



Research Article

Perfluorooctanoic acid dominates the molecular-level effects of a mixture of equal masses of perfluorooctanoic acid and perfluorooctane sulfonic acid in earthworm

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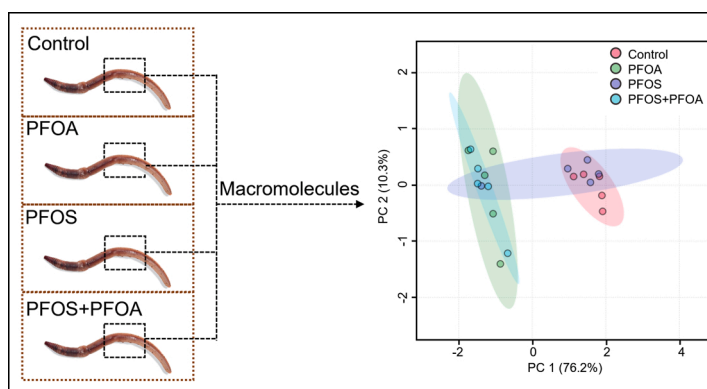
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HIGHLIGHTS

- The molecular effects of a PFAS mixture were probed with FTIR and GC-TOF-MS.
- The negative effect of PFOS-PFOA binary mixture in earthworm was not additive.
- BSAF of PFOS increased while that of PFOA decreased when in a mixture.
- PFAS mixture affected biological pathways related to amino acid and energy metabolism.
- PFOA dominated the molecular-level impact of the binary PFAS mixture.

GRAPHICAL ABSTRACT



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ABSTRACT

Per- and polyfluoroalkyl substances (PFAS) are an important class of emerging contaminants in the environment. Most studies on the impact of PFAS mixtures considered phenotypic endpoints, which may not adequately reflect the sublethal effects on organisms. To fill this knowledge gap, we investigated the subchronic impact of environmentally relevant concentrations of perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS)—as individual compounds and a mixture (PFOS+PFOA)—on earthworm (*Eisenia fetida*), using phenotypic and molecular endpoints. PFAS decreased the survival (12.2–16.3%), biomass (9.0–9.8%), and reproduction (15.6–19.8%) of *E. fetida* after 28 d of exposure. The bioaccumulation of PFOS after 28 d increased (from 2790.7 ng/g-dw to 5224.9 ng/g-dw) while that of PFOA decreased (from 780.2 ng/g-dw to 280.5 ng/g-dw) when *E. fetida* was exposed to the mixture compared to the individual compounds. These bioaccumulation trends were partly attributed to changes in the soil distribution coefficient (K_d) of PFOS and PFOA when present in the

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mixture. Eighty percent of the (p and $FDR < 0.05$) altered metabolites after 28 d were similarly perturbed by both PFOA and PFOS+PFOA. The pathways dysregulated are related to the metabolism of amino acids, energy, and sulfur. We showed that PFOA dominates the molecular-level impact of the binary PFAS mixture.

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) are a diverse class of highly fluorinated synthetic compounds with useful properties such as chemical stability and surfactant capabilities [1]. PFAS have been used in many consumer and industrial products such as aqueous fire-fighting foams (AFFFs), textiles, paints, and lubricants [1–3]. Release of PFAS into the environment occurs during all stages of products' life cycle, and has generated concern due to PFAS' tendency to bioaccumulate, exert toxicity, and persist in the environment [4,5]. Soil is an important sink for PFAS [6,7]. PFAS may be emitted to soil directly (e.g., through AFFF application) or indirectly (e.g., via atmospheric deposition) [6–8]. PFAS-enabled products often contain multiple PFAS or precursors that degrade into several PFAS [2,3,9]. As a result, soils typically contain PFAS mixtures [6,9], with perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS) being two of the most detected PFAS in soil [6–8].

Although the ecotoxicity of PFOS and PFOA have been widely studied, most studies considered the individual compounds [10]. In the few cases where PFAS mixtures have been studied, additive [11–13], antagonistic [13–15], and synergistic [12,13,16] responses have been reported. For instance, Bursian and coworkers found that PFOS was more toxic than PFOA to Japanese quail, and the toxicity of PFOS and PFOA mixture was additive [11]. Similarly, Flynn et al. observed additive or synergistic effects of PFOS and PFOA in American bullfrogs larva [12]. Meanwhile, additive, synergistic, and antagonistic effects were observed when different molar ratios of PFOS and PFOA mixtures were exposed to zebrafish [13]. Despite the importance of soil as a major sink for PFAS, studies on the impact of PFAS mixtures on soil organisms are scarce. There are currently two studies in which PFOS and PFOA mixtures were exposed to earthworm [17,18]. In one of the studies, PFAS exposure was performed via contact of earthworm with contaminated filter papers for 2 d, which does not adequately represent real-world exposure scenarios [17]. The other study was more focused on the role of microplastics as PFAS vectors [18].

Earthworms constitute 60–80% of soil animal biomass and acquire soil contaminants via absorption through their skin and/or ingestion [19,20]. Earthworms also perform important ecological functions. For instance, they are at the base of the food chain in soils and can cause biomagnification of PFAS in higher organisms [4,19]. Earthworms are also considered ideal organisms for studying the impact of soil pollutants [4,19,21]. The concentration ranges of PFOS and PFOA in uncontaminated (0.003–162 ng/g and 0.01–123.6 ng/g, respectively) and contaminated (0.4–460,000 ng/g and 2–50,000 ng/g, respectively) soils are well below their lethal concentrations (LC) to earthworms [6,8,22]. The 14-d LC_{50} value of PFOS and PFOA for *Eisenia fetida* is 365,000–478,000 ng/g-dw and 760,000–1,000,000 ng/g-dw, respectively [19, 21]. Therefore, determining the sublethal effects of PFAS mixtures to earthworms is important for a holistic risk assessment.

Alterations of the molecules that make up the cells, tissues, or organs of organisms (e.g., via metabolomics) can clearly reveal sublethal effects of contaminants, which may not be obvious using phenotypic endpoints [23–25]. However, this approach has rarely been used to assess the impacts of PFAS mixtures on organisms. The main objective of this study was to investigate the subchronic individual and combined effects of PFOS and PFOA on *E. fetida*, using common endpoints adopted in environmental toxicology studies (mortality, growth, and reproduction), as well as monitoring the molecular scale effects. For the first time, the effects of a PFAS mixture at the molecular level were evaluated using Fourier-transform infrared (FTIR) spectroscopy and mass

spectrometry-based targeted metabolomics. We hypothesized that PFOS will dominate any observed perturbation in the macromolecules/metabolites of *E. fetida*'s tissues due to its higher bioaccumulation and toxicity (compared to PFOA) [4,19,21,26].

2. Materials and methods

2.1. Exposure of *E. fetida* to PFAS in soil

The study was set up according to the OECD 222 guideline [27]. The soil we used had a pH of 7.1, a water holding capacity (WHC) of $17.94 \pm 1.29\%$, a cation exchange capacity (CEC) of 9.8 ± 0.1 cmol/kg soil, an anion exchange capacity (AEC) of 13.8 ± 1.2 cmol/kg soil, and an organic carbon fraction of $2.45 \pm 0.24\%$ [20]. Stocks of PFOS ($C_8H_{17}O_3S$) and PFOA ($C_7F_{15}COOH$), both purchased from Alfa Aesar (Ward Hill, MA), were prepared in LC-MS-grade methanol (Thermo Scientific, Waltham, MA) and thoroughly mixed into 500 g of soil in polypropylene pots to achieve (1) 200 ng/g of PFOS, (2) 200 ng/g of PFOA, or (3) a mixture of 200 ng/g PFOS and 200 ng/g PFOA (denoted PFOS+PFOA). Measured PFOS concentration in soil after spiking was 121.5 ± 15.2 ng/g and 114.2 ± 11.6 ng/g in the PFOS-only and PFOS+PFOA treatments, respectively; while PFOA concentration was 122.1 ± 9.2 ng/g and 129.7 ± 10.2 ng/g in the PFOA-only and PFOS+PFOA treatments, respectively. These concentrations are similar to the maximum concentrations of PFOS and PFOA detected in uncontaminated soils [6], which makes them environmentally relevant. Some of the spiked PFAS likely adsorb to the polypropylene pots as revealed by our recent study [28]. For the control, a similar amount of LC-MS-grade methanol (with no PFAS) was mixed into 500 g of soil. There were 5 replicates per treatment and two sets of each treatment were prepared (one set was sacrificed for day 7 bioaccumulation and molecular studies while the other set was kept for 28 d). The methanol in the control and PFAS-contaminated soils was allowed to completely volatilize (which was confirmed gravimetrically), then the soil water content was adjusted to 60% of its WHC using deionized (DI) water (18.2 MΩ cm, Milli-Q Ultrapure Water Systems). This WHC was maintained throughout the study. Similar to our previous study [20], 10 adult *E. fetida* with clitellum (obtained from The Worm Farm, Durham, CA) were added to each pot (average weight $\pm$ standard deviation = 398.8 ± 13.5 mg per earthworm per pot), and kept in a light- and temperature-controlled room (16:8 lights:dark cycle; 20 ± 2 °C). Feed (5 g) was added to the soil surface every 7 d for 28 d.

2.2. Phenotypic effect of PFAS on *E. fetida*

E. fetida survival was determined every 7 d for 28 d by direct counting. Earthworms were classified as dead when they did not respond to a gentle mechanical stimulus [19]. On day 28, the surviving organisms were gently washed with DI water to remove any soil attached to their skin, gently dabbed with paper towel to dry, and weighed. The initial average mass of *E. fetida* in each pot was compared to the final average mass to determine the effects of PFAS on biomass. To evaluate PFAS effect on reproduction, after the surviving earthworms were removed on day 28, the soil was kept for another 28 d. The number of juveniles in each pot was determined thereafter [27].

2.3. Bioaccumulation and molecular-scale effects of PFAS on *E. fetida*

We sacrificed one set of earthworms after 7 d of PFAS exposure to determine the short-term molecular-scale impact of PFAS, while the

other set was analyzed after 28 d (to determine subchronic effects). On both days, we flash-froze surviving earthworms in liquid nitrogen and then lyophilized them using a Labconco Freezone 4.5 (Kansas, MO). PFAS was extracted from a fraction of the lyophilized tissues and quantified via liquid chromatography tandem mass spectrometry (LC-MS/MS). See the details of the tissue PFAS extraction and LC-MS/MS analysis in the [Supporting Information](#) (SI) Section S1 and [Table S1](#). In order to determine the biota-soil accumulation factors (BSAFs), PFAS were also extracted from soil after 7 and 28 d and quantified (see details in Section S2).

For molecular analyses, we performed FTIR spectroscopic analysis on another fraction of lyophilized tissues using a Jasco FT/IR-4700-ATR-PRO ONE (Section S3). Targeted metabolomics was performed using the remaining lyophilized tissue fraction on a gas chromatography-time of flight mass spectrometry (GC-TOF/MS) platform as summarized in Sections S4-S5. We targeted 27 metabolites, including 19 proteinogenic amino acids (lysine, cysteine, phenylalanine, tyrosine, methionine, leucine, aspartate, glutamate, asparagine, glutamine, isoleucine, threonine, glycine, valine, tryptophan, alanine, proline, histidine, and serine), and the main intermediates of the tricarboxylic acid (TCA) cycle (citric acid, isocitric acid, malic acid, pyruvic acid, aconitic acid, succinic acid, fumaric acid, and α -ketoglutarate). The metabolites were selected based on their importance to earthworms. For instance, proteins constitute 11% earthworm tissues on average, only lower than water (69%) and dry matter (16%) ([Fig. S1](#)) [29,30]. Similarly, the TCA cycle is central to many biochemical pathways in respiring organisms [31,32].

2.4. Data and statistical analyses

All (except metabolomics) data were tested for normality and homogeneity of variance using Shapiro-Wilk and Levene methods, respectively. Normality and homogeneity of variance were accepted when $p > 0.05$. Significance of differences among the treatments were determined using ANOVA followed by two-sample Tukey's tests for parametric datasets or Kruskal-Wallis test by rank for non-parametric dataset. These analyses were performed using SPSS Statistics (version 25.0). Metabolomics data were analyzed using MetaboAnalyst 5.0. Data were normalized, transformed, and scaled prior to univariate and multivariate analyses. Univariate and multivariate analyses were performed to identify perturbed metabolites in the tissues of PFAS-exposed *E. fetida*. Twenty metabolites that were identified by ANOVA and Fisher LSD as significantly altered at p and false discovery rate (FDR) < 0.05 were used for biological pathway analysis using the KEGG pathway metabolic library of *Caenorhabditis elegans*. Significantly dysregulated pathways were identified based on enrichment and topology (impact) analyses [33].

3. Results and discussions

3.1. Effect of PFAS on survival and biomass of *E. fetida*

Exposure of *E. fetida* to environmentally relevant concentrations of PFOS or PFOA negatively impacted their survival, but the effects of their binary mixture (PFOS+PFOA) on survival was not additive ([Fig. 1a](#)). Overall, the difference in the number of live organisms between the

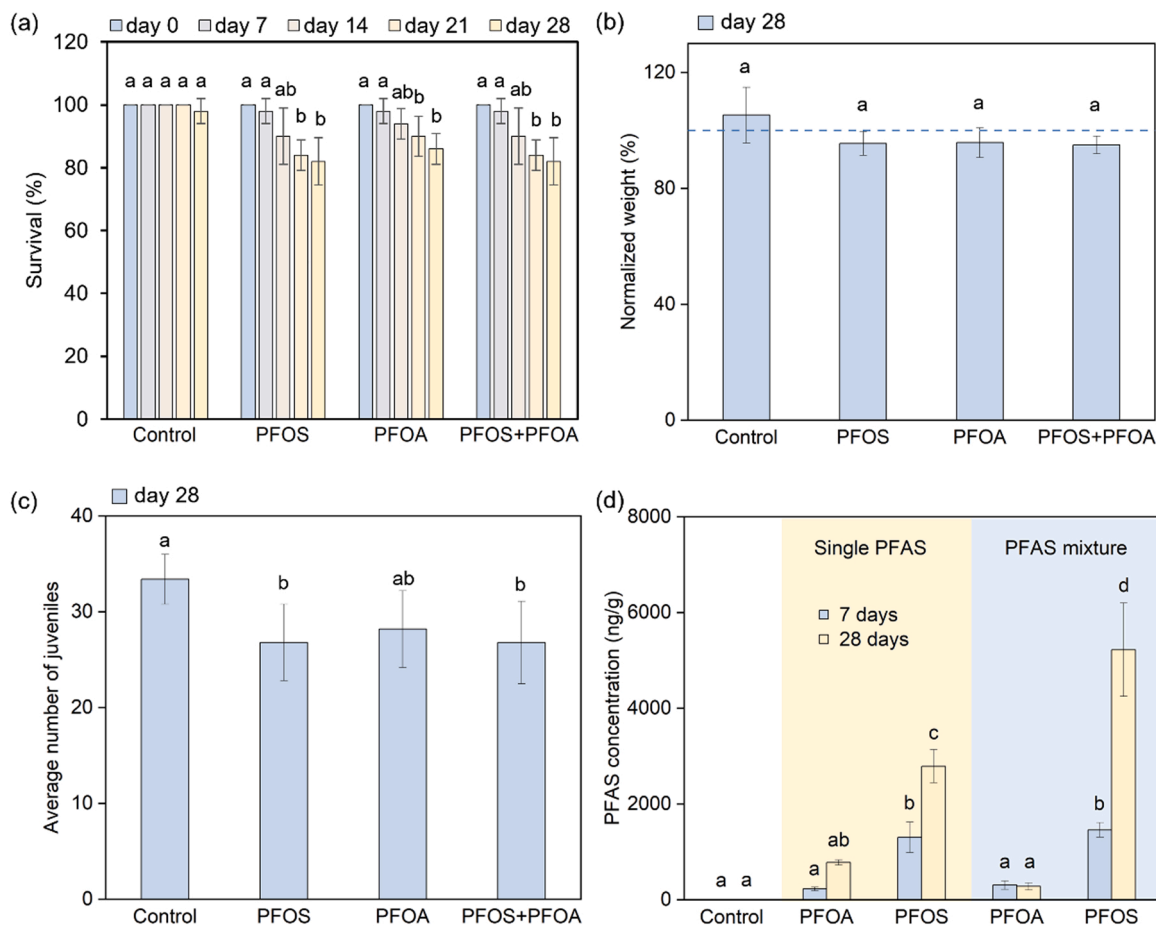


Fig. 1. Phenotypic effects of PFAS on *E. fetida*. (a) Earthworm survival; (b) Normalized weight of *E. fetida* after 28 d. The dash line represents 100%, whereby the day 28 weight equals the initial weight; (c) Average number of juveniles after 28 d; and (d) Concentration of PFAS in *E. fetida* after 7 and 28 d. The same alphabet in each panel indicates no statistical significance at $p < 0.05$; $n = 5$; soil pH = 7.1.

control and PFAS-exposed groups was not significant ($p > 0.05$) until after 14 d, which implies that short-term toxicity studies in soil might underestimate the effects of these PFAS on the survival of *E. fetida*. By day 28, the survival of the PFOS-exposed group ($82 \pm 7.5\%$) was slightly, but not significantly ($p = 0.397$) lower than that of the PFOA-exposed group ($86 \pm 4.9\%$). There was also no significant difference in survival (after 28 d) between organisms exposed to PFOS+PFOA ($82 \pm 7.5\%$) and those exposed to PFOS ($p = 1.000$) or PFOA ($p = 0.397$), despite a much higher total PFAS exposure concentration.

Our finding of minimal mortality of *E. fetida* upon exposure to PFOA or PFOS was expected, considering that the exposure concentration tested was several orders of magnitude below the LC_{50} for the compounds [19,21]. We however expected a substantially higher mortality from exposure to PFOS than to PFOA by day 28 based on reported higher toxicity of PFOS to *E. fetida* (although at higher PFAS exposure concentrations) [19,21]. This is the first study to report the impact of a PFOS+PFOA mixture on the survival of *E. fetida*, and our finding of antagonistic effects agrees with observations in zebrafish at certain PFOS/PFOA ratios [13]. It is necessary to test other ratios of PFOS/PFOA to gain a broader understanding of how interactions between the two compounds in soil possibly influence their impact on soil organisms such as *E. fetida*.

The biomass of surviving *E. fetida* was affected by PFAS exposure. While the average weight of the control group increased by 5.3% after 28 d, it decreased by 4.5% and 4.2% in earthworms exposed to PFOS and PFOA for 28 d, respectively. A stronger impact on biomass would be expected from exposure to the PFOS+PFOA mixture if its effect was additive, but we observed 5% average weight decrease in that condition. Overall, the differences in normalized biomass between the control organisms and the PFAS-exposed groups were not statistically significant ($p = 0.096$, 0.118 , and 0.075 for PFOS-, PFOA-, and PFOA+PFOS-exposed group, respectively), as shown in Fig. 1b. This finding agrees with the observation of Joung et al. who reported a slight (11.7–17.7%) weight loss in *E. fetida* exposed to PFOA or PFOS for 14 d, although weight loss was also observed in the control group in that study [19]. The mortality and loss of biomass we observed in this study suggests that *E. fetida* experienced some stress by the presence of low levels of PFAS in the soil.

3.2. Effect of PFAS on reproduction of *E. fetida*

Earthworms are at the base of the food chain in soil, serving as food for rodents, birds, snakes, frogs, etc. [4,20]. Therefore, a decrease of earthworm's population has important ecological impact in terrestrial communities. In addition to their impact on survival, the effect of PFAS on *E. fetida* population was also assessed using reproduction as an endpoint. The average number of juveniles decreased significantly from 33.4 ± 2.6 (in control) to 26.8 ± 4.0 ($p = 0.024$) in PFOS-exposed soil (Fig. 1c). The decrease in the average number juveniles observed in PFOA-exposed soil (28.2 ± 4.0) was however not significantly different from the control ($p = 0.059$) or PFOS-exposed ($p = 0.631$) group. The effect of PFOS+PFOA did not seem additive based on the average number of juveniles, 26.8 ± 4.3 , which although was statistically ($p = 0.029$) lower than in control group, was not statistically different from the PFOS-exposed ($p = 0.917$) or PFOA-exposed ($p = 0.231$) groups.

We normalized the number of juveniles in each treatment by the number of surviving adult earthworms, to see if the decreased juveniles in the PFAS-exposed groups was mainly due to decreased survival of the adult earthworms or if the fecundity of the surviving earthworms was affected by PFAS exposure. We found that decreased juvenile in the soils contaminated with PFAS was primarily due to decreased survival: Average number of juveniles/surviving adult in the PFOS- (3.26 ± 0.33 ; $p = 0.488$), PFOA- (3.29 ± 0.50 ; $p = 0.679$), and PFOS+PFOA-exposed (3.26 ± 0.35 ; $p = 0.486$) groups was not significantly different from the control (3.41 ± 0.23), as shown in Fig. S2. The average juvenile/

surviving adult decreased slightly with PFAS exposure in the conditions tested in this study, and it remains to be seen if the decrease will become significant at higher exposure concentrations and/or time.

3.3. Bioaccumulation of PFAS in *E. fetida*

Accumulation of PFAS in earthworms is important as it can lead to biomagnification of the compounds in organisms at higher trophic levels. After 7 d of exposure to PFAS through soil, we detected a slightly, but insignificantly ($p = 0.397$) higher average PFOS concentration in *E. fetida* exposed to the PFOS+PFOA mixture (1460.0 ± 168.8 ng/g-dw) than those in soil contaminated with only PFOS (1303.0 ± 355.1 ng/g-dw), as shown in Fig. 1d. Similarly, the PFOA body burden of *E. fetida* was slightly, but insignificantly ($p = 0.134$) higher in organisms exposed to PFOS+PFOA (307.4 ± 98.0 ng/g-dw) than those only exposed to PFOA (228.3 ± 40.1 ng/g-dw). The higher bioaccumulation of both PFAS (and total PFAS) in *E. fetida* exposed to PFOS+PFOA did not result in substantially higher negative impacts (discussed in Sections 3.1 and 3.2). The higher bioaccumulation of PFOS than PFOA in both individual and mixture exposures agrees with our expectation based on reported higher uptake rate and lower elimination rate of PFOS compared to PFOA [26]. The average BSAFs (in kg-OC/kg-dw) for PFOS and PFOA after 7 d (0.206 ± 0.027 and 0.047 ± 0.010 , respectively) was well below the average reported for the PFAS in earthworms (1.113 and 0.275, respectively) [4].

In the individual compound exposure scenario, the concentration of PFOS in *E. fetida* increased by a factor of 2.1 by day 28 (compared to day 7) to 2790.7 ± 388.3 ng/g-dw while the PFOA body burden increased by a factor of 3.4 to 780.2 ± 61.2 ng/g-dw. Interestingly, we found a significantly ($p = 0.002$) higher PFOS concentration in the tissues of *E. fetida* exposed to the PFOS+PFOA mixture for 28 d (PFOS = 5224.9 ± 974.7 ng/g-dw) than when the organism was exposed to only PFOS for the same time (PFOS = 2790.7 ± 347.3 ng/g-dw). In contrast, the PFOA body burden was significantly ($p = 0.0004$) lower in *E. fetida* exposed to the PFOS+PFOA mixture for 28 d (PFOA = 280.5 ± 74.0 ng/g-dw) than when exposed to only PFOA for 28 d (PFOA = 780.2 ± 54.8 ng/g-dw). There was no significant ($p = 0.22$) difference between *E. fetida*'s PFOA body burden on days 7 and 28 when exposed to PFOS+PFOA mixture, which suggests that PFOA net bioaccumulation from soils contaminated with the mixture stopped before or around 7 d. The average day 28 PFOS BSAFs (in kg-OC/kg-dw) increased from 0.595 ± 0.051 in PFOS-only group to 1.027 ± 0.150 in the mixture while that of PFOA decreased from 0.159 ± 0.009 in PFOA-only group to 0.052 ± 0.012 in the mixture.

The reason for the differences in PFAS bioaccumulation in *E. fetida* when exposed to mixture vs. individual compounds is unclear, but we suspect it may partly have to do with changes in the partitioning behavior of PFOA and PFOS when mixed. In a recent study we observed increased adsorption of perfluoroalkyl carboxylic acids (e.g., PFOA) to plastic surfaces when present in a PFAS mixture than when in solution by themselves, while the contrary was true for perfluoroalkyl sulfonic acids (such as PFOS) [28]. Decreased adsorption of PFOS to solid surfaces (such as the pot and soil) when present in a mixture would lead to a higher bioavailability to *E. fetida*, and thus a higher uptake. To test the hypothesis that the partitioning of PFAS changes when in a mixture compared to when in individual solutions, we determined the soil distribution coefficient (K_d) of PFOA and PFOS using (1) individual solutions of PFOS and PFOA, and (2) a mixture of equal masses of PFOS and PFOA (see details in Section S6). As shown in Fig. S3, the K_d of PFOA increased by 4% from an average of 2.06 L/kg in individual PFOA system to 2.14 L/kg when present in the mixture. In contrast, the K_d of PFOS decreased by 12% from 8.58 L/kg when by itself to 7.55 L/kg when present in the mixture. Although neither of these changes in K_d was statistically significant ($p > 0.05$), we believe they partly contributed to higher bioaccumulation of PFOS and lower bioaccumulation of PFOA in *E. fetida* exposed to the PFOS+PFOA mixture.

3.4. Effect of PFAS on the tissue biomolecules of *E. fetida*

Exposure to toxicants often changes the biochemical profile of organisms; hence, FTIR analysis of tissues or organisms can be used as a biomarker for assessing toxicity [34,35]. We analyzed the tissues of surviving *E. fetida* (5 replicates per treatment) on days 7 and 28 with FTIR in order to see if their exposure to PFAS resulted in any biomolecular changes. A typical FTIR spectrum and band identification is shown in Fig. 2a and Table S2, respectively [36,37]. It should be noted that each spectrum was transformed to clearly see changes in molecular fingerprints of the tissues (Fig. S4). Principal component analysis (PCA) of the transformed spectra of the tissues obtained after 7 d revealed no separation between the control and the PFAS-exposed *E. fetida* tissues obtained after 7 d (Fig. 2b), which indicates that PFAS had limited effects after 7 d.

Unlike the 7-d samples, however, we observed a clear separation between the control and all the PFAS-exposed groups (along PC 1) when the whole body tissues obtained after 28 d were analyzed (Fig. 2c). Two PCs cumulatively accounted for 61.5% of the total variance with the PC 1 explaining 43.9% of the total variance and PC 2 explaining 17.6%. Considering the 95% confidence range, although the PFOS-, PFOA-, and PFOS+PFOA-exposed groups overlapped, the PFOS-exposed tissue was clearly different from the other two PFAS treatments along the PC 2. This suggests that the (mechanism of the) effect of PFOS on the biomolecules of *E. fetida* may be different from that of PFOA and PFOS+PFOA, both of which may be similar. In addition, the result suggests that PFOS+PFOA

likely has no additive molecular effects on *E. fetida*. The absence of biomolecular effects of PFAS on *E. fetida* after 7 d of exposure, despite their bioaccumulation, agrees with our observation of no significant phenotypic impact on the organism after the same time. Similarly, there were clear molecular and phenotypic effects after 28 d of PFAS exposure, showing that acute toxicity studies may not adequately capture the effects of PFAS on *E. fetida*.

To get a better understanding of the underlying causes of differentiation in the tissues of *E. fetida* tissue after 28 d, we determined the bands (wavenumbers) with the highest square loadings, which represent the features that are altered and mainly responsible for the observed separation along the PCs [38]. We found that the bands/peaks around 1150–1400 cm^{-1} (Amide III/ PO_2^-) and 1500–1800 cm^{-1} (Amide I/Amide II) were predominantly responsible for the separation between control and PFAS-exposed organisms (Fig. S5). This result can also be visualized with the loading plot (Fig. 2d), in which the light blue and deep red dots (which represent bands at 1200–1800 cm^{-1}) were concentrated at the extremes (higher loading/more important) of the PC 1 while the darker blue dots (which represent bands <1200 cm^{-1}) were closer to the center of PC 1 (lower square loading/less important). This finding of alteration of amide bands by PFAS further underscores the need to target proteinogenic amino acids for targeted metabolomics.

3.5. Effect of PFAS on targeted metabolites of *E. fetida*

Metabolites are the end-products of cellular regulatory processes. As

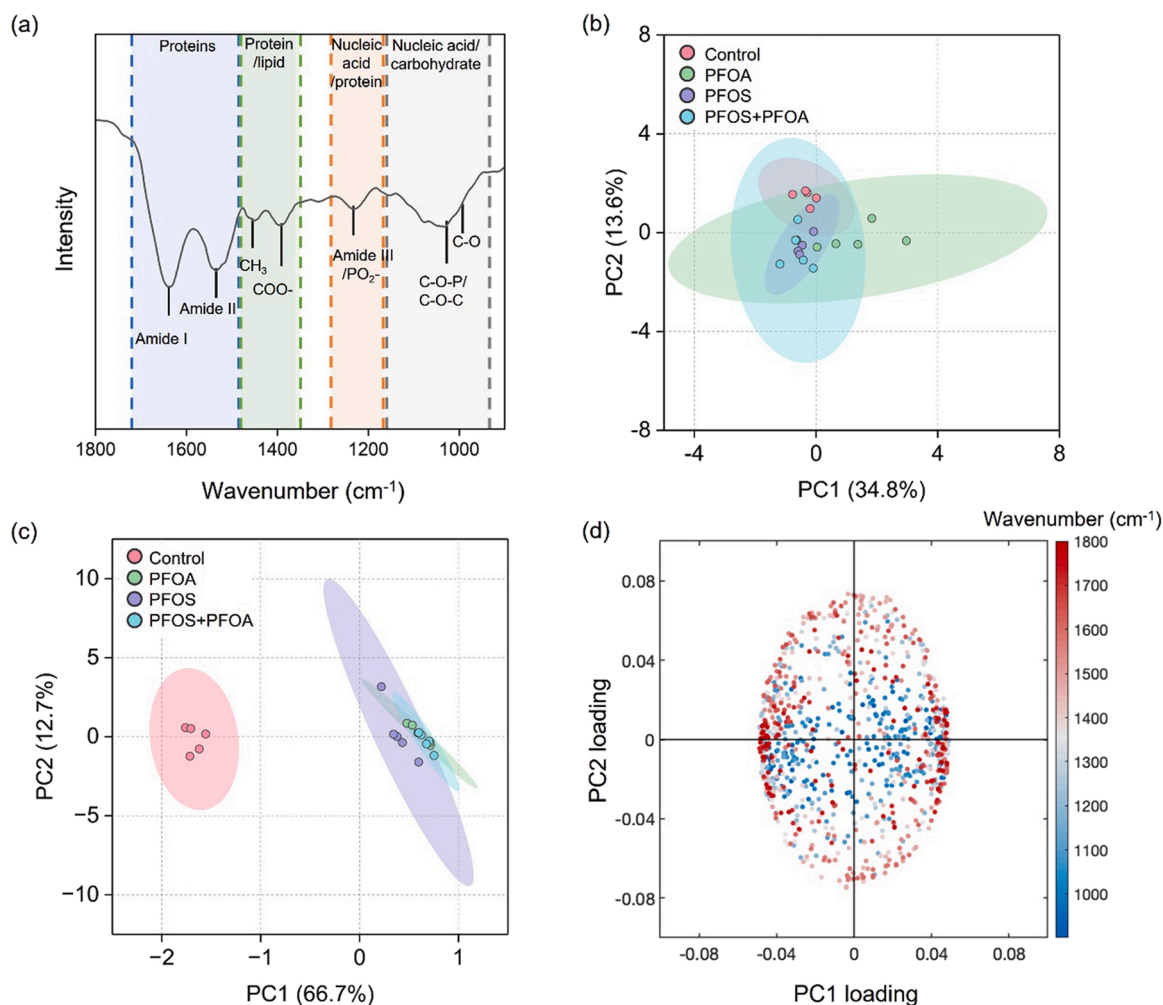


Fig. 2. (a) A typical FTIR spectrum and peak identification of *E. fetida* tissue. PCA score plots of the transformed FTIR spectra of *E. fetida* tissues obtained after (b) 7 d and (c) 28 d. (d) PCA loading plot of the day 28 FTIR *E. fetida* tissue spectra. The ellipses in the PCA score plots represent the 95% confidence range; $n = 5$.

a result, they offer insight into the effects of stressors on organisms, and reflect the (adaptive and adverse) response of organisms to stresses [25, 39,40]. Metabolite profiling has been used to study the impact of PFAS in plant and animal models [5,39,41]. Based on *t* test, none of the 27 metabolites quantified was significantly ($p > 0.05$) altered when *E. fetida* was exposed to PFAS for 7 d (compared to control). After 28 d of exposure, 0, 21, and 22 metabolites were significantly (p and FDR < 0.05) affected by exposure to PFOS, PFOA, and PFOS+PFOA, respectively (Tables S3–S5). As shown in Tables S3 and S5, alanine and tyrosine (in PFOS-treated group) and serine (in PFOS+PFOA-treated group) had $p < 0.05$ but FDR > 0.05 and were overall considered insignificant. Similarly, fold change analysis (cutoff was set at 1.5; see Tables S6–S8) and volcano plots (cutoff was set at 1.5; see Fig. S6–S8) revealed disrupted metabolites after 28 d of exposure and largely agreed with *t* test (that is, PFOA and PFOS+PFOA have similar, stronger effect than PFOS).

When we compared the metabolites obtained on day 28 from the control and PFAS-exposed organisms using ANOVA, 20 out of the 27 targeted metabolites were significantly (p and FDR < 0.05) different (Figs. 3 and S9 and Table S9). Of the 20 differentiated metabolites, 16 (80% of total altered) were altered by both PFOA and PFOS+PFOA in a statistically similar manner (which was not observed in the PFOS-exposed group), showing the dominance of PFOA in the metabolic effects of the PFOS+PFOA mixture. Some of the metabolites (amino acids) that were upregulated by the exposure of *E. fetida* to PFOA and PFOS+PFOA (and not PFOS)—such as tryptophan, methionine, glycine, lysine, and tyrosine—are non-enzymatic antioxidants [42,43]. This suggests that subchronic PFOA exposure likely caused oxidative stress in *E. fetida*. PFOS significantly affected only 3 metabolites: glutamate, succinic acid, and aconitic acid. Overall, the impact of PFOS on the metabolic profile of *E. fetida* was minimal, and did not align with the impact of the PFOS+PFOA mixture.

Upregulation of glutamate and succinic acid when *E. fetida* was exposed to PFOS, which we observed in this study, was also reported by Lankadurai et al. when they exposure *E. fetida* to PFOS [44]. In another study by the same group, significant upregulation of glutamate was also observed when *E. fetida* was exposed to PFOS (which is similar to our observation in this study) and PFOA (which is contrary to our results) [17]. Upregulation of amino acids like tyrosine, lysine, phenylalanine, methionine, leucine, glycine, and valine was reported by Kim and workers when *C. elegans* was exposed to PFOA, similar to what we observed after 28 d exposure of *E. fetida* to PFOA and PFOS+PFOA [45]. We were unable to compare our PFOS+PFOA findings to existing studies as the only other earthworm-based metabolomics study of PFAS mixture did not report the effects of the mixture on specific metabolites [17].

Multivariate analyses, PCA and hierarchical clustering heatmaps, revealed that the metabolites from the tissues of *E. fetida* groups exposed to PFAS for 7 d did not differ from the control group (Fig. S10). On the other hand, the PCA results obtained after 28 d of exposure to PFAS (Fig. 4a) revealed a complete overlap of PFOA- and PFOS+PFOA-exposed groups, which were both separated from the control and PFOS-exposed groups. Most of the replicates in the control and PFOS-exposed groups overlapped, which contrasts with the observation of Lankadurai and coworkers who found a clear separation of both PFOA- and PFOS-exposed *E. fetida* from the control in their 2-d contact tests [17]. The separation of the PFOA- and PFOS+PFOA-exposed groups from the PFOS-exposed and control groups observed in our study is clearly illustrated in the 3D score plot between PCs 1, 2, and 3 (Fig. 4b). Four PCs cumulatively accounted for 92.8% of the total variance, with the PC 1 explaining 76.2% of the total variance and PC 2 explaining 10.3%.

Similarities in the impact of exposure to PFOA and PFOS+PFOA for 28 d were confirmed by hierarchical clustering heatmaps, which showed that metabolites of both PFOA- and PFOA+PFOS-exposed earthworms were similarly altered, while those from the control group and PFOS-exposed groups were largely similar (Fig. 4c). Overall, the multivariate analyses of the days 7 and 28 FTIR spectroscopy and metabolomics data agree: While there were no separations in the tissue biomolecules

obtained from day 7 tissues, PCA analysis of day 28 FTIR spectra suggested similarities in the effects of PFOA and PFOS+PFOA exposure on the biomolecules of *E. fetida* (Fig. 2c). These similarities were confirmed by chemometric analysis of targeted metabolites. As a result, we concluded that the molecular-level effects of the PFOS+PFOA mixture was mainly caused by PFOA, despite its lower bioaccumulation in the tissue of *E. fetida* when present in the mixture. Thus, we rejected our hypothesis that PFOS will dominate any observed perturbation in the macromolecules/metabolites of *E. fetida*'s tissues despite its higher bioaccumulation potential.

3.6. Effect of PFAS on biological pathways of *E. fetida*

Based on a combination of enrichment and topology (impact) analyses of significantly altered day 28 metabolites, identical pathways were dysregulated by PFOA (Fig. S11a) and PFOS+PFOA (Fig. S11b). The pathways affected are related to amino acid, energy, and sulfur metabolisms (Fig. 5). Amino acids are converted to pyruvate, α -ketoglutarate, succinate, fumarate, and oxaloacetate, which are intermediate substrates in the TCA cycle [46]. It is thus not surprising that PFOA and PFOS+PFOA disrupted pathways related to both amino acid and energy metabolism. The impact of PFOS on the biological pathways of *E. fetida* was minimal (as shown by the very low scores from enrichment analysis) and different from those of PFOA and PFOS+PFOA (Fig. S11c). The result of pathway analysis further strengthens the conclusion that PFOA dominates the molecular-level impact of the PFOS+PFOA mixture to *E. fetida* in soil.

3.6.1. Amino acid biosynthesis and metabolism

Exposure of *E. fetida* to PFAS for 28 d significantly altered 12 out of the 19 proteinogenic amino acids that were quantified (Fig. 3; Table S8). These amino acids are involved in a wide range of essential physiological activities, and their disruption could indicate dysregulation of proteins and enzymes, DNA damage, and oxidative stress [47,48]. As shown in Fig. S11, the specific amino acid-related pathways that were affected include phenylalanine, tyrosine and tryptophan biosynthesis (by exposure to PFOA, PFOS+PFOA, and less likely, PFOS); D-glutamine and D-glutamate metabolism (by exposure to PFOA, PFOS+PFOA, and less likely, PFOS); tyrosine metabolism (by exposure to PFOA and PFOS+PFOA); and alanine, aspartate and glutamate metabolism (by exposure to PFOA and PFOS+PFOA).

In the phenylalanine, tyrosine and tryptophan biosynthesis pathway, phenylalanine, which is synthesized from phenylpyruvate (Fig. 5), was upregulated when *E. fetida* was exposed to PFOA and PFOS+PFOA. Phenylalanine is a precursor for tyrosine, which was also upregulated (Fig. 3). Tyrosine is a precursor for catecholamines (dopamine, norepinephrine, and epinephrine), which are produced by organisms in response to physical stress [49]. Therefore, the upregulation of the amino acids in the phenylalanine, tyrosine and tryptophan biosynthesis upon exposure to PFAS suggests that *E. fetida* may have produced endogenous metabolites that help it to cope with stress arising from the elevated PFAS exposure. In addition to catecholamines, tyrosine is also a precursor for other important metabolites, including acetoacetic acid, pyruvic acid, and fumaric acid, which are intermediates in the TCA cycle. Upregulation of the tyrosine metabolism pathway may also be related to need for energy metabolism upregulation due to PFAS exposure [31,48]. Conversely, the alanine, aspartate and glutamate metabolism pathway, which also produces intermediate metabolites in the TCA cycle (fumaric acid, pyruvic acid, and succinic acid) was down-regulated by PFOA and PFOS+PFOA exposure.

D-glutamine and D-glutamate metabolism was impacted by the presence of PFOA and (possibly) PFOS, individually and in the mixture (Fig. S11). Glutamine stimulates protein synthesis and inhibits protein degradation [50]. The metabolism of glutamine to produce glutamate and α -ketoglutarate was negatively impacted by the exposure of *E. fetida* to PFOA and PFOS+PFOA. Interestingly, glutamate synthesis from

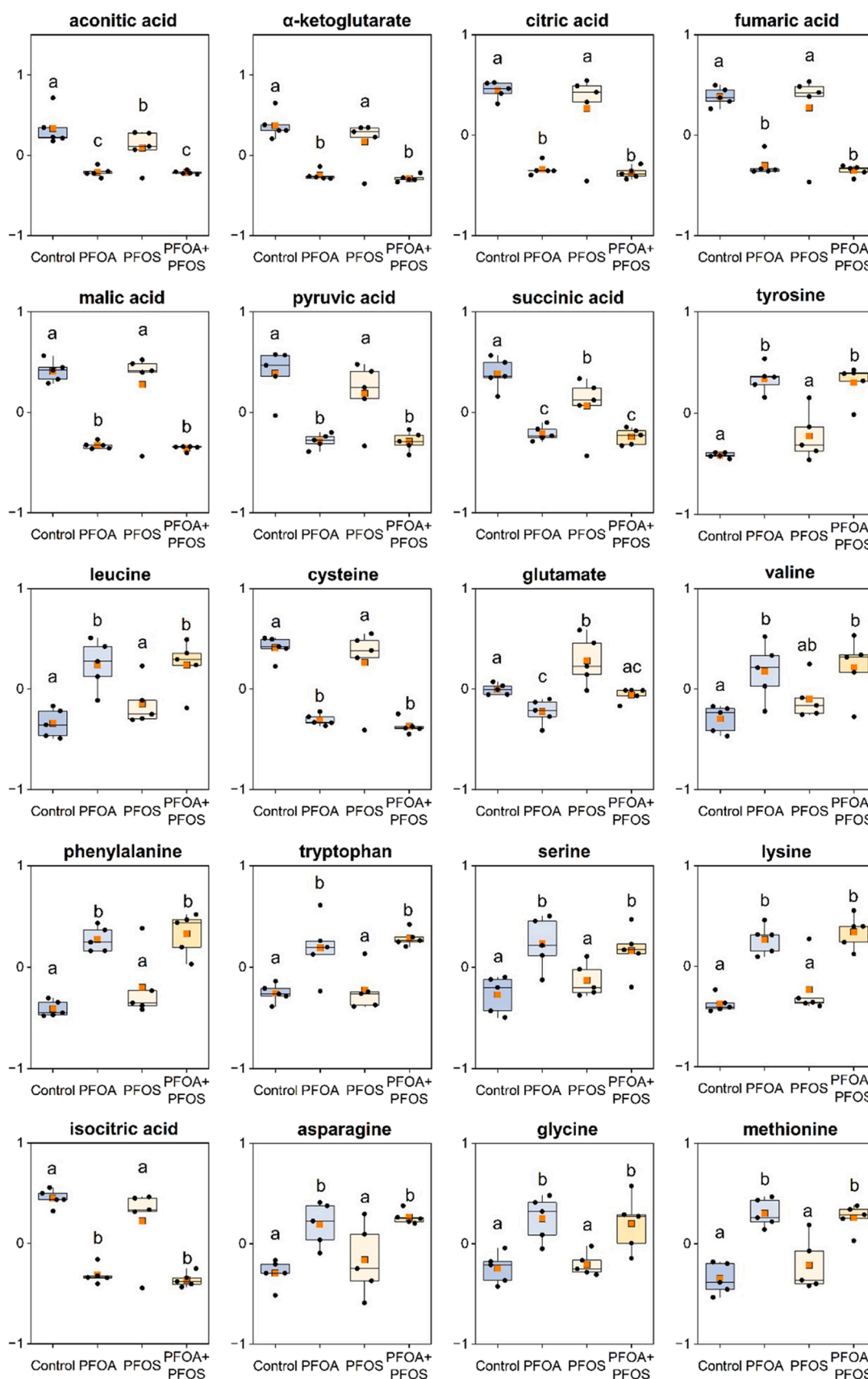


Fig. 3. Boxplot of relative abundance of significantly affected metabolites when *E. fetida* was exposed to PFOS, PFOA, and PFOS+PFOA for 28 d. The black dots represent individual concentrations of metabolites while the black horizontal lines within the boxes represents the median concentration. The mean concentration of each group is indicated with an orange square while the notches indicate the 95% confidence intervals around the median. Letters represent significant difference at $p < 0.05$; $n = 5$.

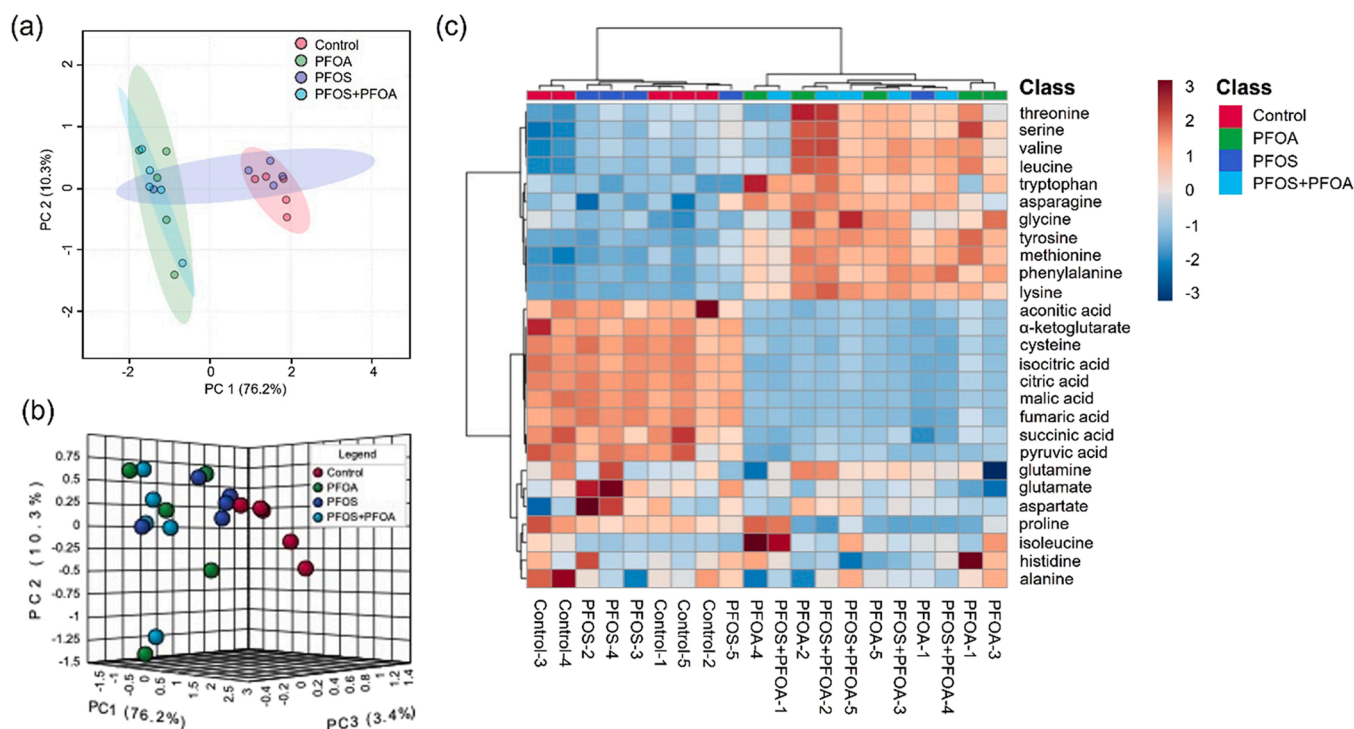


Fig. 4. (a) PCA score plot; (b) 3D score plot between PCs 1, 2, and 3; and (c) hierarchical cluster analysis heatmap for metabolites extracted from *E. fetida* for 28 d. The ellipses in the PCA score plots represent the 95% confidence range; $n = 5$.

glutamine was upregulated in the presence of PFOS, which is similar to the effect of PFOS on the glutamate of Northern leopard frog larvae's brain at GS 46 (metamorphosis) [51]. We however found no significant impact on α -ketoglutarate abundance by PFOS. In contrast, PFOA and PFOS+PFOA downregulated α -ketoglutarate, a key metabolite that determines the overall rate of the TCA cycle in organisms [50]. In addition, α -ketoglutarate is also a source of glutamate. Glutamate is one of the primary excitatory neurotransmitters in the central nervous system, and it undergoes oxidative degradation in neurons and astroglia under stress conditions [52,53]. Perturbed glutamate level was found to induce neurotoxicity in *C. elegans* [52]. The upregulation of glutamate in *E. fetida* by PFOS may have enhanced the arginine biosynthesis pathway, and the arginine and proline metabolism pathway, but their upregulation was not statistically significant (and not further explored in this study).

3.6.2. Carboxylic acid metabolism

The pathways dysregulated under this class—the TCA cycle, and glyoxylate and dicarboxylate metabolism—are both related to energy metabolism [43]. There were no significant differences in the levels of the TCA cycle intermediates in *E. fetida* that were exposed to PFOS for 28 d (relative to control), except for succinic acid that was downregulated (Fig. 3). In contrast, all the TCA cycle intermediates targeted in this study were significantly ($p < 0.05$) downregulated after 28 d of exposure to PFOA and PFOS+PFOA. The consistency in the downregulation of all the TCA cycle metabolites is surprising because several of the metabolites can be synthesized through multiple pathways, some of which we found were upregulated. For instance, pyruvic acid and fumaric acid can be produced through the tyrosine metabolism pathway. However, the upregulation of tyrosine (reported earlier) did not compensate for the low levels of pyruvic acid and fumaric acid. It is likely that the dysregulation of the TCA cycle originated from stress response and the need to maintain homeostasis [43]. Upregulation of amino acids, such as tyrosine, phenylalanine, leucine, and valine, was likely to offset the need for energy metabolism as earthworms experiencing oxidative stress may not obtain sufficient energy from

carbohydrates and fats [48].

Glyoxylate and dicarboxylate metabolism has similar intermediates as the TCA cycle so it is unsurprising that it was dysregulated by the exposure of *E. fetida* to PFOA and PFOS+PFOA. All the quantified metabolites that are associated with this pathway (aconitic acid, glutamate, citric acid, isocitric acid, and succinic acid) were downregulated upon exposure of *E. fetida* to PFOA and PFOS+PFOA, except glycine, which was upregulated. The glyoxylate and dicarboxylate metabolism pathway encompasses several reactions, including the glyoxylate cycle (in which fatty acids are converted to carbohydrates for energy production) and propanoyl-CoA metabolism (in which propanoyl-CoA is converted to succinyl-CoA, an important intermediate in the TCA cycle).

3.6.3. Sulfur metabolism

Sulfur metabolism was impacted by exposure to PFOA and PFOS+PFOA, which led to a significant downregulation of cysteine, a thiol containing proteinogenic amino acid. Cysteine is biosynthesized from serine and methanethiol (from the methionine metabolism pathway). Interestingly, both methionine and serine were upregulated by the exposure of *E. fetida* to PFOA and PFOS+PFOA. Downregulation of cysteine is therefore due to its faster catabolism than anabolism when *E. fetida* was exposed to PFOA and PFOS+PFOA.

The cysteine thiol group is nucleophilic and easily oxidized, which gives cysteine strong antioxidant properties [42]. In fact, cysteine is more easily oxidized than most other amino acids, and the oxidation may lead to ATP production (energy conservation). Cysteine is also an important precursor for the antioxidant, glutathione, which can prevent damage to cells caused by reactive oxygen species (ROS), free radicals, and peroxides. More so, cysteine is important for pantothenate and coenzyme A (CoA) biosynthesis, and pyruvic acid synthesis. Pantothenate is a precursor for the synthesis of CoA, which plays an important role in the synthesis of phospholipids, the synthesis and degradation of fatty acids, and the TCA cycle. Pyruvic acid is also an important intermediate in the TCA cycle (for energy production), and it is important in the synthesis of amino acids such as alanine. Elevated catabolism of cysteine in the whole-body tissue of *E. fetida* due to PFOA and PFOS+PFOA

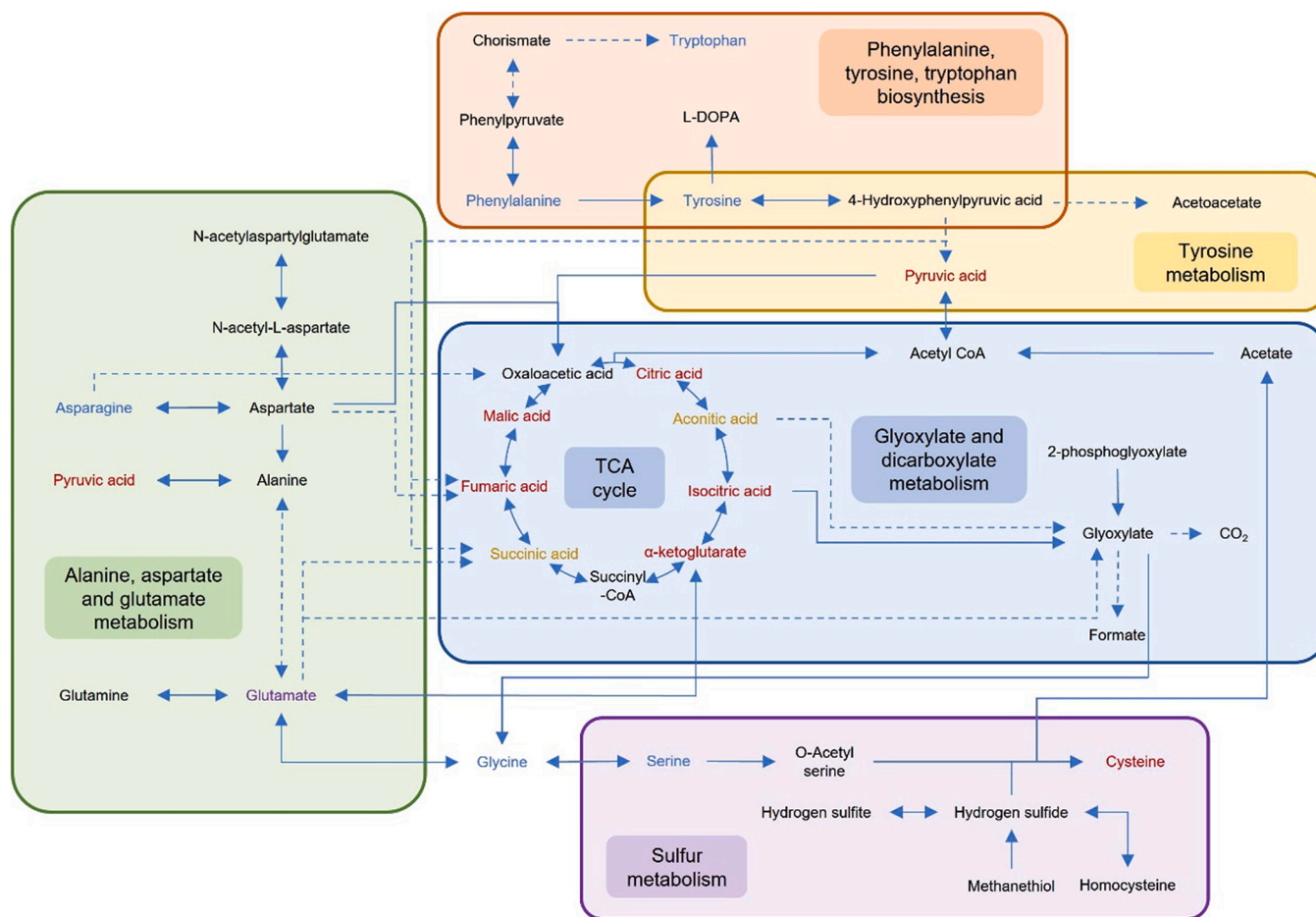


Fig. 5. Main pathways and relevant metabolites dysregulated by exposure of *E. fetida* to PFAS for 28 d. The dotted arrows mean multi-steps while the solid arrows mean one step between two metabolites. The double-side arrows indicate bidirectional while the one-way arrows indicate unidirectional transformation. Metabolites with blue font color were upregulated in PFOA- and PFOS+PFOA-exposed groups; metabolites with red font color were downregulated in PFOA- and PFOS+PFOA-exposed groups; metabolites with yellow font color were downregulated in PFOA-, PFOS- and PFOA+PFOS-exposed groups; and metabolites with purple font color were upregulated in PFOS-exposed group but downregulated in PFOA-exposed group.

exposure was therefore likely a response (directly or indirectly through glutathione synthesis) to oxidative stress, which was discussed earlier. We also cannot rule out the possibility of heightened cysteine catabolism as a response to the dysregulated TCA cycle. This may be to produce CoA and/or pyruvic acid, which both play important roles in the cellular energy metabolic process.

3.7. Conclusion and limitations

We exposed earthworms to PFOA and PFOS—the two most detected PFAS in background soils—as individual compounds and an equal-mass mixture. There was no significant phenotypic or molecular-level impact in the first 7 days of exposure, showing that acute studies may not be adequate for the risk assessment of environmentally relevant concentrations of PFAS. By day 28, however, substantial negative impacts were observed. Surprisingly, the impact of the PFAS mixture was antagonistic. More surprisingly, bioaccumulation of PFOS and PFOA in earthworm was strikingly different when the same concentration was introduced to soil as individual compounds compared to when introduced as a mixture: The average biota-soil accumulation factors (BSAFs, in kg-OC/kg-dw) calculated after 28 d of exposure increased from 0.595 ± 0.051 in PFOS-only group to 1.027 ± 0.150 in the mixture while that of PFOA decreased from 0.159 ± 0.009 in PFOA-only group to 0.052 ± 0.012 in the mixture.

Co-occurrence of PFOA and PFOS in an equal-mass mixture led to a slight (4%) increase in the K_d of PFOA and a substantial (12%) decrease

in the K_d of PFOS compared to when each PFAS was present in soil alone. The magnitude of this mixture effect on PFAS K_d will likely vary with the molar ratio of the compounds in the mixture. In any case, we believe that the mixture impact on K_d played an important role in the stark differences we observed in PFOS and PFOA bioaccumulation when *E. fetida* was exposed to the mixture compared to the individual compounds. Therefore, the existence of PFAS in mixtures may be important enough to influence their fate and effects in soils, and potentially plays a role in the success of different remediation strategies. PFAS studies should increasingly consider mixtures rather than individual compounds.

PFOA dominated the effects of the mixture on the metabolic profile of *E. fetida*. It is essential to note that this finding cannot be generalized for all PFAS or even PFOS and PFOA mixtures in soil because the molar ratio of the compounds that make up the mixture will likely determine the type and extent of mixture effects. As shown here, PFAS bioaccumulation in earthworms (and perhaps other organisms) in the natural environment where PFAS occur as mixtures cannot be accurately predicted based on partitioning coefficients obtained from individual compounds. A mechanistic understanding of the interactions of PFAS in mixtures, and how those interactions influence their partitioning among different phases is therefore required.

CRediT authorship contribution statement

Ziwei Han: Methodology, Data curation, Investigation, Validation, Formal analysis, Writing – original draft, Writing – review & editing.

Bolaji F. Oyeyemi: Data curation, Investigation, Resources, Software, Validation, Writing – original draft. **Jenny E. Zenobio:** Conceptualization, Methodology, Investigation. **Omobayo A. Salawu:** Methodology, Investigation, Validation. **Adeyemi S. Adeleye:** Conceptualization, Methodology, Supervision, Project administration, Resources, Software, Writing – original draft, Writing – review & editing.

Environmental Implication

PFAS are persistent, bioaccumulative, and toxic. Most existing toxicity studies considered the environmental impact of one PFAS, using phenotypic endpoints. However, PFAS typically exist in soil in mixtures and at sub-lethal concentrations. In this study, we investigated the subchronic impact of a mixture of PFOS and PFOA (the most detected PFAS in soil) on earthworms, using phenotypic and molecular endpoints. We compared the impacts of the binary PFAS mixture to those of the individual compounds at similar exposure concentrations and showed the importance of each PFAS in the mixture.

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. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jhazmat.2023.131718](https://doi.org/10.1016/j.jhazmat.2023.131718).

References

- [1] Buck, R.C., et al., 2011. Perfluoroalkyl and polyfluoroalkyl substances in the environment: terminology, classification, and origins. *Integr Environ Assess Manag* 7 (4), 513–541.
- [2] Schultes, L., et al., 2018. Per- and polyfluoroalkyl substances and fluorine mass balance in cosmetic products from the Swedish market: implications for environmental emissions and human exposure. *Environ Sci: Process Impacts* 20 (12), 1680–1690.
- [3] Glüge, J., et al., 2020. An overview of the uses of per- and polyfluoroalkyl substances (PFAS). *Environ Sci: Process Impacts* 22 (12), 2345–2373.
- [4] Burkhard, L.P., Votava, L.K., 2023. Review of per- and polyfluoroalkyl substances (PFAS) bioaccumulation in earthworms. *Environ Adv* 11, 100335.
- [5] Ofoegbu, P.C., et al., 2022. Impacts of perfluorooctanesulfonic acid on plant biometrics and grain metabolomics of wheat (*Triticum aestivum* L.). *J Hazard Mater Adv* 7, 100131.
- [6] Brusseau, M.L., Anderson, R.H., Guo, B., 2020. PFAS concentrations in soils: background levels versus contaminated sites. *Sci Total Environ* 740, 140017.
- [7] Wang, Q., et al., 2018. Occurrence and distribution of perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) in natural forest soils: a nationwide study in China. *Sci Total Environ* 645, 596–602.
- [8] Söregård, M., et al., 2022. Spatial distribution and load of per- and polyfluoroalkyl substances (PFAS) in background soils in Sweden. *Chemosphere* 295, 133944.
- [9] Moody, C.A., Field, J.A., 2000. Perfluorinated surfactants and the environmental implications of their use in fire-fighting foams. *Environ Sci Technol* 34 (18), 3864–3870.
- [10] Hoskins, T.D., et al., 2022. An environmentally relevant mixture of perfluorooctanesulfonic acid and perfluorohexanesulfonic acid does not conform to additivity in northern leopard frogs exposed through metamorphosis. *Environ Toxicol Chem* 41 (12), 3007–3016.
- [11] Bursian, S.J., et al., 2021. The subacute toxicity of perfluorooctane sulfonate and/or perfluorooctanoic acid and legacy aqueous film-forming foams to Japanese quail (*Coturnix japonica*) chicks. *Environ Toxicol Chem* 40 (3), 695–710.
- [12] Flynn, R.W., et al., 2019. Acute and chronic effects of perfluoroalkyl substance mixtures on larval American bullfrogs (*Rana catesbeiana*). *Chemosphere* 236, 124350.
- [13] Ding, G., et al., 2013. Combined Effects of PFOS and PFOA on Zebrafish (*Danio rerio*) Embryos. *Arch Environ Contam Toxicol* 64 (4), 668–675.
- [14] Dennis, N.M., et al., 2021. Species- and tissue-specific avian chronic toxicity values for perfluorooctane sulfonate (PFOS) and a binary mixture of PFOS and perfluorohexane sulfonate. *Environ Toxicol Chem* 40 (3), 899–909.
- [15] Menger, F., et al., 2020. Behavioural effects and bioconcentration of per- and polyfluoroalkyl substances (PFASs) in zebrafish (*Danio rerio*) embryos. *Chemosphere* 245, 125573.
- [16] Yang, H.-B., et al., 2019. Antioxidant defence system is responsible for the toxicological interactions of mixtures: a case study on PFOS and PFOA in *Daphnia magna*. *Sci Total Environ* 667, 435–443.
- [17] Lankadurai, B.P., Simpson, A.J., Simpson, M.J., 2012. ¹H NMR metabolomics of *Eisenia fetida* responses after sub-lethal exposure to perfluorooctanoic acid and perfluorooctane sulfonate. *Environ Chem* 9 (6), 502–511.
- [18] Sobhani, Z., et al., 2021. Microplastics as a vector of toxic chemicals in soil: enhanced uptake of perfluorooctane sulfonate and perfluorooctanoic acid by earthworms through sorption and reproductive toxicity. *Environ Technol Innov* 22, 101476.
- [19] Joung, K.-E., et al., 2010. Toxicological effects of PFOS and PFOA on earthworm, *Eisenia fetida*. *Environ Anal Health Toxicol* 25 (3), 181–186.
- [20] Han, Z.W., et al., 2021. Emerging investigator series: immobilization of arsenic in soil by nanoscale zerovalent iron: role of sulfidation and application of machine learning. *Environ Sci-Nano* 8 (3), 619–633.
- [21] Zheng, X.-q., et al., 2016. Growth inhibition and DNA damage in the earthworm (*Eisenia fetida*) exposed to perfluorooctane sulfonate and perfluorooctanoic acid. *Chem Ecol* 32 (2), 103–116.
- [22] Rankin, K., et al., 2016. A North American and global survey of perfluoroalkyl substances in surface soils: distribution patterns and mode of occurrence. *Chemosphere* 161, 333–341.
- [23] Zhao, L., et al., 2016. Metabolomics to detect response of lettuce (*Lactuca sativa*) to Cu (OH) 2 nanopesticides: oxidative stress response and detoxification mechanisms. *Environ Sci Technol* 50 (17), 9697–9707.
- [24] Zhao, L., et al., 2017. Metabolomics reveals Cu(OH)₂ nanopesticide-activated anti-oxidative pathways and decreased beneficial antioxidants in spinach leaves. *Environ Sci Technol* 51 (17), 10184–10194.
- [25] Huang, Y., et al., 2019. Antioxidant response of cucumber (*Cucumis sativus*) exposed to nano copper pesticide: quantitative determination via LC-MS/MS. *Food Chem* 270, 47–52.
- [26] Wen, B., et al., 2015. Bioavailability of perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) in biosolids-amended soils to earthworms (*Eisenia fetida*). *Chemosphere* 118, 361–366.
- [27] OECD, Test No. 222: Earthworm Reproduction Test (*Eisenia fetida*/*Eisenia andrei*). 2016.
- [28] Zenobio, J.E., et al., 2022. Adsorption of per- and polyfluoroalkyl substances (PFAS) to containers. *J Hazard Mater Adv* 7, 100130.
- [29] Zhenjun, S., et al., 1997. Earthworm as a potential protein resource. *Ecol Food Nutr* 36 (2–4), 221–236.
- [30] Sugimura, K.-i., et al., 1984. Nutritional value of earthworms and grasshoppers as poultry feed. *Jpn Poult Sci* 21 (1), 1–7.
- [31] Nelson, D.L., A.L. Lehninger, and M.M. Cox, *Lehninger principles of biochemistry*. 2008: Macmillan.
- [32] Liu, W., et al., 2022. Metabolic alterations in alga *Chlamydomonas reinhardtii* exposed to nTiO₂ materials. *Environ Sci: Nano* 9 (8), 2922–2938.
- [33] Xia, J., Wishart, D.S., 2011. Web-based inference of biological patterns, functions and pathways from metabolomic data using MetaboAnalyst. *Nat Protoc* 6 (6), 743–760.
- [34] Aja, M., et al., 2014. FT-IR spectroscopy as a sentinel technology in earthworm toxicology. *Spectrochim Acta Part A: Mol Biomol Spectrosc* 120, 534–541.
- [35] Andre, J., et al., 2010. Molecular genetic differentiation in earthworms inhabiting a heterogeneous Pb-polluted landscape. *Environ Pollut* 158 (3), 883–890.
- [36] Guhra, T., et al., 2020. Earthworm mucus contributes to the formation of organo-mineral associations in soil. *Soil Biol Biochem* 145, 107785.
- [37] Tourinho, P.S., et al., 2021. Microplastic fibers influence Ag toxicity and bioaccumulation in *Eisenia andrei* but not in *Enchytraeus crypticus*. *Ecotoxicology* 30 (6), 1216–1226.
- [38] Abdi, H., Williams, L.J., 2010. Principal component analysis. *WIREs Comput Stat* 2 (4), 433–459.
- [39] Yao, X., et al., 2019. An overview of omics approaches to characterize the effect of perfluoroalkyl substances in environmental health. *TrAC Trends Anal Chem* 121, 115367.
- [40] Liu, L., et al., 2022. Study on toxicity effects of environmental pollutants based on metabolomics: a review. *Chemosphere* 286, 131815.
- [41] Beale, D.J., et al., 2022. A review of omics-based PFAS exposure studies reveals common biochemical response pathways. *Sci Total Environ* 845, 157255.
- [42] Xu, N., Chen, G., Liu, H., 2017. Antioxidative categorization of twenty amino acids based on experimental evaluation. *Molecules* 22 (12), 2066.

- [43] Wu, B., Zhu, L., Le, X.C., 2017. Metabolomics analysis of TiO₂ nanoparticles induced toxicological effects on rice (*Oryza sativa* L.). *Environ Pollut* 230, 302–310.
- [44] Lankadurai, B.P., et al., 2013. ¹H NMR-based metabolomic analysis of sub-lethal perfluorooctane sulfonate exposure to the earthworm, *eisenia fetida*, in soil. *Metabolites* 3 (3), 718–740.
- [45] Kim, H.M., et al., 2020. Omics approach reveals perturbation of metabolism and phenotype in *Caenorhabditis elegans* triggered by perfluorinated compounds. *Sci Total Environ* 703, 135500.
- [46] Reeds, P.J., et al., 1997. Integration of amino acid and carbon intermediary metabolism: studies with uniformly labeled tracers and mass isotopomer analysis. *Eur J Pediatr* 156 (1), S50–S58.
- [47] Dani, V.D., et al., 2019. Comparison of metabolomic responses of earthworms to sub-lethal imidacloprid exposure in contact and soil tests. *Environ Sci Pollut Res* 26 (18), 18846–18855.
- [48] Xu, Q., et al., 2022. Is selenium beneficial or detrimental to earthworm? Growth and metabolism responses of *Eisenia Fetida* to Na₂SeO₃ exposure. *Sci Total Environ* 807, 150770.
- [49] Zhu, W., et al., 2005. Tyrosine and tyramine increase endogenous ganglionic morphine and dopamine levels in vitro and in vivo: cyp2d6 and tyrosine hydroxylase modulation demonstrates a dopamine coupling. *Med Sci Monit* 11 (11), BR397–BR404.
- [50] Wu, N., et al., 2016. Alpha-ketoglutarate: physiological functions and applications. *Biomol Ther* 24 (1), 1–8.
- [51] Foguth, R.M., et al., 2020. Single and mixture per- and polyfluoroalkyl substances accumulate in developing Northern leopard frog brains and produce complex neurotransmission alterations. *Neurotoxicology Teratol* 81, 106907.
- [52] Katz, M., et al., 2019. Glutamate spillover in *C. elegans* triggers repetitive behavior through presynaptic activation of MGL-2/mGluR5. *Nat Commun* 10 (1), 1882.
- [53] Mahmoud, S., et al., 2019. Astrocytes maintain glutamate homeostasis in the CNS by controlling the balance between glutamate uptake and release. *Cells* 8 (2), 184.