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Toxicity and bioaccumulation of the fluorosurfactant cC_6O_4 in the earthworm *Eisenia foetida* (Savigny, 1826)

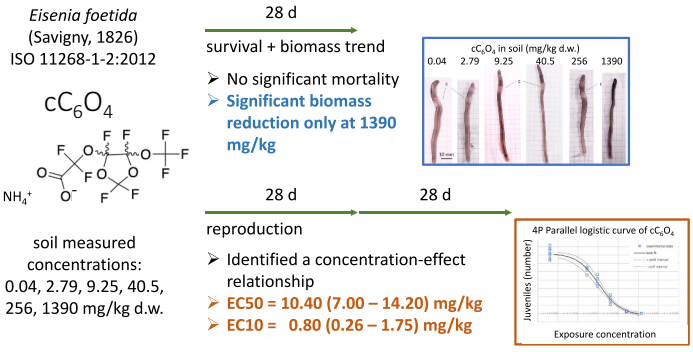
Elisa Chiara Bizzotto [a,*], Giovanni Libralato [b], Silvia Breda [a], Antonietta Siciliano [b], Petra Scanferla [a], Marco Vighi [c], Antonio Marcomini [d]

[a] Foundation Ca' Foscari University, Ca' Dolfin, Calle Larga Ca' Foscari Dorsoduro 3859/A, Venice 30123, Italy
[b] Department of Biology, University of Naples Federico II, Via Vicinale Cupa Cintia 26, 80126 Napoli, Italy
[c] IMDEA Water Institute, Science and Technology Campus of the University of Alcalá, Avenida Punto Com 2, 28805 Alcalá de Henares, Madrid, Spain
[d] Department of Environmental Sciences, Informatics and Statistics, Ca' Foscari University of Venice, via Torino 155, 30172 Venice-Mestre, Italy

HIGHLIGHTS

- The paper describes the first ecotoxicological data of cC_6O_4 on terrestrial invertebrates.
- cC_6O_4 does not cause mortality on earthworms; reproduction is a more sensitive endpoint.
- Adverse effects only occur at levels that are much higher than realistic soil concentrations.
- The bioaccumulation potential of cC_6O_4 in earthworms is extremely low.

GRAPHICAL ABSTRACT



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ABSTRACT

Cyclic C_6O_4 (cC_6O_4, CAS number 1190931–27-1) is a perfluoralkyl ether PFAS used as a polymerization aid in the synthesis of fluoropolymers and produced in Italy since 2011 as substitute of PFOA. To date, available ecotoxicological information on cC_6O_4 is related to regulatory requirements and limited to data on aquatic organisms, while the information on the effects for terrestrial organisms is completely lacking. This work reports the first ecotoxicological data of cC_6O_4 on terrestrial invertebrates: short- and long-term toxicity of cC_6O_4 on *Eisenia foetida* (Savigny, 1826), exposed to spiked soil under laboratory conditions, was investigated evaluating the earthworm survival and growth (observed after 7, 14 and 28 days of exposure), and reproduction (observed after an exposure period of 56 days). Furthermore, also bioaccumulation was investigated (28 days of exposure); overall results are discussed in comparison with literature data available for legacy PFAS. cC_6O_4 did not cause significant mortality on earthworms, for any of the tested concentrations and exposure periods (NOEC: > 1390 mg/kg d.w.), while the reproduction (measured as juveniles production) appears to be a more sensitive endpoint (EC50: 10.4 mg/kg d.w., EC10: 0.8 mg/kg d.w.). The observed adverse effects occur at levels significantly higher than realistic soil concentrations and cC_6O_4 appears to be less toxic than PFOA and PFOS. As for

* Corresponding author.
E-mail address: elisachiara.bizzotto@unive.it (E.C. Bizzotto).

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bioaccumulation, the results indicate a negligible bioaccumulation potential of cC_6O_4, whose Biota-Soil Bioaccumulation Factors (BSAF) are significantly lower than all other considered PFAS.

1. Introduction

Per- and polyfluorinated alkyl substances (PFAS) are a complex class of chemicals with one or more fully fluorinated methyl or methylene carbon atom (with no H/Cl/Br/I atom attached to it) (OECD, 2021), which includes thousands of compounds mainly xenobiotic (i.e., not existing before their production by the chemical industry) used in both industrial and consumer products. The general definition of PFAS is therefore wide and, depending on their molecular structure, PFAS can be grouped in different classes (OECD, 2021); among these, perfluoroalkyl acids (PFAA) are considered the terminal degradation products of the PFAS family and most research and environmental surveys are focused on their presence and distribution (Butt et al., 2014).

The extensive use of PFAS, together with their persistence (primarily due to the high stability of the C—F bond) contributed to determine global environmental contamination (Giesy and Kannan, 2002). Additionally, although research is still ongoing, current knowledge suggests that exposure to PFAS may lead to various adverse health outcomes (Fenton et al., 2021). Based on concerns regarding the potential risks and the lack of knowledge on toxicological profiles, distribution, and properties of many PFAS, in the last two decades many national and international agreements have been developed to control and limit PFAS emissions (ECHA, 2022). However, for some specific uses, these substances still appear useful for the functioning of today's society and there is the need to find suitable alternatives (Glüge et al., 2020). Recently, alternative chemicals designed to replace the most concerning PFAS currently in use, have been developed. Among them, cyclic C_6O_4 (cC_6O_4) was registered and patented by Solvay in 2011, to cope with the need to replace PFOA, one of the most critical PFAA, detected worldwide, representing a threat for human and environmental health (Fujii et al., 2007). In the last few years, cC_6O_4 has been subject of growing attention and monitoring activities (Bizzotto et al., 2023); more recently, the substance has been included in the "sum of PFAS" parameter established for drinking water by the Legislative Decree 18/2023, released in Italy for the implementation of the EU Directive 2020/2184.

A review on the available knowledge on environmental and ecotoxicological characteristics of cC_6O_4 have been recently published (Bizzotto et al., 2023). The available information on the aquatic environment includes short and long term ecotoxicity data on bacteria, algae, crustaceans, and fish, as well as some data on bioaccumulation. Moreover, a long term mesocosm experiment has also been performed (Rico et al., 2023). On the contrary, the information on the effects for terrestrial organisms is completely lacking.

Earthworms represent one of the most important soil invertebrates for their ecological functions such as nutrient cycling, energy transfer, soil formation and maintenance of its quality and structure (Smidova et al., 2021); furthermore, earthworms reside at the base of food chain and constitute a considerable part of natural nourishment of numerous species (Burkhard and Votava, 2023). In general terms, the uptake of chemicals in earthworms is supposed to be through dermal contact with interstitial water, and active ingestion of soil and absorption of contaminants through the gut. Once in the body, different pathways may occur to the chemical, such as metabolization, excretion, bioaccumulation (and eventually sequestration) in other organs, or transportation to the sites of toxic action (Lanno et al., 2004).

Therefore, the evaluation of toxicity and bioaccumulation process in earthworm is important for a proper evaluation of potential ecological risk posed by chemical substances in terrestrial ecosystems.

The scope of this study was to investigate the potential toxicity and the bioaccumulation potential of cC_6O_4 in the earthworm *Eisenia foetida* (Savigny, 1826) exposed to spiked soil under laboratory conditions. Short- and long-term toxicity of cC_6O_4 on *E. foetida* was investigated according to the procedure described in the guideline ISO 11268:2012a including both survival and growth (observed after 7, 14 and 28 days of exposure), and reproduction (observed after an exposure period of 56 days). Furthermore, bioaccumulation was investigated analyzing earthworms collected after 28 days of exposure to spiked soils. To the best of our knowledge, this is the first study evaluating the toxicity and bioaccumulation behavior of cC_6O_4 in terrestrial invertebrates.

2. Materials and methods

2.1. Chemicals

The cC_6O_4 (CAS 1190931–27-1; molecular formula: C6H4F9NO6; complete chemical name: acetic acid, 2,2-difluoro-2-[[2,2,4,5-tetrafluoro-5-(trifluorometoxy)-1,3-dioxolan-4-l]oxy]-,ammonium salt) was gently provided by Solvay Specialty Polymers Italy S.p.A. (batch number: 07040 S, cC_6O_4 concentration in water 39.1 % weight/weight). The experimental activity used ultra-pure water as solvent. Solubility of cC_6O_4 in water is >667 g/L and the critical micellar concentration (CMC) is 36–40 g/L; when in aqueous solution, it completely dissociates in the anionic form.

Besides the very high solubility in water, other properties of environmental relevance are the very low vapour pressure (7.5E-5 Pa at 25 °C), low n-Octanol/Water partition coefficient (log K_{ow} = 1.3) and low Organic Carbon adsorption coefficient (log K_{oc} = 1.04) (Bizzotto et al., 2023). The compound is persistent in water, but no experimental data are available on the persistence in soil.

2.2. Soil preparation and spiking

The soil used in the experiments with oligochaete was collected from the wild from a clean site far from anthropic activities using a stainless-steel shovel and a plastic storage box made of plastic certified food contact material. The soil was used for all experimental activities after sieving (4 mm, stainless-steel sieves) and stored at laboratory room condition until use. The same soil (unspiked) was also used for husbandry practices.

The natural soil properties measured included: pH, texture, water content, water holding capacity, cationic exchange capacity, organic carbon. The values of natural soil properties and the methods used are reported in Table 1-SM in the Supplementary Material.

According to the objective of the study, the unspiked and spiked soils (dilution series of contaminated soil) were prepared with the same natural soil.

The cC_6O_4 was added to the natural soil via dilution in the deionized water that was used to wet the soil samples to reach from 40 % to 60 % of the total water holding capacity (WHC), corresponding to a measured moisture content of ~53–54 %. For the spiking procedure Gilson pipette and pipette tips in polypropylene were used. The required amount of cC_6O_4 was pipetted from the stock solution to the volume of ultra-pure distilled water necessary to reach the required WHC (i.e., 270 mL of ultrapure distilled water every 500 g of soil) and then transferred to the target soil. Soil test matrices were manually mixed in a glass bowl with a stainless-steel spatula and reinforced amide plastic (labelled as food contact material) spoons for 10 min to ensure a complete substrate homogenization. Soil test matrices were transferred to the final testing holder with reinforced amide plastic (labelled as food contact material) spoons; all holders were glass-made. Every testing concentration was performed by spiking 4 kg of natural soil and splitting this quantity into

Table 1
Results of the toxicity test performed on *Eisenia foetida* exposed to cC_6O_4.

Exposure period	Endpoint	NOEC mg/Kg d.w.	LOEC mg/Kg d.w.	EC50 mg/Kg d.w.
7 days	Mortality	> 1390	–	–
	Growth	256	1390	>1390
14 days	Mortality	> 1390	–	–
	Growth	256	1390	>1390
28 days	Mortality	>1390	–	–
	Growth	256	1390	>1390
56 days	Reproduction	0.8 (EC10)	2.79	10.4

Table 2
Results of long-term toxicity studies conducted on cC_6O_4 and on individual PFAA in earthworm exposed to spiked soil under controlled lab conditions. All values refer to PFAS concentration in soil (mg/kg d.w.).

Chemical	Exposure period	Endpoint	NOEC/ EC10	LOEC	LC50/ EC50	Ref.
cC_6O_4	7, 14, 28 days	Survival	≥ 1390			This study
PFOA	7 days	Survival	500		656	Kwak et al., 2020
			500		1000.8	Joung et al., 2010
	14 days	Survival	540°		759.6	Zheng et al., 2016
					812	Wang et al., 2022
					811.42	Yuan et al., 2017
	28 days	Survival	≥100			He et al., 2016
PFOS	14 days	Survival	77	141	373	Sindermann et al., 2002
			160		365.4	Joung et al., 2010
					540.97	Yuan et al., 2017
			400°		478	Zheng et al., 2016
PFBS, PFHxS, PFHpA, PFNA	21 days	Survival	≥100			Karnjanapiboonwong et al., 2018

° Estimated from raw data

8 doses of 500 g (on a dry weight (d.w.) basis). Specifically, 4 replicates were used for the short-term test (7 + 7 days) and 4 replicates for the long-term test (28 + 28 days). A graphical summary of the experimental setting is reported in the Supplementary Material. The same procedure was followed for the control soil which was spiked with pure water and not added with cC_6O_4. Soil test matrices were prepared at least 24 h prior to the test start. The pH for each soil test matrix (one container per concentration) was determined at the beginning and end of the test.

Both short-term and long-term toxicity tests were performed evaluating 5 exposure scenarios (C1-C5) plus the control (C0) (Table 2-SM). To verify and validate the tested exposure scenario, pooled samples of the tested soils were collected at the beginning of the test (T0) and at the end of the day 56 exposure period (T56) for chemical analysis of cC_6O_4; all soil samples were stored at −20 °C for chemical analysis.

Additionally, to test the mobility of cC_6O_4 in terrestrial environment, soils from the C1, C4 and C5 exposure scenarios were used to prepare leachates according to UNI EN 14735:2005 with a soil: water ratio 1:10 (leaching medium: water as established in the ISO 6341:2012b). The leachate, prepared by stirring the soil and aqueous phase for 24 h, was stored after centrifugation (4000 *g*) in two Falcon of 50 mL each.

2.3. Earthworm exposure and toxicity testing

The short-term and long-term toxicity tests with *E. foetida* were carried out according to ISO 11268-1-2:2012. For each concentration (negative control and spiked soils), the percent mortality and the percent loss/increase in biomass of the adults after four weeks, and the number of offsprings produced after another period of four weeks were provided.

The experimental design was composed of two exposure periods: i) up to 28 days for mortality and bioaccumulation in adults; ii) after 28 days, earthworms were removed, and the soil exposure was prolonged for other 28 days for counting the number of juveniles per treatment at the end of the experiment (further details in SM).

At day 0, for each test container and the control container(s), ten worms (*E. foetida*) were prepared, washed, and gently wiped (using absorbent paper). The homogeneity of the test population was verified by weighing each worm individually to avoid systematic errors in distributing the worms to the test containers and to ensure that the weight minimum threshold of 300 mg per individual was respected, including the maximum limit set at 600 mg.

Having ensured homogeneity and weight within the thresholds, batches of 10 worms were selected, weighed, and placed in each test container. Batches of worms were assigned to test containers using a randomization procedure.

Covered containers were randomly placed in the test enclosure.

At day 0 and every 7 days up to day 14 for short-term test and up to day 21 for long-term test, approximately 5 g of food was spread on the soil surface of each container and moistened with deionized water (about 5-6 mL per container). As food source, experience has shown that cow manure can be a suitable food. Self-collected cow manure from cows grazing in the wild was used. The manure was air-dried, finely ground, and pasteurized before use. Each batch of food was successfully used to feed a non-test worm culture prior to using in toxicity testing.

After the exposure period (7, 14 and 28 days), the live worms were counted. The dead worms, if visible (a worm is dead if it displays no reaction to a pin prick applied to its anterior side), were removed. All symptoms observed on the animals were registered. Additionally, the mass of living worms (for each container), the water content (in one control container) and the pH (in one container per test concentration) were determined; finally, number of offsprings produced after another period of four weeks (day 56) were registered.

In order to evaluate bioaccumulation, earthworms exposed to scenarios C0, C1 and C4 were collected for chemical analysis at the end of the 28 days test; the organisms were cleaned from residual earth by soaking three times in ultrapure deionized water and then placed on moistened paper for 24 h to allow gut purging. Biological samples were then stored at −20 °C for the subsequent analysis.

2.4. Analysis of cC_6O_4 in soil and biological samples

The soil, biota and eluate samples were shipped to a qualified analytical laboratory (Mérieux Nutrisciences Italia, Resana, Italy) for chemical analysis of cC_6O_4. All chemical analysis were performed after the conclusion of the overall tests on *E. foetida*.

Aqueous samples (eluates) were diluted with methanol and analyzed by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) according to the method ASTM D7979:2020.

The determination of cC_6O_4 in soil and in biological matrices was conducted applying methods developed and validated internally by Mérieux Nutrisciences (further details in Supplementary Material); both the methods are based on LC-MS/MS detection. For soil, the method is based on ASTM D7968:2017. Soil samples are previously dried in oven at 105 °C and sieved at 2 mm; extraction is then performed using methanol and water under alkaline condition; chemical analysis was run in triplicate. For biological samples, the method is based on FDA Foods Program Compendium of Analytical Laboratory Methods (Method number C-010.01). Briefly, biological sample are shaken for 30 s with water and 15 % sodium chloride solution; then, acetonitrile is added, and the samples are shaken vigorously for 8 min. The extract is then centrifuged; the organic layer is transferred in another tube and dried with magnesium sulfate. After another centrifugation, the extract is purified with charcoal, concentrated to dryness, then dissolved with a solution containing internal reference material and analyzed by LC-MS/MS.

Limit of quantification (LOQ) for cC_6O_4 in soil, biological tissues and eluates are respectively 0.4 μg/kg d.w., 1 μg/kg w.w. and 100 ng/L.

cC_6O_4 was determined instrumentally as anion; all concentrations of cC_6O_4 reported in this study refer to the anionic form.

2.5. Data analysis

The percent mortality (observed at 7, 14 and 28 days), the percent loss/increase in biomass of the adults (observed at 7, 14 and 28 days) and the number of offsprings produced after another period of four weeks were registered for each concentration. For the mortality endpoint, percent effects (if any) were transformed to probits or logits allowing the estimation of a straight-line model and limiting the number of parameters to be estimated. When two consecutive dilutions or concentrations at a ratio less than or equal to 2 give only 0 % and 100 % mortality, the two values were sufficient to indicate the range within which the LC50 falls.

Effect data were analyzed for normality and homogeneity of variance. If data were normally distributed and homoscedastic, parametric methods for data analysis were used like one way analysis of variance (ANOVA) and subsequent post-hoc analysis. Otherwise, non-parametric methods were considered (Kruskal-Wallis), if data transformations failed to meet normality and homoscedasticity criteria. Results were expressed in milligrams per kilogram of dry soil (considering the mean value of soil concentrations measured at T0 and T56) and median effect concentration (if any) of the test substance.

With regard to bioaccumulation, different Biota-Soil Bioaccumulation Factors (BSAF) were calculated dividing earthworm concentration (reported on wet weight) by 1) soil concentrations expressed on dry weight ($BSAF_{w.w./d.w.}$), soil concentration expressed on wet weight (thus, considering the soil water content, $BSAF_{w.w./w.w.}$) and 3) soil concentration normalized on the fraction of organic carbon (f_{OC}) ($BSAF_{w.w./OC}$). All BSAF were determined considering the mean values of soil concentrations measured at T0 and T56.

Since chemical analysis on earthworm were performed on 3 samples from different replicates of the same exposure scenarios, BSAFs are expressed as means with standard deviation.

The mobility of cC_6O_4 in soil was evaluated comparing the concentrations measured in eluates with values estimated assuming that all the cC_6O_4 present in soils was released in the aqueous phase during eluate preparation.

3. Results and discussion

3.1. Toxicity of cC_6O_4 in earthworm and comparative assessment

3.1.1. Results of the toxicity test

The use of spike test is widely used to evaluate chemical behavior and toxicity under controlled lab conditions. However, it is recognized that spiking procedures are not simple and several factors can introduce interferences to experiments (Northcott and Jones, 2000; Fuchsman and Barber, 2000); in this sense, in order to overcome any imprecisions potentially occurred during the soil spiking, cC_6O_4 concentrations were measured both before and after the toxicity testing, to verify the exposure scenarios and if any chemical loss mechanisms occurred during the course of the test.

The results of chemical analysis on the tested soils sampled at the beginning (T0, day 0) and end of the exposure period (T56, day 56) are reported in Table 2-SM. As first observation, a residual concentration of cC_6O_4 was detected in the control, probably due to a cross-contamination event occurred during the sample preparation; in any case, results of the overall toxicity test can be considered valid since the control samples always respected the validity criteria set by the guideline ISO 11268:2012a, both for the short- and long-term tests (Table 3-SM in the Supplementary Material). Secondly, measured concentrations appear reasonably stable during the test; therefore, toxicity testing results are here discussed referring to measured concentrations expressed as mean values of cC_6O_4 measured in soil at T0 and T56.

The toxicity testing was composed of two exposure periods: i) up to 28 days for mortality and growth in adults; ii) 28 days more for counting the number of juveniles per treatment at the end of the experiment. Overall results of the toxicity testing are reported in Table 1; additional information is reported in Supplementary Material (Table 4-SM, 5-SM and Fig. 1-SM).

With regard to mortality, cC_6O_4 did not cause significant effects on earthworms, for any of the tested concentrations; therefore, the LC50 is assumed greater than the maximum tested concentration (1390 mg/kg d.w.) (Table 1).

However, one specific symptom was observed apart death. After 14 and 28 days of exposure to cC_6O_4, earthworms exposed at 1390 mg/kg d.w. of cC_6O_4 appeared significantly smaller and thinner than at lower concentrations (compared to the negative control weight), without clitellum (as evidenced in Fig. 2-SM). On the contrary, earthworms exposed from 2.79 mg/kg d.w. up to 256 mg/kg d.w. of cC_6O_4 did not show any specific morphological alteration (on a qualitative "by eye" approach). The trend of biomass loss, observed at 28 days, is displayed

Table 3
Concentration of cC_6O_4 in soil (mean value of concentrations measured at the beginning and at the end of the test) and in earthworms collected after 28 days of exposure to cC_6O_4. The table also reports the water content of the tested soil (mean value of soil moisture measured at the beginning and after 28 days of exposure).

Sample ID	cC_6O_4 in soil (mg/kg d.w.)	Soil moisture (%)	cC_6O_4 in *E. foetida* (mg/kg w.w.)		
			Rep 1	Rep 2	Rep 3
C0	0.04	54.5	<0.00047	<0.00041	<0.00049
C1	2.79	53.2	0.00101 ± 0.0004	0.0036 ± 0.0013	0.00155 ± 0.0006
C4	256	54.6	0.048 ± 0.017	0.0105 ± 0.0036	0.01 ± 0.0034

Table 4
Biota Soil Accumulation Factors (BSAF) estimated for cC_6O_4 in earthworms collected after 28 days of exposure to cC_6O_4.

Sample ID	$BSAF_{ww/dw}$ (= $Cworm_{ww}/Csoil_{dw}$)	$BSAF_{ww/ww}$ (= $Cworm_{ww}/Csoil_{ww}$)	$BSAF_{ww/OC}$ ($Cworm_{ww}/(Csoil_{dw} / f_{OC})$)
C0	–	–	–
C1	$7.37*10^{-4} \pm 4.91*10^{-4}$	$1.57*10^{-3} \pm 1.05*10^{-3}$	$2.6*10^{-5} \pm 1.8*10^{-5}$
C4	$8.92*10^{-5} \pm 8.51*10^{-5}$	$1.97*10^{-4} \pm 1.88*10^{-4}$	$3.2*10^{-6} \pm 3.1*10^{-6}$

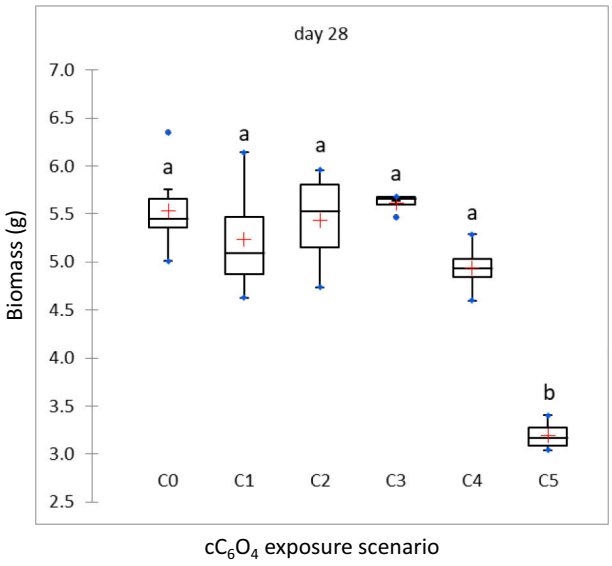

Fig. 1. Box plot evidencing the trend of biomass loss (g) at day 28 according to the considered exposure scenario (measured concentration, C0 = control; C1 = 2.79 mg/kg; C2 = 9.25 mg/kg; C3 = 40.5 mg/kg; C4 = 256 mg/kg; C5 = 1390 mg/kg); equal letters indicate data non significantly different ($p < 0.05$, post-hoc Tukey's test); black line = median, red cross = mean, 1st and 3rd quartile = upper and lower part of the box, whisker = standard deviation, star = outlier, dot = lower or higher value.

in Fig. 1, while the trends measured at 7 and 14 days are reported in Supplementary Material (Fig. 1-SM).

Considering overall results, only the biomass measured at the highest exposure scenario (1390 mg/kg d.w. of cC_6O_4) was significantly different from the effects generated by the lower concentrations. Thus, the biomass of worms exposed for 7, 14 and 28 days at 1390 mg/kg d.w. of cC_6O_4 was significantly impaired compared to the lower concentrations of cC_6O_4 and to the negative control. Specifically, the weight after 28 days of exposure to 1390 mg/Kg d.w. of substance was averagely >1.5 times lower compared to that of the negative control (considering the mean values, the weight of worms exposed to 1390 mg/kg was approximately 56 % of worms weight in the control) (Fig. 1). The concentration determining the 50 % biomass loss is therefore >1390 mg/kg d.w. (Table 1).

With regard to the reproduction test, results about the count of juveniles at the end of the prolonged exposure period (56 days) are summarized in Fig. 2 and fully reported in the Supplementary Material. Substance cC_6O_4 showed to be effective in completely inhibiting the reproduction of *E. foetida* at the highest tested concentration (1390 mg/kg d.w.). Such results are in accordance to the qualitative observations conducted in the first exposure period, evidencing the absence of the clitellum after 14 and 28 days of exposure at 1390 mg/kg d.w. A concentration-response relationship was evidenced in Fig. 2.

Considering that the average reproduction in the negative controls was of 358 juveniles (Table 5-SM in Supplementary Material), the median effective concentration (EC50) should reduce the population of juveniles to the target value of 179 juveniles. Operating on raw data (i.e., effects from each replicate per single treatment concentration) and considering the 4 parameters logistic curve regression eq. $Y = -12.24 + (361.19 + 12.24)/(1 + (X/11.04)\hat{}0.80)$, ($R^2 = 0.974$, MSE = 613) generated from the best fit of the observations, the EC50 was set at 10.40 mg/kg of cC_6O_4 (7.00–14.20 as confidence interval at 95 %) (Fig. 2), while the EC10 was set at 0.80 mg/kg of cC_6O_4 (0.26–1.75 as confidence interval at 95 %) (Table 1).

Data from the reproduction test can be evaluated also as the reduction in the mean juveniles production compared to the negative control. At the lowest investigated concentration (2.79 mg/kg d.w.), the reduction rate in juveniles production was of 27 %, increasing to 45 %, 77 %, 97 %, and 100 % at 9.25, 40.5, 256, and 1390 mg/kg d.w. respectively.

3.1.2. Comparative assessment

To compare the toxicity of cC_6O_4 for oligochaetes with that of other PFAA (with focus on perfluoroalkyl carboxylic acids, PFCA, and perfluoroalkyl sulfonic acids, PFSA), a literature search was carried out selecting studies with similar endpoints (survival, growth, and reproduction) and experimental design (toxicity test on *Eisenia* sp. exposed to spiked soils under lab conditions); results are displayed in Table 2. To date, only few studies investigated the toxicity of PFAS towards terrestrial invertebrates and given the scarcity of data, it is not yet possible to evaluate the potential ecotoxicological differences related to different PFAS groups (Ankley et al., 2020). Although these limitations, some preliminary consideration can be done based on available data, with special focus on PFOS and PFOA (Kwak et al., 2020; Joung et al., 2010; Yuan et al., 2017; Zheng et al., 2016; Sindermann et al., 2002; Wang et al., 2022; Karnjanapiboonwong et al., 2018; He et al., 2016; Xu et al., 2013).

Most of the available studies are related to 14-days toxicity tests (endpoint: survival, Table 2) conducted with PFOA and PFOS; all studies refer to nominal concentrations, except the study of Sindermann et al. (2002), that represents also the only one conducted in compliance with Good Laboratory Practice standards. As also observed for aquatic organisms (Bizzotto et al., 2023), PFOS is more toxic than PFOA; however, while for aquatic organisms the differences for the same endpoints (determined for PFOS and PFOA) are often greater than an order of magnitude, in the case of earthworms the differences are quite small (generally less than a factor of 3), with LC50 values in the range 365–540 mg/kg d.w. for PFOS and 656–1000 mg/kg d.w. for PFOA. In comparison, cC_6O_4 appears less toxic than PFOS and PFOA, since no effect on survival was observed at the maximum tested concentration (28-day NOEC for survival: 1390 mg/kg d.w.). This observation is also consistent with the bioaccumulation potential; as better detailed in section 3.2, the BSAF determined for cC_6O_4 appears significantly lower than PFOS and PFOA (as well as the other considered PFAS), suggesting a relationship between the low toxicity and low capability to bioaccumulate into the organism.

Considering the endpoint related to the growth, for cC_6O_4 biomass loss was observed only at the maximum tested concentration (LOEC = 1390 mg/kg d.w., NOEC = 256 mg/kg d.w.) (Table 1). As a comparison, very few observations are available for PFOA and PFOS; the limited data available in literature suggest that, for earthworms exposed to these compounds, the growth is an endpoint less sensitive than the survival (Joung et al., 2010; Zheng et al., 2016; Sindermann et al., 2002), although some studies documented slight effects on growth in earthworms exposed to sublethal concentrations of PFAA (He et al., 2016; Xu et al., 2013; Karnjanapiboonwong et al., 2018). As example, He et al.

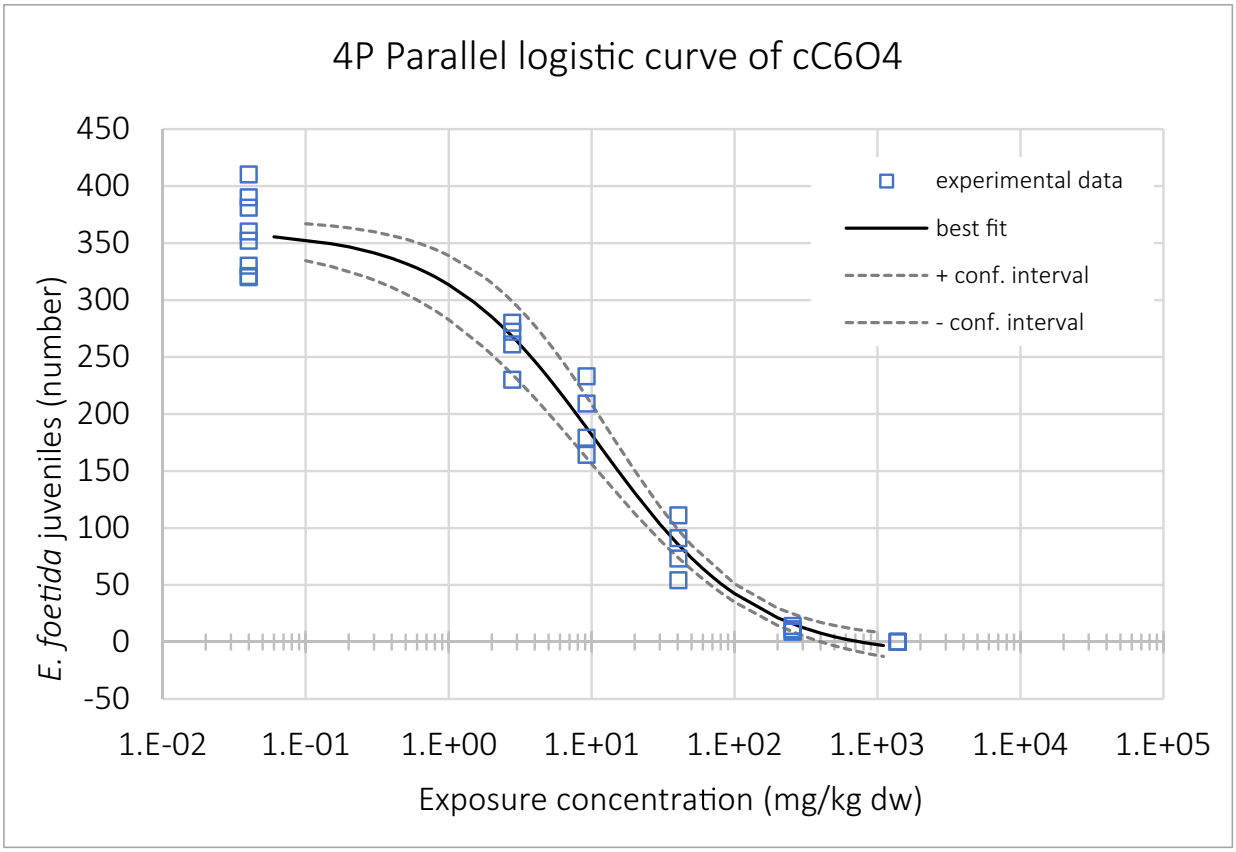

Fig. 2. Concentration-response curve of cC_6O_4 at day 56 on raw experimental data and the modelled data (± 95 % confidence interval) considering the x-axis in the log representation.

(2016) investigated the PFOA sublethal toxicity (PFOA up to 100 mg/kg d.w.) in a 28-day spiked test conducted on two different soils; authors reported absence of relevant effect (<10 %) on growth of earthworm cultured in one of the tested soil while in the second soil they observed a slight weight loss (~15–25 %) in earthworms exposed to PFOA concentrations of 50–100 mg/kg d.w. Another study (Xu et al., 2013) reported that sublethal concentration of PFOS can determined a growth inhibition on earthworms; however, for prolonged exposure period (42 days), only earthworms exposed to the highest treatment of PFOS (120 mg/kg d.w.) were significantly different from the control group.

As regards other PFAAs, only one study was found in the literature which investigated the toxicity of PFBS, PFHxS, PFHpA, PFNA in oligochaetes exposed for 21 days (Karnjanapiboonwong et al., 2018); this study, which evaluated a relatively small range of concentrations (0–100 mg/kg d.w.), essentially showed no mortality and the presence of a slight reduction in growth for oligochaetes exposed to 100 mg/kg d.w. of PFHpA and PFNA.

Finally, with regard to the endpoint reproduction, effects of PFAA on earthworm are substantially unknown and, to the best of our knowledge, there is not a reliable dataset useful for a direct comparison with cC_6O_4.

In summary, the overall available data related to the endpoint survival suggest an increasing trend of toxicity from cC_6O_4 to PFOA and PFOS; additionally, the toxicity testing done on cC_6O_4 indicates that, for this substance, the reproduction endpoints are more sensitive than those related to survival and growth. However, it must be noted that, for all these substances, toxicity on earthworms is relatively low, especially when compared with realistic environmental concentrations. With special regard to cC_6O_4, all the effect thresholds determined in the toxicity test on earthworm (including those for reproductive endpoints) are significantly higher than the expected environmental concentrations. As term of comparison, the cC_6O_4 concentrations measured in soils sampled near the industrial plant of Spinetta Marengo (and therefore to be considered close to the maximum realistically conceivable at a global level), are in the range of a few μg/kg d.w. (unpublished data, presented by Valsecchi in the SETAC Europe 2022), orders of magnitude lower than the lowest NOEC value determined in this study. Therefore, the possibility of a risk for terrestrial invertebrates is unlikely even at the highest environmental concentrations measured.

3.2. *Bioaccumulation of cC_6O_4 in earthworm and comparative assessment*

To evaluate bioaccumulation, earthworms exposed to scenarios C0, C1 and C4 were collected for chemical analysis only at the end of the 28 days exposure period. Therefore, the experimental design did not allow the confirmation of steady-state conditions. However, according to Burkhard and Votava (2023), an exposure time of 21–28 days may be suitable for the attainment of a steady-state condition for PFOA and PFOS while this is unlikely for PFAS larger than C-9. Therefore, a steady-state condition after 28 days of exposure may be assumed for cC_6O_4.

Measured concentrations of cC_6O_4 in earthworms exposed to the control and two different soil treatments are reported in Table 3, while BSAF are shown in Table 4. cC_6O_4 was not detected in earthworms exposed to the control soil (although a residual concentration was detected in the soil, 0.04 mg/kg d.w.), while organisms exposed to soil treatment C1 and C4 (2.79 and 256 mg/kg d.w. of cC_6O_4 in soil) showed cC_6O_4 values respectively in the range 0.001–0.004 and 0.010–0.048 mg/kg w.w. (Table 3).

Fig. 3 shows the comparison between the $BSAF_{ww/OC}$ determined for cC_6O_4 and literature data available for other PFAAs (specifically, carboxylic and sulfonic acids, PFCA and PFSA). Specifically, the BSAFs reported in Fig. 3 as term of comparison with cC_6O_4 refer to values

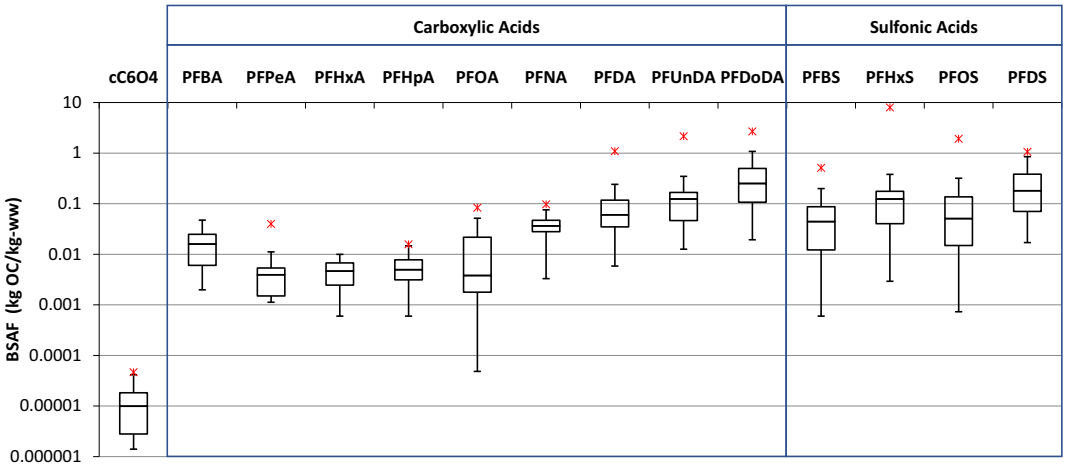

Fig. 3. Boxplots of BSAF (Biota-Soil Accumulation Factors) determined for cC_6O_4 (considering overall replicates investigated in this study) and perfluoro-carboxylic (PFCA) and -sulfonic (PFSA) acids in laboratory studies on Eisenia sp. BSAF values reported in the figure are expressed as $BSAF_{ww/OC}$ (Kg-OC/kg ww). PFCA and PFSA are ordered from left to right by increasing length of the aliphatic chain. The lines in the boxes are median, ends of the boxes the 25th and 75th percentiles; the ends of the whisker are set at 1.5*IQR (Inter Quartile range) above the 75th percentile and below the 25th percentile. The Minimum or Maximum values falling outside this range are shown as outliers. PFBA = Perfluorobutanoic acid, PFPeA = perfluoropentanoic acid; PFHxA = perfluorohexanoic acid; PFHpA = perfluoroheptanoic acid; PFOA = perfluorooctanoic acid; PFNA = perfluorononanoic acid; PFDA = perfluorodecanoic acid; PFUnDA = perfluoroundecanoic acid; PFDoDA = perfluorododecanoic acid, PFBS = perfluorobutane sulfonic acid; PFHxS = perfluorohexane sulfonic acid; PFOS = perfluorooctane sulfonic acid; PFDS = perfluorodecane sulfonic acid.

determined dividing earthworms concentrations by soil concentration normalized on the organic carbon content; the $BSAF_{ww/OC}$ for PFAAs were extrapolated from the recent review of Burkhard and Votava (2023), selecting values measured in laboratory studies on *Eisenia* sp. with an exposure period of at least 21 days and standard exposure condition (Zhao et al., 2013, 2014, 2016, 2018; Navarro et al., 2016; Amundsen et al., 2008; Braunig et al., 2019; Das et al., 2013; He et al., 2016; Jarjour et al., 2022; Rich et al., 2015; Sobhani et al., 2021; Wang et al., 2022; Wen et al., 2015; Karnjanapiboonwong et al., 2018; Munoz et al., 2020).

Overall results highlight the extremely low bioaccumulation potential of cC_6O_4 in earthworms. As reported by Burkhard and Votava (2023), $BSAF_{ww/OC}$ determined for the carboxylic acids and sulfonic acids, although low, tend to increase with increasing carbon chain length; in comparison, cC_6O_4 in earthworm presents BSAF significantly lower (2–3 orders of magnitude) than PFCA and PFSA, suggesting radically different uptake and elimination rate compared to what observed for the other PFAA. The reduced bioaccumulation potential of cC_6O_4 is confirmed also by the $BSAF_{ww/ww}$ values (thus, considering the water content present in earthworms and also in soils); since it is expected that water represents the main vector of cC_6O_4 in the organisms, the $BSAF_{ww/ww}$ represent a proper metric to evaluate cC_6O_4 bioaccumulation. Specifically, a $BSAF_{ww/ww}$ value of 1 indicates a condition of equilibrium, i.e., that concentrations in the organisms are equal to the concentration in the environmental matrix (in this case, the soil, including both the solid and aqueous phase), while values lower than 1 indicate that the elimination process (eg. excretion, metabolization) are predominant over those of uptake; the values determined for cC_6O_4 in earthworms are order of magnitude lower than 1, thus indicating absence of bioaccumulation probably due to efficient elimination process. Available data do not allow to provide a clear explanation for the low BSAF values observed for cC_6O_4 in *Eisenia* sp.; possible hypotheses include an enhanced depuration kinetic of cC_6O_4 in earthworms, that could present for this substance a greater depuration and excretion capacity compared to PFCA and PFSA (characterized by a lower water solubility and higher Log K_{ow} than cC_6O_4).

Additionally, it cannot be excluded a reduced uptake of cC_6O_4 from the tested soil, due to chemical structure and/or to the characteristics of the soil. However, it should be noted that the analyses on eluates confirmed the high solubility of cC_6O_4 in water, since the concentrations measured in the eluates are consistent with a release equal to 100 % of the cC_6O_4 present in the spiked soils used for the eluate preparation (Fig. 3-SM in Supplementary Material). These observations are in line with the physico-chemical properties of the substance, being the cC_6O_4 highly soluble in water (water solubility >667 g/L) and with a low Organic Carbon adsorption coefficient (log K_{oc} 1.04) (Bizzotto et al., 2023), and highlight the high potential of cC_6O_4 to migrate into the environment by transport with water.

Furthermore, the BSAFs measured for cC_6O_4 in *E. foetida* decline with increasing concentrations in the soil, similarly to what observed in other PFAA study on earthworm (Wen et al., 2015; Zhao et al., 2013, 2014) and to collective data assembled in Burkhard and Votava (2023). A similar behavior has also been observed in bioconcentration factors (BCF) measured in aquatic organisms exposed to cC_6O_4 in a mesocosm study (Rico et al., 2023). To date, the mechanisms for BSAFs declining with increasing PFAA exposure concentrations is not still understood and represent a data gap (Burkhard and Votava, 2023). Further studies are requested to better understand and predict bioaccumulation process of PFAA, and cC_6O_4 as well, with focus on metabolic pathways and uptake and elimination kinetics in organisms.

4. Conclusions

The results of the present work contribute to cover the gap on the effects of cC_6O_4 on terrestrial organisms. Additional information will be provided by the results of tests on terrestrial plants that are currently under development.

Overall results indicate a very low bioaccumulation potential and toxicity of cC_6O_4 in earthworms, particularly considering that the lowest tested concentration (exposure scenario C1) is about three orders of magnitude higher than the highest concentrations measured in the topsoil sampled very close to the industrial production site of cC_6O_4. It follows that even the most sensitive adverse effect tested on earthworms (reduction of reproduction) have been observed only at concentrations that are very far from environmentally realistic concentrations.

A comparison with the data currently available in scientific literature indicate that, although in general, the toxicity of PFAS (PFCA o PFSA) on earthworms is relatively low, cC_6O_4 is less toxic than other examined

PFAS. As for bioconcentration and bioaccumulation, measured values of BSAF are orders of magnitude lower than all other considered PFAS, probably due to a very high capability for depuration and excretion.

In conclusion, the results of this study substantially confirm, for terrestrial invertebrates, the relatively low toxicity and negligible bioaccumulation potential of cC_6O_4 that has been already demonstrated for the freshwater ecosystems.

CRediT authorship contribution statement

Elisa Chiara Bizzotto: Conceptualization, Data curation, Funding acquisition, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Giovanni Libralato:** Formal analysis, Investigation, Resources, Visualization, Writing – original draft, Writing – review & editing. **Silvia Breda:** Data curation. **Antonietta Siciliano:** Investigation. **Petra Scanferla:** Funding acquisition, Project administration. **Marco Vighi:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing. **Antonio Marcomini:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Author and co-authors reports financial support was provided by Solvay Specialty Polymers Italy S.p.A. Marco Vighi reports a relationship with Solvay Specialty Polymers Italy S.p.A. that includes: consulting or advisory.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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