other aspects due to the presence of PPCPs and EDCs (Buta et al., 2021) in sewage sludge. Although there are no current legislations regarding the levels of organic micropollutants in sewage sludge for agricultural use, it is essential to conduct studies related to minimizing the potential environmental and agriculture hazards, including the problems related to PPCPs and EDCs (Petrie et al., 2014).

Vermicomposting represents an environmentally friendly waste management approach that utilizes earthworms and microorganisms to convert biodegradable organic waste into valuable bio-fertilizers under

* Corresponding author.
E-mail address: gari@af.czu.cz (B. Dume).

https://doi.org/10.1016/j.jclepro.2023.137869
Received 27 February 2023; Received in revised form 31 May 2023; Accepted 19 June 2023
Available online 24 June 2023

aerobic conditions (Soobhany, 2019). At the same time, sewage sludge could be used as one of a suitable substrate for the vermicomposting process (Rorat et al., 2019). Due to its high tolerance for environmental variables such as toxic substances contained in sewage sludge, pH, moisture, and temperature, as well as its acceptance of a wide variety of feeds, a high growth rate, and the capability of converting biomass into stable products, the *epigeic* earthworm specie *Eisenia andrei* seems to be optimal for a vermicomposting process (Yadav and Garg, 2016). However, if we would like to apply vermicomposting for sewage sludge treatment, it is necessary to search for suitable co-substrates with which it will be optimal to mix the sewage sludge before the starting vermicomposting process. The straw pellets provide a favourable environment for earthworms by increasing the porosity of the composted material and allowing for better aeration and moisture retention. Suthar (2009) compared wheat straw, cow dung, and digested slurry as bulking agents in the vermicomposting of vegetable-market solid waste and found that earthworms preferred wheat straw over other materials.

Previous studies have primarily focused on measuring the concentrations of PPCPs and EDCs in the influent and effluent of wastewater treatment plants (WWTPs) (Gago-Ferrero et al., 2015). Some studies have also explored the occurrence and distribution of PPCPs and EDCs in sewage sludge (Sun et al., 2016). Selected specific previous researches have also investigated the removal efficiency of selected organic micropollutants by earthworms in soil (Shi et al., 2020). However, there is a lack of comprehensive research on the influence of earthworms on the behaviour of PPCPs and EDCs and on the potential ability of earthworms to support the removal of these pollutants during vermicomposting of sewage sludge. This study's novelty lies in its focus on the evaluation of the potential participation of earthworms in removing PPCPs and EDCs from sewage sludge during vermicomposting. This way, this study could fill the existing gap in knowledge regarding the advanced processes applicable for the minimizing of PPCPs and EDCs introduction into the environment. Consequently, reasonable development of more sustainable and effective sewage sludge management strategies could be supported. Based on the available data analysed, 12 selected concrete pollutants belonging to PPCPs and EDCs were monitored during vermicomposting process of the substrate containing the sewage sludge in different mixtures with straw pellets using *Eisenia andrei*. The targeted PPCPs and EDCs concentration was monitored in the treated substrate during the treatment process. Moreover, it was also measured in earthworm tissues to enhance the objectivity of the research.

2. Materials and methods

2.1. Initial raw materials and earthworms

The experiments used freshly deposited sewage sludge collected from a WWTP in a small town in the Czech Republic (3,500 population equivalents). The WWTP was operated on the mechanical-biological principle with an activation process applied for biological (secondary) treatment. The sludge was digested under aerobic conditions. Before being used in the experiment, it was kept at 4 °C for one week. Dried straw pellets were provided by Granofyt Ltd. Company (Chrášťany, Czechia) with a diameter of 10 mm. Because of the low moisture content of the straw pellet, it was mixed with hot water (60 °C) at a 1:4 (w/v) ratio before experimental use. Earthworms were collected from a private vermiculture stock in the Czech Republic with grape marc substrate as survival media. Of the determined organic micropollutants, only 6.05 ng g^{-1} of caffiene (CAF) and 2.24 ng g^{-1} of telmisartan (TE) were detected in earthworm tissues. The selected physicochemical properties of the initial materials and organic micropollutants before vermicomposting are presented in Table 1.

Table 1
Physicochemical properties of the initial materials and organic micropollutants before vermicomposting.

Parameters	Sewage sludge	Straw pellet	Earthworm
Dry matter (%)	13.3 ± 0.19	21.2 ± 0.56	
pH-H_2O	6.9 ± 0.03	8.3 ± 0.52	
Electrical conductivity (mS/cm)	0.617 ± 0.11	0.68 ± 0.07	
Total carbon (%)	32.9 ± 0.26	42.59 ± 0.36	
Total nitrogen (%)	5.36 ± 0.03	0.80 ± 0.12	
C/N ratio	6.1 ± 0.04	53.67 ± 7.60	
Micropollutants (ng g^{-1})			
Bisphenol A	88.78 ± 18	nd	nd
Caffeine	141.81 ± 12.5	10.23 ± 2.56	6.05 ± 0.69
Carbamazepine	38.51 ± 0.73	nd	nd
Cetirizine	78.95 ± 0.69	nd	nd
Citalopram	440 ± 2.84	nd	nd
Diclofenac	284.22 ± 9.8	nd	nd
Ibuprofen	87.33 ± 6.68	nd	nd
Mirtazapine	63.26 ± 2.83	1.75 ± 0.07	nd
Sulfapyridine	15.22 ± 1.03	1.69 ± 0.04	nd
Telmisartan	10,161.60 ± 226	3.74 ± 0.20	2.24 ± 0.11
Triclosan	543.24 ± 36	nd	nd
Venlafaxine	33.97 ± 3.74	1.67 ± 0.03	nd

nd = not detected, values indicate mean ± standard error (n = 3).

2.2. Experimental set-up

The experiment included eight variants with three replications at different mixing proportions of sewage sludge (SS) and straw pellet (SP) with (+EW) and without earthworms (-EW): (T1) 100% SS (+EW); (T2) 100% SS (-EW); (T3) 75% SS + 25% SP (+EW); (T4) 75% SS + 25% SP (-EW); (T5) 50% SS + 50% SP (+EW); (T6) 50% SS + 50% SP (-EW); (T7) 25% SS + 75% SP (+EW); (T8) 25% SS + 75% SP (-EW). Table S1 shows the composition of vermicomposting/composting materials in various proportions. In all variants, the additive material was homogenized and transferred to worm bins (40 × 40 × 15 cm) for 120 days of vermicomposting/composting. The substrate (3 L grape marc) containing earthworm was placed into the tray from the side to avoid earthworm mortality and to allow earthworms to return to optimum conditions (Hanc et al., 2022). The average density of earthworm (*E. andrei*) in the substrate was 126 pieces per litter, with each piece weighing 0.2g. The vermicomposting/composting process was carried out at a constant temperature of 22 °C. The moisture level of the material was maintained at around 70%–80% of the wet mass during vermicomposting/composting by spraying the surface with water every two days. The experiment was carried out at the Faculty of Agrobiology, Food, and Natural Resources experimental station in Cerveny Ujezd, Czech University of Life Sciences Prague.

2.3. Laboratory analysis

2.3.1. Analysis of selected chemical properties

Representative samples of about 150 g wet weight per variant were taken on days 0 and 120, freeze-dried at −25 °C, lyophilised, and ground to analyse selected chemical properties. Another 30 g sample was taken from each variant and kept at 4 °C to determine pH and electrical conductivity (EC). According to BSI EN 15933(2012), the pH-H_2O and EC were determined using a WTW pH 340i and WTW cond 730 (1:5 w/v dry basis). Total carbon (TC) and total nitrogen (TN) were determined using an elemental analyser (CHNS Vario MACRO cube, Elementar Analysensysteme V3.1.1, Hanau, Germany).

2.3.2. Extraction and analysis of PPCPs and EDCs

The PPCPs and EDCs in the samples were analysed using LC-MS/MS after they had been homogenized. Subsequently, 1–2 g samples were moved to an extraction cell and placed in an accelerated solvent extractor (ASE, Dionex). The extraction process included preheating the methanol solvent and the cell to 80 °C and performing three cycles with 5-min fixed intervals between each cycle. The evaporated extracts were spun in a centrifuge at 6000g for 10 min, and the supernatants were collected and transferred to 2 mL vials for further analysis. The Agilent 1260 infinity liquid chromatography system and Agilent 6470 LC/TQ triple quadrupole mass detector were used to examine the samples. Separation was carried out using a Poroshell 120 EC-C18 column (2.7 m, 3 mm × 100 mm, Agilent) and a Poroshell 120 EC-C18 pre-column (2.7 m, 3 mm × 5 mm, Agilent), both of which were heated to 40 °C. The mobile phase was made up of phase A (0.5 mM ammonium fluoride in MQ water plus 0.01% formic acid, LC-MS grade) and phase B (100% methanol, LC-MS grade). The elution schedule of the gradient was such that the % phase B was as follows (time [min]): 0, 5; 4, 50; 6, 50; 18, 100; 21, 100; 22, 5, and 23, 5. The mobile phase had a flow rate of 0.4 mL/min, the duration of the run was 23.50 min, and the amount injected was 2 L. The matrix effect was diminished by the use of automatic standard additions of 1, 5, and 25 ng/mL to measure the samples. Innemanová et al. (2022) utilized MassHunter Source Optimizer and Workstation Optimizer (Versions 10.0, SR1, Agilent) to optimize the mass spectrometric parameters. After the experiment, the analyses were carried out at the Institute of Microbiology of the Czech Academy of Sciences. The analysis was done as part of a planned procedure called "scheduled analysis". 32 organic micropollutants were identified in SS. However, only 12 organic micropollutants, 11 of which were PPCPs: caffeine (CAF), carbamazepine (CBZ), cetirizine (CETI), citalopram (CITA), diclofenac (DCF), ibuprofen (IBF), mirtazapine (MIRT), sulfapyridine (SPD), telmisartan (TE), triclosan (TCS), venlafaxine (VEN), and one was an EDC: bisphenol A (BPA). The reduction percentage (R %) of each variant was calculated for the concentrations of all PPCPs and EDCs using the following equation (Biel-Maeso et al., 2019).

$$R(\%) = \frac{Xi - Xf}{Xi} \quad (1)$$

Where Xi is the concentration of organic micropollutants on the initial (day 0) variants (ng g^{-1}), and Xf denotes the same for the final concentration of organic micropollutants after 120 days of vermicomposting/composting.

2.3.3. Vermidegradation and vermiaccumulation of PPCPs and EDCs

The influence of earthworms on degradation was tested by developing evaluation parameters. The influence of earthworms (vermidegradation (VD)) was determined by calculating the percentage difference between the degradation efficiency (DE) with earthworms (+EW) and the degradation efficiency without earthworms (-EW) (Zeb et al., 2020).

$$VD = (DE(+EW) - DE(-EW)) * 100 \quad (2)$$

$$DE\ (+EW) = 1 - \frac{(+EW)}{\text{Input raw material}} \quad (3)$$

$$DE\ (-EW) = 1 - \frac{(-EW)}{\text{Input raw material}} \quad (4)$$

The influence value indicates how much more significant the reduction in micropollutant concentration was with the use of earthworms compared to the variant without earthworms. The bioconcentration factor (BCF) was calculated by dividing the average concentration of micropollutants in earthworms by the average concentration of micropollutants in vermicomposted material to determine vermiaccumulation (Suthar and Gairola, 2014).

$$BCF = \frac{Concentrations\ in\ earthworms}{Concentrations\ in\ the\ substrate} \quad (5)$$

2.4. Statistical analyses

To ensure that the data were normally distributed and homogeneous, the Shapiro-Wilk and Bartlett tests were used. A one-way variance analysis (ANOVA) was used to determine whether earthworms significantly influenced the concentrations of PPCPs and EDCs during SS vermicomposting. A Tukey test based on the mean differences was applied in a post-hoc analysis to identify the significant variations. The principal component analysis (PCA) was employed to evaluate the relations between the organic micropollutants and specific chemical parameters. The PCA was applied to the variables eigenvalues, variance (%), and cumulative (%) were used to measure the correlation between the variables. The Pearson correlation coefficient (r) was used to analyse the relationships between organic micropollutants and chemical characteristics. The statistical analyses used R version 4.0.2 and STATISTICA 12 software (StatSoft, Tulsa, USA). The significance level of statistical test was set at $p < 0.05$.

3. Results and discussion

3.1. Selected chemical characteristics of vermicomposted sewage sludge

Table 2 presents the initial and final properties of eight different variants. When compared to the initial, the pH of all variants decreased

Table 2
Initial and final selected chemical characterization of different variants.

Variants	pH-H_2O		
	Initial (day-0)	(+EW) (day-120)	(-EW) (day-120)
100% SS	6.9 ± 0.03	5.26 ± 0.49^{a}	5.62 ± 0.31^{a}
75% SS + 25% SP	7.3 ± 0.11	5.61 ± 0.51^{a}	4.99 ± 0.04^{a}
50% SS + 50% SP	7.6 ± 0.25	5.25 ± 0.68^{a}	4.95 ± 0.32^{a}
25% SS + 75% SP	7.9 ± 0.11	5.83 ± 0.46^{a}	5.08 ± 0.20^{a}
Variants	EC (mS/cm)		
	Initial (day-0)	(+EW) (day-120)	(-EW) (day-120)
100% SS	0.617 ± 0.11	3.01 ± 0.56^{a}	2.30 ± 0.35^{a}
75% SS + 25% SP	0.633 ± 0.08	2.77 ± 0.68^{a}	2.09 ± 0.44^{a}
50% SS + 50% SP	0.649 ± 0.06	2.31 ± 0.63^{a}	2.65 ± 0.07^{a}
25% SS + 75% SP	0.664 ± 0.05	2.19 ± 0.41^{a}	2.55 ± 0.18^{a}
Variants	%TC		
	Initial (day-0)	(+EW) (day-120)	(-EW) (day-120)
100% SS	32.9 ± 0.26	28.96 ± 1.37^{c}	25.40 ± 0.03^{b}
75% SS + 25% SP	35.36 ± 0.23	30.55 ± 0.65^{bc}	29.17 ± 0.71^{c}
50% SS + 50% SP	37.77 ± 0.24	32.66 ± 0.32^{ab}	30.87 ± 0.17^{c}
25% SS + 75% SP	40.18 ± 0.29	34.74 ± 0.44^{a}	35.12 ± 0.28^{a}
Variants	%TN		
	Initial (day-0)	(+EW) (day-120)	(-EW) (day-120)
100% SS	5.36 ± 0.03	3.30 ± 0.25^{a}	3.36 ± 0.15^{a}
75% SS + 25% SP	1.98 ± 0.21	3.18 ± 0.25^{a}	2.98 ± 0.14^{a}
50% SS + 50% SP	1.34 ± 0.07	2.92 ± 0.07^{a}	3.16 ± 0.10^{a}
25% SS + 75% SP	1.05 ± 0.05	2.98 ± 0.14^{a}	3.07 ± 0.00^{a}
Variants	C/N ratios		
	Initial (day-0)	(+EW) (day-120)	(-EW) (day-120)
100% SS	6.14 ± 0.04	8.88 ± 0.81^{a}	7.61 ± 0.33^{c}
75% SS + 25% SP	18.03 ± 1.92	9.74 ± 0.86^{a}	9.83 ± 0.21^{b}
50% SS + 50% SP	28.17 ± 1.43	11.20 ± 0.20^{a}	9.80 ± 0.27^{b}
25% SS + 75% SP	38.36 ± 2.03	11.71 ± 0.60^{a}	11.45 ± 0.09^{a}

Mean value followed by different letters is statistically different at ($p < 0.05$). Values indicate mean ± standard error (n = 3). (+EW) = vermicompost with earthworms, (-EW) = compost without earthworms, SS = sewage sludge, SP = straw pellet.

significantly ($F = 4.12$, $p < 0.05$); however, the reduction in pH value after 120 days of processing was statistically not significantly different ($p > 0.05$) among the variants, both with (+EW) and without earthworms (-EW) (Table 2).

The pH change during vermicomposting/composting may be attributed to some different processes, including the conversion of organic nitrogen into nitrites and nitrates via mineralization and nitrification (Sharma and Garg, 2019), the conversion of organic phosphorus into orthophosphates, and the bioconversion of organic material into intermediate species such as low-molecular-weight organic acids and humic acids (Karmegam et al., 2019). Similar pH reductions were found when composting and vermicomposting sewage sludge, crop straw, municipal solid waste, and livestock manure (Singh and Suthar, 2012). The initial EC value was significantly increased ($F = 3.80$, $p < 0.05$); however, there was no significant ($p > 0.05$) difference in the reduction of EC value after 120 days of vermicomposting among the variants with and without earthworms (Table 2). The gradual increase in EC could be attributed to the release of minerals in the form of cations and anions during substrate decomposition within vermicomposting processes (Ramnarain et al., 2019). The breakdown of organic matter in the vermicompost, which released minerals such as exchangeable Ca, Mg, K, and P in their accessible forms as cations, likely caused the increased EC in this study during vermicomposting, supporting the findings of (Dume et al., 2022).

TC was significantly reduced ($p < 0.05$) in the earthworm variants, with reductions of 12%, 14%, 14%, and 14%; however, in non-earthworm variants, TC was reduced by 23%, 18%, 18%, and 13% for 100% SS, 75% SS +25% SP, 50% SS + 50% SP, and 25% SS +75% SP, respectively (Table 2). In their study, Rini et al. (2020) observed a decrease in TC after 45 and 90 days of vermicomposting of solid waste from indigenous and exotic cow breeds using *epigeic* earthworms (*Perionyx excavatus and Eudrilus eugeniae*). Esmaeili et al. (2020) reported a reduction in TC after 45 days of combined composting and vermicomposting of pistachio waste (PW) mixed with cow dung (CD) in various ratios. Dume et al. (2022) also reported a reduction in TC during the vermicomposting of hydrolysed chicken feather residues for 120 days using *Eisenia andrei*. Microbial activity releases CO_2 due to a decreased TC, indicating that organic compounds are being biodegraded and mineralized in the variants (Ravindran et al., 2015). Microorganisms consume carbon to generate energy for their activities (Khatua et al., 2018). TN decreased by 38% in the 100% SS variant with earthworms and 37% in the no-earthworm variant, whereas TN increased by 61%, 118%, and 184% in earthworm variants and 51%, 136%, and 192% in non-earthworm variants for 75% SS + 25% SP, 50% SS + 50% SP, and 25% SS + 75% SP, respectively (Table 2). However, greater values were recorded in the earthworm-free variants than in the earthworm-containing variants, possibly due to organic carbon loss during composting (Huang et al., 2004). During vermicomposting of agricultural residues using *E. fetida* for 60 days, TN increased by 19.5%–152% (Jadia and Fulekar, 2008). TN content increased in tea prunings by 30.5%–51.3% after 30 days of vermicomposting with *Eudrilus euginae* (Pramanik et al., 2016). According to Dume et al. (2022), vermicomposting with *Eisenia andrei* earthworms increased TN by 42.3%–56.9% for 120 days. In comparison, vermicomposting hydrolysed chicken feather residues (HCFR) without the presence of earthworms increased TN by 56.4%–61.4% (Dume et al., 2022). Kaushik and Garg (2004) reported that vermicomposting of textile mill sludge combined with cow dung and agricultural residues using *E. fetida* for 11 weeks resulted in vermicompost with 2–3 times more TN than initial feedstocks. After 60 days of vermicomposting, Sudkolai and Nourbakhsh (2017) discovered that TN was 1.6 times greater in cow dung vermicompost and three times greater in wheat residue vermicompost than the feedstocks using *E. fetida*. A decrease in organic C in the form of CO_2 and the addition of N by earthworms in the form of mucus, nitrogenous excretory substances, and growth-stimulating hormones could be responsible for greater N levels in vermicompost.

The C/N ratio decreased in both earthworm and non-earthworm variants, with an overall reduction of 46%, 60%, and 69% in earthworm variants and 45%, 65%, and 70% in non-earthworm variants for 75% SS + 25% SP, 50% SS + 50% SP, and 25% SS + 75% SP, respectively. In contrast, the C/N ratio increased in both earthworm and non-earthworm variants for the 100% SS variant. This could be due to the lesser TN in this 100% SS variant (Table 2). The C/N ratio indicates compost maturity because it reflects stability and mineralization rates during the processes (Arumugam et al., 2018).

Increasing TN content and organic matter degradation also contribute to the decreased C/N ratio (Devi and Khwairakpam, 2020). Zhi-wei et al. (2019) found that using *Eisenia fetida* for 45 days reduced the C/N ratio of rice straw and kitchen waste vermicompost by 58.5–71.9%. Soobhany et al. (2015) found that vermicomposting organic solid wastes with *Eudrilus eugeniae* for 10 weeks reduced the C/N ratio by 41.5–48.4%. Boruah et al. (2019) observed that using *E. fetida* for 45 days reduced the C/N ratio by 91.1% in citronella bagasse and paper mill sludge vermicomposting. Biruntha et al. (2020) also found that the C/N ratio was reduced by 48.8%, during vermicomposting of different organic materials (seaweed, sugarcane trash, coir pith, and vegetable waste) with cow dung using *Eudrilus eugeniae* for 50 days. Vermicomposting with *E. andrei* earthworms decreased the C/N ratio by 65.8%–67.2% over 120 days, while vermicomposting HCFR without earthworms increased the C/N ratio by 61.7%–67.9% (Dume et al., 2022).

3.2. PPCPs and EDCs concentration in vermicomposted sewage sludge

The concentrations of PPCPs and EDCs, including bisphenol A (BPA), caffeine (CAF), carbamazepine (CBZ), cetirizine (CETI), citalopram (CITA), diclofenac (DCF), ibuprofen (IBF), mirtazapine (MIRT), sulfapyridine (SPD), telmisartan (TE), triclosan (TCS), and venlafaxine (VEN), are presented in Fig. 1. The concentrations of PPCPs and EDCs decreased from the initial concentration (day 0) to the final concentration after 120 days in the final products (vermicomposts/composts). The concentrations varied significantly among the variants ($F = 9.64$, $p < 0.001$ for CAF, $F = 12.50$, $p < 0.001$ for CBZ, $F = 4.53$, $p < 0.05$ for CETI, $F = 4.17$, $p < 0.05$ for DCF, $F = 6.21$, $p < 0.01$ for CITA, $F = 5.97$, $p < 0.01$ for MIRT, $F = 4.07$, $p < 0.05$ for SPD, $F = p < 0.01$ for TCS). Some PPCP and ED concentrations; however, did not differ significantly among the variants ($F = 1.67$, $p > 0.05$ for BPA, $F = 1.91$, $p > 0.05$ for IBF, $F = 1.50$, $p > 0.05$ for TE, $F = 2.06$, $p > 0.05$ for VEN). In the variants that included earthworms (+EW), the concentrations of PPCPs and EDCs varied as follows: BPA (16–59 ng g^{-1}), CAF (25–48 ng g^{-1}), CBZ (16–33 ng g^{-1}), CETI (20–60 ng g^{-1}), CITA (127–388 ng g^{-1}), DCF (3.0–11 ng g^{-1}), IBF (0–7.8 ng g^{-1}), MIRT (4.8–29 ng g^{-1}), SPD (1.6–2.7 ng g^{-1}), TE (4,099–8,257 ng g^{-1}), TCS (9.6–227 ng g^{-1}), and VEN (11–32 ng g^{-1}). In the variants without earthworms (-EW), the concentrations ranged from BPA (18–71 ng g^{-1}), CAF (25–53 ng g^{-1}), CBZ (19–38 ng g^{-1}), CETI (20–56 ng g^{-1}), CITA (146–444 ng g^{-1}), DCF (3.9–12 ng g^{-1}), IBF (2.0–8.8 ng g^{-1}), MIRT (7.38–32 ng g^{-1}), SPD (1.8–3.9 ng g^{-1}), TE (3,211–9,130 ng g^{-1}), TCS (47–454 ng g^{-1}), and VEN (12–48 ng g^{-1}) (dw) (Fig. 1, Table S2). CAF, DCF, IBF, MIRT, SPD, and TCS concentrations were reduced from their initial concentration in all variants, and the reduction percentages with respect to the initial variants (+EW) were: CAF (25–66%), DCF (94–97%), IBF (83–100%), MIRT (42–61%), SPD (61–84%), and TCS (58–90%), and in variants (-EW) were: CAF (25–62%), DCF (92–97%), IBF (79–89%), MIRT (29–49%), SPD (55–78%), and TCS (17–51%). However, BPA (53%), CBZ (14%), CETI (38%), CITA (12%), and VEN (7%) showed reductions only in the 100% SS variant (+EW) and increased in the remaining variants (BPA: 1–4%, CBZ: 29–144%, CETI: 17–46%, CITA: 17–68%, and VEN: 33–46%). In the 100% SS variant (-EW), BPA (45%), CBZ (1%), CETI (42%), CITA (25%), and VEN (20%) showed reductions and increased in the other variants (BPA: 17–23%, CBZ: 45–183%, CETI: 9–48%, CITA: 55–93%, and VEN: 9–241%). Additionally, the 50% SS +

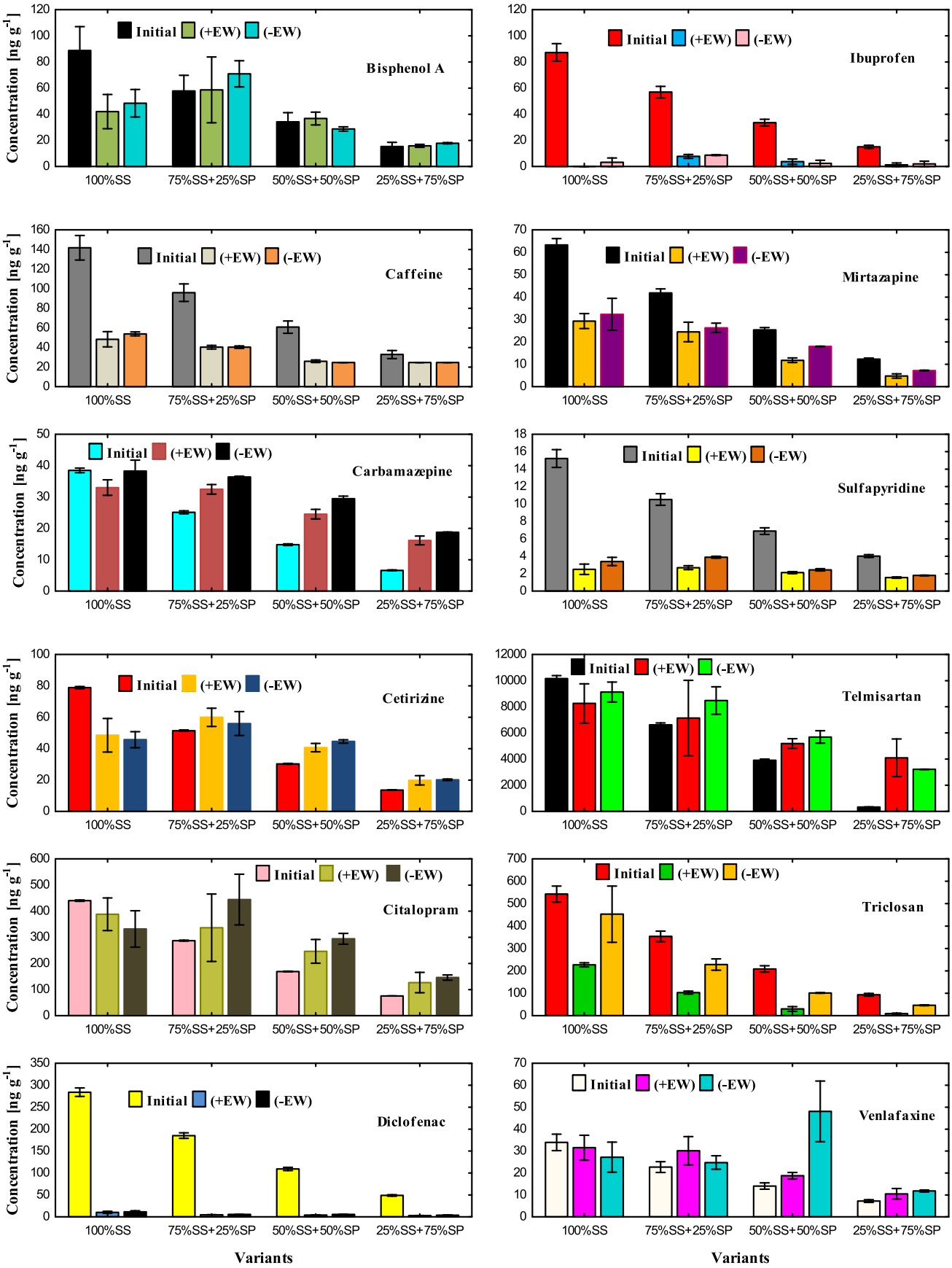


Fig. 1. PPCP and EDC concentrations of different variants at initial input, in final products with earthworms (+EW), and without earthworms (-EW). The bars indicate the standard error of the mean (n = 3).

50% SP variant (+EW) showed a 16% reduction in BPA (Table 3). The decrease in PPCP and EDC concentrations was thought to be caused by bioaccumulation in earthworm tissue during vermicomposting, in their intestine, or by skin absorption. However, a decrease in vermicompost's weight and volume may increase in PPCP and EDC concentration (Mazzeo et al., 2023). According to a report by Hammer and Palmowski (2021), the efficiency of micropollutant removal can differ based on the particular substances and research conducted. The range of removal can fall anywhere between nearly complete to no or insignificant removal. To categorize this range, Hammer and Palmowski (2021) have divided it into five groups: insignificant removal (0–20%), low removal (20–40%), medium removal (40–60%), high removal (60–80%), and very high removal (80%). A medium (53%) removal efficiency of BPA was achieved in 100% SS variant (+EW); however, insignificant to medium (16–45%) removal efficiency was achieved in variant (-EW). From (25–66%), removal efficiency of CAF was achieved in the variant (+EW) and (-EW) (25–62%). The maximum removal efficiency for CBZ in the 100% SS variant (+EW) was 14%, and 1% in the variant (-EW), which is in the range of insignificant removal. A low (38%) removal efficiency of CETI was achieved in 100% SS variant (+EW); however, a medium (42%) removal efficiency was achieved in 100% SS variant (-EW) variant, and an insignificant (12%) and low (25%) of CITA was achieved respectively in variants (+EW) and (-EW). Very high removal efficiency (≥80%) for DCF (94–96%) in the variants (+EW); however, (92–97%) in the variants (-EW), and IBP (83–100%) in the variants (+EW); however, (79–89%) in variants (-EW) was achieved. A medium (42–61%) removal efficiency of MIRT was achieved in variants (+EW); however, low to medium (29–49%) removal efficiency was achieved the variants (-EW). High to very high (61–84%) of SPD in variants (+EW) and medium to high (55–78%) in the variants (-EW) were achieved. 100% SS variants (+EW) (19%) and (-EW) (10%) achieved insignificant removal efficiency of TE, and VEN also achieved insignificant removal efficiency in these variants (+EW) (7%) and (-EW) (20%). Medium to high (58–90%) of TCS in variants (+EW); however, insignificant to medium (17–51%) in variants (-EW) removal efficiency was recorded (Table 3). These present findings, show some inconsistency regarding the removal efficiency of certain PPCPs and EDCs during vermicomposting of SS. It is important to note that the removal efficiency of PPCPs and EDCs could also be influenced by factors such as the type and amount of microorganisms present, the organic loading rate, the retention time, and the system's temperature (Shi et al., 2020). Therefore, it is necessary to conduct more studies under different experimental conditions to understand better the fate and behaviour of organic micropollutants during vermicomposting of SS. No clear and sufficient similar studies that have been published to date. However, Innemanová et al. (2022) conducted a study on the removal efficiency of PPCP and EDC during vermicomposting of dewatered SS under outdoor conditions for one year. Nevertheless, the behaviour of these compounds was not extensively elaborated upon. Moreover, the experiment was conducted outdoors, which could have been impacted by various external factors such as temperature and humidity. According to findings reported by Hammer and Palmowski (2021), CBZ removal efficiency was insignificant during anaerobic sludge digestion. Furthermore, Taboada-Santos et al. (2019) reported a high removal during anaerobic digestion of SS for 115 days. In contrast, other studies (Samaras et al., 2014) achieved very high removal (≥80%) for DCF and IBF during anaerobic digestion of SS for 113 days. Phan et al. (2018) reported that TCS removal efficiencies varied from no removal to high removal during anaerobic digestion. Currently, no environmental legislation limits exist for CAF, CETI, CITA, MIRT, SPD, TE, and VEN. However, the EU has established a legislation limit in SS for CBZ (100 ng g^{-1}), DCF (1,000 ng g^{-1}), IBF (10, 000 ng g^{-1}), TCS (1,000 ng g^{-1}), and (20,000 ng g^{-1}) dw (European Union, 2019). It should be noted that these restrictions are subject to change and may differ based on the regulatory body and country in issue. It is also crucial to remember that some organic micropollutants may not have legal limitations but may still have negatively impact on human health and the environment.

As shown in Table 3, some PPCP and EDC concentrations were reduced in the final products of particular variants. The average negative reduction percentages (R%) of CBZ, CETI, CITA, TE, and VEN showed that the concentrations of PPCP and EDC had increased in both variants (+EW) and (-EW). The increase in some concentrations of PPCP during vermicomposting/composting could be due to the transformation of these compounds into other forms that were not measured in the study. Additionally, some compounds could have been released from the sewage sludge due to the breakdown of organic matter during the processes. Furthermore, the presence of earthworms during vermicomposting could have also contributed to the increased concentration of some compounds by altering the microbial activity and organic matter decomposition rate (Mazzeo et al., 2023), resulting in the formation of new compounds or the release of previously bound compounds (Innemanová et al., 2022). The total average concentrations of BPA, CAF, DCF, IBF, MIRT, SPD, and TCS were reduced by an average of 10, 52, 96, 90, 53, 72, and 76%, respectively and the reduction percentage (R%) value ranged from 10% for BPA to 96% for DCF in variants (+EW); however, 5, 51, 95, 86, 39, 65, and 39%, respectively and the average (R%) value ranged from 5% for BPA to 95% for DCF in variants (-EW). BPA, CAF, DCF, IBF, MIRT, SPD, and TCS reductions were higher in variants (+EW) than in variants (-EW) by 5, 2, 1, 4, 14, 7, and 37%, respectively (Table 3). In general, the reduction in PPCPs and EDCs revealed that their absorption/accumulation in earthworms outweighed the volume reduction effect during processes, and the additive materials

Table 3
PPCP and EDCs reduction percentage in the final products after 120 days of processing (n = 3).

Variants (+EW)	Reduction percentage (R %)											
	BPA	CAF	CBZ	CETI	CITA	DCF	IBF	MIRT	SPD	TE	TCS	VEN
100%SS	53	66	14	38	12	96	100	54	84	19	58	7
75%SS+25%SP	−1	58	−29	−17	−17	97	86	42	74	−6	71	−33
50%SS+50%SP	−8	57	−66	−34	−46	96	83	53	69	−32	86	−33
25%SS+75%SP	−4	25	−144	−46	−68	94	91	61	61	−1150	90	−46
Average	10	52	−56	−15	−30	96	90	53	72	−292	76	−26
Variants (-EW)	BPA	CAF	CBZ	CETI	CITA	DCF	IBF	MIRT	SPD	TE	TCS	VEN
100%SS	45	62	1	42	25	96	89	49	78	10	17	20
75%SS+25%SP	−23	58	−45	−9	−55	97	85	37	63	−28	36	−9
50%SS+50%SP	16	59	−99	−47	−74	95	89	29	65	−46	51	−241
25%SS+75%SP	−17	25	−183	−48	−93	92	79	41	55	−888	50	−63
Average	5	51	−82	−16	−49	95	86	39	65	−238	39	−73

BPA = bisphenol A, CAF = caffeine, CBZ = carbamazepine, CETI = cetirizine, CITA = citalopram, DCF = diclofenac, IBF = ibuprofen, MIRT = mirtazapine, SPD = sulfapyridine, TE = telmisartan, TCS = triclosan, VEN = venlafaxine, (+EW) = variants with earthworms, (- EW) = variants without earthworms, SS = sewage sludge, SP = straw pellet.

enhanced the PPCP and EDC removal efficiency even further (Zeb et al., 2020), and also due to microbial degradations and adsorption of these chemical substances onto organic matter of compost (Dubey et al., 2022).

3.3. PPCP and EDC concentrations in earthworm tissues

Earthworm tissues initially contained only 6.05 ng g^{-1} of CAF and 2.24 ng g^{-1} of TE. However, at the end of vermicomposting, the following seven PPCPs were detected at higher concentrations in the final earthworm tissues: CBZ, CETI, DCF, CAF, CITA, TCS, and TE (Fig. 2). CAF concentration at the end vermicomposting was not detected in the variant of 100% SS, while it increased from 6.05 ng g^{-1} to 23.58 ng g^{-1} (74%) for the 75% SS + 25% SP variant, from 6.05 ng g^{-1} to 11.33 ng g^{-1} (47%) for the 50% SS + 50% SP variant, and from 6.05 ng g^{-1} to 17.78 ng g^{-1} (66%) for the 25% SS + 75% SP variant. The variants with 100% SS, 75% SS + 25% SP, 50% SS + 50% SP, and 25% SS + 75% SP showed a significant increase in TE concentration, ranging from 2.24 to 373.9 ng g^{-1} (99%), 2.24–104.7 ng g^{-1} (98%), 2.24–266.3 ng g^{-1} (99%), and 2.24–116.8 ng g^{-1} (98%), respectively. Other PPCPs that increased were CBZ (5.5–15.9 ng g^{-1}), CETI (6.3–10.9 ng g^{-1}), DCF (5.5–15.5 ng g^{-1}), CITA (16.9–38.2 ng g^{-1}), TCS (8.4–42 ng g^{-1}), and TE (104.7–373.9 ng g^{-1}) (Table S2). The maximum reductions in PPCPs were observed in the 100% SS variant. The highest concentration of PPCP in earthworm tissue was TE; however, BPA, IBF, MIRT, SPD, and VEN were not found in earthworm tissues for all variants. It is therefore concluded that the earthworms had reached the excretion period during vermicomposting, which saw the egestion of accumulated PPCPs and EDCs from their bodies (Zeb et al., 2020). Additionally, the results of PPCPs and EDCs pointed to the possibility of PPCPs and EDCs, essential

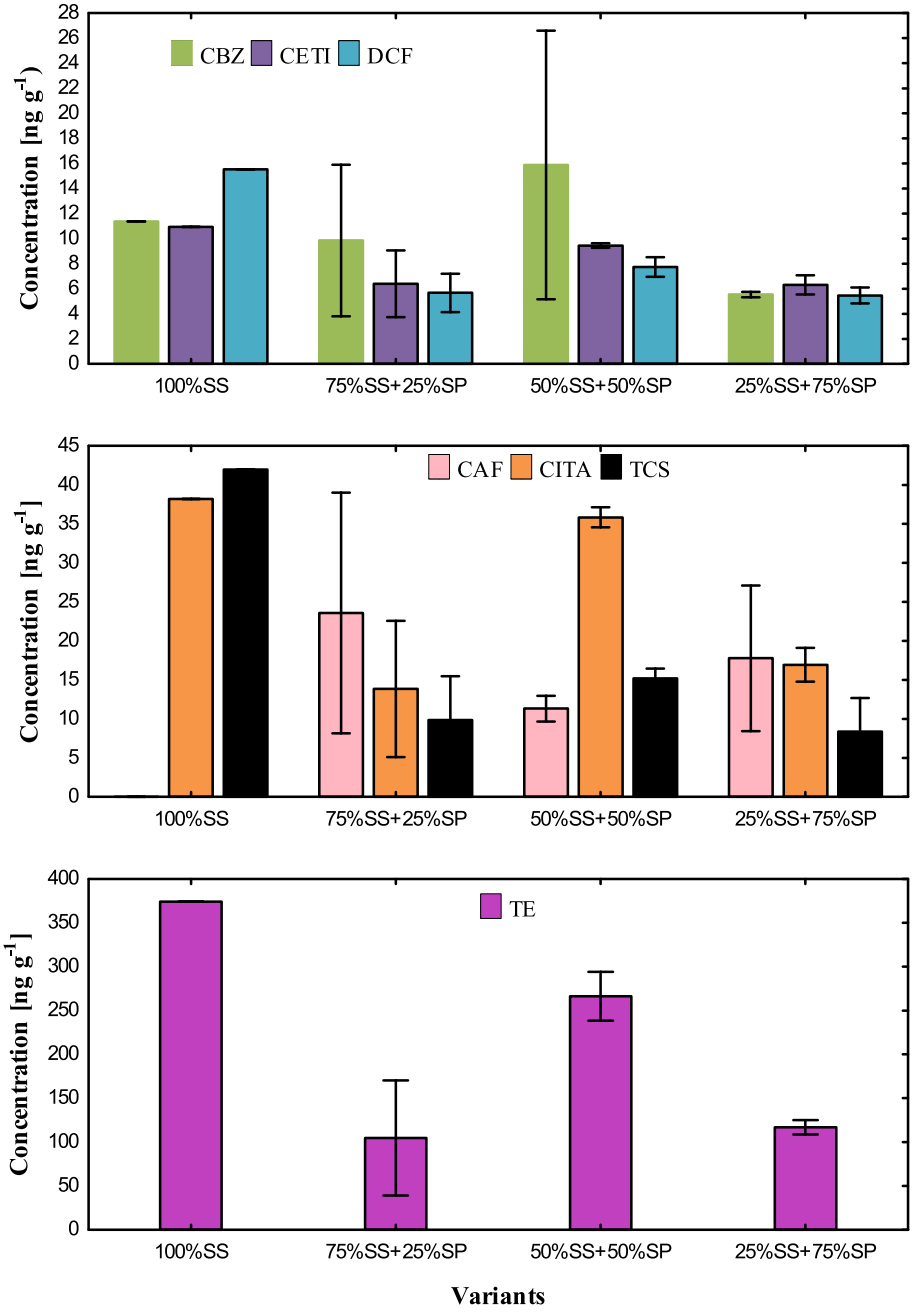


Fig. 2. Concentrations of PPCP found in earthworm tissues. The bars indicate the standard error of the mean (n = 3). A high standard error of the mean indicates that there was some amount of variability in the data.

components of bio-fertilizers, being detoxified by earthworms through metabolisation (Table S2); however, and further histological analysis is needed to validate this hypothesis (Zeb et al., 2020). Because of the reasonably consistent relationships between the concentrations of certain pollutants in earthworms, earthworms accumulate a significant amount of PPCPs and EDCs in their tissues and may be a useful biological indicator of contamination. The earthworm's interaction with local edaphic factors such as pH, organic matter content, enzyme activities and are mainly responsible for the accumulating PPCPs and EDCs (Zeb et al., 2020). TC reduction also results in the formation of intermediate metabolites and acids (humic acids), which lower the pH of the sludge mixtures (Zziwa et al., 2021).

3.4. The influence of earthworms on degradation of PPCPs and EDCs

Vermidegradation is the process by which various pollutants in earthworms are degraded using enzymes such as CYP450 and peroxidase or by gut microbes, also known as 'vermin-endophytes' which are microbes, bacteria, or fungi that live within earthworm tissues without causing any disease. It is one of the pathways of vermicomposting (Zeb et al., 2020). Vermidegradation is primarily concerns removing of organic micro-pollutant compounds such as PPCPs and EDCs (Bhat et al., 2018). Fig. 3 indicates the vermidegradation of some PPCP and EDC. The 100% SS variant had the most earthworms influence (efficiency of degradation) for TCS, with (43%). BPA was second, at (15%), followed by CBZ (14%), TE (8.7%), SPD (6.4%), IBF (5.4%), CAF (4.2%), MIRT (4.3%), and DCF (0.5%). Conversely, three PPCPs (CITA, CETI, and VEN) had negative vermidegradation percentages, with (−13%), (−4%), and (−12%), respectively. CITA (37%) had the most earthworms influence (efficiency of degradation) in the 75% SS + 25% SP variant, followed by TCS (36%), TE (22%), CBZ (16%), SPD (12%), MIRT (4%), IBF (2%), and DCF (0.3%). However, CETI (−8%), VEN (−21%), BPA (−3%), and TE (−262%) had negative vermidegradation percentages, indicating that these PPCPs and EDCs are resistant to vermidegradation (Haiba et al., 2018). Overall, the negative vermidegradation of PPCPs and EDCs highlights the complexity of the environmental fate and impact of these emerging pollutants. Further research is needed to fully understand the factors that influence the effectiveness of earthworms in degrading PPCPs and EDCs and to develop effective strategies for their removal and degradation in the environment.

The variant of 50% SS +50% SP exhibited the most significant percentage of vermidegradation of VEN (193%), followed TCS (35%), CBZ (34%), CITA (28%), MIRT (24%), TE (13%), CETI (13%), SPD (4.5%), and DCF (1.2%), whereas, BPA (−23%), CAF (−2.4%), and IBF (−1.6%) showed negative values (Table S4). The variant with 25% SS +75% SP had percentage of vermidegradation of TCS (40%), CBZ (38%), CITA (25%), VEN (20%), MIRT (19%), BPA (16%), IBF (11%), SPD (6%), CETI (3%), and DCF (2%) (Fig. 3). The negative percentage of vermidegradation for some PPCPs and EDCs implies that the final concentrations of PPCPs and EDCs found in vermicompost were more significant than the initial input materials, which implied that the earthworms did not influence on the degradation of PPCPs and EDCs during vermicomposting and this difference might be due to the extremely high concentration in the variant without earthworms (Shi et al., 2020). Table 4 summarizes the sum of PPCP and EDC concentrations in the initial variant, as well as the sum of these concentrations in the variant at the end of processing for both the (+EW) and (-EW) variants.

The summarised data shows how the concentrations of all determined substances changed during the experiment. The variants with 75% SS + 25% SP had the most earthworm influence on the degradation of targeted organic micropollutants (20.3%), followed by the variant with 50% SS + 50% SP (14.2%) (Table 4). These findings suggest that more research is needed to assess the influence of earthworms on organic micropollutants including PPCPs and EDCs.

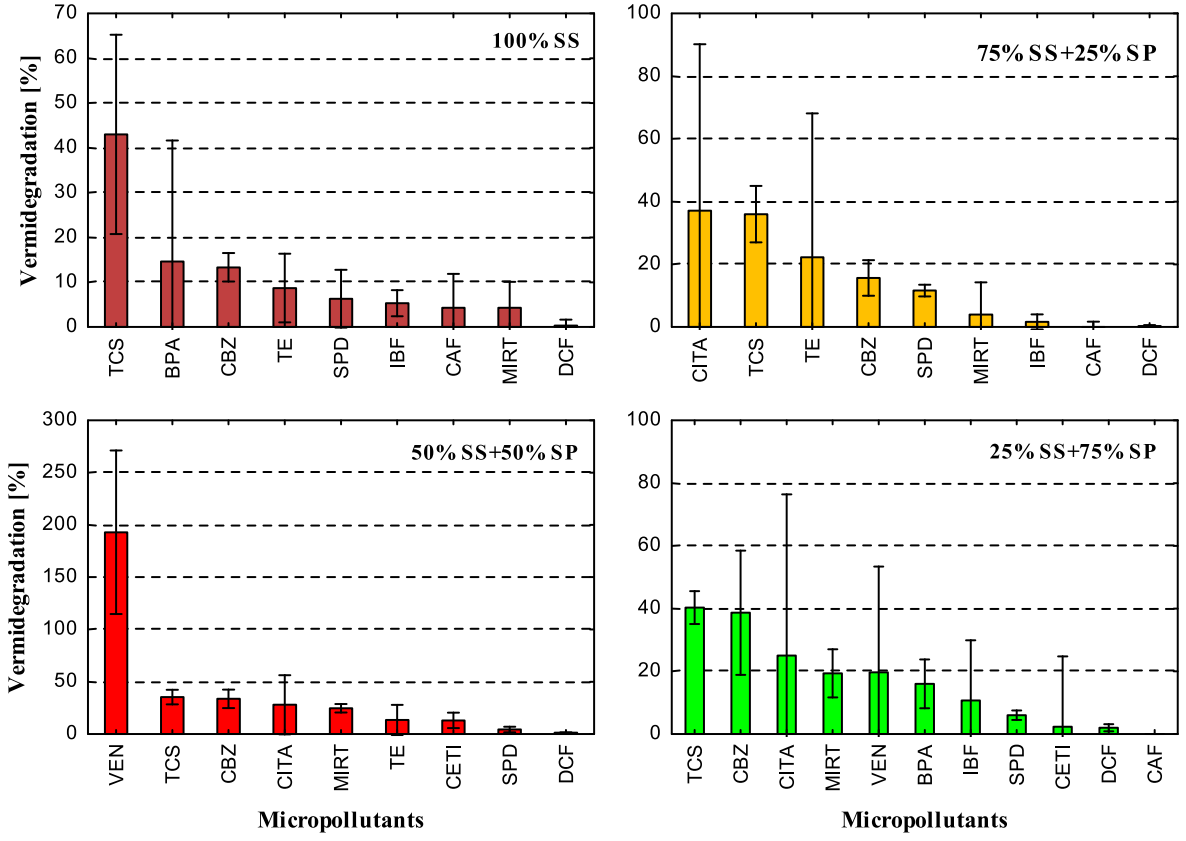


Fig. 3. Influence of earthworms on the degradation of PPCPs and EDCs in different variants. The bars indicate the standard error of the mean (n = 3). A high standard error of the mean indicates that there was some amount of variability in the data.

Table 4
A summary of the degradation efficiencies of PPCPs and EDCs.

Variants	Concentration [ng g^{-1} dw]			Efficiency of degradation [%]		
	Σ Initial	Σ (+EW)	Σ(-EW)	(+EW)	(-EW)	Influence of earthworms (%)
100% SS	11977	9119	10179	24	15	8.9
75% SS + 25% SP	7817	7839	9423	−0.28	21	20.3
50% SS + 50% SP	4617	5637	6293	−22	−36	14.2
25% SS + 75% SP	651	4333	3512	−566	−439	−126.1

Σ Initial = summation of all PPCP concentrations in the initial input materials, Σ (+EW) = summation of all PPCP concentrations in the final vermicompost (with earthworms), Σ (-EW) = summation of all PPCP concentrations in the final product (without earthworms), SS = sewage sludge, SP = straw pellet. dw = dry weight.

3.5. Vermiaccumulation of PPCP and EDC

Shi et al. (2020) explain that vermiaccumulation is the process by which earthworms absorb and retain pollutants, leading to a decrease in the concentration of substances like PPCPs and EDCs in SS. To quantify this assimilation of PPCPs and EDCs into earthworm tissues, the bio-concentration factor (BCF) can be used. The concentrations of PPCPs and EDCs in earthworm tissues were recorded by examining earthworm samples before and after vermicomposting. The vermiaccumulation percentage varied for all PPCPs among the variants; however, maximum vermiaccumulation of caffeine was CAF (72%), CBZ (65%), CETI (32%), CITA (16%), DCF (183%), TE (5%), and TCS (118%) (Fig. 4).

The presence of organic micropollutants in SS is proportional to the level of organic micropollutants in wastewater. The BCF was indicated in the following manner: DCF > TCS > CAF > CBZ > CETI > CITA > TE (Table S3). *Eisenia andrei*, a species of earthworm, has the ability to ingest and process pollutants during vermicomposting. This includes the process of grinding and digestion, allowing for the absorption of these pollutants through the intestinal tract into the worm tissues. This process is known as nutrient uptake and is further facilitated by epidermal uptake, both of which allow for earthworms to acquire organic micropollutants. Vermicomposting has been found to be effective in reducing the concentration of organic micropollutants in SS, thus addressing the issue. However, a new question arises about how to handle the earthworms that have accumulated organic micropollutants in their bodies, as highlighted by Shi et al. (2020). This is troubling for two reasons. Separation of vermicomposting earthworms is not difficult; this is often accomplished simply by adding fresh material where earthworms naturally crawl. However, earthworms can still be found in vermicompost or other materials; they are simply separated from the final product. When careful separation of earthworms from impurities and matrixes becomes economically viable, a problem arises because these methods are typically time-consuming. This represents the second issue in dealing with earthworms. The spectrum of use for uncontaminated earthworms is broad; however, there is currently no use for earthworms with high PPCP and EDC bioaccumulation. One option is not to separate the earthworms but to leave the earthworm population in vermicompost. However, this option has limitations in terms of earthworm bioaccumulation limits. These organisms will vermiaccumulate PPCP and EDC to a certain level, after which the concentration of pollutants inside the organism will either stop increasing or the organism will die. In both cases, this means that earthworms' ability to degrade PPCP and EDC is reduced. Earthworms' ability to degrade PPCP and EDC is reduced in both cases. Measurable influence earthworms may be possible only if new earthworms are used in each situation (Zeb et al., 2020).

3.6. Worm reproduction and growth

Growth rate, earthworm number (*E. andrei*), and cocoons in the vermicompost process in different variants are shown in Table S5. *E. andrei* exhibited significant ($p < 0.05$) variations in the number of earthworm pieces/kg in the vermicomposted material and also the number of cocoons/kg in the vermicomposted material (Table S5). The initial weight and amount of earthworms were 0.2 g/piece and 125 pieces/kg of vermicomposted material, respectively. The initial earthworms weighed 25 g per kilogram of vermicomposted material. After 120 days, the variant with 25% SS + 75% SP contained the maximum number of cocoons (178 pieces/kg), and the 50% SS + 50% SP variant contained the minimum (59 pieces/kg). The results indicate that, despite some mortality, there was an increase in the number of earthworms in some variants. This increase was more significant in the variant with 50% SS + 50% SP than in the other variants, and worm mass was also more significant in this variant. This could be due to the presence of nutrients for worm growth in the additive material, which makes this variant (50% SS + 50% SP) a favourite feed for earthworms (Pérez-Godínez et al., 2017). *E. andrei* produced more cocoons in the variant with 25% SS + 75% SP than in the other variants. The additive material is a carbon source, a vital determinant of earthworm production initiation, and might explain differences in cocoon production levels among the variants. A higher carbon content additive material promotes growth and reproduction by providing earthworms with an adequate amount of organic matter. Higher carbon source of additive material appears to a significant impact cocoon production (Sonmezdag et al., 2017).

3.7. Principal component analysis (PCA)

Fig. 5 shows the principal component analysis of 12 organic micropollutants and the correlation between organic micropollutants and select chemical parameters. Principal component analysis (PCA) was used to evaluate the relationships between the PPCP and EDC (BPA, CAF, CBZ, CETI, CITA, DCF, IBF, MIRT, SPD, TE, TCS, and VEN) and selected chemical parameters (pH, EC, TC, TN, and C/N ratio), and plotted PC1 with PC2. The PCA analysis was designed to compare all of the investigated parameters, focusing on exciting relationships. The relationship between the variables was determined by analysing their eigenvalues, variance (%), and cumulative (%) criteria. The principal component (PC) accounted for 60.11% of the variance, 7.98 of the eigenvalue and was dominant for the variables pH, TC, and C/N ratio. All 12 PPCP and EDC were negatively correlated with pH, TC, and C/N ratios and positively correlated with EC and TN. PC2 accounted for 14.07% of the variance and 2.8 of the eigenvalue. All PPCPs and EDCs dominated this component and were positively correlated with TN, apart from IBF, which was negatively correlated with TN. VEN also had a significantly positive correlation with EC ($r = 0.4177$, $p < 0.05$) except for SPD, IBF, DCF and TCS, which negatively correlated with EC. TE was significantly correlated with EC ($r = 0.4696$, $p < 0.05$) and TN ($r = 0.7057$, $p < 0.001$), whereas CITA was significantly correlated with EC ($r = 0.5751$, $p < 0.01$), and TN ($r = 0.6514$, $p < 0.01$); however, CITA ($r = -0.4115$, $p < 0.05$) and TE ($r = -0.5228$, $p < 0.01$) had a significantly negative correlation with pH (Table S6).

As a result of TC reduction, the formation of intermediate metabolites and acids (humic acids) reduces the pH of the sludge mixtures. PPCP and EDC accumulation in tissues is a distinct phenomenon. Each PPCP and EDC exhibits a distinct physiological mechanism of assimilation and excretion during its metabolism in the earthworm's gut. As a result, higher TC and C/N values result in better PPCP and EDC degradation. The degradation of PPCP and EDC is not affected by pH. According to Dubey et al. (2022), the degradation of PPCP and EDC is not influenced by pH. Bacteria tend to favour high carbon and C/N ratios for breaking down PPCPs and EDCs, whereas fungi prefer environments with high pH and nitrogen levels.

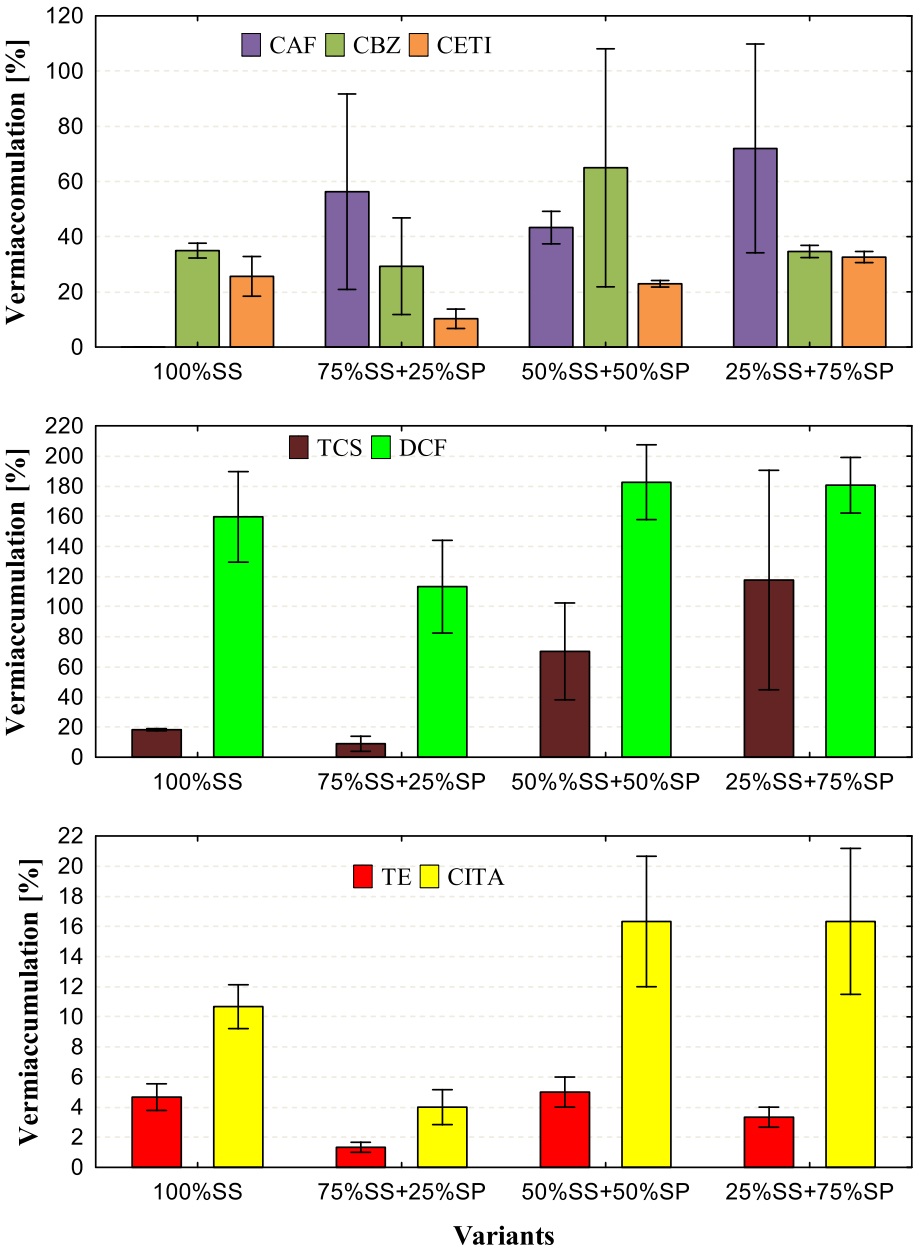


Fig. 4. Vermiaccumulation of some PPCPs during SS vermicomposting in different variants. The bars indicate the standard error of the mean (n = 3). A high standard error of the mean indicates that there was some amount of variability in the data.

3.8. Current research challenges and future perspectives

Although earthworms are known to contribute to the degradation of PPCPs and EDCs in SS, the specific enzymes and metabolic pathways involved in this process are not fully understood (Shi et al., 2020). Identifying these mechanisms is crucial for optimising earthworm-based treatment and developing more effective methods for removing PPCP and EDC levels in SS. Identifying these pathways to optimize earthworm-based treatment systems and developing more effective strategies for lowering PPCP and EDC levels in SS is critical. Temperature, moisture content, and other organic matter can all impact activity and the rate of PPCP and EDC degradation (Zeb et al., 2020). Understanding these impacts is critical for optimising earthworm-based treatment systems and forecasting their efficacy in various environmental situations. Although earthworm-based treatment systems have shown promise in the laboratory, assessing their viability at an industrial or municipal scale is critical. This includes determining the economic viability of large-scale earthworm culture and the possibility of combining earthworm-based treatment systems with existing wastewater treatment infrastructure. Therefore, the following concerns must be addressed.

1. Earthworms employed for SS vermicomposting may accumulate specific organic micropollutants from the SS, such as PPCPs and EDCs. If these earthworms are utilized in soil or other applications, these micropollutants may be transferred to the new environment. As a result, it is critical to appropriately manage or treat earthworms to remove toxins before employing them in other applications. One method is to submit the earthworms to a procedure known as "phytoremediation," in which they are fed plants capable of absorbing and breaking down toxins in their bodies (Zheng et al., 2022).
2. Developing earthworm-based treatment systems for wider usage: While earthworm-based treatment systems have shown promise in

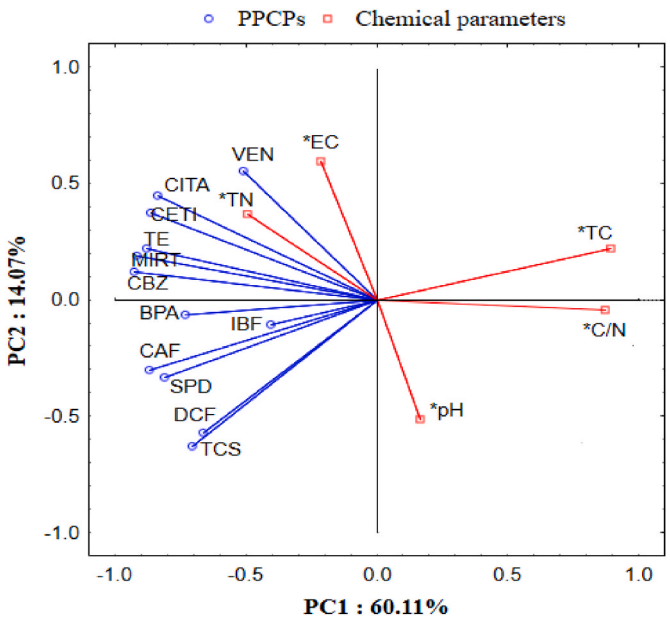


Fig. 5. Principal components (PC) of PPCP and EDC and along with their correlation with selected chemical parameters (pH, EC, TC, TN, and C/N ratio). BPA = bisphenol A, CAF = caffeine, CBZ = carbamazepine, CETI = cetirizine, CITA = citalopram, DCF = diclofenac, IBF = ibuprofen, MIRT = mirtazapine, SPD = sulfapyridine, TE = telmisartan, TCS = triclosan, VEN = venlafaxine, TC = total carbon; TN = total nitrogen, C/N = carbon to nitrogen ratio.

lowering PPCP and EDC levels in SS, more effective and scalable methods are needed for general application. Optimising the conditions for earthworm activity, developing new strains of earthworms that are more efficient at degrading pollutants, and integrating earthworm-based treatment systems with existing wastewater treatment infrastructure could all be part of this.

3. Developing new analytical methods for monitoring pollutant degradation in SS: This can help to make monitoring more efficient and cost-effective. Future studies could focus on developing new real-time methods for monitoring pollution levels, such as employing nanoparticles or sophisticated imaging techniques.
4. Evaluating the long-term viability of earthworm-based treatment systems: While earthworms can effectively reduce pollutant levels in SS, it is critical to evaluate the long-term viability of these systems. This involves comprehending the effects of repeated cycles of earthworm digestion and the potential accumulation of pollutants in earthworm tissues.

4. Conclusion

It was hypothesised that earthworms could remove the PPCPs and EDCs due to bioaccumulation of these chemicals in earthworm tissue during vermicomposting. According to this assumption, variants with earthworms reduced some PPCPs and EDCs such as BPA, CAF, DCF, IBF, MIRT, SPD, and TCS more effectively than variants without earthworms. However, the concentrations of CBZ, CETI, CITA, TE, and VEN increased in both variants with and without earthworms. Furthermore, the reduction in the weight and volume of end product (vermicompost/compost) may result in an increase in the concentration of these selected organic micropollutants. In all variants with and without earthworms, a very high removal efficiency of DCF and IBF was achieved. Therefore, from this finding, earthworms have shown great promise in removing selected PPCP and EDC from sewage sludge. Simultaneously, it is strongly suggested to perform further research oriented to the development of more effective and sustainable methods for removing organic micropollutants from sewage sludge.

CRediT authorship contribution statement

Bayu Dume: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Visualization. **Aleš Hanč:** Conceptualization, Formal analysis, Resources, Data curation, Writing – original draft, Methodology, Supervision, Project administration, Funding acquisition. **Pavel Švehla:** Conceptualization, Methodology, Supervision, Formal analysis, Resources, Data curation, Writing – original draft. **Pavel Michal:** Sample and data collection. **Vojtěch Pospíšil:** Formal analysis. **Alena Grasserová:** Formal analysis. **Tomáš Cajthaml:** Project administration, Review, Editing. **Abraham Demelash Chane:** Sample and data collection. **Abebe Nigussie:** Review, Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

Financial support for this work was provided by the Ministry of Agriculture of the Czech Republic under the NAZV project number QK1910095. The authors would like to thank Christina Baker Starrman (https://cbsciedit.com/) for revision of the English text.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jclepro.2023.137869.

References

Arumugam, K., Renganathan, S., Babalola, O.O., Muthunarayanan, V., 2018. Investigation on paper cup waste degradation by bacterial consortium and *Eudrilus eugeniae* through vermicomposting. Waste Manage. (Tucson, Ariz.) 74, 185–193.

Bhat, S.A., Singh, S., Singh, J., Kumar, S., Vig, A.P., 2018. Bioremediation and detoxification of industrial wastes by earthworms: vermicompost as powerful crop nutrient in sustainable agriculture. Bioresour. Technol. 252, 172–179.

Biel-Maeso, M., Corada-Fernández, C., Lara-Martín, P.A., 2019. Removal of personal care products (PCPs) in wastewater and sludge treatment and their occurrence in receiving soils. Water Res. 150, 129–139.

Biruntha, M., Karmegam, N., Jeyaprakasam, A., Selvi, B.K., Paul, J.A.J., Balamuralikrishnan, B., Chang, S.W., Ravindran, B., 2020. Vermiconversion of biowastes with low-to-high C/N ratio into value added vermicompost. Bioresour. Technol. 297, 122398.

Boruah, T., Barman, A., Kalita, P., Lahkar, J., Deka, H., 2019. Vermicomposting of citronella bagasse and paper mill sludge mixture employing *Eisenia fetida*. Bioresour. Technol. 294, 122147.

British Standards Institution (BSI) European Standard (EN) 15933, 2012. Sludge, Treated Biowaste and Soil Determination of pH.

Buta, M., Hubeny, J., Zieliński, W., Harnisz, M., Korzeniewska, E., 2021. Sewage sludge in agriculture–the effects of selected chemical pollutants and emerging genetic resistance determinants on the quality of soil and crops–a review. Ecotoxicol. Environ. Saf. 214, 112070.

Devi, C., Khwairakpam, M., 2020. Bioconversion of *Lantana camara* by vermicomposting with two different earthworm species in monoculture. Bioresour. Technol. 296, 122308.

Dubey, M., Rajpal, A., Vellanki, B.P., Kazmi, A.A., 2022. Occurrence, removal, and mass balance of contaminants of emerging concern in biological nutrient removal-based sewage treatment plants: role of redox conditions in biotransformation and sorption. Sci. Total Environ. 808, 152131.

Dume, B., Hanc, A., Svehla, P., Michal, P., Solcova, O., Chane, A.D., Nigussie, A., 2022. Nutrient recovery and changes in enzyme activity during vermicomposting of hydrolysed chicken feather residue. Environ. Technol. 43, 1–27.

Esmaeili, A., Khoram, M.R., Gholami, M., Eslami, H., 2020. Pistachio waste management using combined composting-vermicomposting technique: physico-chemical changes and worm growth analysis. J. Clean. Prod. 242, 118523.

European Union, 2019. Regulation (EU) 2019/1021 of the European Parliament and of the Council of 20 June 2019 on Persistent Organic Pollutants (Text with EEA Relevance). http://data.europa.eu/eli/reg/2019/1021/oj.
Gago-Ferrero, P., Borova, V., Dasenaki, M.E., Thomaidis, N.S., 2015. Simultaneous determination of 148 pharmaceuticals and illicit drugs in sewage sludge based on ultrasound-assisted extraction and liquid chromatography-tandem mass spectrometry. Anal. Bioanal. Chem. 407, 4287–4297.
Haiba, E., Nei, L., Herodes, K., Ivask, M., Lillenberg, M., 2018. On the degradation of metformin and carbamazepine residues in sewage sludge compost. Agron. Res. 16, 696–707.
Hammer, L., Palmowski, L., 2021. Fate of selected organic micropollutants during anaerobic sludge digestión. Water Environ. Res. 93, 1910–1924.
Hanc, A., Dume, B., Hrebeckova, T., 2022. Differences of enzymatic activity during composting and vermicomposting of sewage sludge mixed with straw pellets. Front. Microbiol. 12, 3892.
Huang, G.F., Wong, J.W.C., Wu, Q.T., Nagar, B.B., 2004. Effect of C/N on composting of pig manure with sawdust. Waste Manage. (Tucson, Ariz.) 24, 805–813.
Hu, X., Shi, W., Wei, S., Zhang, X., Yu, H., 2021. Identification of (anti-)androgenic activities and risks of sludges from industrial and domestic wastewater treatment plants. Environ. Pollut. 268, 115716.
Innemanová, P., Grasserová, A., Cajthaml, T., 2022. Pilot-scale vermicomposting of dewatered sewage sludge from medium-sized wastewater treatment plant (WWTP). Detritus 18, 35.
Jiang, L., Li, Y., Chen, Y., Yao, B., Chen, X., Yu, Y., Yang, J., Zhou, Y., 2023. Pharmaceuticals and personal care products (PPCPs) in the aquatic environment: biotoxicity, determination and electrochemical treatment. J. Clean. Prod. 135923.
Jadia, C.D., Fulekar, M.H., 2008. Vermicomposting of vegetable waste: a biophysicochemical process based on hydro-operating bioreactor. Afr. J. Biotechnol. 7, 3723–3730.
Karmegam, N., Vijayan, P., Prakash, M., John Paul, J.A., 2019. Vermicomposting of paper industry sludge with cow dung and green manure plants using *Eisenia fetida*: a viable option for cleaner and enriched vermicompost production. J. Clean. Prod. 228, 718–728.
Kaushik, P., Garg, V.K., 2004. Dynamics of biological and chemical parameters during vermicomposting of solid textile mill sludge mixed with cow dung and agricultural residues. Bioresour. Technol. 94, 203–209.
Khatua, C., Sengupta, S., Balla, V.K., Kundu, B., Chakraborti, A., Tripathi, S., 2018. Dynamics of organic matter decomposition during vermicomposting of banana stem waste using *Eisenia fetida*. Waste Manage. (Tucson, Ariz.) 79, 287–295.
Mazzeo, D.E.C., Dombrowski, A., Oliveira, F.A., Levy, C.E., Oehlmann, J., Marchi, M.R. R., 2023. Endocrine disrupting activity in sewage sludge: screening method, microbial succession and cost-effective strategy for detoxification. J. Environ. Manag. 330, 117207.
Menon, N.G., Mohapatra, S., Padhye, L.P., Tatiparti, S.S.V., Mukherji, S., 2020. Review on Occurrence and Toxicity of Pharmaceutical Contamination in Southeast Asia. Emerging Issues in the Water Environment during Anthropocene: A South East Asian Perspective, pp. 63–91.
Nunes, N., Ragonezi, C., Gouveia, C.S.S., Pinheiro de Carvalho, M.A.A., 2021. Review of sewage sludge as a soil amendment in relation to current international guidelines: a heavy metal perspective. Sustainability 13, 2317.
Pérez-Godínez, E.A., Lagunes-Zarate, J., Corona-Hernández, J., Barajas-Aceves, M., 2017. Growth and reproductive potential of *Eisenia fetida* (Sav) on various zoo animal dungs after two methods of pre-composting followed by vermicomposting. Waste Manage. (Tucson, Ariz.) 64, 67–78.
Petrie, B., Barden, R., Kasprzyk-Hordern, B., 2014. A review on emerging contaminants in wastewaters and the environment: current knowledge, understudied areas and recommendations for future monitoring. Water Res. 72, 3–27.
Phan, H.V., Wickham, R., Xie, S., McDonald, J.A., Khan, S.J., Ngo, H.H., Guo, W., Nghiem, L.D., 2018. The fate of trace organic contaminants during anaerobic digestion of primary sludge: a pilot scale study. Bioresour. Technol. 256, 384–390.
Pramanik, P., Safique, S., Jahan, A., Bhagat, R.M., 2016. Effect of vermicomposting on treated hard stem leftover wastes from pruning of tea plantation: a novel approach. Ecol. Eng. 97, 410–415.
Ramnarain, Y.I., Ansari, A.A., Ori, L., 2019. Vermicomposting of different organic materials using the *epigeic* earthworm *Eisenia fetida*. Int. J. Recycl. Org. Waste Agric. 8, 23–36.
Ravindran, B., Contreras-Ramos, S.M., Sekaran, G., 2015. Changes in earthworm gut associated enzymes and microbial diversity on the treatment of fermented tannery waste using *epigeic* earthworm *Eudrilus eugeniae*. Ecol. Eng. 74, 394–401.
Rini, J., Deepthi, M.P., Saminathan, K., Narendhirakannan, R.T., Karmegam, N., Kathireswari, P., 2020. Nutrient recovery and vermicompost production from livestock solid wastes with *epigeic* earthworms. Bioresour. Technol. 313, 123690.
Rorat, A., Courtois, P., Vandenbulcke, F., Lemiere, S., 2019. Sanitary and environmental aspects of sewage sludge management. In: Industrial and Municipal Sludge. Butterworth-Heinemann, pp. 155–180.
Samaras, V.G., Stasinakis, A.S., Thomaidis, N.S., Mamais, D., Lekkas, T.D., 2014. Fate of selected emerging micropollutants during mesophilic, thermophilic and temperature co-phased anaerobic digestion of sewage sludge. Bioresour. Technol. 162, 365–372.
Schug, T.T., Johnson, A.F., Birnbaum, L.S., Colborn, T., Guillette Jr., L.J., Crews, D.P., Collins, T., Soto, A.M., Vom Saal, F.S., McLachlan, J.A., Sonnenschein, C., 2016. Mini review: endocrine disruptors: past lessons and future directions. Mol. Endocrinol. 30, 833–847.
Sharma, K., Garg, V.K., 2019. Recycling of lignocellulosic waste as vermicompost using earthworm *Eisenia fetida*. Environ. Sci. Pollut. Res. 26, 14024–14035.
Shi, Z., Liu, J., Tang, Z., Zhao, Y., Wang, C., 2020. Vermiremediation of organically contaminated soils: concepts, current status, and future perspectives. Appl. Soil Ecol. 147, 103377.
Singh, D., Suthar, S., 2012. Vermicomposting of herbal pharmaceutical industry waste: earthworm growth, plant-available nutrient and microbial quality of end materials. Bioresour. Technol. 112, 179–185.
Sonmezdag, A.S., Kelebek, H., Selli, S., 2017. Characterization and comparative evaluation of volatile, phenolic and antioxidant properties of pistachio (*Pistacia vera L.*) hull. J. Essent. Oil Res. 29, 262–270.
Soobhany, N., 2019. Insight into the recovery of nutrients from organic solid waste through biochemical conversion processes for fertilizer production: a review. J. Clean. Prod. 241, 118413.
Soobhany, N., Mohee, R., Garg, V.K., 2015. Experimental process monitoring and potential of *Eudrilus eugeniae* in the vermicomposting of organic solid waste in Mauritius. Ecol. Eng. 84, 149–158.
Sudkolai, S.T., Nourbakhsh, F., 2017. Urease activity as an index for assessing the maturity of cow manure and wheat residue vermicomposts. Waste Manage. (Tucson, Ariz.) 64, 63–66.
Sun, Q., Li, M.Y., Ma, C., Chen, X.Q., Xie, X.Q., Yu, C.P., 2016. Seasonal and spatial variations of PPCP occurrence, removal and mass loading in three wastewater treatment plants located in different urbanization areas in Xiamen, China. Environ. Pollut. 208, 371–381.
Suthar, S., Gairola, S., 2014. Nutrient recovery from urban forest leaf litter waste solids using *Eisenia fetida*. Ecol. Eng. 71, 660–666.
Suthar, S., 2009. Vermicomposting of vegetable-market solid waste using *Eisenia fetida*: impact of bulking material on earthworm growth and decomposition rate. Ecol. Eng. 35, 914–920.
Taboada-santos, A., Braz, G.H.R., Fernandez-gonzalez, N., Carballa, M., Lema, J.M., 2019. Thermal hydrolysis of sewage sludge partially removes organic micropollutants but does not enhance their anaerobic biotransformation. Sci. Total Environ. 690, 534–542.
Thomas, A.R., Kranert, M., Philip, L., 2020. Fate and impact of pharmaceuticals and personal care products during septage co-composting using an in-vessel composter. Waste Manage. (Tucson, Ariz.) 109, 109–118.
Yadav, A., Garg, V.K., 2016. Vermiconversion of biogas plant slurry and parthenium weed mixture to manure. Int. J. Recycl. Org. Waste Agric. 5, 301–309.
Zeb, A., Li, S., Wu, J., Lian, J., Liu, W., Sun, Y., 2020. Insights into the mechanisms underlying the remediation potential of earthworms in contaminated soil: a critical review of research progress and prospects. Sci. Total Environ. 740, 140145.
Zhi-Wei, S., Tao, S., Wen-Jing, D., Jing, W., 2019. Investigation of rice straw and kitchen waste degradation through vermicomposting. J. Environ. Manag. 243, 269–272.
Zheng, Y., Sun, Z., Liu, Y., Cao, T., Zhang, H., Hao, M., Chen, R., Dzakpasu, M., Wang, X. C., 2022. Phytoremediation mechanisms and plant eco-physiological response to micro-organic contaminants in integrated vertical-flow constructed wetlands. J. Hazard Mater. 424, 127611.
Zziwa, A., Jjagwe, J., Kizito, S., Kabenge, I., Komakech, A.J., Kayondo, H., 2021. Nutrient recovery from pineapple waste through controlled batch and continuous vermicomposting systems. J. Environ. Manag. 279, 111784.

Supplementary material

Influence of earthworms on the behaviour of organic micropollutants in sewage sludge

Bayu Dume[*a], Aleš Hanč[a], Pavel Švehla[a], Pavel Michal[a], Vojtěch Pospíšil[a], Alena Grasserová[b,c], Tomáš Cajthaml[b,c], Abraham Demelash Chane[a], Abebe Nigussie[d]

[a]*Czech University of Life Sciences, Faculty of Agrobiology, Food, and Natural Resources, Department of Agro-Environmental Chemistry and Plant Nutrition, Kamycka 129, Prague 16500, Czech Republic.*

[b]*Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic*

[c]*Institute for Environmental Studies, Faculty of Science, Charles University in Prague, Czech Republic*

[d]*Jimma University, College of Agriculture, 307, Jimma, Ethiopia*

*Corresponding author

E-mail address: gari@af.czu.cz

Table S1: Composition of vermicomposting materials in different proportions

Variants	SS (%)	SS(kg)	SP (%)	SP (kg)	Mixing ratio	Total weight Material (kg)	Earthworm substrate (L)
T1	100	9	0	0	4:0	9	3
T2	100	9	0	0	4:0	9	0
T3	75	6.75	25	2.25	3:1	9	3
T4	75	6.75	25	2.25	3:1	9	0
T5	50	4.5	50	4.5	2:2	9	3
T6	50	4.5	50	4.5	2:2	9	0
T7	25	2.25	75	6.75	1:3	9	3
T8	25	2.25	75	6.75	1:3	9	0

SS = sewage sludge, SP = straw pellets

Table S2: PPCP and EDC concentration at initial and after 120 days of processing in all variants

	BPA (ng g^{-1})				CAF (ng g^{-1})			
Variants	Initial	(+EW)	(-EW)	EWs	Initial	(+EW)	(-EW)	EWs
100%SS	89±18	42±13^{ab}	48±10.5^{ab}	nd	142±12.5	48±7.8^{a}	53±2.0^{a}	0±0.0^{b}
75%SS+25%SP	58±12	59±25^{a}	71±10.0^{a}	nd	96±8.9	40±1.8^{ab}	40±1.3^{b}	23.6±15^{a}
50%SS+50%SP	34±7.0	37±4.9^{b}	29±1.6^{b}	nd	61±6.6	26±1.3^{b}	25±0.0^{c}	11.3±1.7^{ab}
25%SS+75%SP	15±3.2	16±1.15^{c}	18± 0.43^{b}	nd	33±4.2	25±0.0^{b}	25±0.0^{c}	17.8±9.4^{ab}
	CBZ (ng g^{-1})				CETI(ng g^{-1})			
Variants	Initial	(+EW)	(-EW)	EWs	Initial	(+EW)	(-EW)	EWs
100% SS	39±0.73	33±2.5^{a}	38±3.5^{a}	11.4±0^{b}	79±0.69	49±11^{ab}	46±5.1^{b}	10.9±0^{a}
75%SS+ %SP	25±0.48	32±1.5^{ab}	36±0.25^{ab}	9.86±6^{b}	51±0.45	60±5.9^{a}	56±7.6^{a}	6.39±2.7^{a}
50%SS+50%SP	15±0.28	25±1.5^{b}	29±0.83^{b}	15.9±11^{a}	30±0.25	41±2.7^{ab}	45±1.1^{b}	9.44±0.18^{a}
25%SS+75%SP	6.6±0.13	16±1.4^{c}	19±0.10^{c}	5.5±0.2^{b}	14±0.12	20±3.0^{b}	20±3.0^{c}	6.3±0.76^{a}
	CITA(ng g^{-1})				DCF(ng g^{-1})			
Variants	Initial	(+EW)	(-EW)	EWs	Initial	(+EW)	(-EW)	EWs
100%SS	440±2.84	388±62.3^{a}	332±70^{ab}	38.2±0^{b}	284±9.8	11±2.29^{a}	12±2.5^{a}	15.5±0^{a}
75%SS+25%SP	287±1.85	337±129^{a}	444±97^{a}	13.9±9^{b}	185±6.4	5.0±0.1^{b}	5.6±0.3^{b}	5.67±1.5^{b}
50%SS+50%SP	169±1.09	247±45^{b}	294±21^{ab}	35.8±1^{b}	109±3.4	4.3±0.3^{b}	5.6±0.5^{b}	7.74±0.78^{b}
25%SS+75%SP	76±0.49	127±37^{c}	146±10^{b}	16.9±2^{b}	49±1.7	3.0±0.1^{b}	3.9±0.5^{b}	5.5±0.63^{b}
	IBF(ng g^{-1})				MIRT(ng g^{-1})			
Variants	Initial	(+EW)	(-EW)	EWs	Initial	(+EW)	(-EW)	EWs
100%SS	87±6.68	0.0±0.0^{b}	3.3±3.26^{a}	nd	63±2.83	29±3.36^{a}	32±7.1^{a}	nd
75%SS+25%SP	57±4.36	7.8±1.50^{a}	8.8±0.32^{a}	nd	42±1.83	24±4.4^{ab}	26±2.1^{a}	nd
50%SS+50%SP	34±2.57	3.8±2.04^{ab}	2.4±2.39^{a}	nd	25±1.05	12±1.01^{b}	18±0.1^{ab}	nd
25%SS+75%SP	15±1.15	1.4±1.36^{ab}	2.0±2.0^{a}	nd	12±0.44	4.8±1.0^{c}	7.3±0.1^{b}	nd
	SPD(ng g^{-1})				TE(ng g^{-1})			
Variants	Initial	(+EW)	(-EW)	EWs	Initial	(+EW)	(-EW)	EWs
100%SS	15±1.03	2.5±0.60^{a}	3.4±0.47^{ab}	nd	10162±226	8257±1510^{a}	9130±762^{a}	373.9±0^{a}
75%SS+25%SP	11±0.66	2.7±0.22^{a}	3.9±0.11^{a}	nd	6628±147	7138±2884^{b}	8477±1050^{ab}	104.7±66^{c}
50%SS+50%SP	6.9±0.38	2.1±0.11^{a}	2.4±0.13^{ab}	nd	3910±87	5191±370^{c}	5692±475^{b}	266.3±28^{b}
25%SS+75%SP	4.0±0.16	1.6±0.07^{a}	1.8±0.04^{c}	nd	325±7.2	4099±1434^{d}	3211±17^{c}	116.8±8.3^{c}
	TCS(ng g^{-1})				VEN(ng g^{-1})			
Variants	Initial	(+EW)	(-EW)	EWs	Initial	(+EW)	(-EW)	EWs
100%SS	543±36	227±9.1^{a}	454±125^{a}	42±0^{a}	34±3.74^{a}	32±5.65^{a}	27±6.83^{ab}	nd
75%SS+25%SP	354±23	103±6.6^{b}	228±25^{ab}	9.9±5.6^{b}	23±2.44^{b}	30±6.45^{ab}	25±3.06^{ab}	nd
50%SS+50%SP	209±14	30±10.7^{c}	102±1.66^{b}	15.2±1^{b}	14±1.43^{ab}	19±a1.52^{b}	48±13.8^{a}	nd
25%SS+75%SP	94±6.2	9.6±2.02^{c}	47±1.68^{b}	8.4±4^{b}	7.2±0.64^{c}	11±2.37^{b}	12±0.43^{b}	nd

BPA = bisphenol A, CAF = caffeine, CBZ = carbamazepine, CETI = cetirizine, CITA = citalopram, DCF = diclofenac, IBF = ibuprofen, MIRT = mirtazapine, SPD = sulfapyridine, TE = telmisartan, TCS = triclosan, VEN = venlafaxine. Mean value followed by different letters is statistically different at ($p < 0.05$). Values indicate mean ± standard error (n = 3). (+EW) = vermicompost with earthworms, (-EW) = compost without earthworms, SS= sewage sludge, SP = straw pellet. EWs = earthworms, ns = not detected.

Table S3: Bio-concentration factors (BCF) for PPCPs after 120 days of vermicomposting

	Bio-Concentration Factors (BCF) for PPCPs						
Variants	CAF	CBZ	CETI	CITA	DCF	TE	TCS
100% SS	0	0.35	0.26	0.10	1.60	0.05	0.19
75% SS	0.56	0.29	0.10	0.04	1.13	0.01	0.09
50% SS	0.43	0.65	0.23	0.16	1.83	0.05	0.70
25% SS	0.72	0.35	0.32	0.16	1.81	0.03	1.18

CAF = caffeine, CBZ = carbamazepine, CETI = cetirizine, CITA = citalopram, DCF = diclofenac, TE = telmisartan, TCS = triclosan

Table S4: Vermidradation (%) of PPCPs and ED in given variants of sewage sludge

Variants	Vermidegradation (%)											
	BPA	CAF	CBZ	CETI	CITA	DCF	IBF	MIRT	SPD	TE	TCS	VEN
100%SS	15	4.2	14	-4	-13	0.5	5.4	4.3	6.4	8.7	43	-12
75%SS+25%SP	-3	0	16	-8	37	0.3	2	4	12	22	36	-21
50%SS+50%SP	-23	-2.4	34	13	28	1.2	-1.6	24	4.5	13	35	193
25%SP+75%SP	16	0	38	3	25	2	11	19	6	-262	40	20

BPA = bisphenol A, CAF = caffeine, CBZ = carbamazepine, CETI = cetirizine, CITA = citalopram, DCF = diclofenac, IBF = ibuprofen, MIRT = mirtazapine, SPD = sulfapyridine, TE = telmisartan, TCS = triclosan, VEN = venlafaxine.

Table S5: Number, weight of *Eisenia andrei* and number of cocoons after 120 days of vermicomposting

Variants	No. of earthworms pieces /kg	Weight of earthworms g/kg	No. of cocoons/kg
100% SS	32 ± 30^{b}	11.4 ± 10.9^{a}	64 ± 38^{b}
75% SS + 25% SP	$59\pm14a^{b}$	14 ± 2.9^{a}	62 ± 23^{b}
50% SS +50% SP	120 ± 8.7^{a}	29 ± 4.7^{a}	59 ± 3.3^{b}
25% SS + 75% SP	73 ± 2.9^{ab}	15 ± 1.1^{a}	178 ± 118^{a}

Mean value followed by different letters is statistically different at ($p < 0.05$). Values indicate mean ± standard error (n = 3), SS= sewage sludge.

Table S6: Pearson correlation coefficients(r) between PPCPs and selected chemical parameters

Variables	pH	EC	TN	TC	C/N
SPD	-.0057	-.2187	.0173	-.7595***	-.5425**
VEN	-.2655	.4177*	.4341*	-.4400*	-.5344**
CBZ	-.1960	.1730	.4965*	-.8918***	-.8621***
CETI	-.2546	.3104	.4325*	-.6618***	-.6814***
MIRT	-.2329	.3445	.6238**	-.8538***	-.9007***
CAF	.0522	.0983	.4702*	-.8702***	-.8232***
BPA	.0368	.0385	.1549	-.4825*	-.4337*
IBF	.0490	-.1950	-.1943	-.2679	-.1036
DCF	.2550	-.1166	.2126	-.7677***	-.6394**
TCS	.1135	-.1297	.2304	-.8257***	-.6898***
TE	-.4115*	.4696*	.7057***	-.7217***	-.8706***
CITA	-.5228**	.5751**	.6514**	-.5731**	-.7499***

Correlation coefficients indicated with *, **, and *** were statistically significant ($p<0.05$, $p<0.01$, and $p<0.001$, respectively).

Waste Management 174 (2024) 496–508

Contents lists available at ScienceDirect

Waste Management

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

New insights into vermiremediation of sewage sludge: The effect of earthworms on micropollutants and vice versa

Alena Grasserová [a,b], Natividad I.N. Pacheco [b,c,d], Jaroslav Semerád [b], Alena Filipová [b], Petra Innemanová [a,e], Aleš Hanč [f], Petra Procházková [b], Tomáš Cajthaml [a,b,*]

[a] Institute for Environmental Studies, Faculty of Science, Charles University, Benátská 2, Prague 2, 12801, Czech Republic
[b] Institute of Microbiology of the Czech Academy of Sciences, Vídeňská 1083, Prague 4, 14220, Czech Republic
[c] First Faculty of Medicine, Charles University, Kateřinská 32, Prague 2, 12108, Czech Republic
[d] Laboratory of Ecotoxicology, Institute of Environmental Sciences, University of Castilla-La Mancha, 45004 Toledo, Spain
[e] DEKONTA a.s, Dřetovice 109, Stehelčeves, 27342, Czech Republic
[f] Department of Agro-Environmental Chemistry and Plant Nutrition, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamýcká 129, Prague 6, 16500, Czech Republic

ARTICLE INFO

Keywords:
Vermicomposting
Per/polyfluoroalkyl substances
Pharmaceuticals and personal care products
Bioaccumulation
Vermiremediation
Cytotoxicity

ABSTRACT

Vermicomposting represents an environmentally friendly method for the treatment of various types of biowastes, including sewage sludge (SS), as documented in numerous studies. However, there are few papers providing insights into the mechanisms and toxicity effects involved in SS vermicomposting to present a comprehensive overview of the process. In this work, the vermiremediation of SS containing various micropollutants, including pharmaceuticals, personal care products, endocrine disruptors, and per/polyfluoroalkyl substances, was studied. Two SSs originating from different wastewater treatment plants (WWTP1 and WWTP2) were mixed with a bulking agent, moistened straw, at ratios of 0, 25, 50, and 75% SS. *Eisenia andrei* earthworms were introduced into the mixtures, and after six weeks, the resulting materials were subjected to various types of chemical and toxicological analyses, including conventional assays (mortality, weight) as well as tissue- and cell-level assays, such as malondialdehyde production, cytotoxicity tests and gene expression assays. Through the vermiremediation process significant removal of diclofenac (90%), metoprolol (88%), telmisartan (62%), and triclosan (81%) was achieved. Although the concentrations of micropollutants were substantially different in the original SS samples, the micropollutants vermiaccumulated to a similar extent over the incubation period. The earthworms substantially eliminated the present bacterial populations, especially in the 75% SS treatments, in which the average declines were 90 and 79% for WWTP1 and WWTP2, respectively. To the best of our knowledge, this is the first study to investigate the vermiremediation of such a large group of micropollutants in real SS samples and provide a thorough evaluation of the effect of SS on earthworms at tissue and cellular level.

1. Introduction

Sewage sludge (SS), the end product of wastewater treatment plants (WWTPs), often contains potentially toxic substances, such as heavy metals (Carbonell and Pro, 2009), organopollutants (pharmaceuticals and personal care products (PPCPs), endocrine disruptors (EDs), halogenated substances, per/polyfluoroalkyl substances (PFASs; Semerád et al., 2020a)) or pathogens (Singh and Agrawal, 2008). However, SS also represents a valuable material containing large quantities of carbon, nitrogen, phosphorous and micronutrients, and is often used as a fertilizer on agricultural lands (Singh and Agrawal, 2008). In 2021, 33.6% of the 196 577 t dry weight (dw) SS produced in the Czech Republic was directly utilized in agriculture, making land application of sludge the second most common method of SS disposal following composting (Hudcová et al., 2019). Prior to application to agricultural land, SS must meet the European Union legislation limits, which cover only heavy metals; limits for other pollutants are established on a country-wide basis or might be lacking altogether. As a result, risks related to the transfer of non-regulated toxic pollutants to soil, surface water, and groundwater remain, and these contaminants can subsequently be taken up by plants (Passuello et al., 2010) and organisms (da Silva Souza et al., 2020), contaminating the food chain. Although micropollutants (as well

* Corresponding author.
E-mail address: cajthaml@biomed.cas.cz (T. Cajthaml).

https://doi.org/10.1016/j.wasman.2023.12.016
Received 13 June 2023; Received in revised form 26 October 2023; Accepted 8 December 2023
Available online 20 December 2023

as many new emerging pollutants) are detected at very low concentrations due to their constant introduction into the environment and their persistence, they can be found across the globe (Luo et al., 2014). Their presence has often been associated with adverse effects, such as endocrine disruption, short- and long-term toxicities, and antibiotic resistance in microbes (Bhatt et al., 2022).

Vermicomposting takes advantage of earthworm and microorganism activities to decompose organic matter (Samal et al., 2019) and to biodegrade organic substances (Grasserová et al., 2020). This process represents one of the vast variety of techniques used to eliminate toxic substances from SS and is called vermiremediation (Shi et al., 2020). Vermiremediation has been previously employed for eliminating heavy metals (He et al., 2016), petroleum oil (Chachina et al., 2016) or other organic compounds (Rodriguez-Campos et al., 2014) from soil or SS. In contrast, the number of studies investigating the vermiremediation of micropollutants/emerging pollutants from SS is scarce. Kinney et al. (2008) observed an accumulation of pharmaceuticals and other anthropogenic waste indicators (AWIs) in field-collected earthworms from an agricultural soil previously amended with biosolids or swine manure. The same authors evaluated the bioaccumulation of AWIs in *Eisenia andrei* upon exposure to soil amended with fresh or aged biosolids (Kinney et al., 2012). Rivier et al. (2019) studied the transfer of six organic pollutants to the earthworm *Aporrectodea caliginosa*. Innemanová et al. (2022) investigated the ability of *Eisenia andrei* to reduce the concentration of PPCPs in SS under field conditions. Dume et al. (2023) observed vermiremediation of selected PPCPs and EDs after introducing *Eisenia andrei* to SS mixed with straw. The abovementioned studies provide an insight into micropollutant vermiremediation. However, more comprehensive research is needed not only to assess the extent of micropollutant elimination that occurs during this process but also to clarify the processes underlying the elimination and ecotoxicity of SS to ensure optimal conditions are established for micropollutant vermiremediation.

As earthworms constitute the greatest proportion of the total soil biomass and are essential for maintaining soil structure and function, they are often used as soil health indicators/model organisms in soil studies (Babić et al., 2016). Researchers mostly monitor conventional endpoints such as mortality, weight loss, reproduction rate or avoidance behavior. Recently, scientists have started examining earthworm ecotoxicity at the tissue/cellular level using variety of biomarkers. In these studies, enzymatic activity (García-Gómez et al., 2014), specific protein inhibition and lipid peroxidation (Babić et al., 2016) or the quantity of coelomocytes (earthworm immune cells) and the riboflavin content (Suleiman et al., 2017) are monitored. Earthworms have three types of coelomocytes: eleocytes, granular amoebocytes (GA), and hyaline amoebocytes (HA), which float freely in the coelomic cavity (Bilej et al., 2010). Amoebocytes are involved in a broad range of defense mechanisms, such as phagocytosis (Homa et al., 2016), and can therefore be used as another toxicity indicator at the cellular level (Semerád et al., 2020b).

In the present study, we investigated the vermiremediation of 88 micropollutants (namely, 37 pharmaceuticals and personal care products, and 14 endocrine disruptors – collectively referred to as PPCPs in the text and 37 per/polyfluoroalkyl substances – referred to as PFASs) in two SSs originating from different WWTPs. The SS was mixed with a bulking agent, moistened straw, at four different ratios, and the mixture was treated with *Eisenia andrei* earthworms for six weeks. At the end of the experiment, the substrate mixture and the earthworms were analyzed to investigate the fate of the selected micropollutants. Moreover, microbial phospholipid fatty acid (PLFA) analysis was performed to monitor bacterial and fungal biomass. In addition, a battery of toxicity tests was performed to investigate the influence of SS exposure on earthworms. These tests encompassed conventional (mortality, weight) as well as tissue- and cell-level methods, such as malondialdehyde determination, cytotoxicity tests (viability, apoptosis, necrosis, reactive oxygen species (ROS) production, and phagocytosis) and gene expression assays. To the best of our knowledge, this is the first study in which the vermiremediation of such a broad group of micropollutants (including PFASs) from real SS samples was examined and the influence of SS on earthworms was assessed in-depth on a cellular level using hyaline and granular amoebocytes to evaluate the overall process from multiple perspectives.

2. Materials and methods

2.1. Earthworms and in vivo exposure

Clitellated *E. andrei* earthworms from our laboratory were used for the experiment. The species was previously confirmed by analyzing species-specific primers (Dvořák et al., 2013). All earthworms weighed approximately 200–300 mg. The substrate for the experiment consisted of SS mixed with moistened straw in four different proportions: 0, 25, 50, and 75% SS, each in triplicate. Stabilized SS was obtained from two different WWTPs (WWTP1 and WWTP2) located in the Czech Republic. WWTP1 has a population equivalent of 6 thousand and employs aerobic stabilization, and WWTP2 has a population equivalent of 33 thousand and utilizes anaerobic stabilization. Previously autoclaved straw pellets (Granofyt, Czech Republic) were soaked in Milli-Q water to 65% water holding capacity and used as a bulking agent to achieve the desired ratio of SS – 0% SS (0SS), 25% SS (25SS), 50% SS (50SS), and 75% SS (75SS). Characterization of these materials can be found in Table S1. The stabilized SS was mixed with moistened straw in polypropylene buckets using an overhead shaker GFL 3040 (11 RPM, 1 h; Germany). The homogenized mixture was placed in mesh textile sacks, and adult *E. andrei* earthworms of known weight were added (20 earthworms per 200 g of the mixture). In addition, control sacks without earthworms were established. To maintain suitable conditions for vermicomposting (i.e., humidity, temperature), the experiment was conducted in an isolated box.

The experiment lasted six weeks. Thereafter, the animals were collected, counted, washed with distilled water, gently dried with a clean paper towel, and weighed. The pH of the control and vermicompost substrate mixture was measured with an SI400 pH meter equipped with a LanceFET probe (Sentron, Netherlands). The substrate was frozen, lyophilized, and stored for subsequent xenobiotic and PLFA analyses. To clean their intestines, the earthworms for xenobiotic analyses were left on moistened filter paper for two days. Then, the earthworms were washed with distilled water, sacrificed by deep-freezing, and lyophilized.

Coelomocyte extraction of cleaned earthworms for cell analyses was carried out directly after the experiment. Malondialdehyde and mRNA levels (samples stored in DNA/RNA Shield (Zymo Research)) were determined in samples stored at −80 °C.

2.2. Chemical analysis

Metals were extracted using microwave-assisted acid digestion according to a previously published protocol (Pacheco et al., 2022). The analyzed metals were Ag, As, Cd, Co, Cr, Cu, Ni, Pb, Se, Sr, and Zn. Pb was used as an internal standard for micropollutant concentration correction to compensate for the loss of the substrate in the control and vermicomposted SS mixtures at the end of the experiment (Covino et al., 2016).

PPCPs (Innemanová et al., 2022), as well as PFASs (Semerád et al., 2020a), were analyzed using liquid chromatography-mass spectrometry (LC-MS). Previously published methods were followed with only slight modifications for PPCPs analysis. Briefly, the ASE 200 (Dionex) extraction cell was filled with 2 g of the sample (in the case of an earthworm sample less, approximately 0.6 g) and extracted with methanol. The mobile phase for PPCPs analysis consisted of phase A: 0.5 mM ammonium fluoride (LC-MS grade; Honeywell, USA) in Milli-Q + 0.01% formic acid (LC-MS grade; Honeywell, USA) and phase B: 100%

methanol (LC-MS grade; Honeywell, USA). The gradient elution program was as follows (time [min]/% phase B): 0/5; 0.5/5; 3.17/50; 4.5/50; 12.5/100; 14.5/100; 15.17/5; 15.83/5. The mobile phase flow rate was 0.6 mL·min^{-1}, the run time was 17.50 min, and the injection volume was 2 μL. The ion source temperature was set to 180 °C. To suppress matrix effects, the samples were analyzed with standards additions of 1, 5, and 25 or 5, 25, and 125 ng·mL^{-1}. The parameters for the mass spectrometer were optimized using MassHunter Workstation Optimizer and Source Optimizer (both Version 10.0, SR1; Agilent Technologies, USA), and the list of analyzed compounds can be found in Table S2. The bioaccumulation factor (BAF) for the accumulation of an individual micropollutant in earthworms was calculated according to the following equation: *BAF = concentration (earthworm tissue; ng·g^{-1} dw)/concentration (substrate mixture; ng·g^{-1} dw)*. The values of the n-octanol/water partition coefficient (usually expressed as log K_{ow}) were obtained from pubchem.ncbi.nlm.nih.gov and were calculated according to the equation K_{ow} = *concentration (compound in n-octanol)/concentration (compound in water)*.

Estimation of the microbial biomass was performed according to a previously published protocol (Šnajdr et al., 2008).

2.3. Toxicological assays

Cytotoxicity assays on earthworm coelomocytes were performed according to previously published methods with slight changes (Navarro Pacheco et al., 2021a). A 60% RPMI 1640 medium (3:2 with PBS 3:2; *v:v*) and PBS (3:2; *v:v*) were prepared according to Navarro Pacheco et al. (2021a).

For the ROS assay, the plate was centrifuged (150 g, 10 min, 4 °C), the RPMI 1640 medium was removed carefully, and the cells were washed with 100 μL of PBS. Thereafter, 100 μL of 2′7′-dichlorofluorescein diacetate (DCF-DA; 1:1000 (*v:v*) in PBS 3:2; Sigma-Aldrich, Germany) was applied to the cells, and the plate was kept in the dark for 15 min. The cells were washed twice with PBS and analyzed.

For the apoptosis and necrosis assay, the plate was centrifuged (150 g, 10 min, 4 °C), the RPMI 1640 medium was removed, and the cells were washed twice with 100 μL of annexin binding buffer (5x concentrate diluted 1:4 with Milli-Q (*v:v)*; Thermo Fisher Scientific, Czech Republic). Then, 30 μL of Annexin V (Alexa Fluor 647; Thermo Fisher Scientific, Czech Republic) was applied to the cells, and the plate was kept in the dark for 15 min. Afterward, 100 μL of annexin binding buffer was added, and the cells were analyzed.

For the phagocytosis assay, the plate was centrifuged (150 g, 10 min, 4 °C), the RPMI 1640 medium was removed, and 100 μL of medium was added. Thereafter, Fluoresbrite fluorescent microbeads (1 μm diameter; Polysciencies, Inc., United Kingdom) were added at a quantity of 100 beads per 1 cell. The plate was then incubated for 18 h in the dark at 17 °C. After incubation, the cells were washed twice with PBS and analyzed.

Prior to flow cytometry analysis, the cells were transferred to microtubes (Alpha Laboratories Ltd, United Kingdom). All cells except the non-propidium iodide controls were stained with 10 μL of propidium iodide (PI; 1 mg·L^{-1}; Sigma-Aldrich, Germany). The PI control without the fluorescent probe was also analyzed. The cells were analyzed with an LSR II flow cytometer (BD Biosciences, USA). The flow cytometer settings (forward and side scatter) were adjusted to measure each coelomocyte subtype as well as the fluorescent probe. The data obtained were analyzed in FlowJo software (version 9.9.4; BD Biosciences, USA).

The levels of malondialdehyde (MDA), a marker of oxidative stress, were determined according to a previously published protocol (Semerád et al., 2018) with slight changes for the extraction of MDA from the earthworm tissue (Pacheco et al., 2022).

Quantification of the mRNA levels of manganese superoxide dismutase (MnSOD), copper–zinc superoxide dismutase (CuZnSOD), lumbricin, and fetidin-lysenin genes was performed according to a previously published method (Roubalová et al., 2018). The selected primer sequences can be found in the work of Navarro Pacheco et al. (2021b). Gene expression values were calculated according to the Livak method (Taylor et al., 2019). *RPL17* and *RPL13* (reference genes) were used as internal controls for gene expression normalization. Non-template controls were included in the gene expression analyses. The reported value is the mean of three experiments (± standard deviation), each performed in duplicate.

2.4. Statistical analyses

All statistical analyses ($P < 0.05$) were performed using OriginPro 2019b software (9.6.5.169; USA). The data were tested for normality prior to each analysis using the Shapiro-Wilk test, and non-parametric Kruskal-Wallis ANOVA or one-way ANOVA with means comparison was performed using Tukey's test.

3. Results and discussion

3.1. Earthworm weight and mortality

After six weeks of incubation, the individual earthworm weight increase was proportional to the increase in the ratio of SS in the mixture. This trend was observed for both WWTPs. In the case of WWTP1 SS mixture, the average wet weight of the earthworms increased by 57, 106, and 129% for 25SS, 50SS, and 75SS, respectively, as shown in Figure S1. Similarly, the earthworm weight increased by 13 and 34% for 50SS and 75SS, respectively, in the WWTP2 SS mixture. Earthworm growth indicated that a sufficient content of nutrients was available in the SS used. In contrast, the earthworms in the 0SS (moistened straw material) and 25SS WWTP2 treatments lost weight, which indicates a depletion of nutrients during the six-week experimental period. All changes were statistically significant ($P < 0.05$). While some authors observed an increase in earthworm weight after exposure to SS, supporting our results, e.g., Courtois et al. (2021) found increases between 34 and 176%, and Havranek et al. (2017) noted a 40% increase; others detected a decrease, e.g., Urionabarrenetxea et al. (2022) found an up to 47% decrease and Wen et al. (2015) reported an up to 40% decrease. These discrepancies are most likely due to the composition of the SS used, which can include nutrients as well as harmful substances, and due to the earthworm species used and other experimental conditions (Vafa et al., 2016).

At the end of the experiment, some earthworms were missing: they either escaped or died and subsequently decomposed. The mortality data are shown in Table S3. There was not significant difference in the number of earthworms at the beginning and at the end of the experiment. Likewise, in terms of mortality, the treatments with the highest level of SS added from both WWTPs did not show significant differences ($P < 0.05$) from those with 0% SS added.

3.2. Substrate pH

The addition of SS to moistened straw caused a shift in pH from slightly acidic to neutral for 0SS and 25SS, while the acidity increased in 50SS and 75SS for treatments containing SS from both WWTPs (Table S3). During vermicomposting, the pH of the mixture dropped from the initial value of 6.9 ± 0.2 to 5.6 ± 0.6 in 75SS WWTP1 as well as from 6.9 ± 0.1 to 6.0 ± 0.6 and 5.1 ± 0.5 in 50SS WWTP2 and 75SS WWTP2, respectively (statistically significant changes, $P < 0.05$). The same trend was observed in the case of control samples (no earthworm activity). The pH drop observed during the process of vermicomposting is in accordance with other published data. Contreras-Ramos et al. (2005) observed a shift in pH from 8.4 to 7.9; Georgi et al. (2022) recorded a shift from 6.1 to 5.7, and Ludibeth et al. (2012) reported a shift from 6.02 to 5.65–5.82. The shift to more acidic pH probably occurred because microbial activity produced compounds such as CO_2, organic acids, NO_3^-, and NO_2^-.

3.3. Heavy metals

In the case of both WWTP SS mixtures, the total concentration of heavy metals increased after six weeks of vermicomposting as well as in the controls, which probably occurred due to a loss in substrate weight and volume. Nevertheless, the concentrations were low and were below the legislation limits (data not shown). An increase in the concentration of heavy metals after vermicomposting was observed in earlier published works. Bhat et al. (2013) observed an increase in Zn, Cu, Fe, and Mn concentrations after the vermicomposting of dyeing sludge from textile mills. Georgi et al. (2022) reported an increase in Cu, Cr, Ni, and Fe and a decrease in Zn content after the vermicomposting of municipal SS. Vig et al. (2011) detected an increase in Cu, Fe, Mn, and Zn when tannery sludge was vermicomposted with cattle dung.

Interestingly, there was almost no difference in the heavy metal concentration in earthworm bodies in the 0SS, 25SS, 50SS, and 75SS treatments after vermicomposting (data not shown). However, as stated by Innemanová et al. (2022), the monitoring of heavy metals during the process is not completely relevant. Since earthworm population turnover occurs, heavy metals are released into the substrate during earthworm decomposition. Therefore, it is important to analyze heavy metals in the starting material and to compare the values with the legislation requirements. In our case, in the treatments with the highest amount of SS (75SS), the concentration of all analyzed metals would comply with the Czech legislation limit for the agricultural land application of treated SS (Ministry of the environment of the Czech Republic., 2021).

3.4. Micropollutant vermiremediation

3.4.1. Pharmaceuticals and personal care products (PPCPs)

Out of the 51 PPCPs analyzed, 22 were found in WWTP1 SS (Figure S2a). The sums of PPCPs in the initial mixture were 2509 ± 117, 4542 ± 115, and 6925 ± 402 ng·g^{-1} dw for 25SS, 50SS, and 75SS, respectively. Telmisartan was detected at the highest concentrations by far, followed by triclosan, citalopram, azithromycin, and cetirizine. A significant decrease in the PPCPs sum was observed both in the vermicomposted and control substrates after six weeks ($P < 0.05$). The total concentrations of PPCPs in the vermicompost were 1298 ± 256, 2353 ± 443, and 4451 ± 472 ng·g^{-1} dw for 25SS, 50SS, and 75SS, respectively. In the WWTP2 SS mixture, 24 PPCPs were found (Fig. S2b). Overall, the PPCPs concentrations in the initial mixtures were 1646 ± 108, 3485 ± 133, and 5197 ± 371 ng·g^{-1} dw for 25SS, 50SS, and 75SS, respectively. Similarly, telmisartan was the most abundant PPCP, followed by triclosan, bisphenol A, citalopram, and cetirizine. A significant decrease in the PPCPs sums was observed after six weeks of vermicomposting ($P < 0.05$); the concentrations decreased to 528 ± 40, 1769 ± 123 and 2741 ± 65 for 25SS, 50SS, and 75SS, respectively. However, a similar decrease was noted in control without earthworms except in the 50SS mixture, in which the concentration was significantly higher than that in the vermicompost ($P < 0.05$). The concentration of most of the detected PPCPs decreased during the vermicomposting process. The decline in PPCPs amounts was more distinct in vermicompost than in the control SS mixture that did not contain earthworms. However, the differences were not significant in most cases, as shown in Fig. 1 ($P < 0.05$), which presents the concentration of the individual compounds in 25SS samples. Statistically significant differences relative to the control with no earthworms were observed for four compounds, diclofenac, metoprolol, telmisartan, and triclosan with average decreases of 90, 88, 62, and 81%, respectively. These PPCPs (except metoprolol), together with others, were also found in the earthworm bodies; thus, the decrease can partially be attributed to vermiaccumulation. In contrast, hydrochlorothiazide was significantly eliminated in the control without earthworms. A list of vermiaccumulated PPCPs, together with their application, log K_{ow}, and calculated BAF, can be found in Table 1 and Table S4. BAFs decreased when the SS amount added was increased, which is in accordance with other studies (Rivier et al., 2019). The most vermiaccumulated PPCPs were caffeine and diclofenac, with BAFs of 1.93 and 1.61 for 25SS WWTP1 and 2.13 and 1.21 for 25SS WWTP2, respectively. The log K_{ow} of PPCPs detected in earthworms ranged from −0.1 (caffeine) to 7.7 (telmisartan), and 7 out of 10 vermiaccumulated compounds had log K_{ow} values greater than 3. Spearman's correlation test showed no correlation between BAF and log K_{ow} values (Table S5; $P > 0.34$), which is in accordance with the work of Kinney et al. (2008).

Despite the significantly different content of PPCPs in 75SS WWTP1 and 75SS WWTP2 at the start of the experiment, there were no significant differences in the amount of accumulated PPCPs in the earthworm bodies (Fig. 2a), suggesting that this process is determined by the ability of earthworms to take up pollutants rather than by the concentration of PPCPs in the surrounding environment. Triclosan was found to accumulate in earthworms in many studies, and documented BAF values range from 0.5 to 1334. Chen et al. (2020) reported BAFs reaching 11 in *Eisenia fetida* and 0.6 in *Metaphire guillelmi*; Chevillot et al. (2018) reported a value of 2 in *Eisenia andrei*; Havranek et al. (2017) found a value of 10.9 in *Dendrobaena veneta*; Kinney et al. (2008) observed a value of 27 in unspecified field-collected earthworms; Pannu et al. (2012) noted a value of 10 in unspecified earthworm species and; Rivier et al. (2019) found a value of 1334 in *Aporrectodea caliginosa*. The concentration of diclofenac in the substrate decreased after vermicomposting in a study by Carter et al. (2016), and the biota-sediment accumulation factor (BSAF) in earthworms ranged from 1.01 to 12.36, based on soil type. Innemanová et al. (2022) observed a significant decrease in the concentrations of caffeine, metoprolol, and telmisartan ($P < 0.01$) in a pilot-scale vermicomposting experiment with SS.

3.4.2. Perfluoroalkyl and polyfluoroalkyl substances (PFASs)

Out of the 37 analyzed PFASs, 12 were found in the WWTP1 SS mixture (Fig. S3a). Their total sum concentrations were 21 ± 2, 45 ± 2, and 67 ± 5 ng·g^{-1} dw for 25SS, 50SS, and 75SS, respectively. The PFASs present in the highest concentration were perfluorooctane sulfonic acid (PFOS), followed by perfluoro-n-decanoic acid (PFDA), and n-ethylperfluoro-1-octanesulfonamidoacetate (nEtFOSAA). No clear trend was observed in the PFASs sum concentration between the beginning and end of the experiment. The total concentrations were 16 ± 5, 30 ± 5, and 59 ± 33 ng·g^{-1} dw for 25SS, 50SS, and 75SS, respectively. The WWTP2 SS mixture contained 12 out of 37 analyzed PFASs (Fig. S3b). The initial sums of their concentrations were 11 ± 2, 19 ± 2, and 26 ± 3 ng·g^{-1} dw for 25SS, 50SS, and 75SS, respectively. Once again, the most abundant compound was PFOS, followed by 7:3 fluorotelomer acid (73 FTA), PFDA, and perfluoro-n-dodecanoic acid (PFDoDA). The total concentration of PFASs decreased during the vermicomposting process. At the end, the sum concentrations were 3 ± 0, 9 ± 0, and 11 ± 1 ng·g^{-1} dw for 25SS, 50SS, and 75SS, respectively. The decrease in the total concentrations in the control sacks without earthworms showed a similar trend. PFASs are very persistent compounds and cannot be fully decomposed by biological processes. However, they can be transformed or taken up by organisms, accumulating in the organisms' bodies. This might be a probable reason for the drop in the concentrations of PFASs in our study. For instance, 73 FTA was significantly transformed in both vermicompost and control SS mixtures ($P < 0.05$), and its possible biotransformation was demonstrated in other studies (Butt et al., 2014). The average earthworm:substrate weight ratio of the PFASs sum at the end of the experiment was 1:7, 1:12, and 1:47 in 25SS, 50SS, and 75SS WWTP1 and 1:7, 1:10, and 1:6 in 25SS, 50SS, and 75SS WWTP2, respectively. A list of vermiaccumulated PFASs, their log K_{ow} values, and their calculated BAFs can be found in Table 2. The PFASs with BAF > 3 in either of the vermicomposted WWTP SS mixtures were perfluoro-n-heptanoic acid (PFHpA), PFDA, perfluoro-n-undecanoic acid (PFUnDA), PFDoDA, perfluoro-n-tridecanoic acid (PFTrDA) and PFOS. The PFASs with the highest BAFs were PFTrDA, PFOS and PFDoDA, which were found to have the highest BAFs in previously published studies: Navarro et al. (2016) reported a BAF for PFDoDA of 198; Zhao et al. (2014) reported BAFs reaching 5.19; and Zhao et al. (2013)

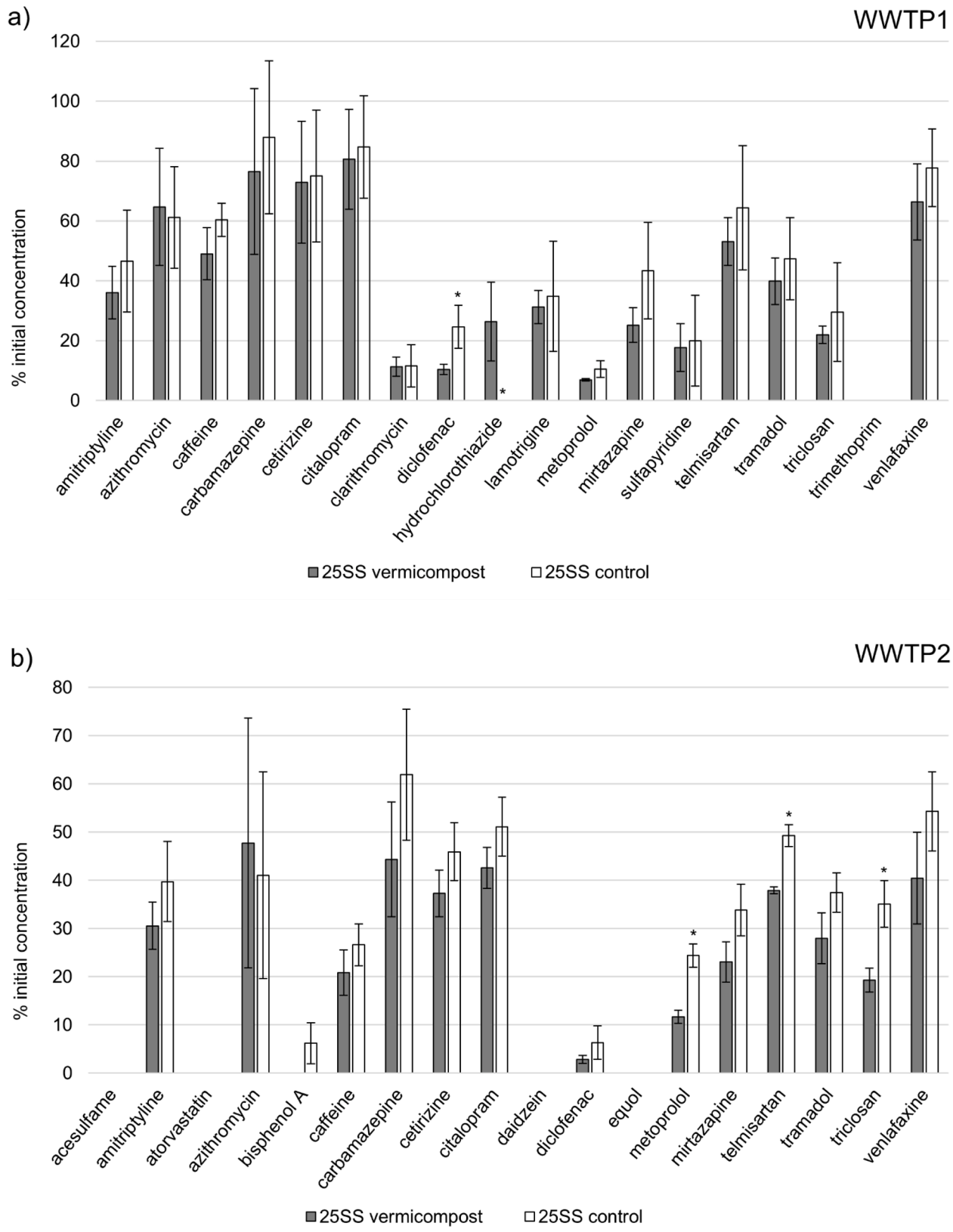


Fig. 1. Concentrations of the individual PPCPs in the 25SS vermicompost and the 25SS control (no earthworms) for WWTP1 (a) and WWTP2 (b) after six weeks of vermicomposting expressed as % of the initial concentration. The asterisks represent significant differences ($P < 0.05$). The columns represent the averages and the error bars represent standard deviations (n = 3). PPCPs = pharmaceuticals and personal care products; 25SS = substrate containing 25% sewage sludge and 75% straw; WWTP1 = wastewater treatment plant 1; WWTP2 = wastewater treatment plant 2.

Table 1
PPCPs found in the SS mixtures and their BAFs. The BAF values were calculated according to the following equation: *BAF = concentration (earthworm tissue; ng·g^{-1} dw)/ concentration (substrate mixture; ng·g^{-1} dw).*

		WWTP1				WWTP2			
PPCPs	**log K_{ow}**	**Bioaccumulated**	**BAF 25SS**	**BAF 50SS**	**BAF 75SS**	**Bioaccumulated**	**BAF 25SS**	**BAF 50SS**	**BAF 75SS**
acesulfame[2]	−1.3	X	–	–	–	X	–	–	–
amitriptyline	4.9	X	–	–	–	X	–	–	–
atorvastatin	6.4	X	–	–	–	X	–	–	–
azithromycin	4.0	✓	0.00	0.00	0.03	X	–	–	–
bisphenol A[2]	3.3	X	–	–	–	✓	NC	0.00	0.43
bisphenol F[2]	2.9	X	–	–	–	X	–	–	–
caffeine	−0.1	✓	1.93	0.67	0.48	✓	2.13	0.75	0.39
carbamazepine	2.5	✓	0.00	0.06	0.13	✓	0.14	0.02	0.05
cetirizine	1.7	✓	0.09	0.06	0.05	✓	0.18	0.05	0.07
citalopram	3.7	✓	0.12	0.10	0.09	✓	0.05	0.07	0.07
clarithromycin	3.2	X	–	–	–	X	–	–	–
daidzein	2.6	X	–	–	–	X	–	–	–
diclofenac	4.5	✓	1.61	1.52	0.74	✓	1.21	0.39	0.19
equol[2]	3.2	X	–	–	–	X	–	–	–
genistein	2.8	X	–	–	–	X	–	–	–
hydrochlorothiazide[1]	−0.1	X	–	–	–	X	–	–	–
ibuprofen[1]	4.0	✓	–	–	NC	X	–	–	–
lamotrigine	2.6	X	–	–	–	X	–	–	–
metoprolol	1.9	X	–	–	–	X	–	–	–
mirtazapine	2.9	X	–	–	–	X	–	–	–
sulfapyridine	0.4	X	–	–	–	X	–	–	–
telmisartan	7.7	✓	0.03	0.03	0.02	✓	0.05	0.03	0.02
tramadol	1.4	X	–	–	–	X	–	–	–
triclosan	4.8	✓	0.25	0.16	0.10	✓	0.28	0.11	0.10
trimethoprim	0.9	X	–	–	–	X	–	–	–
venlafaxine	3.2	X	–	–	–	X	–	–	–

WWTP1 = wastewater treatment plant 1; WWTP2 = wastewater treatment plant 2; PPCPs = pharmaceuticals and personal care products; K_{ow} = octanol–water partition coefficient; BAF = bioaccumulation factor; SS = sewage sludge; 25SS = substrate containing 25% sewage sludge and 75% straw; 50SS = substrate containing 50% sewage sludge and 50% straw; 75SS = substrate containing 75% sewage sludge and 25% straw; 1 - PPCP found only in the SS mixture of WWTP1; 2 - PPCP found only in the SS mixture of WWTP2; NC = not calculated (was found in the earthworms but the concentration was below the quantification limit of the substrate mixture); ✓ - vermiaccumulated; X – not vermiaccumulated.

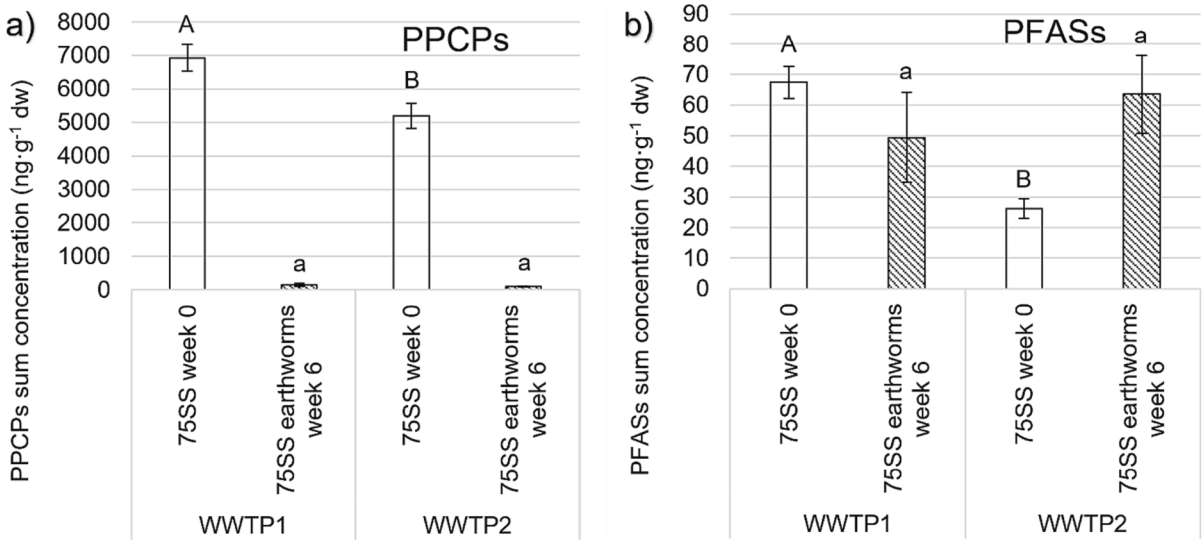


Fig. 2. Concentrations of PPCPs (a) and PFASs (b) in the 75SS starting mixture with WWTP1 and WWTP2 SS (capital letters represent significant differences; $P < 0.05$) and concentrations in earthworms after six weeks of vermicomposting (lowercase letters represent significant differences; $P < 0.05$). The columns represent the averages, and the error bars represent standard deviations (n = 3). PPCPs = pharmaceuticals and personal care products; PFASs = per/polyfluoroalkyl substances; WWTP1 = wastewater treatment plant 1; WWTP2 = wastewater treatment plant 2; SS = sewage sludge; 75SS = substrate containing 75% sewage sludge and 25% straw.

reported BAFs reaching 0.5 (as BSAF). The BAF values for PFOS were 3.72 and 8.16 in the WWTP1 and WWTP2 25SS mixtures, respectively. These values match those published previously by Navarro et al. (2017): 3.89 and 6.09; Wen et al. (2015): 1.54 to 4.12; and Zhao et al. (2014): 2.945 to 4.709 (as BSAF). For perfluoro-n-octanoic acid (PFOA), a lower BAF (2.75) was observed than that of PFOS with an identical carbon chain length. This outcome supports the trend previously mentioned by Zhao et al. (2013, 2014), that the BAFs of perfluorosulfonate acids are generally greater than those of perflurocarboxylic acids of equal perfluorinated chain length. Navarro et al. (2016) and Wen et al. (2015) also observed higher BAFs for PFOS (21 and 1.54–4.12, respectively) than for PFOA (2.2 and 0.52–1.34, respectively). The log K_{ow} of PFASs

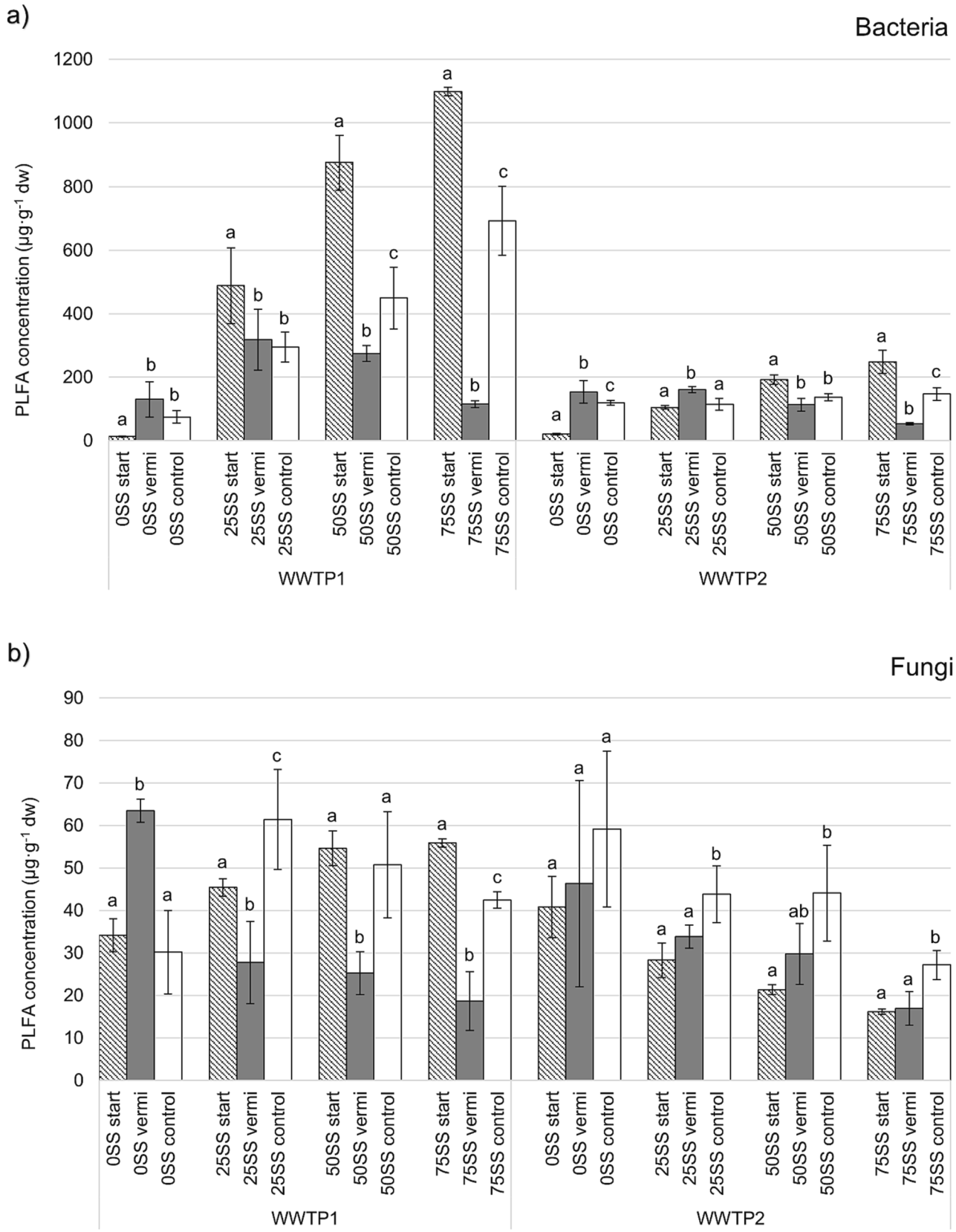


Fig. 3. PLFA concentrations of the total bacterial (a) and fungal (b) biomass. The letters above the columns represent significant differences ($P < 0.05$) in the groups for the three values: *start* (week 0), end *vermi* (vermicompost; week 6), and *control* (no earthworms; week 6). The columns represent the averages, and the error bars represent standard deviations (n = 3). PLFAs = phospholipid fatty acids; WWTP1 = wastewater treatment plant 1; WWTP2 = wastewater treatment plant 2; SS = sewage sludge; 0SS = substrate containing 0% sewage sludge and 100% straw; 25SS = substrate containing 25% sewage sludge and 75% straw; 50SS = substrate containing 50% sewage sludge and 50% straw; 75SS = substrate containing 75% sewage sludge and 25% straw.

Table 2
PFASs found in the SS mixtures and their BAFs. The BAF values were calculated according to the following equation: *BAF = concentration (earthworm tissue; ng·g^{-1} dw)/ concentration (substrate mixture; ng·g^{-1} dw).*

				WWTP1				WWTP2			
PFASs	**Formula**	**log K_{ow}**	**Per F–C**	**Bioaccumulated**	**BAF 25SS**	**BAF 50SS**	**BAF 75SS**	**Bioaccumulated**	**BAF 25SS**	**BAF 50SS**	**BAF 75SS**
PFPeA[2]	$C_5HF_9O_2$	2.7	4	X	–	–	–	X	–	–	–
PFHxA	$C_6HF_{11}O_2$	3.5	5	X	–	–	–	X	–	–	–
PFHpA	$C_7HF_{13}O_2$	4.4	6	X	–	–	–	✓	3.51	3.14	3.94
PFOA	$C_8HF_{15}O_2$	5.2	7	✓	2.75	1.83	0.16	X	–	–	–
PFNA	$C_9HF_{17}O_2$	6.0	8	✓	2.68	1.89	1.21	X	–	–	–
PFDA	$C_{10}HF_{19}O_2$	6.8	9	✓	1.84	1.17	0.86	✓	3.26	1.10	0.98
PFUnDA	$C_{11}HF_{21}O_2$	7.6	10	✓	5.57	2.30	1.62	✓	3.09	0.00	0.00
PFDoDA	$C_{12}HF_{23}O_2$	8.4	11	✓	5.95	3.95	2.81	✓	6.01	2.60	1.95
PFTrDA	$C_{13}HF_{25}O_2$	9.2	12	✓	4.99	9.51	5.36	✓	NC	4.60	4.15
PFTeDA[2]	$C_{14}HF_{27}O_2$	10.0	13	X	–	–	–	✓	NC	NC	NC
PFOS	$C_8HF_{17}O_3S$	4.0	8	✓	3.72	1.83	1.46	✓	8.16	4.42	5.04
PFOSA	$C_8H_2F_{17}NO_2S$	4.8	8	✓	–	NC	NC	✓	–	NC	NC
53 FTA[2]	$C_8H_5F_{11}O_2$	4.2	5	X	–	–	–	✓	–	NC	NC
73 FTA[2]	$C_{10}H_5F_{15}O_2$	6.0	7	X	–	–	–	✓	–	NC	NC
nMetFOSAA[1]	$C_{11}H_6F_{17}NO_4S$	8.8	8	X	–	–	–	X	–	–	–
nEtFOSAA[1]	$C_{12}H_8F_{17}NO_4S$	9.3	8	✓	0.00	0.00	0.51	X	–	–	–

WWTP1 = wastewater treatment plant 1; WWTP2 = wastewater treatment plant 2; PFASs = per/polyfluoroalkyl substances; K_{ow} = octanol–water partition coefficient; Per F-C = number of perfluorinated carbons; BAF = bioaccumulation factor; SS = sewage sludge; 25SS = substrate containing 25% sewage sludge and 75% straw; 50SS = substrate containing 50% sewage sludge and 50% straw; 75SS = substrate containing 75% sewage sludge and 25% straw; PFPeA = perfluoro-n-pentanoic acid; PFHxA = perfluoro-n-hexanoic acid; PFHpA = perfluoro-n-heptanoic acid; PFOA = perfluoro-n-octanoic acid; PFNA = perfluoro-n-nonanoic acid; PFDA = perfluoro-n-decanoic acid; PFUnDA = perfluoro-n-undecanoic acid; PFDoDA = perfluoro-n-dodecanoic acid; PFTrDA = perfluoro-n-tridecanoic acid; PFTeDA = perfluoro-n-tetradecanoic acid; PFOS = perfluorooctane sulfonic acid; PFOSA = perfluorooctane sulfonamide; 53 FTA = 5:3 fluorotelomer acid; 73 FTA = 7:3 fluorotelomer acid; nMetFOSAA = n-methyl-perfluoro-1-octanesulfonamidoacetate; nEtFOSAA = n-ethyl-perfluoro-1-octanesulfonamidoacetate; 1 - PFAS found only in the SS mixture of WWTP1; 2 - PFAS found only in the SS mixture of WWTP2; NC = not calculated (was found in the earthworms but the concentration was below the quantification limit of the substrate mixture); ✓ - vermiaccumulated; X - not vermiaccumulated.

detected in earthworms ranged from 4.0 (PFOS) to 10.0 (perfluoro-n-tetradecanoic acid; PFTeDA). Spearman's correlation test did not demonstrate any significant correlations between BAF and log K_{ow} values (Table S5; $P > 0.23$). This is in contradiction to reports by Zhao et al. (2013) and Navarro et al. (2016), who stated that PFASs uptake by earthworms can be predicted by the log K_{ow} values of the compounds. However, there was a moderate to strong positive correlation found between the individual BAFs and the number of fluorinated carbons in the molecule for PFASs in the WWTP1 SS mixture (Table S5; $r = 0.55$, 0.59, 0.65 and $P = 0.03$, 0.02, 0.01 for 25SS, 50SS, 75SS, respectively) and WWTP2 SS mixture (Table S5; $r = 0.50$, 0.44, 0.40 and $P = 0.07$, 0.15, 0.20 for 25SS, 50SS, 75SS, respectively). Chain-length-dependent uptake of PFASs by earthworms was previously described by other researchers (Rich et al., 2015). Some compounds were found only in earthworm bodies and not in the vermicomposted substrate. Their concentrations increased with an increase in the proportion of SS in the mixture. These compounds were perfluorooctane sulfonamide (PFOSA), PFTeDA, 5:3 fluorotelomer acid (53 FTA), and 73 FTA. This indicates that earthworms can act as living passive samplers that extract compounds from a surrounding material. The highest BAF levels for most of the substances were observed in the 25SS treatment for both WWTP1 and WWTP2. Thus, bioaccumulation of PFASs is not completely concentration-dependent, and the BAFs decrease with increasing PFASs concentration in a substrate, as has been noted by Wen et al. (2015) and Zhao et al. (2013, 2014). These authors stated that the reason probably involved the saturation of PFAS-binding proteins, which disabled further absorption of PFASs into the earthworm bodies. However, we observed the same trend for a diverse group of PPCPs. These findings suggest that the absorption capacity of earthworms for micropollutants is not specific and might be driven by general mechanisms, e.g., the surface area of the earthworm bodies and their gut. The data in Fig. 2b support this theory. It illustrates that while the WWTP1 and WWTP2 75SS mixture contained significantly different amounts of PFASs at week 0, there was no significant difference between the amounts of PFASs accumulated by the earthworms after six weeks of vermicomposting ($P < 0.05$).

3.5. Phospholipid fatty acid (PLFA) analysis

To monitor the microbial biomass at the beginning and end of the vermicomposting process, an analysis of the characteristic cell membrane PLFAs was performed. The increase in the total bacterial biomass (represented by PLFAs) of the initial SS mixture was proportional to the SS amount added (Fig. 3a). During the vermicomposting experiment, bacterial PLFAs decreased, especially in the 75SS mixture, in which the average decline was 90 and 79% for WWTP1 and WWTP2, respectively. The corresponding controls without earthworms showed average decreases of 37 and 41%, respectively. All changes were statistically significant ($P < 0.05$). In the case of WWTP1, the increase in fungal biomass was proportional to the amount of SS added and in the case of WWTP2, the opposite trend was observed (Fig. 3b). For WWTP1, the fungal biomass in 75SS was significantly reduced by 67% and by 24% in the vermicompost and the control, respectively ($P < 0.05$). Vermicomposting of the WWTP2 25SS, 50SS, and 75SS mixtures did not lead to changes in fungal PLFAs; however, there was a significant increase observed in all controls ($P < 0.05$).

The reduction in microbial biomass during vermicomposting is consistent with previously published works stating that the presence of epigeic earthworms has a negative effect on microbial biomass growth. Gómez-Brandón et al. (2011a) observed a decrease in bacterial biomass after the vermicomposting of pig slurry with *Eisenia fetida*. Gómez-Brandón et al. (2011b) reported a large decrease in bacterial biomass after the vermicomposting of cow, pig, and horse manure with *Eisenia andrei*. Villar et al. (2016) observed a reduction in the biomass of all microbes after SS was vermicomposted with *Eisenia andrei*. Fungi are less affected by the action of earthworms since they constitute a smaller fraction of microbes (Gómez-Brandón et al., 2011b) and are mostly present in the form of spores (Domínguez et al., 2010). In a previous study, researchers noted that microbial biomass decrease is proportional to the increase in earthworm quantity and biomass (Aira et al., 2011). This is in accordance with our findings, which show that the earthworms in 0SS lost weight during the vermicomposting process, while the overall microbial biomass increased. Additionally, in the control treatments

without earthworms, the microbial biomass amounts were generally greater than those in the vermicomposted treatments. The same outcome was observed when SS from a malt house was vermicomposted (Hanc et al., 2020). In contrast, the earthworms in the 75SS mixture increased in weight by up to 129% (WWTP1), which resulted in a major decrease in microbial biomass. There can be multiple explanations for the depletion of microbial biomass when earthworms are present. Earthworms can digest bacteria and fungi and use them as sources of energy and/or they can also act as food competitors consuming resources essential for microbes (Domínguez et al., 2010). Additionally, the use of SS as a vermicomposting substrate could be a key reason; the SS could have undergone biological degradation in the WWTP and easily utilized food sources for microorganisms could have been exhausted, leading to their depletion (Villar et al., 2016). However, although the abundance of the microbial population was greatly reduced, the bacterial population that remained was more active due to processes enhanced by earthworms (Gómez-Brandón et al., 2011a). Aira et al. (2011) reported microbial activity was maintained despite the decrease in microbial biomass after the vermicomposting process. Zhao et al. (2018) observed a decline in bacterial biomass in vermicomposted SS containing *Eisenia fetida*. Earthworms also enhanced the growth of fungi and protozoa, resulting in a modification of the microbial community and optimization of sludge stabilization. Nevertheless, further research is needed to ascertain the specific microbial species present in the vermicomposted SS and corresponding control containing no earthworms to estimate possible mechanisms of micropollutant degradation.

3.6. Toxicity bioassays: Malondialdehyde production, cytotoxicity assays, and gene expression

Lipid peroxidation induced by SS exposure was monitored as MDA production in the earthworms. Surprisingly, the MDA levels were significantly higher in 0SS earthworms than in 75SS earthworms for both WWTP SSs used in the experiment (Fig. 4, $P < 0.05$). The concentrations of MDA in 0SS earthworms were 442 ± 98 and 415 ± 172 $nM \cdot g^{-1}$ of wet tissue (wt), while in the 75SS mixture, they were 198 ± 77 and 102 ± 40 $nM \cdot g^{-1}$ wt for WWTP1 and WWTP2, respectively. These outcomes indicate a deterioration in the health of the earthworms that were grown in moistened straw pellets containing no SS or other sources of nutrients. Combined with the observation that the earthworms had lower weights, the results indicate that the earthworms did not thrive and that straw might not be a suitable bulking agent for vermicomposting since it does not provide the nutrients needed for earthworms. In previously published works, SS (Kaur et al., 2020) and wastewater (Mkhinini et al., 2020, 2019) exposure caused higher production of MDA in *Eisenia* spp. earthworms. However, in some studies with continuous monitoring of MDA, a decreasing trend was observed in its production, probably due to the antioxidant effect of enzymes that scavenge ROS, which reduced MDA production (Zhang et al., 2013).

Generally, HAs evinced lower viability and were more prone to apoptosis, necrosis, and phagocytosis, indicating their higher sensitivity. The cell viability of earthworms in SS-enriched treatments remained at the same level as in the WWTP1 0SS (Fig. 5a, $P < 0.05$). There was a significant increase in cell viability in 75SS GA and HA compared to WWTP2 0SS. Contrary to the MDA outcomes, the ROS measured in cells did not show any significant changes except in 0SS and 25SS GA for WWTP1 (Fig. 5b, 75SS WWTP1 was not analyzed due to the low number of earthworms found in the samples). The addition of WWTP1 SS had no significant effect on apoptosis and necrosis, except in early apoptosis, which was significantly increased in 75SS compared to 25SS HA (Fig. 5c, d, e). For WWTP2, fewer cells underwent early and late apoptosis in 75SS than in 0SS (Fig. 5c, d), but more cells underwent necrosis (Fig. 5e). Phagocytosis was not affected by the addition of SS in any of the cell populations (Fig. 5f).

Changes in the mRNA levels of selected molecules were analyzed using the bottom part of the earthworms containing gut tissue (Table 3). The MnSOD and CuZnSOD proteins that protect cells against oxidative stress were not up- or downregulated in SS treatments compared to 0SS, which is in accordance with the outcomes of the ROS assay (Fig. 5b). The antimicrobial protein lumbricin was not affected, while fetidin-lysenin genes were significantly upregulated in all WWTP1 SS treatments, indicating the involvement of defense mechanisms against pathogens (Roubalová et al., 2020). The expression of fetidin-lysenin genes in *E. andrei* is generally higher than that in *E. fetida,* probably due to evolutionary selection (Dvořák et al., 2013).

Various toxicity assays have been employed to monitor the fate of earthworms after exposure to SS; however, the results are ambiguous. Generally, the results are based on conventional endpoints such as mortality and reproduction rather than tissue/cellular level markers (Babić et al., 2016). Additionally, the so-called toxic cocktail effect must be examined in earthworms at environmentally relevant micropollutant concentrations without any additional spikes of pollutants into SS (Zhao et al., 2022). SS toxicity to earthworms can be caused by various pollutants. Generally, earthworms are very sensitive to elevated concentrations of ammonia (Domínguez, 2004), heavy metals (Natal-da-Luz et al., 2009), and pathogens (Ghosh, 2018); however, SS can contain

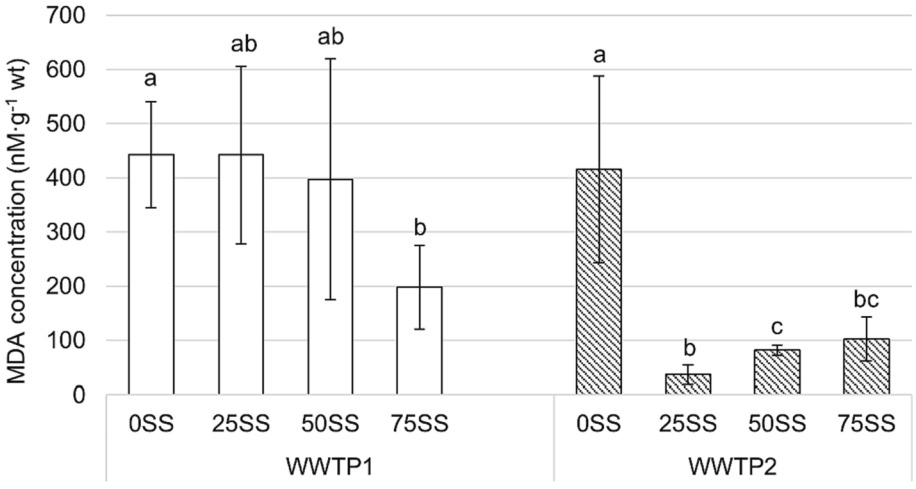


Fig. 4. MDA concentrations in earthworm tissue. The letters above the columns represent significant differences for each WWTP ($P < 0.05$). The columns represent the averages, and the error bars represent standard deviations (n = 3). MDA = malondialdehyde; WWTP1 = wastewater treatment plant 1; WWTP2 = wastewater treatment plant 2; SS = sewage sludge; 0SS = substrate containing 0% sewage sludge and 100% straw; 25SS = substrate containing 25% sewage sludge and 75% straw; 50SS = substrate containing 50% sewage sludge and 50% straw; 75SS = substrate containing 75% sewage sludge and 25% straw.

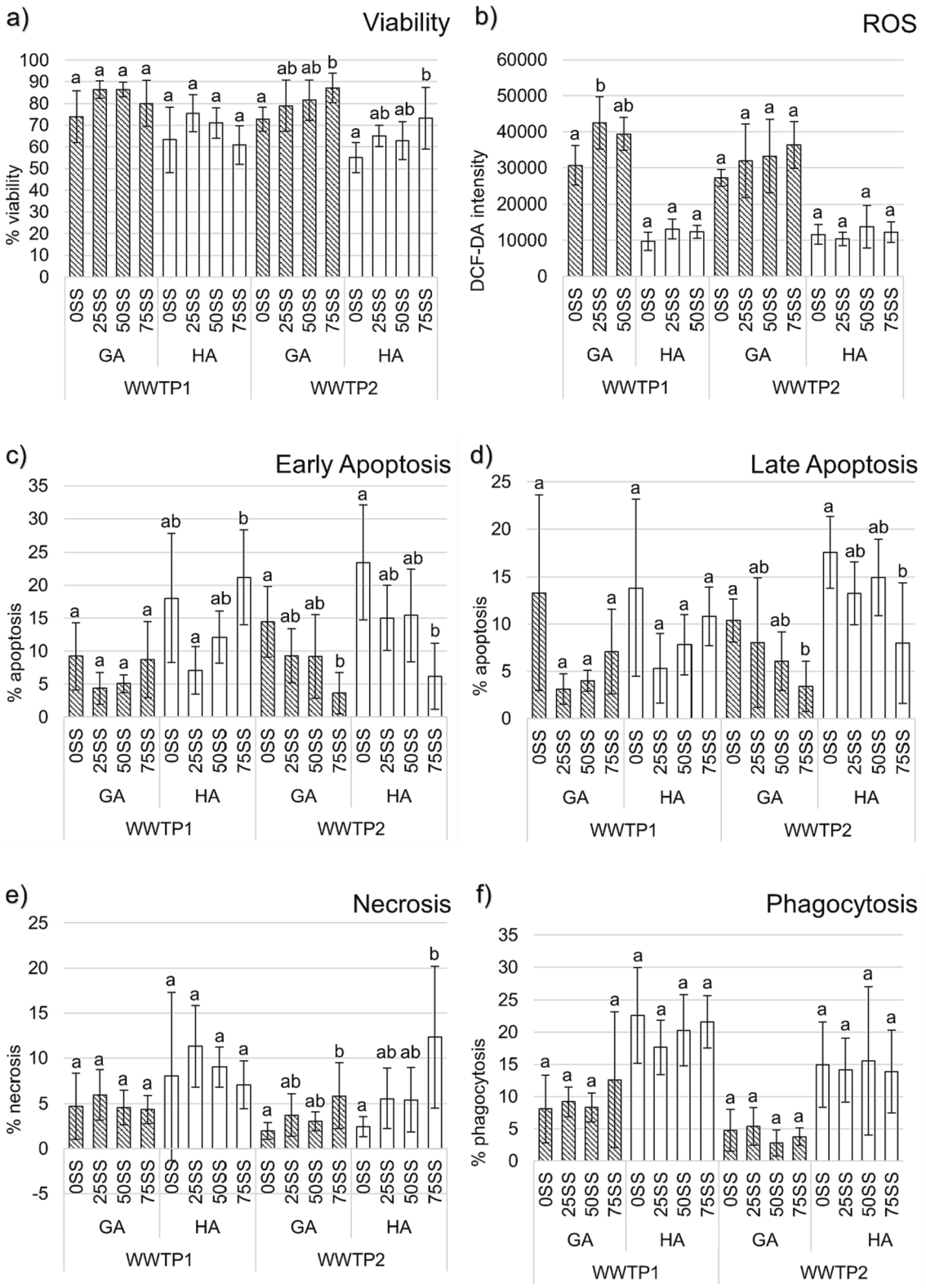


Fig. 5. Cytotoxicity assays: viability (a), ROS (b), early apoptosis (c), late apoptosis (d), necrosis (e), and phagocytosis (f). The letters above the columns represent significant differences for each cell subtype – granular amoebocytes (GA) and hyaline amoebocytes (HA) in WWTP1 and WWTP2 ($P < 0.05$). The columns represent the averages, and the error bars represent standard deviations (n = 5–6). WWTP1 = wastewater treatment plant 1; WWTP2 = wastewater treatment plant 2; SS = sewage sludge; 0SS = substrate containing 0% sewage sludge and 100% straw; 25SS = substrate containing 25% sewage sludge and 75% straw; 50SS = substrate containing 50% sewage sludge and 50% straw; 75SS = substrate containing 75% sewage sludge and 25% straw; ROS = reactive oxygen species; DCF-DA = 2′7′-dichlorofluorescein diacetate.

Table 3
Normalized expression of selected molecule-encoding genes in *E. andrei* after exposure to SS. The asterisks represent significant differences among the averages that are followed by standard deviations (n = 6) of SS mixtures for each WWTP ($P < 0.05$). The gene expression values were calculated according to the Livak method. *RPL17* and *RPL13* (reference genes) were used as internal controls for gene expression normalization.

Molecule		lumbricin	fetidin-lysenin	CuZnSOD	MnSOD
Function		immunity	immunity	oxidative stress	oxidative stress
WWTP1	**0SS**	1.10 ± 0.54	1.44 ± 1.11	1.03 ± 0.27	1.09 ± 0.50
	25SS	1.93 ± 0.67	**6.52 ± 3.36***	1.31 ± 0.34	1.38 ± 0.70
	50SS	1.92 ± 0.35	**5.07 ± 1.38***	0.92 ± 0.18	1.71 ± 0.68
	75SS	1.49 ± 0.70	**5.34 ± 1.99***	1.03 ± 0.60	1.05 ± 0.63
WWTP2	**0SS**	1.02 ± 0.19	1.08 ± 0.45	1.00 ± 0.08	1.07 ± 0.47
	25SS	0.66 ± 0.18	0.94 ± 0.21	0.95 ± 0.29	0.69 ± 0.15
	50SS	1.06 ± 0.54	1.40 ± 0.40	1.33 ± 0.55	1.18 ± 0.44
	75SS	0.76 ± 0.48	1.13 ± 0.58	1.00 ± 0.36	1.15 ± 0.47

WWTP = wastewater treatment plant; WWTP1 = wastewater treatment plant 1; WWTP2 = wastewater treatment plant 2; SS = sewage sludge; 0SS = substrate containing 0% sewage sludge and 100% straw; 25SS = substrate containing 25% sewage sludge and 75% straw; 50SS = substrate containing 50% sewage sludge and 50% straw; 75SS = substrate containing 75% sewage sludge and 25% straw; CuZnSOD = copper–zinc superoxide dismutase; MnSOD = manganese superoxide dismutase.

many other pollutants causing toxic effects (Fijalkowski et al., 2017). Natal-da-Luz et al. (2009) emphasized that the toxicity of SS highly depends on its origin. In their study, SS from the electroplating industry induced an avoidance response in *E. andrei* after one week, whereas urban and olive-processing SS did not. Suleiman et al. (2017) reported no mortality of *E. andrei*, *E. fetida*, and *D. veneta* after exposure to three different SSs for 45 days. Although no statistically significant differences were observed between the earthworm coelomocyte number in controls and after vermicomposting, the number of cells per worm in *E. fetida* and *D. veneta* increased along with increasing SS proportion. In the case of *E. andrei*, the cell populations were not affected, demonstrating the strong detoxification mechanisms of this species. On the other hand, Urionabarrenetxea et al. (2022) reported a decrease in the coelomocyte number and calcein retention, suggesting cell damage in *E. fetida* in landfill soil after three days. Babić et al. (2016) observed increased levels of lipid peroxidation in *E. fetida* on the fourth day of exposure to diluted SS. Subsequently, the levels started decreasing, indicating an activation of compensatory defense mechanisms, which is in agreement with the results in our study. The body wall of the earthworms was disrupted and started thinning after 14 days. The damage was proportional to SS concentration and duration of exposure. In the present study, the authors did not observe any disruption of the body walls of earthworms in 75% SS. There were no significant changes in the riboflavin content of *D. veneta* after 56 days of exposure to municipal SS (Rorat et al., 2013). The coelomocyte number of the earthworms kept in soil with 25% SS addition increased gradually, and reproduction was not disrupted, unlike in 50% SS and 0% SS (only soil). The authors therefore suggest that a moderate amount of SS provided a good source of food and nutrients for the earthworms, which is in agreement with our findings.

4. Conclusion

Earthworms facilitated the composting process by considerably changing SS properties, including its microbial biomass proportion. A wide range of micropollutants was vermiaccumulated with the highest BAFs observed in the 25% SS treatments. Vermicomposted material had significantly lower contents of diclofenac, metoprolol, telmisartan, and triclosan than the control containing no earthworms. As expected, we did not detect any substantial removal of PFASs, except for the transformation of compounds that are not fully fluorinated (e.g., fluorotelomeric acids); moderate bioaccumulation was also observed in the earthworms. An interesting trend was noted for the overall bioaccumulation of the detected micropollutants. Although the original SS samples contained substantially different concentrations of micropollutants, both groups of compounds (PPCPs and PFASs) vermiaccumulated to a similar extent. Considering the very different properties of the micropollutants, these findings indicate that organic micropollutants are absorbed *via* some very general mechanisms controlled by earthworm physiology rather than by specific transporters, as previously suggested for PFASs in the literature. The results of toxicity testing using isolated earthworm cells and selected gene expression were ambiguous. Macroscopic tests for the toxicity of the sludge on earthworm biomass showed positive effects. Based on the results of this study, it is not possible to infer any direct effects of the micropollutants present in the sludge on earthworm physiology. However, it is obvious that the use of immune earthworm cells is a much more sensitive tool to evaluate toxicity effects on organisms, and the appropriateness of this approach is emphasized for further studies.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This research was funded by Charles University [project GA UK No. 370621]; the Ministry of Agriculture of the Czech Republic [project NAZV No. QK1910095]; and the Center for Geosphere Dynamics [project No. UNCE/SCI/006]. The authors would like to acknowledge the Cytometry and Microscopy Facility at the Institute of Microbiology of the ASCR, v.v.i. for their help with flow cytometry analyses and Jaroslav Kukla from the Institute for Environmental Studies at Charles University for performing the analyses of metals.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.wasman.2023.12.016.

References

Aira, M., Gómez-Brandón, M., González-Porto, P., Domínguez, J., 2011. Selective reduction of the pathogenic load of cow manure in an industrial-scale continuous-feeding vermireactor. Bioresour. Technol. 102, 9633–9637. https://doi.org/10.1016/j.biortech.2011.07.115.

Babić, S., Barišić, J., Malev, O., Klobučar, G., Popović, N.T., Strunjak-Perović, I., Krasnići, N., Čož-Rakovac, R., Klobučar, R.S., 2016. Sewage sludge toxicity assessment using earthworm Eisenia fetida: can biochemical and histopathological analysis provide fast and accurate insight? Environ. Sci. Pollut. Res. 23, 12150–12163. https://doi.org/10.1007/s11356-016-6097-3.

Bhat, S.A., Singh, J., Vig, A.P., 2013. Vermiremediation of dyeing sludge from textile mill with the help of exotic earthworm Eisenia fetida Savigny. Environ. Sci. Pollut. Res. 20, 5975–5982. https://doi.org/10.1007/s11356-013-1612-2.

Bhatt, P., Bhandari, G., Bilal, M., 2022. Occurrence, toxicity impacts and mitigation of emerging micropollutants in the aquatic environments: Recent tendencies and

perspectives. J. Environ. Chem. Eng. 10, 107598 https://doi.org/10.1016/j.jece.2022.107598.
Bilej, M., Procházková, P., Šilerová, M., Josková, R., 2010. Earthworm Immunity, in: Söderhäll, K. (Ed.), Invertebrate Immunity, Advances in Experimental Medicine and Biology. Springer US, Boston, MA, pp. 66–79. https://doi.org/10.1007/978-1-4419-8059-5_4.
Butt, C.M., Muir, D.C.G., Mabury, S.A., 2014. Biotransformation pathways of fluorotelomer-based polyfluoroalkyl substances: A review: Biotransformation pathways of fluorotelomer compounds. Environ. Toxicol. Chem. 33, 243–267. https://doi.org/10.1002/etc.2407.
Carbonell, G., Pro, J., 2009. Sewage sludge applied to agricultural soil: Ecotoxicological effects on representative soil organisms. Ecotoxicol. Environ. Saf. 11 https://doi.org/10.1016/j.ecoenv.2009.01.007.
Carter, L.J., Ryan, J.J., Boxall, A.B.A., 2016. Effects of soil properties on the uptake of pharmaceuticals into earthworms. Environ. Pollut. 213, 922–931. https://doi.org/10.1016/j.envpol.2016.03.044.
Chachina, S.B., Voronkova, N.A., Baklanova, O.N., 2016. Biological remediation of the petroleum and diesel contaminated soil with earthworms eisenia fetida. Procedia Eng. 152, 122–133. https://doi.org/10.1016/j.proeng.2016.07.642.
Chen, X., Ma, X., Pan, Y., Ji, R., Gu, X., Luo, S., Bao, L., Gu, X., 2020. Dissipation, transformation and accumulation of triclosan in soil-earthworm system and effects of biosolids application. Sci. Total Environ. 712, 136563 https://doi.org/10.1016/j.scitotenv.2020.136563.
Chevillot, F., Guyot, M., Desrosiers, M., Cadoret, N., Veilleux, É., Cabana, H., Bellenger, J., 2018. Accumulation and sublethal effects of triclosan and its transformation product methyl-triclosan in the earthworm Eisenia andrei exposed to environmental concentrations in an artificial soil. Environ. Toxicol. Chem. 37, 1940–1948. https://doi.org/10.1002/etc.4156.
Contreras-Ramos, S.M., Escamilla-Silva, E.M., Dendooven, L., 2005. Vermicomposting of biosolids with cow manure and oat straw. Biol. Fertil. Soils 41, 190–198. https://doi.org/10.1007/s00374-004-0821-8.
Courtois, P., Rorat, A., Lemiere, S., Guyoneaud, R., Attard, E., Longepierre, M., Rigal, F., Levard, C., Chaurand, P., Grosser, A., Grobelak, A., Kacprzak, M., Lors, C., Richaume, A., Vandenbulcke, F., 2021. Medium-term effects of Ag supplied directly or via sewage sludge to an agricultural soil on Eisenia fetida earthworm and soil microbial communities. Chemosphere 269, 128761. https://doi.org/10.1016/j.chemosphere.2020.128761.
Covino, S., Fabianová, T., Křesinová, Z., Čvančarová, M., Burianová, E., Filipová, A., Voříšková, J., Baldrian, P., Cajthaml, T., 2016. Polycyclic aromatic hydrocarbons degradation and microbial community shifts during co-composting of creosote-treated wood. J. Hazard. Mater. 301, 17–26. https://doi.org/10.1016/j.jhazmat.2015.08.023.
da Silva Souza, T., Lacerda, D., Aguiar, L.L., Martins, M.N.C., de Oliveira, A., David, J., 2020. Toxic potential of sewage sludge: Histopathological effects on soil and aquatic bioindicators. Ecol. Indic. 111, 105980 https://doi.org/10.1016/j.ecolind.2019.105980.
Domínguez, J., Aira, M., Gómez-Brandón, M., 2010. Vermicomposting: Earthworms Enhance the Work of Microbes, in: Insam, H., Franke-Whittle, I., Goberna, M. (Eds.), Microbes at Work. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 93–114. https://doi.org/10.1007/978-3-642-04043-6_5.
Domínguez, J., 2004. State-of-the-Art and New Perspectives on Vermicomposting Research, in: Edwards, C. (Ed.), Earthworm Ecology. CRC Press, pp. 401–424. https://doi.org/10.1201/9781420039719.ch20.
Dume, B., Hanč, A., Švehla, P., Michal, P., Pospíšil, V., Grasserová, A., Cajthaml, T., Chane, A.D., Nigussie, A., 2023. Influence of earthworms on the behaviour of organic micropollutants in sewage sludge. J. Clean. Prod. 416, 137869 https://doi.org/10.1016/j.jclepro.2023.137869.
Dvořák, J., Mančíková, V., Pižl, V., Elhottová, D., Šilerová, M., Roubalová, R., Škanta, F., Procházková, P., Bilej, M., 2013. Microbial environment affects innate immunity in two closely related earthworm species eisenia andrei and eisenia fetida. PLoS One 8, e79257.
Fijalkowski, K., Rorat, A., Grobelak, A., Kacprzak, M.J., 2017. The presence of contaminations in sewage sludge – The current situation. J. Environ. Manage. 203, 1126–1136. https://doi.org/10.1016/j.jenvman.2017.05.068.
García-Gómez, C., Fernández, M.D., Babin, M., 2014. Ecotoxicological evaluation of sewage sludge contaminated with zinc oxide nanoparticles. Arch. Environ. Contam. Toxicol. 67, 494–506. https://doi.org/10.1007/s00244-014-0070-2.
Georgi, K., Ekaterina, S., Alexander, P., Alexander, R., Kirill, Y., Andrey, V., 2022. Sewage sludge as an object of vermicomposting. Bioresour. Technol. Rep. 20, 101281 https://doi.org/10.1016/j.biteb.2022.101281.
Ghosh, S., 2018. Environmental pollutants, pathogens and immune system in earthworms. Environ. Sci. Pollut. Res. 25, 6196–6208. https://doi.org/10.1007/s11356-017-1167-8.
Gómez-Brandón, M., Aira, M., Lores, M., Domínguez, J., 2011a. Changes in microbial community structure and function during vermicomposting of pig slurry. Bioresour. Technol. 102, 4171–4178. https://doi.org/10.1016/j.biortech.2010.12.057.
Gómez-Brandón, M., Aira, M., Lores, M., Domínguez, J., 2011b. Epigeic earthworms exert a bottleneck effect on microbial communities through gut associated processes. PLoS One 6, e24786.
Grasserová, A., Hanč, A., Innemanová, P., Cajthaml, T., 2020. Composting and vermicomposting used to break down and remove pollutants from organic waste: a mini review. Eur. J. Environ. Sci. 10, 9–14. https://doi.org/10.14712/23361964.2020.2.
Hanc, A., Hrebeckova, T., Pliva, P., Cajthaml, T., 2020. Vermicomposting of sludge from a malt house. Waste Manag. 118, 232–240. https://doi.org/10.1016/j.wasman.2020.08.027.
Havranek, I., Coutris, C., Norli, H.R., Rivier, P.-A., Joner, E.J., 2017. Uptake and elimination kinetics of the biocide triclosan and the synthetic musks galaxolide and tonalide in the earthworm *Dendrobaena veneta* when exposed to sewage sludge: Transfer of sewage sludge xenobiotics to earthworms. Environ. Toxicol. Chem. 36, 2068–2073. https://doi.org/10.1002/etc.3737.
He, X., Zhang, Y., Shen, M., Zeng, G., Zhou, M., Li, M., 2016. Effect of vermicomposting on concentration and speciation of heavy metals in sewage sludge with additive materials. Bioresour. Technol. 218, 867–873. https://doi.org/10.1016/j.biortech.2016.07.045.
Homa, J., Stalmach, M., Wilczek, G., Kolaczkowska, E., 2016. Effective activation of antioxidant system by immune-relevant factors reversely correlates with apoptosis of Eisenia andrei coelomocytes. J. Comp. Physiol. B 186, 417–430. https://doi.org/10.1007/s00360-016-0973-5.
Hudcová, H., Vymazal, J., Rozkošný, M., 2019. Present restrictions of sewage sludge application in agriculture within the European Union. Soil Water Res. 14, 104–120. https://doi.org/10.17221/36/2018-SWR.
Innemanová, P., Grasserová, A., Cajthaml, T., 2022. Pilot-scale vermicomposting of dewatered sewage sludge from medium-sized WWTP. Detritus 35–41. https://doi.org/10.31025/2611-4135/2022.15166.
Kaur, H., Sharma, S., Vijaya, P., 2020. Toxicological effect of Parthenium hysterophorus and milk processing industry sludge on earthworms, Eisenia fetida. Environ. Sci. Pollut. Res. 27, 33464–33473. https://doi.org/10.1007/s11356-019-05222-x.
Kinney, C.A., Furlong, E.T., Kolpin, D.W., Burkhardt, M.R., Zaugg, S.D., Werner, S.L., Bossio, J.P., Benotti, M.J., 2008. Bioaccumulation of pharmaceuticals and other anthropogenic waste indicators in earthworms from agricultural soil amended with biosolid or swine manure. Environ. Sci. Technol. 42, 1863–1870. https://doi.org/10.1021/es702304c.
Kinney, C.A., Campbell, B.R., Thompson, R., Furlong, E.T., Kolpin, D.W., Burkhardt, M.R., Zaugg, S.D., Werner, S.L., Hay, A.G., 2012. Earthworm bioassays and seedling emergence for monitoring toxicity, aging and bioaccumulation of anthropogenic waste indicator compounds in biosolids–amended soil. Sci. Total Environ. 433, 507–515. https://doi.org/10.1016/j.scitotenv.2012.06.097.
Ludibeth, S.-M., Marina, I.-E., Vicenta, E.M., 2012. Vermicomposting of sewage sludge: earthworm population and agronomic advantages. Compost Sci. Util. 20, 11–17. https://doi.org/10.1080/1065657X.2012.10737016.
Luo, Y., Guo, W., Ngo, H.H., Nghiem, L.D., Hai, F.I., Zhang, J., Liang, S., Wang, X.C., 2014. A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. Sci. Total Environ. 473–474, 619–641. https://doi.org/10.1016/j.scitotenv.2013.12.065.
Ministry of the environment of the Czech Republic., 2021. Vyhláška č. 273/2021 Sb., vyhláška o podrobnostech nakládání s odpady. Sbírka zákonů ČR [WWW Document]. URL https://eagri.cz/public/web/mze/legislativa/ostatni/103809054.html (accessed 3.16.23).
Mkhinini, M., Boughattas, I., Bousserhine, N., Banni, M., 2019. Biochemical and transcriptomic response of earthworms Eisenia andrei exposed to soils irrigated with treated wastewater. Environ. Sci. Pollut. Res. 26, 2851–2863. https://doi.org/10.1007/s11356-018-3794-0.
Mkhinini, M., Helaoui, S., Boughattas, I., Amemou, C., Banni, M., 2020. Earthworm Eisenia andrei modulates oxidative stress in bean plants Vicia faba irrigated with treated wastewater. Ecotoxicology 29, 1003–1016. https://doi.org/10.1007/s10646-020-02243-y.
Natal-da-Luz, T., Tidona, S., Jesus, B., Morais, P.V., Sousa, J.P., 2009. The use of sewage sludge as soil amendment. The need for an ecotoxicological evaluation. J. Soils Sediments 9, 246–260. https://doi.org/10.1007/s11368-009-0077-x.
Navarro, I., de la Torre, A., Sanz, P., Pro, J., Carbonell, G., de Martínez, M., los Á, 2016. Bioaccumulation of emerging organic compounds (perfluoroalkyl substances and halogenated flame retardants) by earthworm in biosolid amended soils. Environ. Res. 149, 32–39. https://doi.org/10.1016/j.envres.2016.05.004.
Navarro, I., de la Torre, A., Sanz, P., Porcel, M.Á., Pro, J., Carbonell, G., de Martínez, M., los Á, 2017. Uptake of perfluoroalkyl substances and halogenated flame retardants by crop plants grown in biosolids-amended soils. Environ. Res. 152, 199–206. https://doi.org/10.1016/j.envres.2016.10.018.
Navarro Pacheco, N.I., Roubalova, R., Dvorak, J., Benada, O., Pinkas, D., Kofronova, O., Semerad, J., Pivokonsky, M., Cajthaml, T., Bilej, M., Prochazkova, P., 2021a. Understanding the toxicity mechanism of CuO nanoparticles: the intracellular view of exposed earthworm cells. Environ. Sci. Nano 8, 2464–2477. https://doi.org/10.1039/D1EN00080B.
Navarro Pacheco, N.I., Roubalova, R., Semerad, J., Grasserova, A., Benada, O., Kofronova, O., Cajthaml, T., Dvorak, J., Bilej, M., Prochazkova, P., 2021b. In vitro interactions of TiO2 nanoparticles with earthworm coelomocytes: Immunotoxicity assessment. Nanomaterials 11, 250. https://doi.org/10.3390/nano11010250.
Pacheco, N.I.N., Semerad, J., Pivokonsky, M., Cajthaml, T., Filip, J., Busquets-Fité, M., Dvorak, J., Rico, A., Prochazkova, P., 2022. Effects of silver sulfide nanoparticles on the earthworm Eisenia andrei. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 257, 109355 https://doi.org/10.1016/j.cbpc.2022.109355.
Pannu, M.W., O'Connor, G.A., Toor, G.S., 2012. Toxicity and bioaccumulation of biosolids-borne triclosan in terrestrial organisms. Environ. Toxicol. Chem. 31, 646–653. https://doi.org/10.1002/etc.1721.
Passuello, A., Mari, M., Nadal, M., Schuhmacher, M., Domingo, J.L., 2010. POP accumulation in the food chain: Integrated risk model for sewage sludge application in agricultural soils. Environ. Int. 36, 577–583. https://doi.org/10.1016/j.envint.2010.04.015.
Rich, C.D., Blaine, A.C., Hundal, L., Higgins, C.P., 2015. Bioaccumulation of Perfluoroalkyl Acids by Earthworms Eisenia fetida Exposed to Contaminated Soils. Environ. Sci. Technol. 49, 881–888. https://doi.org/10.1021/es504152d.

Rivier, P.-A., Havranek, I., Coutris, C., Norli, H.R., Joner, E.J., 2019. Transfer of organic pollutants from sewage sludge to earthworms and barley under field conditions. Chemosphere 222, 954–960. https://doi.org/10.1016/j.chemosphere.2019.02.010.

Rodriguez-Campos, J., Dendooven, L., Alvarez-Bernal, D., Contreras-Ramos, S.M., 2014. Potential of earthworms to accelerate removal of organic contaminants from soil: A review. Appl. Soil Ecol. 79, 10–25. https://doi.org/10.1016/j.apsoil.2014.02.010.

Rorat, A., Kacprzak, M., Vandenbulcke, F., Płytycz, B., 2013. Soil amendment with municipal sewage sludge affects the immune system of earthworms Dendrobaena veneta. Appl. Soil Ecol. 64, 237–244. https://doi.org/10.1016/j.apsoil.2012.12.017.

Roubalová, R., Dvořák, J., Procházková, P., Škanta, F., Navarro Pacheco, N.I., Semerád, J., Cajthaml, T., Bilej, M., 2018. The role of CuZn- and Mn-superoxide dismutases in earthworm Eisenia andrei kept in two distinct field-contaminated soils. Ecotoxicol. Environ. Saf. 159, 363–371. https://doi.org/10.1016/j.ecoenv.2018.04.056.

Roubalová, R., Procházková, P., Hanč, A., Dvořák, J., Bilej, M., 2020. Mutual interactions of E. andrei earthworm and pathogens during the process of vermicomposting. Environ. Sci. Pollut. Res. 27, 33429–33437. https://doi.org/10.1007/s11356-019-04329-5.

Samal, K., Raj Mohan, A., Chaudhary, N., Moulick, S., 2019. Application of vermitechnology in waste management: A review on mechanism and performance. J. Environ. Chem. Eng. 7, 103392 https://doi.org/10.1016/j.jece.2019.103392.

Semerád, J., Čvančarová, M., Filip, J., Kašlík, J., Zlotá, J., Soukupová, J., Cajthaml, T., 2018. Novel assay for the toxicity evaluation of nanoscale zero-valent iron and derived nanomaterials based on lipid peroxidation in bacterial species. Chemosphere 213, 568–577. https://doi.org/10.1016/j.chemosphere.2018.09.029.

Semerád, J., Hatasová, N., Grasserová, A., Černá, T., Filipová, A., Hanč, A., Innemanová, P., Pivokonský, M., Cajthaml, T., 2020. Screening for 32 per- and polyfluoroalkyl substances (PFAS) including GenX in sludges from 43 WWTPs located in the Czech Republic - Evaluation of potential accumulation in vegetables after application of biosolids. Chemosphere 261, 128018. https://doi.org/10.1016/j.chemosphere.2020.128018.

Semerad, J., Pacheco, N.I.N., Grasserova, A., Prochazkova, P., Pivokonsky, M., Pivokonska, L., Cajthaml, T., 2020. In Vitro Study of the Toxicity Mechanisms of Nanoscale Zero-Valent Iron (nZVI) and Released Iron Ions Using Earthworm Cells. Nanomaterials 10, 2189. https://doi.org/10.3390/nano10112189.

Shi, Z., Liu, J., Tang, Z., Zhao, Y., Wang, C., 2020. Vermiremediation of organically contaminated soils: Concepts, current status, and future perspectives. Appl. Soil Ecol. 147, 103377 https://doi.org/10.1016/j.apsoil.2019.103377.

Singh, R.P., Agrawal, M., 2008. Potential benefits and risks of land application of sewage sludge. Waste Manag. 28, 347–358. https://doi.org/10.1016/j.wasman.2006.12.010.

Šnajdr, J., Valášková, V., Merhautová, V., Cajthaml, T., Baldrian, P., 2008. Activity and spatial distribution of lignocellulose-degrading enzymes during forest soil colonization by saprotrophic basidiomycetes. Enzyme Microb. Technol. 43, 186–192. https://doi.org/10.1016/j.enzmictec.2007.11.008.

Suleiman, H., Rorat, A., Grobelak, A., Grosser, A., Milczarek, M., Płytycz, B., Kacprzak, M., Vandenbulcke, F., 2017. Determination of the performance of vermicomposting process applied to sewage sludge by monitoring of the compost quality and immune responses in three earthworm species: Eisenia fetida, Eisenia andrei and Dendrobaena veneta. Bioresour. Technol. 241, 103–112. https://doi.org/10.1016/j.biortech.2017.05.104.

Taylor, S.C., Nadeau, K., Abbasi, M., Lachance, C., Nguyen, M., Fenrich, J., 2019. The Ultimate qPCR Experiment: Producing Publication Quality, Reproducible Data the First Time. Trends Biotechnol. 37, 761–774. https://doi.org/10.1016/j.tibtech.2018.12.002.

Urionabarrenetxea, E., Garcia-Velasco, N., Zaldibar, B., Soto, M., 2022. Impacts of sewage sludges deposition on agricultural soils: Effects upon model soil organisms. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 255, 109276 https://doi.org/10.1016/j.cbpc.2022.109276.

Vafa, H.J., Raiesi, F., Hosseinpur, A., 2016. Sewage sludge application strongly modifies earthworm impact on microbial and biochemical attributes in a semi-arid calcareous soil from Iran. Appl. Soil Ecol. 100, 45–56. https://doi.org/10.1016/j.apsoil.2015.11.022.

Vig, A.P., Singh, J., Wani, S.H., Singh Dhaliwal, S., 2011. Vermicomposting of tannery sludge mixed with cattle dung into valuable manure using earthworm Eisenia fetida (Savigny). Bioresour. Technol. 102, 7941–7945. https://doi.org/10.1016/j.biortech.2011.05.056.

Villar, I., Alves, D., Pérez-Díaz, D., Mato, S., 2016. Changes in microbial dynamics during vermicomposting of fresh and composted sewage sludge. Waste Manag. 48, 409–417. https://doi.org/10.1016/j.wasman.2015.10.011.

Wen, B., Zhang, H., Li, L., Hu, X., Liu, Y., Shan, X., Zhang, S., 2015. Bioavailability of perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) in biosolids-amended soils to earthworms (Eisenia fetida). Chemosphere 118, 361–366. https://doi.org/10.1016/j.chemosphere.2014.08.009.

Zhang, Q., Zhu, L., Wang, J., Xie, H., Wang, J., Han, Y., Yang, J., 2013. Oxidative stress and lipid peroxidation in the earthworm Eisenia fetida induced by low doses of fomesafen. Environ. Sci. Pollut. Res. 20, 201–208. https://doi.org/10.1007/s11356-012-0962-5.

Zhao, S., Fang, S., Zhu, L., Liu, L., Liu, Z., Zhang, Y., 2014. Mutual impacts of wheat (Triticum aestivum L.) and earthworms (Eisenia fetida) on the bioavailability of perfluoroalkyl substances (PFASs) in soil. Environ. Pollut. 184, 495–501. https://doi.org/10.1016/j.envpol.2013.09.032.

Zhao, W., Teng, M., Zhang, J., Wang, K., Zhang, J., Xu, Y., Wang, C., 2022. Insights into the mechanisms of organic pollutant toxicity to earthworms: Advances and perspectives. Environ. Pollut. 303, 119120 https://doi.org/10.1016/j.envpol.2022.119120.

Zhao, C., Wang, Y., Wang, Y., Wu, F., Zhang, J., Cui, R., Wang, L., Mu, H., 2018. Insights into the role of earthworms on the optimization of microbial community structure during vermicomposting of sewage sludge by PLFA analysis. Waste Manag. 79, 700–708. https://doi.org/10.1016/j.wasman.2018.08.041.

Zhao, S., Zhu, L., Liu, L., Liu, Z., Zhang, Y., 2013. Bioaccumulation of perfluoroalkyl carboxylates (PFCAs) and perfluoroalkane sulfonates (PFSAs) by earthworms (Eisenia fetida) in soil. Environ. Pollut. 179, 45–52. https://doi.org/10.1016/j.envpol.2013.04.002.

Supplementary material

New insights into vermiremediation of sewage sludge: The effect of earthworms on micropollutants and vice versa

Alena Grasserová [a, b], Natividad I. N. Pacheco [b, c, d], Jaroslav Semerád [b], Alena Filipová [b], Petra Innemanová [a, e], Aleš Hanč [f], Petra Procházková [b], Tomáš Cajthaml [a, b, *]

[a] Institute for Environmental Studies, Faculty of Science, Charles University, Benátská 2, Prague 2, 12801, Czech Republic

[b] Institute of Microbiology of the Czech Academy of Sciences, Vídeňská 1083, Prague 4, 14220, Czech Republic

[c] First Faculty of Medicine, Charles University, Kateřinská 32, Prague 2, 12108, Czech Republic

[d] Laboratory of Ecotoxicology, Institute of Environmental Sciences, University of Castilla-La Mancha, 45004 Toledo, Spain

[e] DEKONTA a.s., Dřetovice 109, Stehelčeves, 27342, Czech Republic

[f] Department of Agro-Environmental Chemistry and Plant Nutrition, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamýcká 129, Prague 6, 16500, Czech Republic

[*] Corresponding author. E-mail address: cajthaml@biomed.cas.cz (T. Cajthaml)

Number of tables: 5

Number of figures: 3

Supplementary Table 1 Characterization of the initial materials – sewage sludge and straw. Values are expressed as average followed by standard deviation (n = 3).

Material	pH	Electrical conductivity [mS·cm^{-1}]	Total carbon [%]	Total nitrogen [%]	C/N ratio
WWTP1 SS	6.9 ± 0.1	0.6 ± 0.1	32.9 ± 0.3	5.4 ± 0.1	6.1 ± 0.1
WWTP2 SS	7.0 ± 0.2	0.5 ± 0.1	31.2 ± 0.2	7.5 ± 0.3	4.5 ± 0.3
Straw pellets	6.1 ± 0.1	0.7 ± 0.1	42.6 ± 0.4	0.8 ± 0.1	53.7 ± 7.6

WWTP1 = wastewater treatment plant 1; WWTP2 = wastewater treatment plant 2; SS = sewage sludge.

Supplementary Table 2 List of analyzed PPCPs and EDs (in the main text collectively referred to as PPCPs).

PPCPs	Quantifier/qualifier transition	Ion polarity	Collision energy [eV]	Fragmentor [eV]	Cell accelerator voltage [V]	Analytical standard manufacturer
acesulfame	162.0 → 82.0	negative	12	65	4	Sigma-Aldrich
	162.0 → 78.0	negative	36	65	4	
acetaminophen	152.1 → 65.0	positive	32	100	3	Sigma-Aldrich
(paracetamol)	152.1 → 110.1	positive	18	100	3	
amitriptyline	278.2 → 233.1	positive	16	120	4	Carl Roth GmbH
	278.2 → 105.1	positive	28	120	4	
atenolol	267.2 → 145.1	positive	28	120	3	Sigma
	267.2 → 56.0	positive	32	120	3	
atorvastatin	559.3 → 440.2	positive	24	165	3	Sigma-Aldrich
	559.3 → 250.1	positive	48	165	3	
azithromycin	749.5 → 591.3	positive	32	210	4	Sigma-Aldrich
	749.5 → 573.3	positive	40	210	4	
caffeine	195.1 → 138.0	positive	20	105	4	Sigma-Aldrich
	195.1 → 110.0	positive	26	105	4	
carbamazepine	237.1 → 194.1	positive	20	120	4	Sigma
	237.1 → 179.1	positive	40	120	4	
carbamazepine-	253.1 → 210.1	positive	12	65	4	Sigma-Aldrich
10,11-epoxide	253.1 → 236.1	positive	8	65	4	
cetirizine	389.2 → 201.0	positive	20	110	3	Alfa Aesar
	389.2 → 165.1	positive	48	110	3	
citalopram	325.2 → 109.1	positive	28	95	4	European
	325.2 → 234.1	positive	32	95	4	Pharmacopoeia
clarithromycin	748.5 → 158.0	positive	32	100	4	Sigma-Aldrich
	748.5 → 590.3	positive	20	100	4	
diclofenac	296.0 → 213.9	positive	40	85	4	Sigma
	296.0 → 179.0	positive	60	85	4	
erythromycin	734.5 → 158.1	positive	32	105	4	Sigma-Aldrich
	734.5 → 576.4	positive	20	105	4	
fluconazole	307.2 → 238.0	positive	16	95	4	Alfa Aesar
	307.2 → 220.0	positive	20	95	4	
furosemide	329.0 → 285.0	negative	12	75	4	European
	329.0 → 205.0	negative	24	75	4	Pharmacopoeia
gabapentin	172.1 → 154.1	positive	14	95	3	Sigma-Aldrich
	172.1 → 55.0	positive	28	95	3	
hydrochlorothiazide	296.0 → 268.9	negative	18	80	3	Alfa Aesar
	296.0 → 205.0	negative	18	80	3	

ibuprofen	205.1 → 161.1	negative	4	65	4	Sigma
	-					
iomeprol	777.9 → 405.0	positive	48	150	4	Ehrenstorfer
	777.9 → 686.8	positive	20	150	4	
ketoprofen	255.1 → 77.0	positive	48	110	3	Sigma
	255.1 → 105.0	positive	28	110	3	
lamotrigine	256.0 → 43.2	positive	44	145	4	Sigma
	256.0 → 211.0	positive	28	145	4	
metoprolol	268.2 → 74.1	positive	24	125	3	Alfa Aesar
	268.2 → 56.0	positive	32	125	3	
mirtazapine	266.2 → 195.1	positive	32	95	4	European
	266.2 → 72.2	positive	20	95	4	Pharmacopoeia
naproxen	231.1 → 185.1	positive	14	100	3	Sigma-Aldrich
	231.1 → 170.1	positive	28	100	3	
omeprazole	346.1 → 198.1	positive	10	95	3	Acros Organics
	346.1 → 136.1	positive	44	95	3	
paraxanthine	181.1 → 124.1	positive	20	90	4	Sigma
	181.1 → 42.0	positive	48	90	4	
saccharine	182.0 → 42.0	negative	20	105	4	Alfa Aesar
	182.0 → 106.0	negative	36	105	4	
sulfamethazine	279.0 → 186.0	positive	15	100	3	Sigma
	279.0 → 124.0	positive	15	100	3	
sulfamethoxazole	254.1 → 65.0	positive	48	100	3	Fluka
	254.1 → 156.0	positive	14	100	3	
sulfanilamide	173.0 → 92.0	positive	20	70	3	Sigma-Aldrich
	173.0 → 156.0	positive	2	70	3	
sulfapyridine	250.1 → 92.0	positive	28	110	3	Fluka
	250.1 → 156.0	positive	18	110	3	
telmisartan	515.2 → 276.1	positive	56	210	4	European
	515.2 → 497.2	positive	40	210	4	Pharmacopoeia
tramadol	264.2 → 58.2	positive	20	95	4	Sigma
	264.2 → 42.2	positive	84	95	4	
triclosan	289.0 → 35.0	negative	8	70	3	Sigma-Aldrich
	289.0 → 37.0	negative	8	70	3	
trimethoprim	291.1 → 230.1	positive	24	140	3	Sigma
	291.1 → 261.1	positive	28	140	3	
venlafaxine	278.2 → 58.2	positive	20	70	4	European
	278.2 → 260.2	positive	12	70	4	Pharmacopoeia

EDs	**Quantifier/qualifier transition**	**Ion polarity**	**Collision energy [eV]**	**Fragmentor [eV]**	**Cell accelerator voltage [V]**	**Analytical Standard Manufacturer**
17alfa-estradiol	271.2 → 145.1	negative	44	160	3	Toronto
	271.2 → 183.1	negative	48	160	3	Research Chemicals, Inc.
17beta-estradiol	271.2 → 145.1	negative	44	160	3	Sigma-Aldrich
	271.2 → 183.1	negative	48	160	3	
bisphenol A (BPA)	227.1 → 212.1	negative	20	115	4	Sigma-Aldrich
	227.1 → 133.0	negative	28	115	4	
bisphenol F (BPF)	199.1 → 105.0	negative	24	125	4	Sigma-Aldrich
	199.1 → 93.0	negative	24	125	4	
daidzein	253.1 → 223.0	negative	40	145	4	Toronto
	253.1 → 208.0	negative	36	145	4	Research Chemicals, Inc.
equilin	267.1 → 265.1	negative	24	145	4	Sigma-Aldrich
	267.1 → 143.0	negative	36	145	4	
equol	241.1 → 119.0	negative	20	110	4	

	241.1 → 121.0	negative	12	110	4	Toronto Research Chemicals, Inc.
estriol	287.2 → 145.0	negative	60	95	4	Sigma-Aldrich
	287.2 → 255.1	negative	60	95	4	
estrone	269.2 → 159.0	negative	32	125	4	Sigma-Aldrich
	269.2 → 143.1	negative	60	125	4	
ethinylestradiol	295.2 → 145.0	negative	48	200	3	Sigma-Aldrich
	295.2 → 159.0	negative	36	200	3	
genistein	269.0 → 133.0	negative	32	140	4	Alfa Aesar
	269.0 → 63.0	negative	28	140	4	
norethindrone	299.2 → 109.1	positive	32	130	4	Sigma-Aldrich
	299.2 → 91.0	positive	56	130	4	
norgestrel	313.2 → 109.1	positive	32	130	3	Supelco
	313.2 → 91.1	positive	48	130	3	
zearalenol	319.2 → 275.2	negative	20	160	3	Toronto Research Chemicals, Inc.
	319.2 → 301.2	negative	24	160	3	

Purity of all analytical standards was ≥ 97 % (except iomeprol with stated purity 94.89 %).

PPCPs = pharmaceuticals and personal care products; EDs = endocrine disruptors.

Supplementary Table 3 Substrate pH at the beginning of the experiment (week 0) and at the end (week 6) for control and vermicompost; Earthworm mortality. Values are expressed as average followed by standard deviation (n = 3). The lowercase letters represent significant differences between week 0, week 6 vermicompost, and week 6 control (without earthworms) for pH value of each SS mixture ($P < 0.05$). The uppercase letters following the values represent significant differences between week 0 and week 6 vermicompost for earthworm number ($P < 0.05$).

	SS mixture	pH start (week 0)	pH vermicompost (week 6)	pH control (week 6)	number earthworms (week 0)	number earthworms (week 6)
WWTP1	0SS	6.1 ± 0.1*a*	7.8 ± 0.2*b*	7.2 ± 0.2*c*	20 ± 0*A*	20 ± 0*A*
	25SS	6.6 ± 0.1*a*	7.2 ± 0.2*b*	6.9 ± 0.3*ab*	20 ± 0*A*	19 ± 2*A*
	50SS	6.7 ± 0.2*a*	6.0 ± 0.7*a*	6.1 ± 0.7*a*	20 ± 0*A*	19 ± 1*A*
	75SS	6.9 ± 0.2*a*	5.6 ± 0.6*b*	6.2 ± 0.1*b*	20 ± 0*A*	16 ± 7*A*
WWTP2	0SS	6.5 ± 0.1*a*	7.4 ± 0.2*b*	7.1 ± 0.5*ab*	20 ± 0*A*	18 ± 3*A*
	25SS	6.8 ± 0.1*a*	6.8 ± 0.5*a*	6.7 ± 0.1*a*	20 ± 0*A*	19 ± 1*A*
	50SS	6.9 ± 0.1*a*	6.0 ± 0.6*b*	5.6 ± 0.4*b*	20 ± 0*A*	20 ± 1*A*
	75SS	6.9 ± 0.1*a*	5.1 ± 0.5*b*	5.4 ± 0.5*b*	20 ± 0*A*	18 ± 2*A*

WWTP1 = wastewater treatment plant 1; WWTP2 = wastewater treatment plant 2; SS = sewage sludge; 0SS = substrate containing 0% sewage sludge and 100% straw; 25SS = substrate containing 25% sewage sludge and 75% straw; 50SS = substrate containing 50% sewage sludge and 50% straw; 75SS = substrate containing 75% sewage sludge and 25% straw.

Supplementary Table 4 Application description of the detected PPCPs and EDs (in the main text collectively referred to as PPCPs).

PPCPs	Application
acesulfame	artificial sweetener

amitriptyline	tricyclic antidepressant
atorvastatin	beta blocker - antihypertensive
azithromycin	macrolide antibiotic
bisphenol A	polycarbonate monomer, thermal printing etc.
bisphenol F	polycarbonate monomer, thermal printing etc.
caffeine	central nervous system stimulant
carbamazepine	anticonvulsant
cetirizine	antihistamine
citalopram	antidepressant
clarithromycin	macrolide antibiotic
daidzein	natural isoflavonoid
diclofenac	nonsteroidal anti-inflammatory drug
equol	isoflavandiol estrogen, metabolite of daidzein
genistein	natural isoflavonoid
hydrochlorothiazide	diuretic
ibuprofen	nonsteroidal anti-inflammatory drug
lamotrigine	antiepileptic
metoprolol	beta blocker - antihypertensive
mirtazapine	antidepressant
sulfapyridine	sulfonamide antibiotic
telmisartan	angiotensin II receptor blocker - antihypertensive
tramadol	opioid analgesic
triclosan	antibacterial and antifungal agent
trimethoprim	antibiotic
venlafaxine	antidepressant

PPCPs = pharmaceuticals and personal care products; EDs = endocrine disruptors.

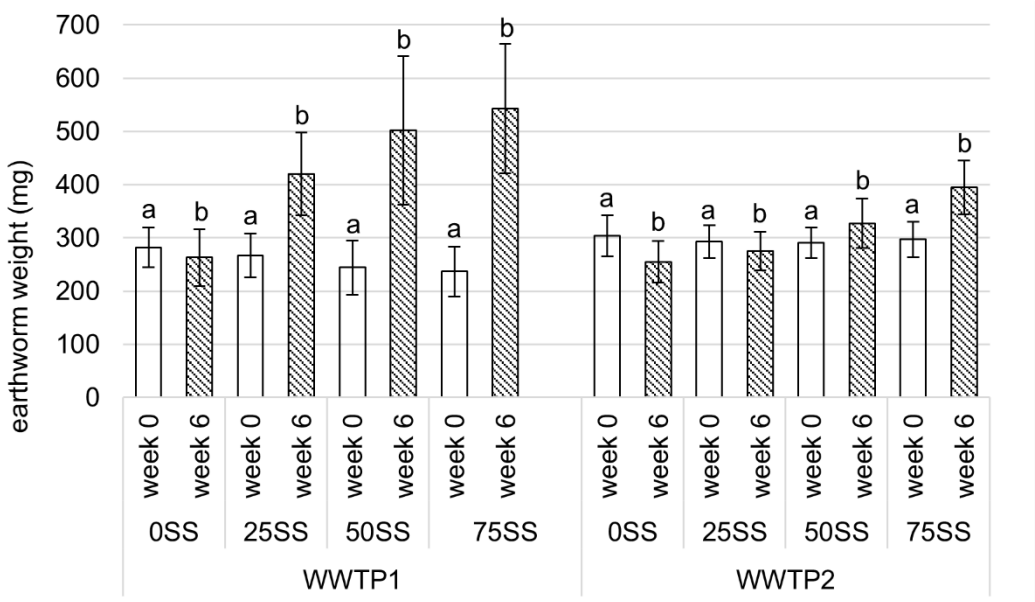
Supplementary Table 5 Correlation analyses coefficients.

	SS	**PPCPs log K_{ow} - BAF**		**PFASs log K_{ow} - BAF**		**PFASs Per F–C - BAF**	
	mixture	Spearman corr.	P-value	Spearman corr.	P-value	Spearman corr.	P-value
WWTP1	25SS	0.19	0.35	0.21	0.43	0.55	0.03
	50SS	0.15	0.46	0.22	0.43	0.59	0.02
	75SS	0.20	0.34	0.33	0.23	0.65	0.01
WWTP2	25SS	0.12	0.57	0.05	0.85	0.50	0.07
	50SS	0.15	0.47	0.09	0.79	0.44	0.15
	75SS	0.18	0.38	0.02	0.94	0.40	0.20

WWTP1 = wastewater treatment plant 1; WWTP2 = wastewater treatment plant 2; PPCPs = pharmaceuticals and personal care products; PFASs = per/polyfluoroalkyl substances; K_{ow} = octanol-water partition coefficient; BAF = bioaccumulation factor; Per F-C = number of perfluorinated carbons; Spearman corr. = Spearman correlation coefficient; SS = sewage sludge; 25SS = substrate containing 25% sewage sludge and 75% straw; 50SS = substrate containing 50% sewage sludge and 50% straw; 75SS = substrate containing 75% sewage sludge and 25% straw.

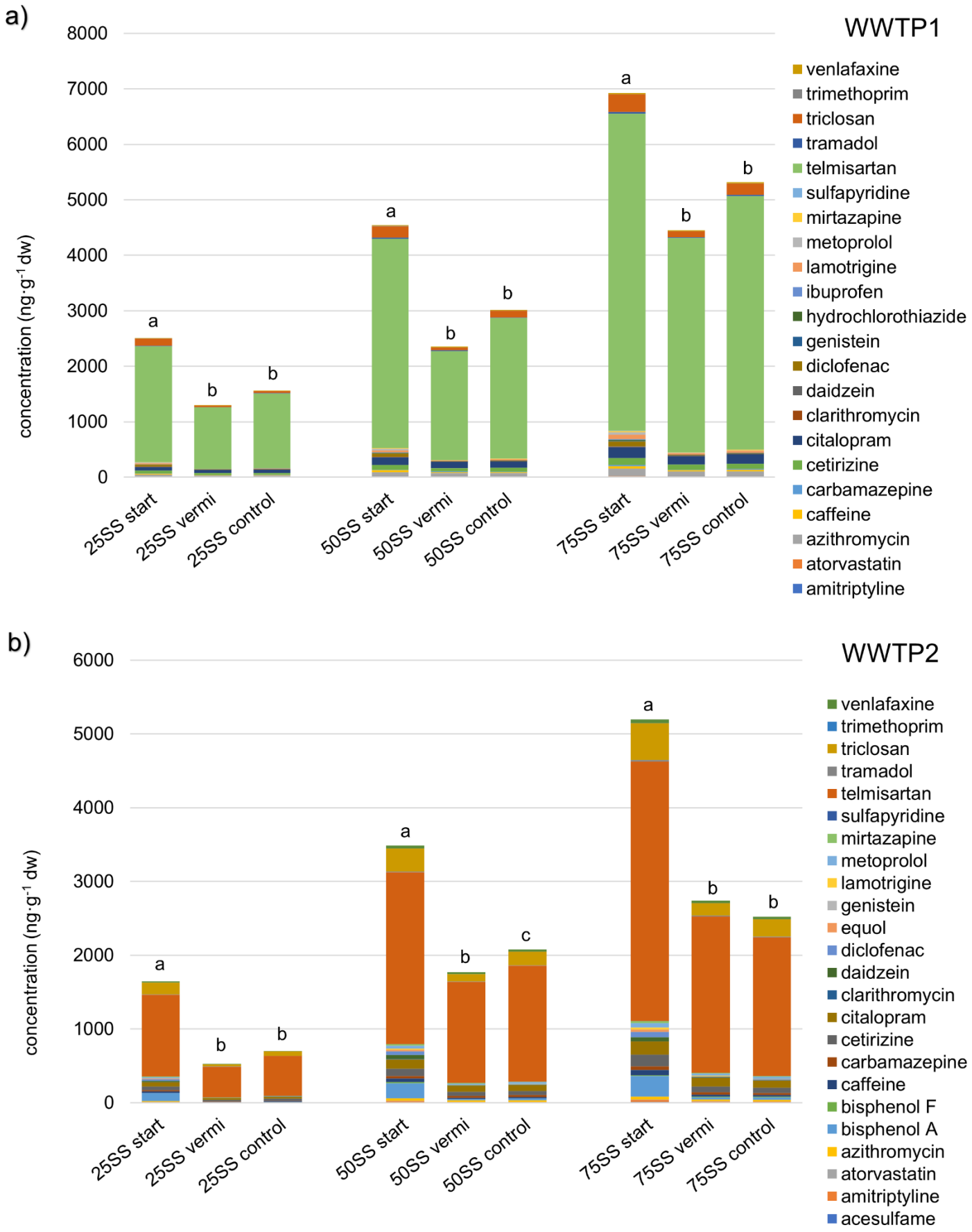
Supplementary Figure 1 Weight of earthworms at the beginning of the experiment (week 0) and at the end (week 6). The letters above the columns represent significant differences between week 0

and week 6 for each WWTP SS mixture ($P < 0.05$). The columns stand for the averages and the error bars represent standard deviations (n = 49–60).



WWTP1 = wastewater treatment plant 1; WWTP2 = wastewater treatment plant 2; SS = sewage sludge; 0SS = substrate containing 0% sewage sludge and 100% straw; 25SS = substrate containing 25% sewage sludge and 75% straw; 50SS = substrate containing 50% sewage sludge and 50% straw; 75SS = substrate containing 75% sewage sludge and 25% straw.

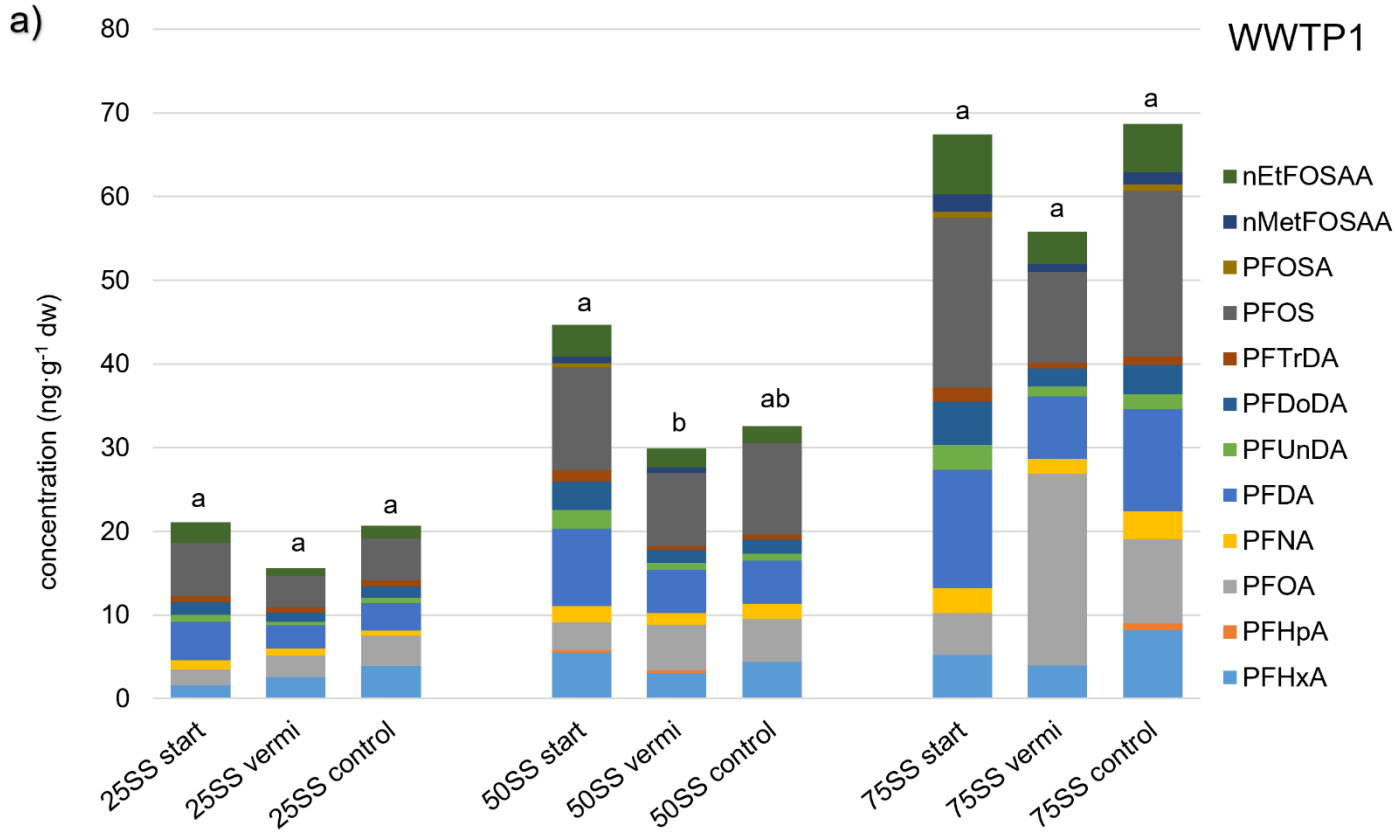
Supplementary Figure 2 PPCPs sum for start, end vermicompost (*vermi*), and end control (without earthworms) in 25, 50, and 75SS mixture for WWTP1 (a) and WWTP2 (b). The letters above the columns represent significant differences of the three values: start, end vermicompost (*vermi*), and end control (no earthworms; $P < 0.05$). The columns stand for the sum averages (n = 3).

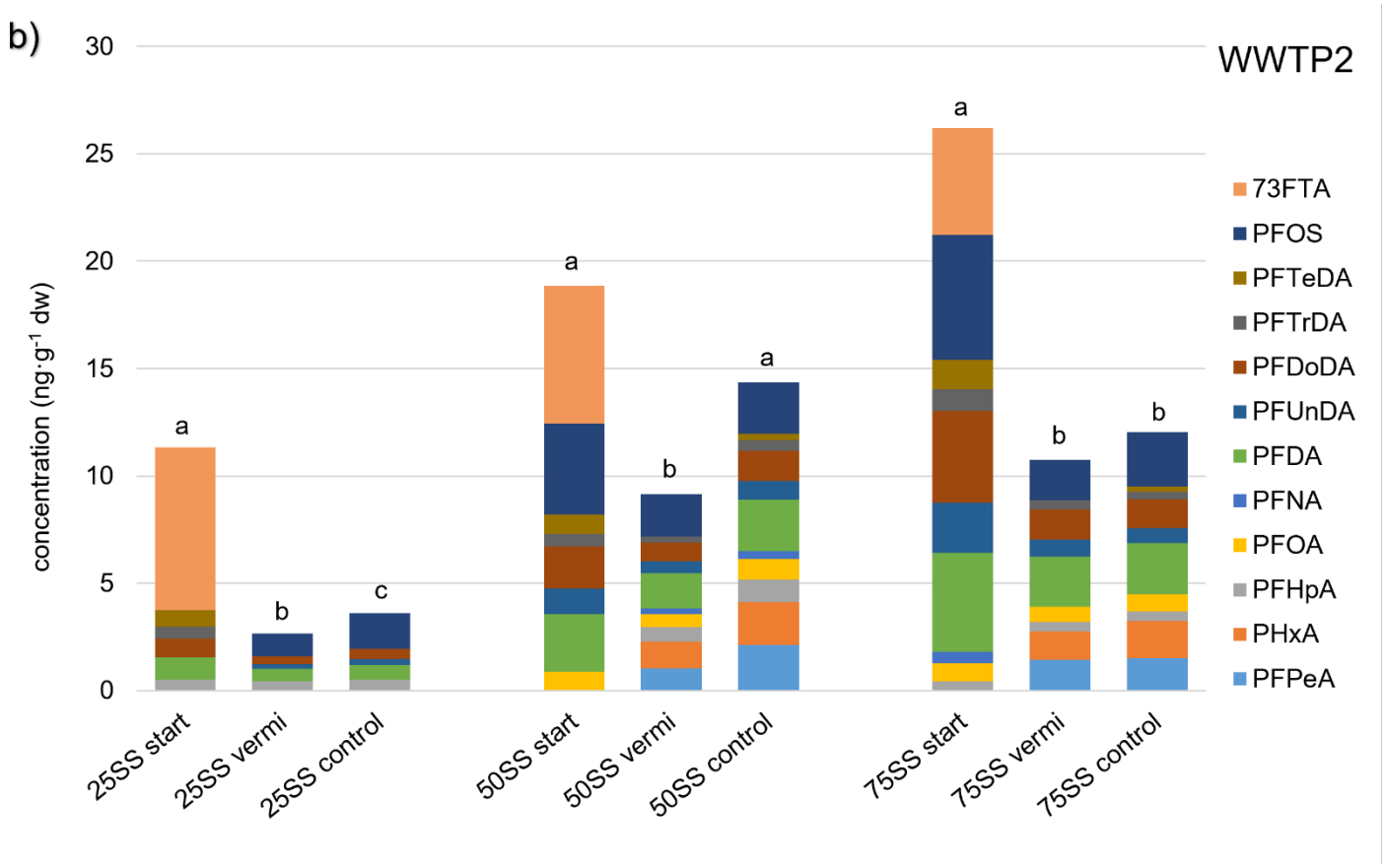


PPCPs = pharmaceuticals and personal care products; WWTP1 = wastewater treatment plant 1; WWTP2 = wastewater treatment plant 2; SS = sewage sludge; 25SS = substrate containing 25%

sewage sludge and 75% straw; 50SS = substrate containing 50% sewage sludge and 50% straw; 75SS = substrate containing 75% sewage sludge and 25% straw.

Supplementary Figure 3 PFASs sum for start, end vermicompost (*vermi*), and end control (without earthworms) in 25, 50, and 75SS mixture for WWTP1 (a) and WWTP2 (b). The letters above the columns represent significant differences of the three values: start, end vermicompost (*vermi*), and end control (no earthworms; $P < 0.05$). The columns stand for the sum averages (n = 3).





PFASs = per/polyfluoroalkyl substances; WWTP1 = wastewater treatment plant 1; WWTP2 = wastewater treatment plant 2; SS = sewage sludge; 25SS = substrate containing 25% sewage sludge and 75% straw; 50SS = substrate containing 50% sewage sludge and 50% straw; 75SS = substrate containing 75% sewage sludge and 25% straw; PFPeA = perfluoro-n-pentanoic acid; PFHxA = perfluoro-n-hexanoic acid; PFHpA = perfluoro-n-heptanoic acid; PFOA = perfluoro-n-octanoic acid; PFNA = perfluoro-n-nonanoic acid; PFDA = perfluoro-n-decanoic acid; PFUnDA = perfluoro-n-undecanoic acid; PFDoDA = perfluoro-n-dodecanoic acid; PFTrDA = perfluoro-n-tridecanoic acid; PFTeDA = perfluoro-n-tetradecanoic acid; PFOS = perfluorooctane sulfonic acid; PFOSA = perfluorooctane sulfonamide; 73 FTA = 7:3 fluorotelomer acid; nMetFOSAA = n-methyl-perfluoro-1-octanesulfonamidoacetate; nEtFOSAA = n-ethyl-perfluoro-1-octanesulfonamidoacetate.