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

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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

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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)

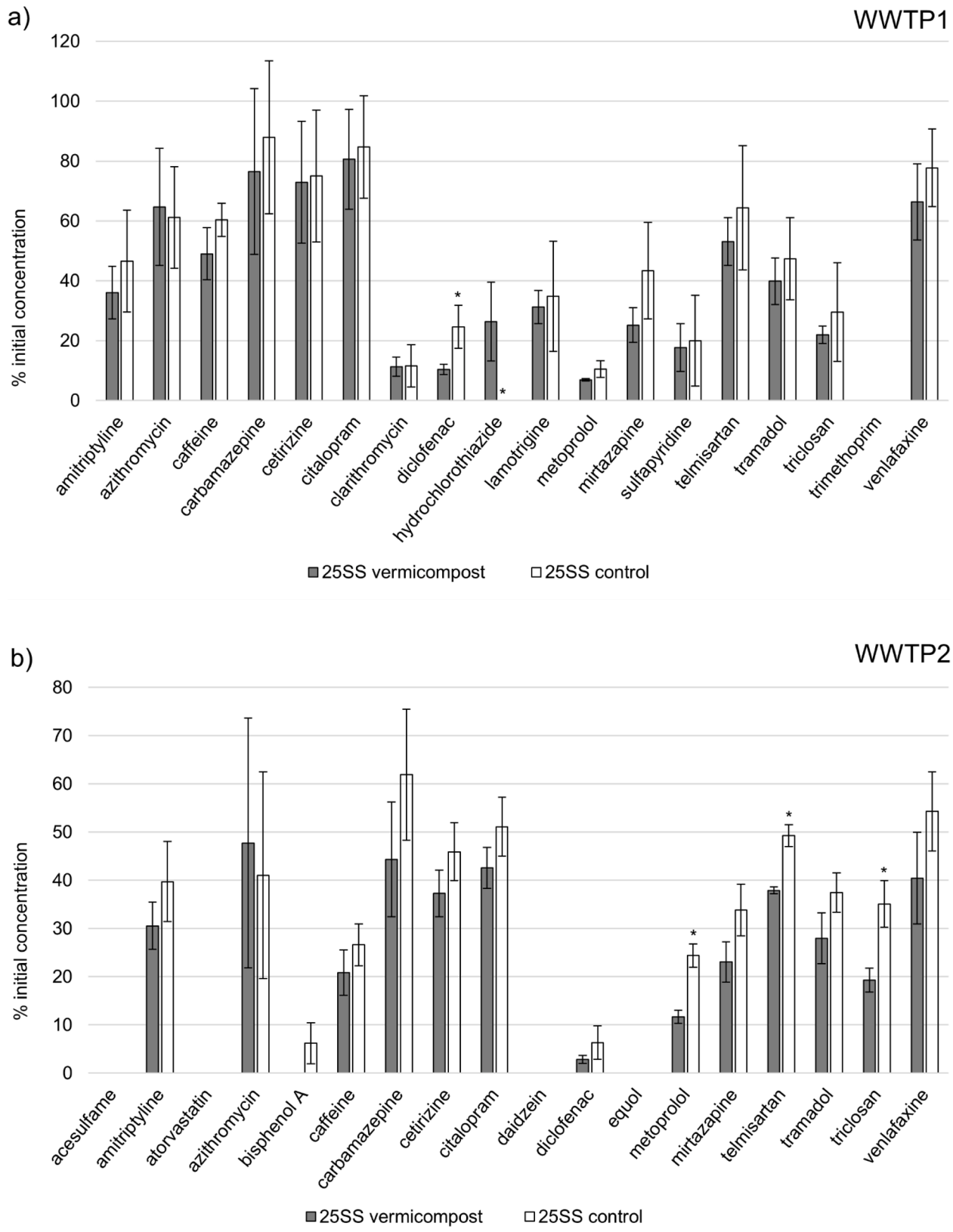

Fig. 1. Concentrations of the individual PPCPs in the 25SS vermicompost and the 25SS control (no earthworms) for WWTP1 (a) and WWTP2 (b) after six weeks of vermicomposting expressed as % of the initial concentration. The asterisks represent significant differences ($P < 0.05$). The columns represent 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.

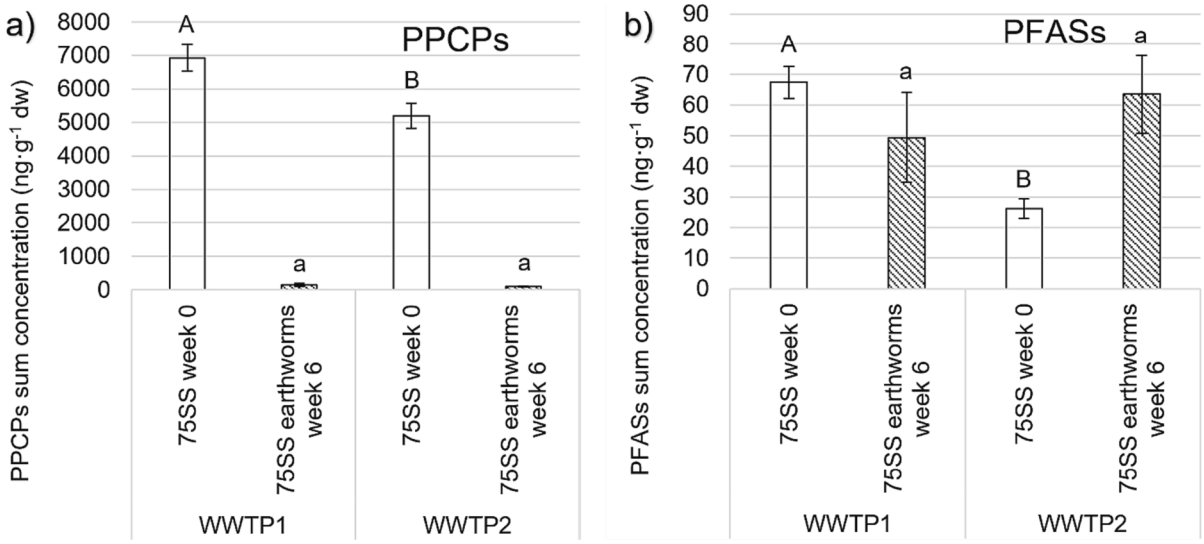

Fig. 2. Concentrations of PPCPs (a) and PFASs (b) in the 75SS starting mixture with WWTP1 and WWTP2 SS (capital letters represent significant differences; $P < 0.05$) and concentrations in earthworms after six weeks of vermicomposting (lowercase letters represent significant differences; $P < 0.05$). 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

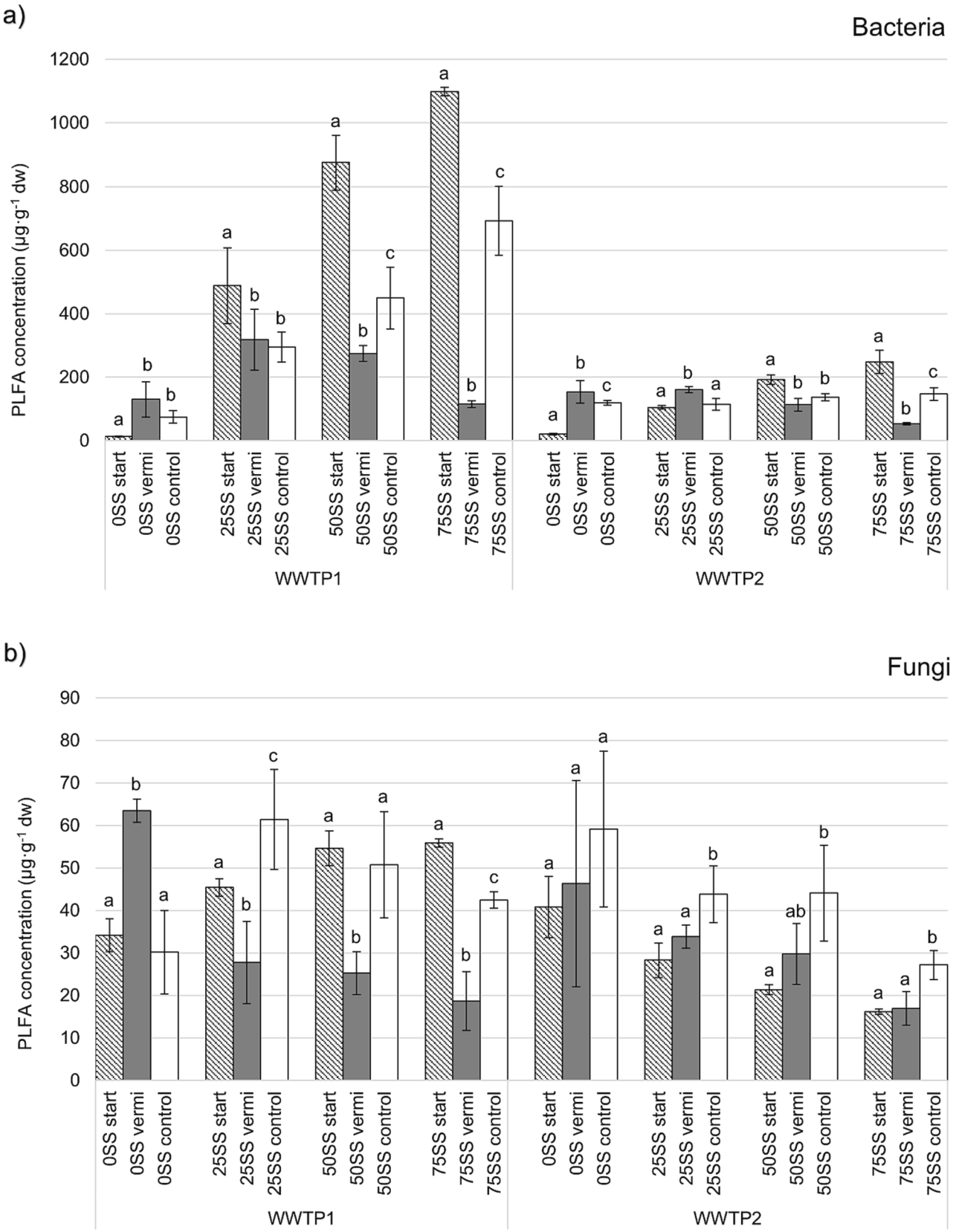

Fig. 3. PLFA concentrations of the total bacterial (a) and fungal (b) biomass. The letters above the columns represent significant differences ($P < 0.05$) in the groups for the three values: *start* (week 0), end *vermi* (vermicompost; week 6), and *control* (no earthworms; week 6). The columns represent 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

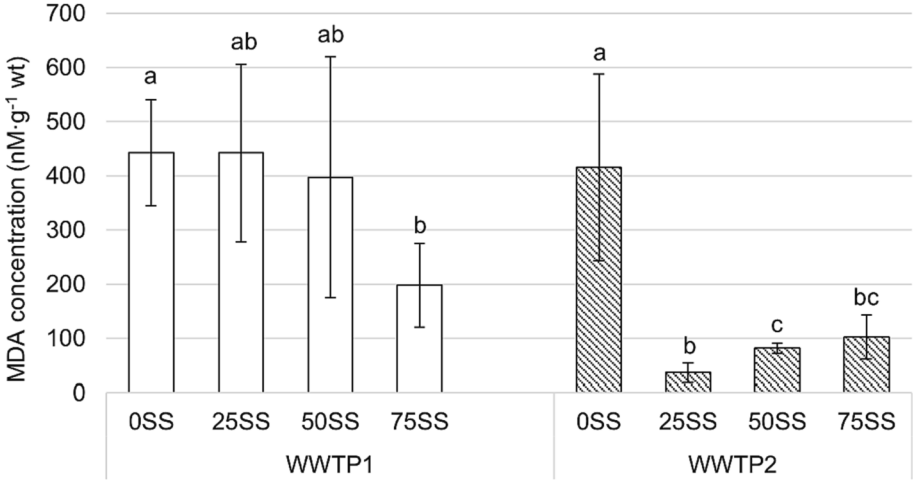

Fig. 4. MDA concentrations in earthworm tissue. The letters above the columns represent significant differences for each WWTP ($P < 0.05$). The columns represent the averages, and the error bars represent standard deviations (n = 3). MDA = malondialdehyde; WWTP1 = wastewater treatment plant 1; WWTP2 = 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.

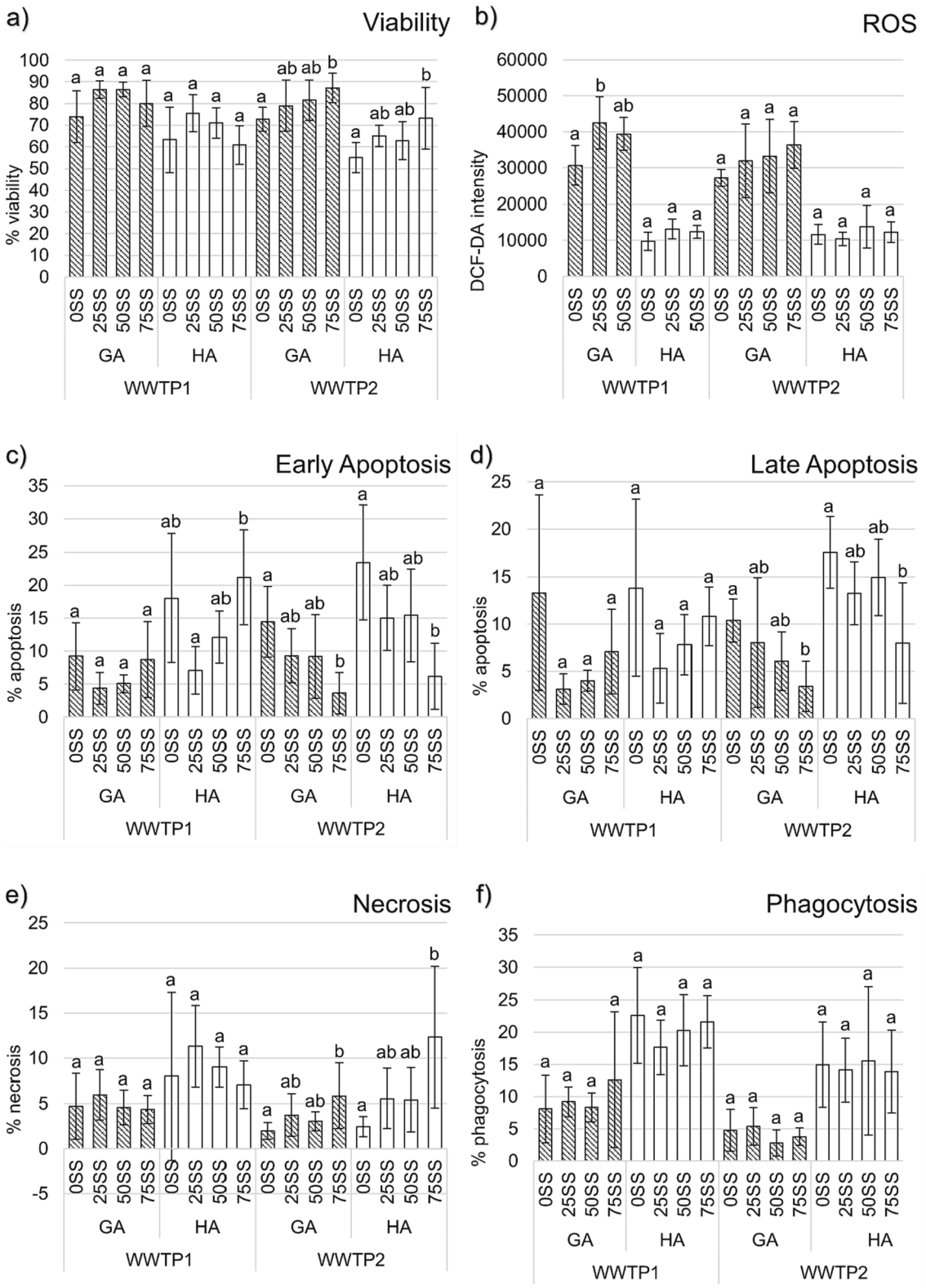

Fig. 5. Cytotoxicity assays: viability (a), ROS (b), early apoptosis (c), late apoptosis (d), necrosis (e), and phagocytosis (f). The letters above the columns represent significant differences for each cell subtype – granular amoebocytes (GA) and hyaline amoebocytes (HA) in WWTP1 and WWTP2 ($P < 0.05$). 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.

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Appendix A. Supplementary data

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

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