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Impacts of a glyphosate-based herbicide on the gut microbiome of three earthworm species (*Alma millsoni, Eudrilus eugeniae* and *Libyodrilus violaceus*): A pilot study

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ABSTRACT

While the impact of glyphosate-based herbicides (GBHs) on earthworms has been studied, little is known about their effects on the earthworm gut microbiome. This study investigated the impact of a GBH on the gut microbial communities of three earthworm species (*Alma millsoni, Eudrilus eugeniae* and *Libyodrilus violaceus*). Earthworm species accommodated in soil were sprayed with 115.49 mL/m^2 of Roundup® Alphée or water. Gut microbiome composition was analysed using 16S rRNA Bacterial Tag–Encoded FLX Amplicon Pyrosequencing (bTEFAP) at the 8th week post-herbicide application. A profound shift in bacterial populationswas observed in all exposed earthworms with *Proteobacteria* becoming the dominant phylum. Affected bacteria were mostly from the genus *Enterobacter*, *Pantoea* and *Pseudomonas*, which together represented approximately 80 % of the total abundance assigned at the genus level in exposed earthworms, while they were present at a minor abundance (~1%) in unexposed earthworms. Although consistent results were observed between the three groups of worm species, it is not possible to generalize these outcomes due to a lack of biological replicates, which does not allow for inferential statistical analysis. Nevertheless, our study is the first to report the effects of a GBH on the earthworm gut microbiome and paves the way for future more comprehensive investigations.

1. Introduction

Glyphosate-based herbicides (GBHs) such as Roundup, manufactured by Monsanto, are the most widely used herbicides for weed control in both agricultural and non-agricultural sectors. Glyphosate, the active ingredient, is mixed with various co-formulants such as surfactants in GBHs [1]. These co-formulants are added to stabilize glyphosate and increase its penetration through the waxy cuticle and translocated to the tissues of growing plants [2].

Glyphosate kills plants by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) in the shikimate pathway, which is known to be a key step in the biosynthesis of phenylalanine, tyrosine and tryptophan essential aromatic amino acids. The shikimate pathway is also involved in the synthesis of other secondary plant metabolites such as ubiquinone and lignans [3]. Since the shikimate pathway is absent in animals, glyphosate is claimed to be one of the safest herbicides by comparison to other pesticides [4]. However, even if glyphosate does not interfere with aminoacid biosynthesis in animals, it can inhibit the shikimate pathway ofmicroorganisms inhabiting their gut [5]. In addition, several studies have documented toxic effects of glyphosate in animals and humans,which were not mediated by the shikimate pathway, but due to the ability of glyphosate to generate oxidative stress damage by interfering with mitochondrial metabolism in laboratory rats [6], zebrafish *Danio rerio* [7], *Caenorhabditis elegans* [8], or human peripheral blood mononuclear cells [9].

Earthworms are the most abundant soil invertebrates by biomass in most agro-ecosystems, and through their activities, they improve nutrient availability in soil and enhance macroporosity that increases

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Fig. 1. Experimental set up (A) with the three species of earthworm *Libyodrilus violaceus* (B),*Eudrilus eugeniae* (C) and *Alma millsoni* (D).

soil water dynamics and aeration [10]. The interaction of earthworms with their micro-environment is an important parameter affecting plant growth [11]. Earthworms can be affected by agricultural chemicals such as pesticides [12] or toxic contaminants in fertilizers [13]. Studies have demonstrated adverse effects of GBHs on earthworms, some of which include reduction in sperm number in *Lumbricus terrestris* [14] and stunted growth in *Eisenia fetida* [15]. In our recent study, earthworms (*Alma millsoni, Eudrilus eugeniae* and *Libyodrilus violaceous*) exposed to a GBH showed the potential to vermiremediate (i.e. remove organic contaminants from soils) as evidenced by decontamination of herbicide residues in soil [16]. This was supported by another study, which showed that glyphosate degradation rate is significantly higher in biomixtures that contained earthworms [17].

The earthworm gut is a transient habitat for soil microflora and accommodates a broad range of microorganisms with diverse functions. An increasing number of studies have characterized the composition and the ecological functions of the earthworm gut microbial communities. For instance, a comparative study of gut microbiota profiles of earthworm (*E. fetida*) fed on nutrient-rich and nutrient-poor substrates showed a core microbiome that appeared in all the samples and varied with substrates [18]. A species-specific distinct microbiome in four different earthworm species and life forms from a grassland soil was identified by Sapkota and colleagues [19]. In addition, Mathipi and colleagues identified the diversity of the microbial community and their metabolic potential from the gut content of four earthworm species from a diversity hotspot [20].

Few studies have described if agricultural applications of herbicides can affect the gut microbiome of earthworms.The gut microbiome of earthworms exposed to arsenic contamination has been characterized by sequencing of 16S rRNA gene amplicons [21]. Interestingly, high concentrations of arsenic induced a distinct shift in the earthworm gut microbiome compared to that of the surrounding soil. In another study, the effects of triclosan, an antibacterial and antifungal agent, were investigated [22]. It was found that triclosan induced concentration-dependent changes in gut microbiome composition, with some bacterial genera found to be resistant such as *Pseudomonas, Stenotrophomonas,* and *Achromobacter* [22]. Despite the increasing number of studies showing the impact of GBHs on earthworm species, to the best of our knowledge, little is known about the possible effects of this herbicide on their gut microbiome.

Since the shikimate pathway targeted by glyphosate leading to inhibition of aromatic amino acid biosynthesis is widely distributed in microorganisms, glyphosate exposure can affect microbial communities inhabiting the gastrointestinal tract of animals [23]. This was studied in laboratory rats [5], honeybees [24], Japanese quail [25], and Hawaiian green turtles [26].

Given the importance of earthworms and their gut microbiome in the maintenance of healthy soil and the exponential increase in the use of glyphosate, there is an urgent need to investigate the impact of this herbicide on the gut microbiome of earthworms. We therefore undertook a pilot study to investigate the impact of a GBH on the gut microbiome of three earthworm species (*E. eugeniae, A. millsoni* and *L. violaceous*) using a 16S rRNA sequencing approach.

2. Materials and methods

2.1. Experimental set up

This experiment was carried out at the research section of the Animal House of the Department of Zoology and Environmental Biology, Olabisi Onabanjo University, Ago-Iwoye, Ogun State, Nigeria. The experiment was set up in accordance with the method of Gaupp-Berghausen and colleagues [27] as modified by Owagboriaye et al. [28]. We used 120 plastic pails (diameter: 25 cm, height: 30 cm) perforated at their base and filled with soil collected from an arable field of the University research farm. Each pail was planted with three weed plants (*Tridax*

Table 1
Alpha diversity of the gut microbiota in three earthworm species exposed to a GBH. The number of observed OTUs, Faith's Phylogenetic Diversity, and the Shannon diversity indices were determined on a dataset rarefied to 20,000 reads per sample.

Treatment	Earthworm species	Faith PD	Observed	Shannon
Control	*A. millsoni*	112.2	1042	7.1
	E. eugeniae	90.1	886	6.5
	L. violaceus	49.6	322	2.0
GBH	*A. millsoni*	12.7	86	2.9
	E. eugeniae	12.9	83	2.7
	L. violaceus	16.6	133	3.7

procumbense, Ludwigia pasturis and *Pannicum maximum*) (distance between plants in pail: 4.8 cm) commonly found in agricultural areas (Fig. 1A). The weeds, at 5 cm average height, were collected from the University research farm and transplanted into the pails. At two weeks post-transplantation, 3 earthworm species (*A. millsoni, E. eugeniae, L. violaceus*) (Fig. 1B-C) were established in the pails and labeled accordingly. These earthworm species are native to Africa and commonly found in the soils of agricultural, industrial and residential areas in Nigeria [29]. *E. eugeniae* is an epigeic species while *A. millsoni* and *L. violaceus* are endogeic species. A total of 20 well-developed clitellate earthworms of each species were separately introduced into each pail; 20 replicates were established per species, making a total of 60 pails set up for the experiment. All earthworms appeared to be in good health and burrowed into the soil immediately upon being released.

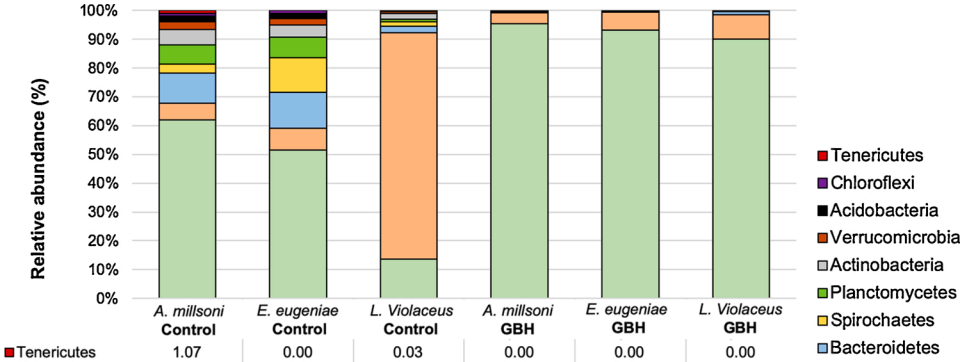
We added ground hay to the pails to provide food for the earthworms. After the 3rd week following transplantation (when *T. procumbense* had attained average height 14 cm, *L. pasturis* was 22 cm and *P. maximum* was 28 cm), each pail was sprayed with 7.2 mL of Roundup® Alphée (7.20 g/l glyphosate; Scotts Celaflor, Mainz, Germany) for two consecutive days as recommended by the manufacturer. With reference to the total area of the pails used (of 25 cm diameter and 30 cm height × 20 pails), each earthworm species treatment in 20 pail replicates, was sprayed with 115.49 mL/m^2 of Roundup® Alphée. A similar set up of each species of the earthworm in 60 pails established without herbicide application were sprayed with water and this served as the control experiment. In a first report of results from this study, we described that wormcasts produced by the three GBH-exposed earthworm species reduced tomato growth, fruit yield and quality [28]. In the follow-up analysis presented here, after the 8th week of post-herbicide application, a total of 3 individual earthworms were collected from each pail (making each sample to be a pool of 60 earthworms of the same species). Gut contents from these groups of worms was obtained by degutting and subjected to 16S rRNA gene sequencing analysis.

2.2. DNA extraction

Total DNA was extracted from the earthworms' gut contents with the Qiagen extraction kit (Qiagen, Crawley, UK) according to manufacturer's guidelines. Prior to polymerase chain reaction (PCR), DNA quantification was performed using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2.3. Sequencing

Amplicon sequencing using next generation technology (bTEFAP®) was carried out at the MR DNA, Shallowater, TX, USA as previously described [30]. The 16S rRNA primer pair, 515 F GTGYCAGCMGCCGCGGTAA / 806R GGACTACNVGGGTWTCTAAT, on the MiSeq via the bTEFAP® DNA analysis was used to evaluate the microbial communities in the gut of the earthworm species. Each sample underwent a single-step 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA, USA) under the following conditions: 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s,53 °C for 40 s and 72 °C for 1 min, after which a final elongation step at 72 °C for 5 min was performed. Following PCR, all amplicon products were mixed in equal concentrations and purified using Agencourt AM Pure beads (Agencourt Bioscience Corporation, MA, USA). Samples were sequenced with the Illumina MiSeq platform.The Q25 sequence data derived from the sequencing process was processed using a proprietary analysis pipeline (www.mrdnalab.com, MR DNA, Shallowater, TX, USA). DNA sequence analyses were then performed using QIIME2 [31].The error-correction algorithm dada2 was used. Sequences were depleted of barcodes and primers. In addition, short sequences <200bp and sequences with ambiguous base calls were removed. Sequences with homopolymer runs exceeding 6bp and chimeras were also removed. Because of the low number of samples in this study, we clustered DNA sequences as Operational taxonomic units (OTUs). They were defined after removal of singleton sequencesand clustering at 3% divergence (97 % similarity) [30]. OTUs were taxonomically classified using BLASTn against a curated NCBI database. After stringent quality sequence curation, a total of 535,241 sequences were parsed and 493,621 were clustered. A total



	A. millsoni Control	E. eugeniae Control	L. Violaceus Control	A. millsoni GBH	E. eugeniae GBH	L. Violaceus GBH
Tenericutes	1.07	0.00	0.03	0.00	0.00	0.00
Chloroflexi	0.72	0.91	0.19	0.02	0.01	0.01
Acidobacteria	2.00	1.91	0.23	0.03	0.02	0.02
Verrucomicrobia	2.79	2.10	0.57	0.06	0.05	0.03
Actinobacteria	5.18	4.29	2.02	0.12	0.09	0.10
Planctomycetes	6.60	7.00	0.88	0.15	0.11	0.17
Spirochaetes	3.19	11.80	1.45	0.02	0.02	0.03
Bacteroidetes	10.27	12.42	2.25	0.42	0.20	1.18
Firmicutes	5.81	7.34	78.41	3.71	6.21	8.45
Proteobacteria	61.23	50.85	13.63	95.43	93.27	89.97

Fig. 2. Relative abundance of total observed phyla in the earthworm species exposed to a GBH. OTUs were aggregated and their total relative abundance reported for *A. millsoni*, *E. eugeniae and L. violaceus*.

of 485,797 sequences identified within the Bacteria and Archaea domains were utilized for final microbiota analyses (Supplementary Table S1). The average reads per sample was 80,966. For alpha and beta diversity analysis, samples were rarefied to 20,000 sequences. Alpha and beta diversity analysis was conducted using QIIME2 [31].

3. Results and discussion

We performed a preliminary study by sequencing amplicons from regions of the 16S rRNA genes in the gut microbiota of three different earthworm species exposed to a GBH. There was a higher microbial diversity among the control earthworms compared to those exposed to the GBH (Table 1). Principal coordinate analysis plot (i.e. beta diversity)

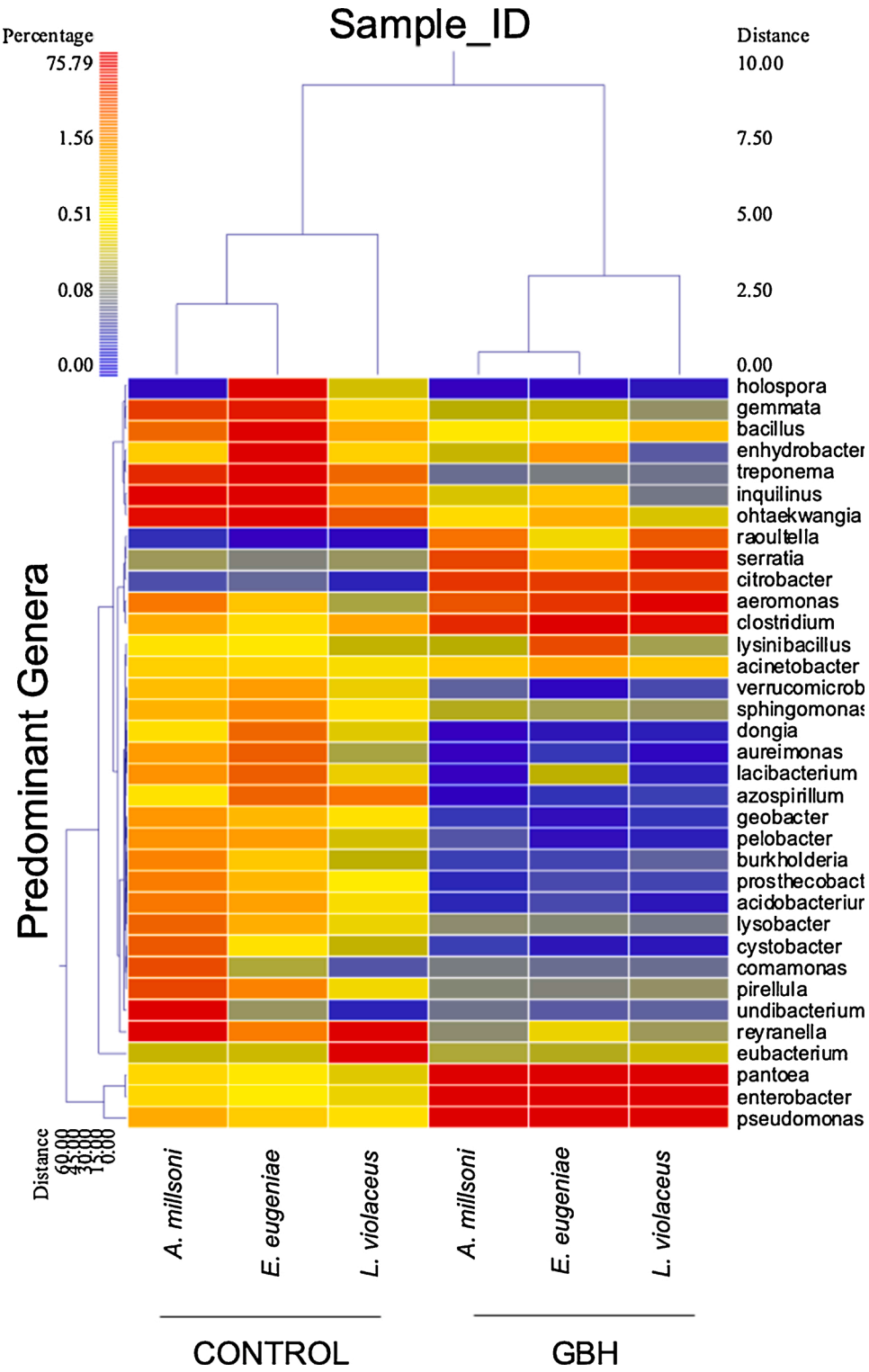


Fig. 3. Hierarchal classification of genus abundance in three earthworm species exposed to a GBH. The most prevalent genera are displayed for *A. millsoni*, *E. eugeniae*, *and L. violaceus exposed to GBH*.

shows that the first component, which separated the three earthworms by treatment groups accounted for 57.8 % of the total variation (Supplementary Fig. 1). Thus, all the exposed earthworm species were more closely related to each other than those in control.

Members of the phyla Spirochaetes, Planctomycetes, Actinobacteria and Verrucomicrobia were present in the gut of the control earthworms but virtually absent in those exposed to the GBH (Fig. 2). Exposure to the GBH caused a marked increase in the relative abundance of the Proteobacteria, which became the dominant phylum (95 % *A. millsoni*; 93 % *E. eudrilus*; 89 % *L. violaceus*) in the three earthworm species.

We then explored the effects of the GBH at lower taxonomic levels. At the family level, the gut microbiome of GBH-exposed earthworms was mostly composed of Enterobacteriaceae (44 % *A. millsoni*; 76 % *E. eudrilus*; 32 % *L. violaceus*) and Pseudomonadaceae (9% *A. millsoni*; 52 % *E. eudrilus*; 36 % *L. violaceus*) while these families were present at low abundance below 1% in unexposed animals. These were mostly from the genus *Enterobacter, Pantoea* and *Pseudomonas*, which together represented approximately 80 % of the total abundance assigned at the genus level (Fig. 3). Although the taxonomic assignment at the species level should be interpreted cautiously because of intrinsic limitations of 16S rRNA gene sequencing, our results highlight that the four species *Enterobacter* spp. *DHL-02, Pseudomonas putida, Pantoea agglomerans* and *Pseudomonas taiwanensis* were consistently found at a high abundance of over 10 % in the gut microbiome of GBH-exposed earthworms while they were a minor component (<1%) in unexposed animals.

Interestingly, a computational analysis of the human gut microbiome predicted that glyphosate might be degraded by some Proteobacteria including *Enterobacter spp*. into usable phosphate using the carbon–phosphorus lyase pathway [32]. Therefore, it is possible that the higher abundance of this phylum in the gut of all the earthworms exposed to the GBH was a response to herbicide residues promoting their survival. Future studies could include *in vitro* culture of bacterial strains isolated from the digestive tracts of earthworms to test to see if they are able to use glyphosate as an energy source.

Although further studies are needed with different brands of GBHs, the effects of glyphosate alone also needs to be investigated. Studies have documented toxicities of GBHs in animals at doses at which glyphosate alone is considered to be safe. For example, using a multi-omics analysis Mesnage and colleagues reported damage to the liver and kidneys of rats exposed to a Roundup GBH [33,34]. We have previously reported nephrotoxicity [35], reproductive toxicity [36] and metabolic alterations [37] of Roundup GBH exposed rats. More recently, it was found that a primary effect of a GBH on the gut microbiome of rats was inhibition of EPSPS of the shikimate pathway highlighted by the accumulation of shikimic acid and 3-dehydroshikimic acid [5].

Agricultural soils and even public playgrounds are frequently contaminated by pesticide mixtures [38]. Pesticides can have greater toxic effects in mixtures than when they are tested individually in earthworms [39] and other animal species such as chicks [40], salmon [41] or on mammalian bone marrow erythrocytes in CD-1 mice [42]. Recent scientific studies have highlighted the need to test real-life exposure scenarios [43]. Earthworms are sentinel species. Effects on their microbiomes could be considered as bioindicators for contamination of soil ecosystems [44] which could inform human health risks associated to the presence of pesticides on public sites [38].

Future studies would also benefit from the use of more advanced methods to characterize gut microbiome composition. Using shotgun metagenomics would allow the determination of taxonomic profiles at the strain level [45]. Since changes in gut microbiome metabolism does not always coincide with changes in bacterial abundance, other 'omics' approaches such as metabolomics or meta-transcriptomics could provide insight into functional aspects of the microbiome [46,47]. An increasing number of studies show that the faecal metabolome can be used to study ecological roles of the gut microbiome including its association to human diseases [48].

We used a GBH dose representative of agricultural dilutions in our study, providing a realistic exposure scenario for earthworms resulting directly from agricultural sprays. Effects could nonetheless be different depending on soil composition as glyphosate can bind to soil particlesat least in part due to its chelation properties [49]. The half-life of glyphosate can also differ depending on physicochemical properties of soils and climatic factors such as temperature. In field studies, the half-life of glyphosate can range between a few days to around 6 months [50]. In soils, glyphosate competes with phosphorus and thus its absorption depends on mineral composition [51]. All these environmental factors will impact GBH availability to earthworms in agricultural settings.

4. Conclusions

Our results suggest that exposure to GBHs could result in a bacterial compositional shift as well as functional changes in the earthworms' gut microbial population and in turn, may disrupt fundamental physiological processes and consequently affect the ecological roles of these animals as previously suggested [52]. Although consistent results were observed between the three test groups of worm species, it is not possible to generalize these outcomes due to a lack of biological replicates, which does not allow for inferential statistical analysis. However, although the conclusiveness is limited, GBH effects are highly consistent within the three earthworm species tested, which paves the way to future more detailed studies.

Author contributions

Conceptualization, F.O.; G.D.; and S.O.; methodology, F.O.; G.D.; and S.O.; validation, F.O.; and G.D.; investigation, F.O.; and MA.; resources, T.A.; and A.A.; data curation, F.O.; writing - original draft preparation, F.O.; writing - review and editing, R.M.; and MNA.; visualization, R.M.; supervision, G.D.; project administration, F.O.; All authors have read and agreed to the published version of the manuscript.

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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.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxrep.2021.03.021.

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