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Sublethal effects of Cu and Cr on *Eisenia andrei*: Life-cycle endpoints and biomarker integration for soil assessment

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A B S T R A C T

Soil contamination by heavy metals poses profound ecological challenges owing to their persistent nature and inherent toxicity. This study aims to assess sublethal effects of copper (Cu) and chromium (Cr) on earthworm *Eisenia andrei* by integrating life-cycle endpoints and biomarkers to develop an early-warning framework for soil contamination and ecological risk assessment. Natural soils were spiked with increasing concentrations of Cu (0 −1600 mg/kg) and Cr (0–200 mg/kg) and organismal responses were evaluated by assessing survival, growth (after 10 or 28 days) and reproduction (after 56 days), and a suite of physiological and biochemical biomarkers, including membrane stability (LMS), accumulation of lipofuscin (LF) and unsaturated neutral lipids (NL), synthesis of metallothioneins, and activities of Ca^{2+}-ATPase (CaATP), glutathione S-transferase, catalase, and acetyl-CoA oxidase (AOX) after 10 days. The reproduction endpoint emerged as the most sensitive indicator of toxicity, with estimated effective concentrations (EC50) of 355 mg/kg for Cu and 150 mg/kg for Cr. Conversely, effects on survival and growth were primarily observed at the highest concentrations. Among biomarkers, LMS and LF were the most sensitive parameters, with EC50 values of 157 and 142 mg/kg, respectively. For Cr, AOX was the most sensitive biomarker (EC50 of 131 mg/kg), followed by LMS. For Cr, AOX was the most sensitive biomarker (EC50 of 131 mg/kg), followed by LMS. The findings highlight that the selected biomarkers can detect sublethal stress from metal contamination prior to observable effects on life-cycle parameters. This study underscores the usefulness of an integrated approach for early detection of soil contamination and proactive ecological management and preservation of soil health.

1. Introduction

Soil contamination poses a significant global concern due to its potential adverse effects on the food chain, influencing the health of plants, animals, and humans (Liang et al., 2017; Global assessment of soil pollution, 2021). The presence of a diverse array of organic and inorganic pollutants including emerging contaminants, many of which possess mutagenic or carcinogenic properties (Manoj et al., 2020; Bayabil et al., 2022) underscores the importance of addressing soil contamination both to ensure ecosystem health and for safeguarding food security and public health.

Among soil pollutants, heavy metals are contaminants of particularly concern, known for their persistence, toxicity, and potential for mutagenicity (Balali-Mood et al., 2021). Heavy metals are disseminated globally via various anthropogenic activities, including industrial emissions, mining operations, painting, agricultural practices, etc (Vareda et al., 2019; Zhao et al., 2022). Although some metal elements, such as copper, manganese, and zinc, are essential for biological processes, they can become detrimental at elevated concentrations (Jomova et al., 2022). In contrast, other heavy metals, such as cadmium, lead, and mercury, do not serve any biological function and are toxic even at low exposure levels (Tchounwou et al., 2012).

In soil copper (Cu) occurs in two oxidation states: Cu(I) and Cu(II), the latter being more mobile in acidic, oxidizing environments (Rudnick, 2003). Cu tends to complex organic matter and to adsorb onto clay minerals and metal oxides, resulting in substantial retention within soils. Natural soil Cu concentrations generally range from 2 to 50 mg/kg (Rudnick, 2003; Albanese et al., 2015; Ballabio et al., 2018). European soil surveys report average Cu levels of 16 mg/kg (FOREGS, (Lado et al., 2008)), 20 mg/kg (GEMAS, (Reimann et al., 2018)), and 16.86 mg/kg

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(LUCAS, (Ballabio et al., 2018)). Agricultural use of Cu-based fungicides is the major anthropogenic source of Cu accumulation in soils (Brunetto et al., 2014). While Cu is an essential micronutrient for plants and animals, excessive levels pose toxicity risks due to its persistence and bioaccumulation potential (Fagnano et al., 2020). The EU Directive 86/278/EEC recommends Cu limits between 40–140 mg/kg in agricultural soils with pH 6–7, although specific national thresholds may vary (Albanese et al., 2015).

Chromium (Cr), primarily occurs in two oxidation states: trivalent chromium (Cr(III)), which is stable and poorly mobile, and hexavalent chromium (Cr(VI)), which is highly mobile, bioavailable, and toxic, especially as chromate (CrO_4^{2-}) under alkaline conditions (Kotaś and Stasicka, 2000). Cr behaviour in soils is influenced by several factors, including parent material, pH, redox potential, and organic matter content (Kabata-Pendias, 2000). While Cr is considered an essential micronutrient with roles in glucose metabolism (Vincent, 2019), Cr(VI) is a known carcinogen and potent oxidant capable of causing adverse effects even at low concentrations (Ray, 2016; Ao et al., 2022; Kapoor et al., 2022). Anthropogenic sources, particularly from industrial activities such as chrome plating, leather tanning, and textile manufacturing, significantly contribute to local Cr contamination (Pushkar et al., 2021). In soils, Cr often complexes with organic matter and Fe or Mn oxo-hydroxides, affecting its mobility and bioavailability (Zulfiqar et al., 2023).

Earthworms are key components of soil fauna useful as effective bioindicators for assessing soil contamination (Calisi et al., 2024) and recognized for their potential in remediation (Xiao et al., 2022). They interact with a variety of organic and inorganic pollutants and play a crucial role in the food web by serving as prey for numerous animals, including birds, mammals, reptiles, and amphibians.

Uptake of heavy metals by earthworms occurs primarily through dermal contact or ingestion (Lanno et al., 2004; Pérès et al., 2011), making them highly susceptible to soil contaminants. Due to their ecological relevance and sensitivity, species such as *Eisenia fetida* and *Eisenia andrei* (following *E. fetida* and *E. andrei*) have been selected as standard test organisms in soil toxicity assessments. These species are employed in established methodologies that evaluate various endpoints, including survival, reproduction, and avoidance behaviour. The short life cycle of *E. andrei* under laboratory conditions (≈ 20 °C, with adequate moisture and food) makes this species ideal for experimental studies. Cocoons hatch after 2–3 weeks, juveniles reach sexual maturity within 6–8 weeks, and the complete life cycle from cocoon to adult spans approximately 8–10 weeks (OECD, 2016).

The use of earthworms for biomonitoring has increasingly included the analysis of biomarkers, i.e. measurable biological changes that occur at molecular, biochemical, cellular, or histological levels in response to individual contaminants or mixtures (Lionetto, 2012; Solsona et al., 2021; Hattab et al., 2023). Monitoring these alterations enables the early evaluation of potential adverse effects, providing a proactive framework for detecting pollution impacts before they manifest as severe ecological consequences. This early-detection capability underscores the critical role of biomarkers as integrative tools in ecotoxicology, environmental biomonitoring, and ecological risk assessment (Viarengo et al., 2007; Shi et al., 2017). The concept of early warning implies that assessing biomarker responses allow predictions about the long-term effects on individual organisms, populations, and communities. A biomarker can be an effective early warning indicator if there is a clear and consistent correlation established between its response, measured after short-term exposure, and long-term ecologically significant effects at higher levels of biological organization (Chambers et al., 2002). However, it is noteworthy that such correlations are often hypothesized rather than empirically demonstrated, and few biomarkers have had their relationships with organism fitness unequivocally established (Forbes et al., 2006; Lam, 2009).

While numerous studies have validated molecular, biochemical, and histological biomarkers in earthworms (Spurgeon et al., 2003; Svendsen et al., 2004; Gastaldi et al., 2007; Gambi et al., 2007; Boughattas et al., 2016), few have directly compared their dose-response relationships with life-cycle endpoints (Clasen et al., 2021; Spurgeon et al., 2000; Reinecke et al., 2002; Moore, 2013). Here, we demonstrate that integrating multiple biomarkers with life-cycle endpoints enhances their effectiveness as early warning systems for predicting soil contamination and assessing ecological risk. Three life-cycle endpoints survival, growth, and reproduction in *E. andrei*, along with seven histochemical and enzymatic biomarkers, were analysed to compare their sensitivity and assess their predictive value as early indicators of Cu or Cr(VI) stress in spiked soils. The biomarkers included lysosomal membrane stability (LMS), neutral lipid (NL) and lipofuscin (LF) accumulation, metallothionein (MT) content, and the activities of Ca^{2+}-ATPase (CaATP), glutathione S-transferase (GST), catalase (CAT), and acetyl-CoA oxidase (AOX).

2. Materials and methods

Two independent experiments were performed at different times, exposing earthworms to soils spiked with Cu (first experiment) and Cr (VI) (second experiment). The life-cycle endpoints and biomarkers assessed in both experiments are summarized in Table 1.

2.1. Earthworms

E. andrei individuals (Bouché, 1972) were obtained from a vermiculture company (Lombricoltura Compagnoni, Como, Italy). Several weeks before the experiments, the worms were incubated in a mixture of peat, cow manure, and soil under the same environmental conditions used during the experiment (temperature: 18 ± 1 °C; illumination: 400–800 lux; photoperiod: 16 hr light / 8 hr dark) and were fed weekly with wheat bran. As suggested by OECD protocols (OECD, 2016), to minimize physiological variability, ensure uniform physiological conditions and reproducible experimental outcomes, individuals within a range of 4 weeks of maturity, similar size and well-developed clitellum were selected for the experiments. They were removed from the culture 24 h before the start of the experiment and kept on moist filter paper at 18 ± 1 °C in the dark, to allow voiding of gut content (OECD, 2016). Mean individual live weight at the beginning of the trials was 466 mg and 364 mg for Cu and Cr(VI) experiment, respectively.

2.2. Soil preparation and spiking with Cu and Cr

The used soil was collected in a lowland rural area (Municipality of Argenta, NE Italy) from a plot that had not received chemical treatments for ten years (pH 7.3, total organic carbon 2.3 %, clay 50 %, silt 33 %, sand 17 %). The soil was oven dried at 60 °C, grinded and sieved at 2 mm.

Experiments were conducted in glass containers (20 × 2 cm, height 8 cm). One week before the start of the exposure, each container was filled with 600 g of dry and sieved soil. Solutions of copper chloride ($CuCl_2$) or potassium dichromate ($K_2Cr_2O_7$) were then mixed with the soil to achieve the desired water content corresponding to the field capacity (approximately 30 % w/w) and the target metal concentrations. The same volume of distilled water was added to the unspiked control

Table 1
Summary of the life cycle endpoints and biomarkers considered in both experiments. Bold indicates assessed only for Cr experiments.

Cu experiments	Cr experiments
Life cycle endpoints:	Life cycle endpoints:
N° of adult alive on day 10 and 28	N° of adult alive on day 10 and 28
Worms weight on day 28	Worms weight on day **10** and 28
N° cocoons and juveniles on day 56	N° cocoons and juveniles on day 56
Biomarkers on day 10	Biomarkers on day 10
LMS, LF, CaATP, MT	LMS, LF, CaATP, MT, **NL, AOX, CAT, GST**

soils. Soil moisture was weekly maintained by determining the weight loss of the containers and replenished with distilled water over the 56-day period.

The choice of the metal experimental concentrations was based on the results of a previous range-finding tests, and the applied nominal concentrations were 0, 25, 50, 100, 200, 400, 800, 1600 mg/kg for Cu and 0, 6.25, 12.5, 25, 50, 100, 200 mg/kg for Cr(VI).

Two sets of containers were prepared: one for life-cycle endpoints and another for histochemical and enzymatic biomarkers. In both series, four replicate containers were established for each Cu and Cr(VI) concentration, as well as for the unspiked soil. Before the start of the exposure, 130 g of wet soil were removed from each container and stored for the chemical analyses, leaving 650 g of wet soil, corresponding to 500 g of dry soil, in the containers.

2.3. *Experimental exposure and life-cycle endpoints measurement*

Groups of 10 individuals were formed, and each group was weighed and randomly assigned to an experimental container. Containers were then covered with transparent perforated polyethylene sheets and immediately placed in an environmental chamber (18 ± 1 °C; 400–800 lux; 16 h light, 8 h dark) (Gastaldi et al., 2007).

The containers for the biomarkers were incubated for 10 days, as indicated by Sforzini et al (Sforzini et al., 2012). and earthworms were not fed. At the end of the exposure living worms were recovered and counted. Four individuals for each concentration (one individual from each replicate) were extracted and immediately processed for LMS analysis. All other worms were kept for one day on moist filter paper in Petri dishes in the dark, to allow voiding of gut content and then processed for further biomarkers analyses. The containers of the life-cycle were further incubated and processed according to the procedure outlined in OECD guidelines (OECD, 2016). Adults were kept in the experimental vessels for 28 days and fed every 7 days with 3 g of wheat bran. On day 28 all adult worms were removed from the containers, counted, weighed, kept for one day on moist filter paper and then analysed for Cu and Cr bioaccumulation. Soil replicates containing any cocoon that had been produced was incubated for further 28 days at the same environmental conditions. Food was provided only at the beginning of the second 28-day period. At day 56 hatched juveniles and empty and full cocoons were counted by hand sorting and then wet sieving the soil. Adult survival (number of individuals alive at the end of the first 28-day period), adult growth (live weight at the end of the first 28-day period), and reproduction (number of cocoons and number of hatched juveniles) were assessed.

2.4. *Biomarker analysis*

A battery of seven biomarkers was evaluated in *E. andrei* to assess sublethal toxic effects. After exposure, four earthworms for each concentration were processed for LMS analysis or were dissected, and tissues were processed immediately or stored at −80 C depending on the endpoint.

LMS was assessed in coelomocytes using the Neutral Red Retention Assay (NRRA). NL and LF contents were quantified in chloragogenous tissue on cryosections stained with Oil Red O or ferric reaction medium, respectively, as described by Gastaldi et al (Gastaldi et al., 2007). MT content was determined spectrophotometrically using the Ellman's reagent according to Viarengo et al (Viarengo et al., 1997). CaATP activity was histochemically measured in cryosections of the post-clitellar intestinal tract as in Gastaldi et al (Gastaldi et al., 2007). Following the preparation protocol reported by Mimeault et al (Mimeault et al., 2006)., CAT and GST activities were assessed in whole-body homogenates by spectrophotometric assays and protein assessed according to Lowry et al (Lowry et al., 1951). AOX activity was quantified via absorbance at 502 nm after substrate incubation (Cancio et al., 1998). Methodological details are provided in the Supplementary Materials.

2.5. *Metal determination in soil and bioaccumulation in earthworms*

To determine Cu and Cr tissue concentrations, the earthworms were placed alive on moist filter papers without food for one day to empty their gut. The earthworm species were washed clean, patted dry, lyophilized, and then digested in a microwave with concentrated HNO_3 and $HClO_4$ (ratio 5:1) in Teflon vessels, evaporated to dryness and recovered with concentrated HCl to a final volume of 25 mL. The total Cu and Cr concentrations were measured by FAAS with a Perkin-Elmer mod. 5000 spectrophotometer. For each exposure condition the earthworm mineralization was run in triplicate. The international reference material IASS-2 (Antarctic krill) was used as monitor for the FAAS analytical accuracy.

The total metal concentrations in soils were measured by X-Ray Fluorescence spectrometry using a PW1480 Spectrometer equipped with a Rh tube. Determinations were made on pressed powder pellets in triplicate; calibrations were made using international reference materials over a concentration range of 5–4000 mg/kg. The analytical technique provided information also on the bulk geochemical composition of the soil sample used in the experiment.

Bioavailable Cu and Cr(VI) were evaluated after DTPA extraction performed following Lindsay and Norwell (Lindsay and Norvell, 1978), mixing 10 g of ground soil with 40 mL of a solution containing 0.005 M DTPA, adjusted to pH 7.3, for 1 hr. Cr(VI) was evaluated using the difenilcarbazide reagent and readings were made using a portable spectrophotometer HACH DR/2010. For calibration, artificial solutions of Cr(VI) were made by mixing $K_2Cr_2O_7$ in DTPA with deionized water. The bioavailable Cu concentrations were measured by FAAS with a Perkin-Elmer mod. 5000 spectrophotometer.

2.6. *Statistics*

The EC50 was estimated for each endpoint by fitting a four parameters logistic model to the experimental data. The equation used was:

$$y = bottom + \frac{(top - bottom)}{1 + \left(\frac{x}{\log_{10}EC50}\right)^{slope}} \tag{1}$$

Where: y = endpoint value; x = $\log_{10}$ of total Cu or Cr concentration; *top* = top asymptote of the curve; *bottom* = bottom asymptote; *slope* = slope parameter, refers to the steepness of the curve (Eq. 1). The bottom parameter was set to 0 for life-cycle endpoints, since at high concentrations of toxicants survival, live weight and reproduction all tend to zero. On the other hand, readings for biomarkers were never below a threshold, corresponding to a positive value for the *bottom* parameter. Following the same methodology, except that $x = \log_{10}$ of the bioaccumulated Cu or Cr concentration we also calculated IEC50 and compared them with the EC50, but only for the life-cycle endpoints. This is because bioaccumulation was measured at 28 days, which was too distant from day 10, when the biomarkers were assessed. The parameters of the equations and their 95 % confidence intervals, were estimated using non-linear regression procedures (Levenberg-Marquardt estimation method) as implemented in Statistica 6 (Statsoft, Tulsa, OK, USA). Lastly, model-based curves were used to examine the relationship between effects on juveniles and biomarker responses. This approach estimates the magnitude of short-term biomarker changes associated with specific long-term reductions in reproduction, effectively addressing questions such as: "*What level of early biomarker alteration corresponds to a 20 % reduction in reproduction at longer exposure durations?*".

3. Results

3.1. Metal in soil and bioaccumulation

The total concentration of Cu measured in the unspiked soil was 35.2 ± 2.7 mg/kg (mean ± standard error) (Table 2). The spiked Cu concentrations exhibited good concordance with the nominal values, particularly when the background concentration was subtracted (Table 2), and both bioavailable and bioaccumulated Cu in earthworm tissues exhibited an approximately linear relationship with total Cu concentrations. Specifically, bioavailable Cu ranged from 5.2 ± 0.4 mg/kg to 405.8 ± 3.4 mg/kg as total Cu concentrations increased from 35.2 to 1873.4 mg/kg. Bioaccumulated Cu remained relatively constant between 15 and 24 mg/kg when soil Cu levels were below 133.7 mg/kg but showed a clear linear increase from 27 mg/kg to 120 mg/kg as soil Cu rose within the 133.7–1873.4 mg/kg range (Table 2). The bioaccumulation factor (BAF) stayed below 1.0 across all tested concentrations, with a pronounced decrease from 0.6 in unspiked soil to 0.06 at the highest total Cu concentration (1873.4 mg/kg) (Table 2).

The total Cr concentration in the unspiked soil was 120.4 ± 3.9 mg/kg (Table 3). A strong correlation was maintained between nominal and total Cr, although deviations became more evident at higher concentrations (Table 3). For total Cr up to 149.5 mg/kg, bioavailable Cr(VI) remained low and relatively stable, ranging from 0.02 to 0.06 mg/kg. In contrast, bioavailable Cr(VI) increased markedly, reaching a maximum of 17.8 mg/kg at higher Cr concentrations (Table 3). Bioaccumulated Cr exhibited an exponential relationship with total Cr concentration, as confirmed by exponential regression analysis ($r^2 = 0.964$; F-test, $P < 0.001$). The bioaccumulation factor (BAF) remained nearly constant (≈ 0.1) within the Cr range of 120.4–149.5 mg/kg, but showed a pronounced increase, rising to 0.2, 0.5, and 1.5 at total Cr concentrations of 172.7, 217.8, and 299 mg/kg, respectively (Table 3).

3.2. Life-cycle endpoints

In Fig. 1 the relationship between life-cycle endpoints and total Cu concentrations is illustrated (raw data in Table S1 of Supplementary Material). Relationship between life-cycle endpoints and bioaccumulated Cu concentrations are available in Figure S1 of Supplementary Material. All endpoints exhibited clear sensitivity to Cu within the tested concentration range. Survival rates remained consistently high, ranging from 90 % to 100 %, in soil containing Cu up to 949.7 mg/kg and 382.8 mg/kg after 10 and 28 days of exposure, respectively (Fig. 1a,b). Then it declined dramatically to approximately 20 % following exposure to 1873.4 mg/kg Cu for both exposure durations. After 28 days, earthworms in unspiked soil reached an average total biomass of 6.0 g, representing a 29 % increase from their initial weight. In contrast, exposure to the highest Cu concentration (1873.4 mg/kg) caused a marked decline in biomass to 0.9 g (Fig. 1d), driven by increased mortality and 8 % reduction in individual weight (average = 453 mg). Unspiked soil replicates produced an average of 47 cocoons and 96 juveniles. Reproduction was already significantly impaired at 382.8 mg/kg Cu and was completely inhibited at the highest tested concentration (Fig. 1e,f). Concentration–response relationships for survival, growth, and reproduction were well described by logistic regression models, all showing high statistical significance (Table 4). Among the assessed endpoints, survival exhibited the highest EC50 values of 1504.2 mg/kg after 10 days and 1408.2 mg/kg after 28 days, followed by growth (1057.2 mg/kg). Reproductive endpoints were the most sensitive, with EC50 values of 511.2 mg/kg for cocoon production and 355.2 mg/kg for juvenile output. Although calculated only for life-cycle endpoints, the IEC50 preserved the same sensitivity ranking as the EC50 but was one order of magnitude lower (Table 4).

The concentration–response relationships for life-cycle endpoints

Table 2

Total and bioavailable Cu in soils spiked at eight nominal concentrations, and Cu bioaccumulated in *Eisenia andrei* after a 28-day exposure. BAF: bioaccumulation factor. Bold indicates background concentration.

Cu nominal concentration (mg/kg)	Total Cu (mg/kg) mean ± SE (*n* = 4–8)	Difference between Total Cu - Background (mg/kg)	Bioavailable Cu (mg/kg) mean ± SE (*n* = 4–8)	Bioavailable / total (%)	Bioaccumulated Cu (mg/kg) mean ± SE (*n* = 2)	BAF (Bioaccumulated / Total)
	35.2 ± 2.7 (Background)		5.2 ± 0.4	14.8	22.0 ± 0.9	0.63
25	59.2 ± 1.9	24.0	14.7 ± 1.5	24.8	23.8 ± 0.4	0.40
50	88.1 ± 18.8	52.9	22.3 ± 5.8	25.3	18.0 ± 1.3	0.20
100	133.7 ± 35.7	98.5	28.8 ± 7.9	21.5	15.8 ± 0.2	0.12
200	255.8 ± 30.8	220.6	59.7 ± 3.9	23.3	27.0 ± 5.9	0.11
400	382.8 ± 46.8	347.6	106.3 ± 10.2	27.8	49.0 ± 6.0	0.13
800	949.7 ± 86.8	914.5	252.4 ± 22.0	26.6	71.8 ± 1.0	0.07
1600	1873.4 ± 97.5	1838.2	405.8 ± 3.4	21.7	119.9 ± 32.4	0.06

Table 3

Total Cr and bioavailable Cr(VI) in soils spiked at seven nominal concentrations, and Cr bioaccumulated in *Eisenia andrei* after a 28-day exposure. BAF: bioaccumulation factor. Bold indicates background concentration.

Cr(VI) nominal concentration (mg/kg)	Total Cr (mg/kg) mean ± SE (n = 6)	Difference between Total Cr - Background (mg/kg)	Bioavailable Cr(VI) (mg/kg) mean ± SE (n = 2)	Bioavailable / Total (%)	Bioaccumulated Cr (mg/kg) mean ± SE (n = 4)	BAF (Bioaccumulated / Total)
	120.4 ± 3.9 (Background)		0.02 ± 0.03	0.02	11.6 ± 2.3	0.10
6.25	127.3 ± 2.9	6.9	0.06 ± 0.08	0.05	12.4 ± 0.9	0.10
12.5	134.4 ± 2.4	14.0	0.02 ± 0.03	0.01	15.8 ± 1.9	0.12
25	149.5 ± 2.3	29.1	0.04 ± 0.00	0.03	21.7 ± 2.5	0.15
50	172.7 ± 7.6	52.3	0.18 ± 0.03	0.10	40.4 ± 7.4	0.23
100	217.8 ± 5.2	97.4	5.5 ± 2.1	2.53	109 ± 20	0.50
200	299 ± 15	178	17.8 ± 4.2	5.95	453 ± 164	1.51

under total Cr exposure are shown in Fig. 2 (raw data in Table S2 of Supplementary Material). Relationship between life-cycle endpoints and bioaccumulated Cr concentrations are available in Figure S2 of Supplementary Material. All endpoints were significantly affected across the tested range. Survival remained at 100 % up to 172.7 mg/kg Cr for both 10- and 28-day exposures but declined sharply to ~10 % at 299 mg/kg (Fig. 2a,b). In unspiked soils, total earthworm biomass slightly increased after 10 days (4.3 g) (Fig. 2c) and by 55 % after 28 days (5.4 g) (Fig. 2d). Growth inhibition began at 149.5 mg/kg Cr, with total biomass dropping to 0.17 g at 299 mg/kg, due to both elevated mortality and reduced individual weights (down to 233 mg, a 42 % decrease from initial values). Reproductive output averaged 8 cocoons and 13 juveniles in controls but declined to 4 cocoons and 6 juveniles at 149.5 mg/kg Cr, with complete inhibition at 172.7 mg/kg (Fig. 2e,f). Logistic regression model well described the concentration–response relationships (Table 5), though reproductive endpoints showed higher variability at low concentrations. Among life-cycle parameters, survival displayed the highest EC50 values (246.4 and 240.4 mg/kg for 10 and 28 days), followed by growth (215.4 and 195.4 mg/kg). Reproduction was the most sensitive endpoint, with EC50 values around 150 mg/kg, although estimates for juvenile production were less precise due to variability (Table 5). For Cr as well, the IEC50, calculated only for life-cycle endpoints, showed the same sensitivity order, with values close to the EC50 for survival but markedly different for reproduction endpoints (Table 5).

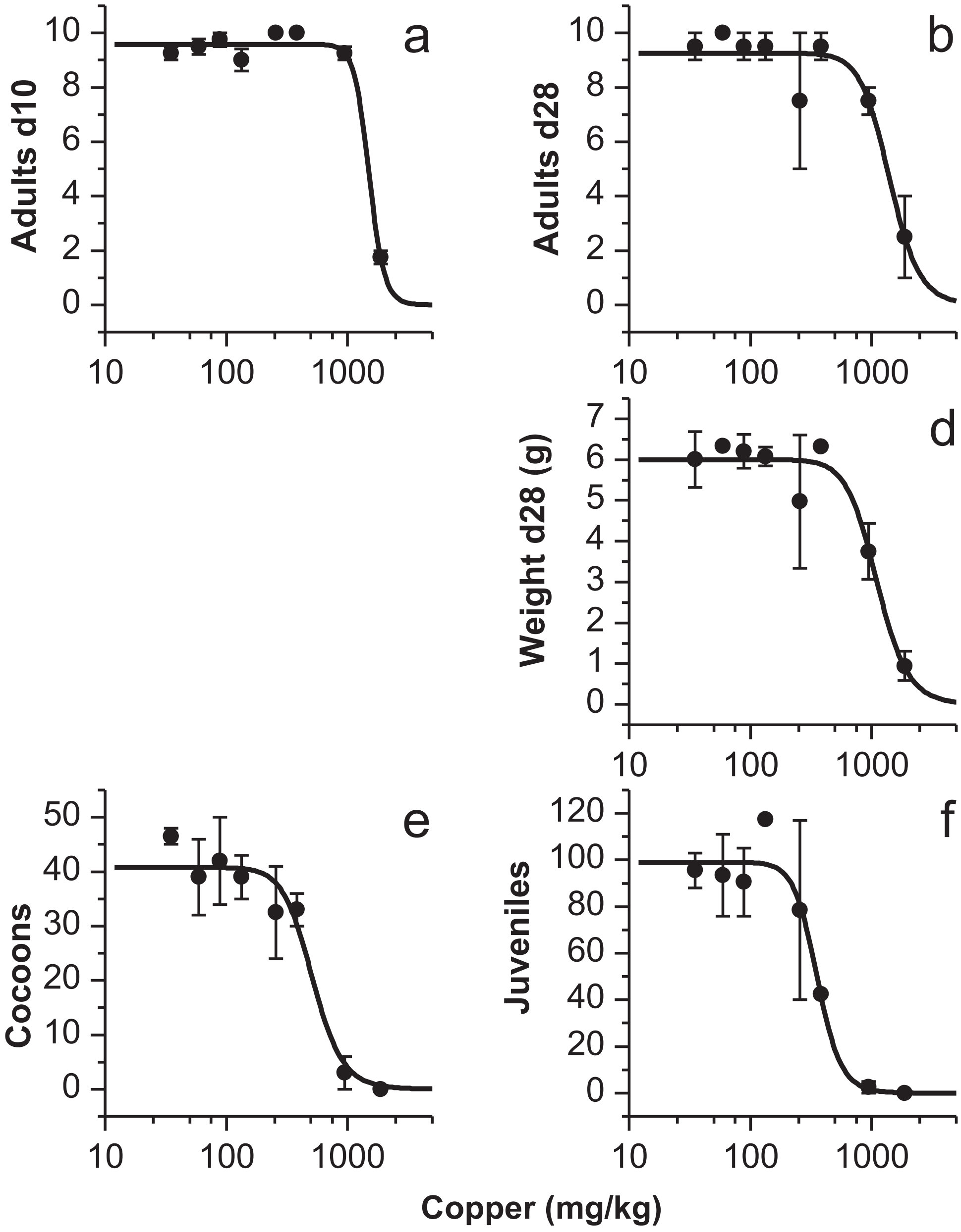

Fig. 1. Relationship between total Cu concentration and life-cycle endpoints. a) Number of adults alive at day 10; b) Number of adults alive at day 28; d) Weight of earthworms at day 28; e) Number of cocoons at day 56; f) Number of hatched juveniles at day 56. Each point is the mean of two replicates, error bars represent standard errors; when not appearing, bars are within symbols. The continuous line represents the logistic model fitted to the observed data. Note: weight of earthworms at day 10 was *not tested.*

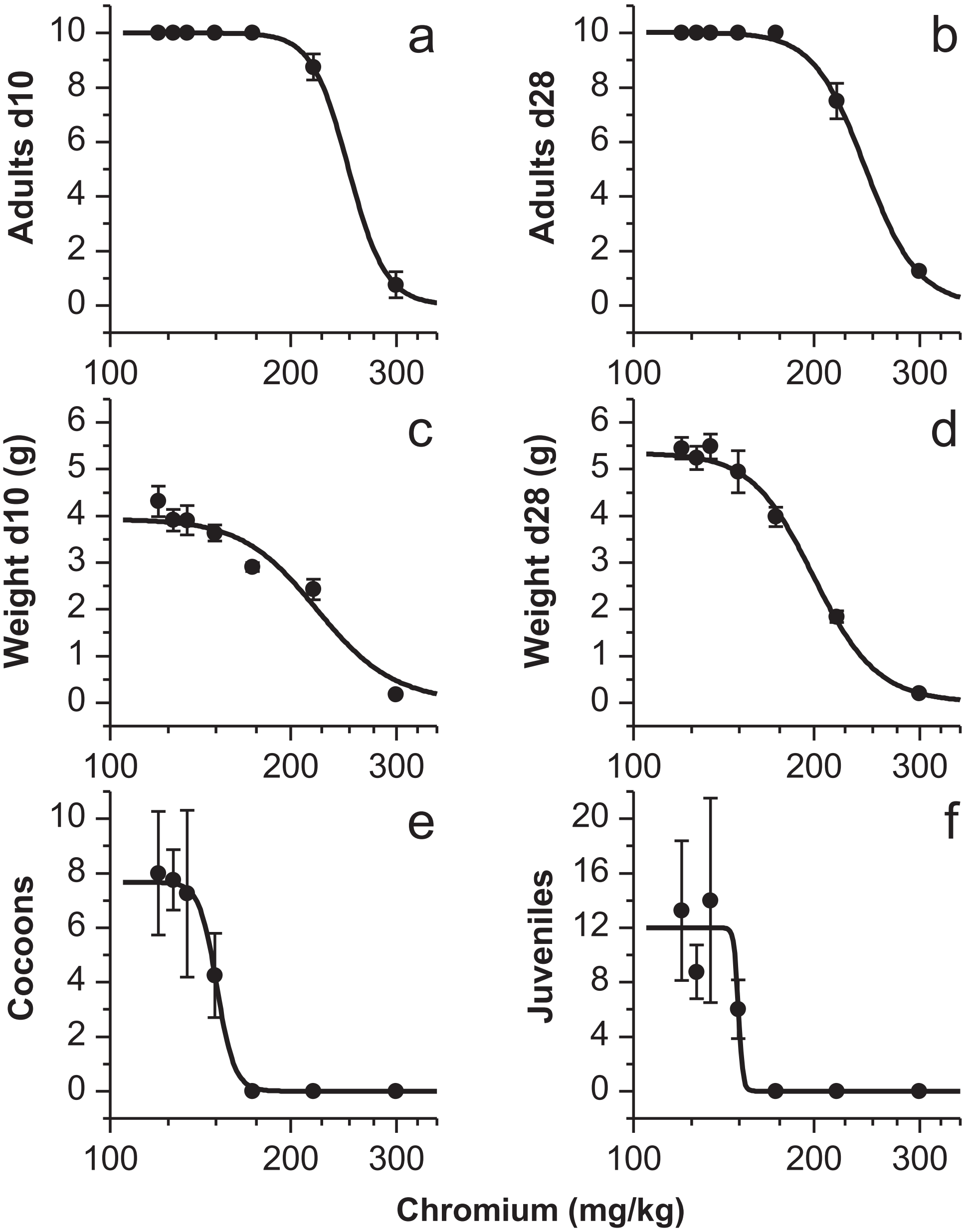

Fig. 2. Relationship between total Cr concentration and life-cycle endpoints. a) Number of adults alive at day 10; b) Number of adults alive at day 28; c) Weight of earthworms at day 10; d) Weight of earthworms at day 28; e) Number of cocoons at day 56; f) Number of hatched juveniles at day 56. Each point is the mean of four replicates, error bars represent standard errors; when not appearing, bars are within symbols. The continuous line represents the logistic model fitted to the observed data.

3.3. Biomarkers

Both LMS and CaATP activity exhibited a decreasing trend with increasing total Cu concentrations (Fig. 3a,c). Conversely, the levels of LF and MT showed an upward trend in response to rising Cu concentrations (Fig. 3b,d). The concentration-response relationships for these biomarkers were effectively modeled using the logistic regression model, highlighting the clarity of the observed trends. The EC50 values for the biomarkers are detailed in Table 4. Specifically, the EC50 values for MT content and CaATP activity were 261.2 mg/kg and 266.2 mg/kg, respectively, while the EC50 values for LMS and LF content were approximately 50 % lower, recorded at 157.2 mg/kg and 142.2 mg/kg, respectively (Table 4). A differential sensitivity of biomarkers in response to Cu exposure occurred with MT and CaATP serving as indicators of higher toxicity thresholds compared to LMS and LF.

The LMS, and the activities of CaATP and AOX decreased with increasing total Cr concentration in spiked soils and the concentration-response relationship was adequately described by the logistic model (Fig. 4a,c,f). Regression line was significant for AOX, highly significant in all other cases (Table 5). The EC50 for CaATP activity was estimated at 145.4 mg/kg. The EC50 values for LMS and AOX activity were lower (135.4 and 131.4 mg/kg, respectively) but affected by a large error as indicated by the wide confidence intervals (Table 5). LF content generally increased with rising total Cr concentrations, reaching a maximum at 172.7 mg/kg (Fig. 4b). The logistic model estimated an EC50 of 140.4 mg/kg. However, at the highest Cr concentration, a decrease in LF content was observed. MT and NL contents (Fig. 4d,e) increased progressively with rising total Cr across the entire tested range. The increases were relevant but did not reach a plateau, preventing EC50 statistical estimation.

After earthworm exposure to Cr, CAT and GST activities showed a bell-shaped concentration response curve (Figs. S3a,b). Enzymatic

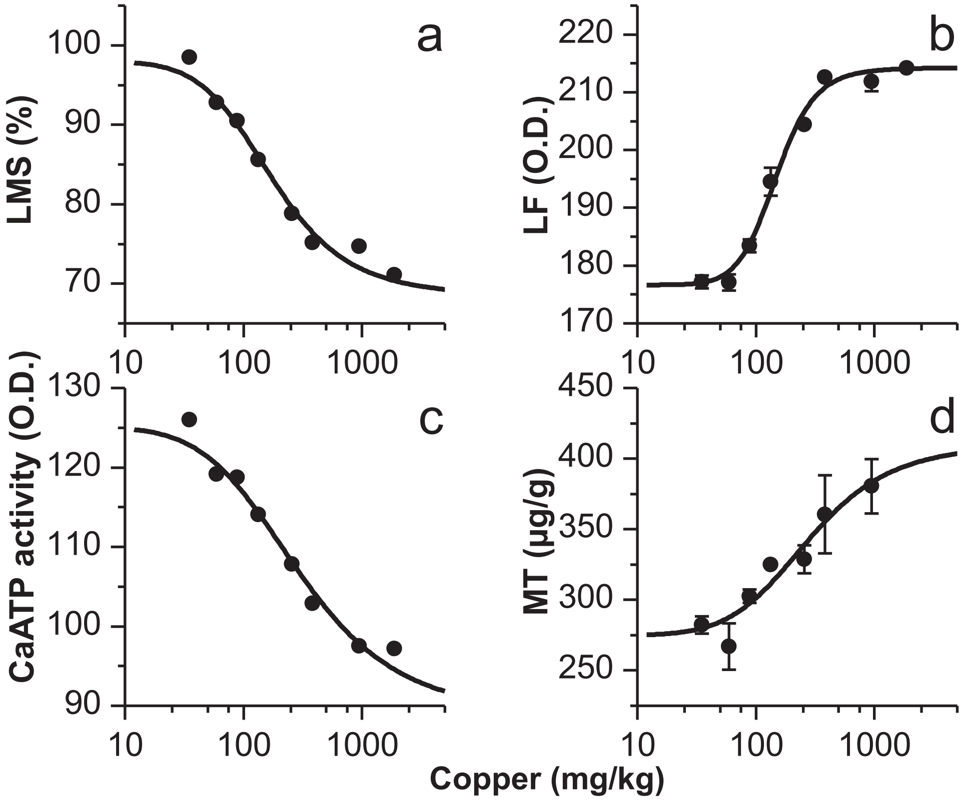

Fig. 3. Relationship between total Cu concentration and biomarkers. a) LMS; b) LF; c) CaATP; d) MT. For LF, each point is the mean of four replicates; for MT, each point is the mean of two replicates, while for LMS and CaATP replicates are not available. Error bars represent standard errors; when not appearing, bars are within symbols. The solid line represents the logistic model fitted to the observed data.

Table 4
Results of non-linear regression (logistic model) applied to the total Cu concentration-response data: EC50 (with 95 % confidence limits), percent of total sum of squares explained (SS) by regression, significance level of the regression (F test) and IEC50 only for life cycle endpoints.

Endpoint	EC50 (mg/kg dry soil)	% explained SS	Significance (p)	IEC50 (mg/kg dry organism)
N° of adults alive at day 10 (survival)	1504 (1365–1657)	96	< 0.001	102 (95–110)
N° of adults alive at day 28 (survival)	1408 (1063–1868)	74	< 0.001	98 (79–121)
Total wet weight of adults alive at day 28 (growth)	1107 (853–1440)	82	< 0.001	82 (66–101)
N° of cocoons at day 56 (reproduction)	511 (357–737)	88	< 0.001	57 (47–68)
N° of hatched juveniles at day 56 (reproduction)	355 (273–465)	84	< 0.001	37 (21–65)
Lysosomal membrane stability (LMS)	157 (131–191)	99	< 0.001	
Lipofuscin content (LF)	142 (125–161)	97	< 0.001	
Ca^{2+} ATPase activity (CaATP)	266 (190–369)	98	< 0.001	
Metallothioneins (MT)	261 (158–449)	81	< 0.001	

activities reached a maximum at about 134.4 mg/kg and then steadily decreased at higher concentrations.

The model-based curves (Fig. 5) illustrate the relationship between early biomarker responses (day 10), and later reproductive effects measured as juvenile production (day 56). Across both Cu and Cr, LF showed the highest sensitivity, followed by LMS and CaATP. Assuming a 20 % reduction in juveniles at day 56 corresponds to approximately 50–65 % biomarker alteration for Cu and 55–75 % for Cr at day 10, depending on the biomarker considered.

4. Discussion

Like other heavy metals, Cu and Cr in soil can cause negative effects at multiple biological levels, from the molecular to the ecosystem scale (Yan et al., 2023; Pelosi et al., 2024). This reflects a fundamental ecotoxicological principle, which states that contaminant-induced stress responses occur hierarchically across biological levels (Spurgeon et al., 2005; Newman MCand CWH, 2008). Toxic compounds first act at the molecular level, triggering cellular responses that may extend to the whole organism. Although population and ecosystem level assessments are ecologically significant, their responses to contaminants are typically delayed (Lam, 2009). In the present study, we evaluated the effects of a broad range of Cu and Cr concentrations in spiked soils on the earthworm species *E. andrei* using biomarkers and life-cycle endpoints.

4.1. Metals in soil and bioaccumulation

The background concentration of Cu in unspiked soil (35 mg/kg; Table 1) aligns with the European average soil concentration reported by Albanese et al (Albanese et al., 2015). and with local values documented by Buscaroli et al (Buscaroli et al., 2021). Similarly, the Cr concentration in unspiked soil (120 mg/kg) corresponds well with local

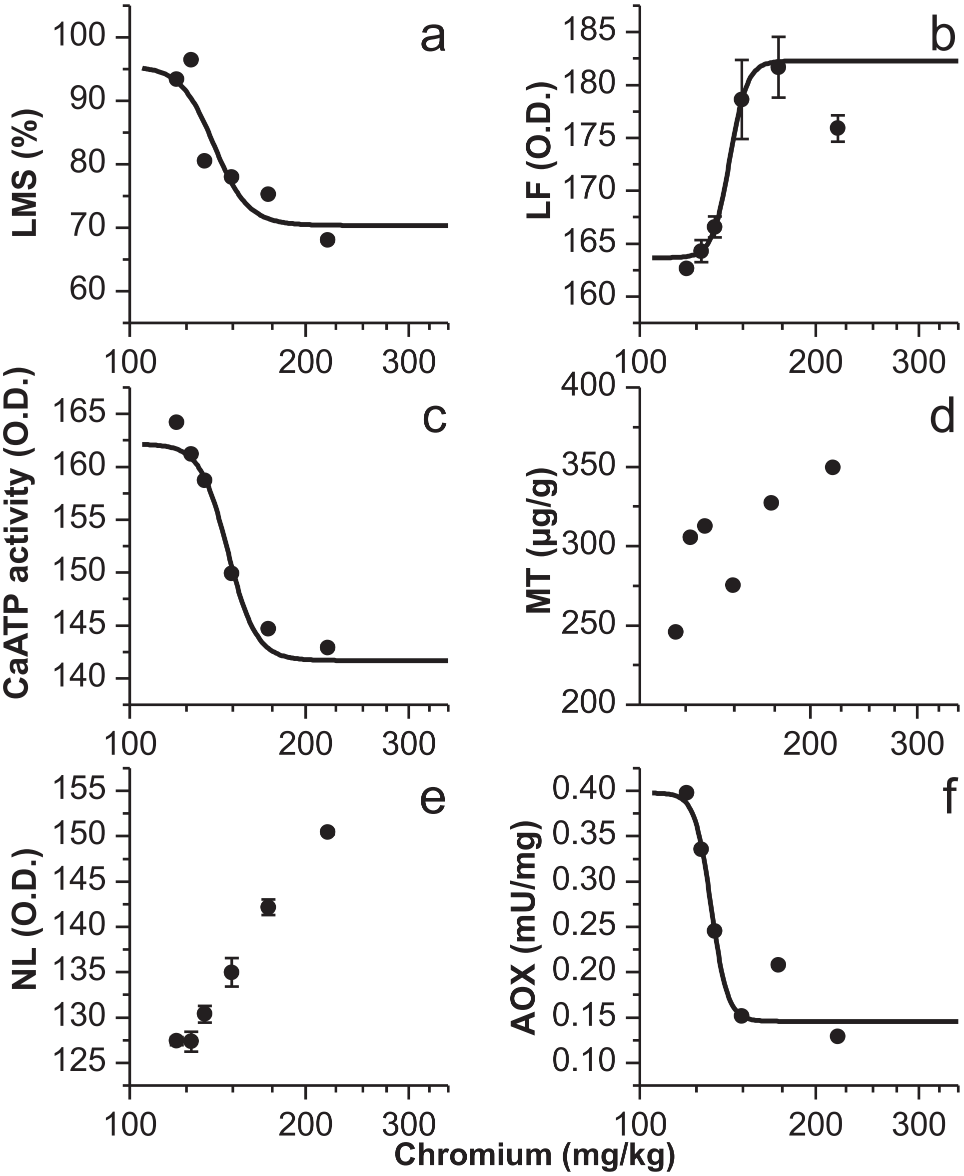

Fig. 4. Relationship between total Cr concentration and biomarkers. a) LMS; b) LF; c) CaATP; d) MT; e) NL; f) AOX. For LF, LMS and CaATP, each point is the mean of four replicates, error bars represent standard errors; when not appearing, bars are within symbols. For LMS, MT and AOX activity, replicates are not available. The solid line represents the logistic model fitted to the observed data.

background levels reported by Amorosi et al (Amorosi et al., 2002, 2014). and Buscaroli et al (Buscaroli et al., 2021).

The bioavailable concentration of Cu showed an increase in response to the total soil concentrations starting from the lowest spiked levels; however, the proportion of bioavailable/total Cu remained relatively stable at around 20–25 % across all spiked concentrations (Table 1). This behaviour is consistent with findings by Guan et al (Guan et al., 2011)., albeit the authors reported a higher proportion of bioavailability at approximately 65 %. Variations in these findings may be attributed to differences in soil type and application of fertilizers.

At low concentrations, bioaccumulated Cu remained below 24 mg/kg but increased markedly to about 120 mg/kg at the highest spiked concentration (Table 1). These results agree with Peijnenburg et al (Peijnenburg et al., 1999)., who reported Cu bioaccumulation levels of 88.2 and 97.1 mg/kg in *E. andrei* exposed to elevated soil Cu concentrations. In contrast, Langdon et al (Langdon et al., 2001). exposed *Lumbricus rubellus* to 725 and 1732 mg/kg of total Cu and observed lower bioaccumulation levels of 44.1 and 85.3 mg/kg, respectively. Marini et al (Marini et al., 2024). found comparable Cu bioaccumulation in *E. andrei*, although tissue Cu declined at exposures of 200 mg/kg or higher. Our study, instead, showed a steady increase in Cu bioaccumulation across the tested concentrations. However, the bioaccumulation factor (BAF) decreased from 0.63 to 0.06 (Table 1), indicating that earthworms may regulate Cu uptake and excretion, resulting into lower bioaccumulation in tissues (Marini et al., 2024; Richardson et al., 2015; Fai et al., 2023; Kilpi-Koski et al., 2020).

In our study, the added Cr(VI) was no more bioavailable at lower concentrations after 28 days (Choppala et al., 2013) (Table 2). Typically, Cr(VI) exists in soil as soluble chromate (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$) anions; however, these forms can become unavailable because of a rapid reduction to Cr(III) in presence of organic matter or Fe^{2+} (Griffin et al., 1977). At elevated Cr concentrations (here $>$ 172.7 mg/kg), the depletion of reducing agents limits the reduction to Cr(III), resulting in increased bioavailable Cr(VI). Generated Cr(III) is characterized by poor solubility and strong adsorption to soil particles, which significantly restricts its mobility and bioavailability (Bartlett and Kimble, 1976).

The Cr bioaccumulated in this study exceeded those reported in previous works, where *E. andrei* was exposed to similar concentrations.

Table 5

Results of non-linear regression (logistic model) applied to the total Cr concentration-response data: EC50 (with 95 % confidence limits), percent of total sum of squares explained (SS) by regression and significance level of the regression (F test).

Endpoint	EC50 (mg/kg dry soil)	% explained SS	Significance (p)	IEC50 (mg/kg dry organism)
N° of adults alive at day 10 (survival)	246 (219–253)	98	< 0.001	204 (179–232)
N° of adults alive at day 28 (survival)	240 (134–246)	98	< 0.001	181 (161–204)
Total wet weight of adults alive at day 10 (growth)	215 (198–235)	85	< 0.001	102 (59–177)
Total wet weight of adults alive at day 28 (growth)	195 (186–204)	94	< 0.001	68 (53–88)
N° of cocoons at day 56 (reproduction)	150 (136–176)	61	< 0.001	22 (17–28)
N° of hatched juveniles at day 56 (reproduction)	149 (127–240)	43	< 0.001	22 (20–24)
Lysosomal membrane stability (LMS)	135 (126–159)	75	0.002	
Lipofuscin content (LF)	140 (133–153)	78	< 0.001	
Ca^{2+} ATPase activity (CaATP)	145 (141–148)	98	< 0.001	
AOX activity (AOX)	131 (129–134)	93	0.017	

For instance, van Gestel et al (van Gestel et al., 1993). reported Cr concentrations ranging from 0.8 to 18 mg/kg, while Peijnenburg et al (Peijnenburg et al., 1999). recorded values between 1.04 and 14.0 mg/kg following comparable exposure conditions. It is worth noting that in previous studies, Cr was applied in the form of Cr(III), whereas in the present study soils were spiked with Cr(VI). Although Cr (VI) was rapidly reduced in this study, at the highest tested concentration approximately 17 mg/kg of Cr(VI) remained bioavailable. As reported by Kilpi-Koski et al (Kilpi-Koski et al., 2020). Cr and Cu rapidly accumulate in *E. andrei* reaching the steady-state within 1 day, despite high elimination rates. They measured an internal Cr concentration of 106 mg/kg after 21 days, similar to the levels observed in this study. The Cr is rapidly taken up via SO_4^{2-} and HPO_4^{2-} channels (Sivakumar and Subbhuraam, 2005), and quickly excreted until the organisms is capable. Later, the reduction from Cr(VI) to Cr(III) can generate reactive oxygen species (ROS) decreasing antioxidant enzyme activity, such as catalase (CAT) and explaining the elevated levels of bioaccumulated Cr observed in this study.

4.2. *Life-cycle endpoints*

Control earthworms demonstrated an average weight increase of 55 % (about 190 mg) over the 28-day period, reflecting a growth rate higher than those observed in previous studies on *E. andrei* or *E. fetida* (Zhou et al., 2013; Žižek and Zidar, 2013). However, the reproductive output of the control group was lower than previous laboratory trials (Zhou et al., 2013; Žižek and Zidar, 2013; González et al., 2013) and below the standards set by the OECD guidelines (OECD, 2016). Spurgeon et al (Spurgeon et al., 2000). also noted reduced cocoon production in *E. fetida*, attributing this to relatively low incubation temperatures of 15 °C. Elevated temperatures are known to promote both growth and reproduction in earthworms (González-Alcaraz and van Gestel, 2016; Presley et al., 1996). This could partially explain our findings, as the current study was conducted at 18 °C, lower than the 20 °C recommended by the OECD guidelines (OECD, 2016). Additionally, the initial weight of the worms may have affected reproduction, as it approached the lower limit of the recommended weight range (250–600 g) specified by the OECD guidelines (OECD, 2016).

Cu resulted in weight loss in earthworms after 28 days of exposure at the highest soil concentrations (Fig. 1a,b), which is consistent with findings from previous studies (van Gestel et al., 1991; Helling et al., 2000). In other investigations, *E. fetida* exposed to Cu for 90 days exhibited substantial impairment on survival, cocoon production, and weight beginning at concentrations as low as 40 mg/kg (Domínguez-Crespo et al., 2012; Yadav et al., 2023). However, it is important to note that in this latter case earthworms were raised on multi-metal contaminated municipal sewage sludge.

Among the assessed life-cycle endpoints, number of cocoon and juveniles, were identified as the most sensitive to Cu exposure (lower EC50 and IEC50 values) and has been well-documented in various studies. Additionally, the EC50 values for cocoon production were comparable to those obtained for *E. fetida* in natural and artificial soils in different European region (Kilpi-Koski et al., 2020; Zhou et al., 2013;

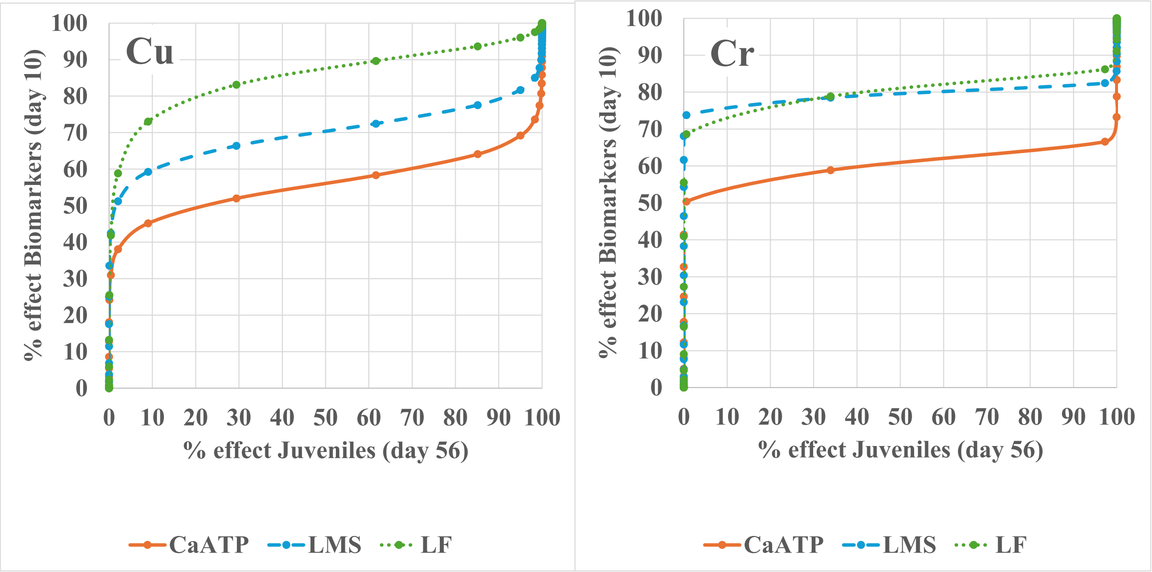

Fig. 5. Model-based curves illustrating the relationship between early biomarker responses (day 10) and later reproductive effects measured as juvenile production (day 56).

Scott-Fordsmand et al., 2000; Caetano et al., 2016).

The Cr soil concentrations exceeding 172.7 mg/kg significantly impacted the survival rates of *E. andrei* in both the 10- and 28- day exposure experiments, in contrast to Kilpi-Koski et al (Kilpi-Koski et al., 2020)., who reported no effects on survival rates even at higher Cr concentrations of up to 1224 mg/kg. Their experiments used artificial soils with 5.8 % organic carbon, over four times higher than in our natural soil. In contrast, Sivakumar and Subbhuraam (Sivakumar and Subbhuraam, 2005) reported survival EC50 values for Cr in soils from India that were tenfold higher, while Cui et al (Cui et al., 2015). identified a 14-day EC50 of 241 mg/kg for *E. fetida*.

Although cocoon and juvenile production was relatively low, a clear concentration–response relationship was evident, making these endpoints the most sensitive life-cycle metrics. The EC50 for Cr in experiments on reproduction were approximately half of the EC50 recorded for growth and survival in this study. Comparable values were reported by Kilpi-Koski et al (Kilpi-Koski et al., 2020). and Lock and Janssen (Lock and Janssen, 2002) for *E. andrei* and *E. fetida*, respectively. Considering the bioaccumulated Cr, the IEC50 for juveniles and cocoons were fully consistent with those reported by Fai et al (Fai et al., 2023)., at around 21 mg/kg of Cr. Reproductive output is a highly sensitive and ecologically relevant parameter; it is frequently among the most impacted endpoints in response to metal contamination (Marini et al., 2024).

When comparing Cu and Cr, the EC50 for Cr were found to be from two to six times lower, indicating that the reproductive capacity of *E. andrei* is more adversely affected by Cr than by Cu. Conversely, Kilpi-Koski et al (Kilpi-Koski et al., 2020). reported that *E. andrei* exhibited greater sensitivity to Cu than to Cr suggesting that organic carbon content in soil can mitigate the toxic effects of Cr, reducing its bioavailability (Eckbo et al., 2022). Comparing EC50 and IEC50 showed consistently lower IEC50 for all life-cycle endpoints, as expected when using bioaccumulated concentrations. However, because organisms regulate their internal concentrations, IEC50 does not fully reflect the physiological effort and stress involved, leading to lower values that do not represent the total experienced stress.

4.3. Biomarkers

The marked reduction in coelomocyte lysosomal membrane stability (LMS), reflected the physiological stress imposed on *E. andrei* by metal exposure. The LMS EC50 for Cu indicated that the health status of earthworms was adversely impacted by metal concentrations present in the soil (Tables 4 and 5). The observed reduction in LMS has been primarily attributed to lipid peroxidation of the membrane, which occurs when cytosolic antioxidant defences are overwhelmed and oxidative agents, such as heavy metals, permeate the lysosomal membranes (Weeks and Svendsen, 1996; Rocco et al., 2011). The decrease in LMS was identified as a reliable and rapid indicator of physiological stress and served as a sensitive early-warning biomarker for potential life-cycle effects (Calisi et al., 2024; Moore, 2013).

The validity of LMS as an early warning signal for Cu impact on *E. andrei* is further corroborated by findings from Pelosi et al (Pelosi et al., 2024). and references therein. Svendsen and Weeks (Svendsen and Weeks, 1997) documented significant reductions in LMS at Cu concentrations of 27 and 40 mg/kg in earthworm tissues, which agrees with 15–30 mg/kg in tissue from this study. Boeri et al (Boeri et al., 2017). observed effects at significantly lower Cu concentrations, but the longer exposure of the test (28 days vs. 10 days) must also be considered.

The chloragogenous tissue of the earthworms was adversely affected by both Cu and Cr following exposure to contaminated soils. A significant accumulation of LF in the lysosomes was induced in the tissue of the exposed earthworms. The EC50 values for both metals were consistent with the LMS values, suggesting that LF can be equally effective as early warning tool for metal pollution in *E. andrei*. Metal accumulation was found to be dose-dependent up to levels of 250 mg/kg for Cu and 50 mg/kg for Cr; however, at the highest concentrations, the LF content remained stable for Cu and decreased for Cr (Figs. 3 and 4). Sforzini et al (Sforzini et al., 2011). suggested that such a decrease may be attributed to the elimination of cells from the chloragogenous tissue by earthworms accumulating insoluble compounds. Lipofuscins, the end-products of membrane lipid peroxidation, accumulate in lysosomes as insoluble granules containing oxidized proteins, lipids, carbohydrates, and metals (Terman and Brunk, 2004). Their accumulation is a reliable indicator of cellular oxidative stress, closely linked to membrane lipid peroxidation and LMS in earthworms. The CaATP activity in the intestinal epithelium of earthworms exposed to Cu and Cr(VI) was inhibited because of heavy metals damage biological membranes and disrupt the function of transporters involved in cellular ion homeostasis (Moore, 2013; Zhang et al., 2016), thereby contributing to cytotoxic effects. Boeri et al., 2017 (Boeri et al., 2017) reported inhibition of CaATP activity in Cr (VI)-exposed earthworms was likely due to both oxidation of the enzyme's sulfhydryl (SH) groups or direct binding of Cr(VI) cations to the SH groups, disrupting or impairing enzyme activity. Gastaldi et al (Gastaldi et al., 2007). identified CaATP activity as the most sensitive biomarker for Cu exposure; however, here it exhibited an EC50 approximately twice as high as those observed for LMS and LF (Table 3). Conversely, earthworm CaATP activity demonstrated high sensitivity to Cr, yielding estimated EC50 values comparable to those for LMS and LF (Table 4). In agreement, previous studies (Boeri et al., 2017; Sforzini et al., 2017) suggested that the inhibition of CaATP may serve as an effective early warning signal for Cr contamination in soils.

The MT increase content in tissues serves as a robust biomarker for indicating metal contamination including both toxic (Cd, Cr, Hg) and essential (Zn, Cu) elements (Viarengo and Nott, 1993; Wang et al., 2013; Gao et al., 2016). In this study, *E. andrei* responded with an increased MT synthesis when exposed to Cu. Hattab et al (Hattab et al., 2023). reported MT concentrations that were tenfold lower, with bioaccumulated Cu levels that were approximately half of those observed in our study. Regarding Cr, while the increase in MT was significant, the logistic model was unable to calculate the EC50 (Fig. 4). The MT concentrations in our study was in close agreement with those recorded by Gao et al (Gao et al., 2016)., with detectable increases in MT occurring even at the lowest Cr concentrations.

The inhibition of AOX activity was pronounced with increasing soil Cr concentrations, resulting in the lowest EC50. This suggests that AOX activity represents a highly sensitive response in earthworms exposed to heavy metals, corroborating the findings by Cataldo et al (Cataldo et al., 2011). Asensio et al (Asensio et al., 2013). reported an initial decrease in AOX activity followed by an increase after 17 days of exposure, suggesting that although metals do not function as peroxisome proliferators, they can induce lipid peroxidation, which subsequently alters peroxisomal function and stimulates AOX activity.

CAT and GST were analysed as key enzymes involved in antioxidant defence and phase II detoxification (Boughattas et al., 2016; Clasen et al., 2021; Maity et al., 2018; Mkhinini et al., 2020). Both enzymes exhibited dose-dependent induction at low Cr concentrations (6–12 mg/kg), followed by a decline at higher levels, ultimately falling below control values. This bell-shaped pattern, commonly reported in metal-exposure studies, likely reflects increased catabolic activity or direct inhibition of enzyme function by toxic compounds (Viarengo et al., 2007; Boughattas et al., 2016; Mkhinini et al., 2020; Zhang et al., 2009). Although CAT and GST may show non-monotonic responses that limit their reliability under certain conditions, they remain valuable indicators of oxidative stress when interpreted within a broader biomarker framework.

4.4. Ecological and regulatory implications

The biomarker's responses demonstrated their capacity to detect physiological impairments prior to alterations in life-cycle endpoints in earthworms exposed to Cu and Cr pollution. The model-based curves (Fig. 5), linking early biomarker responses (day 10) to later reproductive effects (day 56), indicated that relatively modest cellular alterations can foreshadow substantial impacts on reproduction, showing that early sub-lethal responses were strongly predictive of long-term reproductive impairment (Amiard-Triquet et al., 2015).

After 56 days of exposure, the decreased number of cocoons and juveniles were the most sensitive life-cycle endpoints affected (Fig. S4 in supplementary material). The observed reduction in cocoon and juvenile represents a critical ecological concern, as impaired reproduction directly limits population renewal and, consequently, the maintenance of essential soil functions provided by earthworms (Kilpi-Koski et al., 2020).

Based on EC50 values, LMS was the most sensitive biomarker for both Cu and Cr exposure. LF (for Cu) and AOX (for Cr) showed comparable sensitivity, indicating that these biomarkers function as equally effective early-warning indicators. Under Cr exposure, EC50 values for reproduction were not significantly different from those of LMS and AOX (Fig. S4, Table 4), despite their different response times. This highlights the potential of these biomarkers for early detection of contamination levels capable of causing ecological damage, including decreased reproductive performance (Sforzini et al., 2017).

Calisi et al (Calisi et al., 2011)., highlighted the importance of a biomarker-based approach in environmental monitoring, particularly in instances where biomarkers can provide clear early warnings before disturbances manifest at higher ecological levels. However, their study examined only one high Cu concentration reporting that the assessed biomarkers were no more sensitive than life-cycle endpoints. The authors attributed this limited sensitivity to the use of a single maximum concentration, which may have induced acute conditions that reduced the predictive capability of the biomarkers power (Calisi et al., 2011).

On the other hand, by applying a comprehensive contamination gradient for both Cu and Cr, our approach enabled the establishment of clear dose–response relationships. This allowed the identification of the most effective early-warning biomarkers, improving the understanding of their relevance in environmental contamination assessment.

The recent adoption of the European Directive on Soil Monitoring and Resilience (23 October 2025) requires Member States to develop strategies for achieving “healthy soils across Europe by 2050”, including monitoring systems that assess soil physical, chemical, and biological status (European Parliament, 2025). This framework opens the possibility of incorporating biomarkers as early-warning indicators of inorganic contamination and as indicators of soil quality through earthworm-based assays. Our results support the integration of biomarker-based approaches into tiered ecological risk assessments, complementing traditional endpoints such as survival and reproduction with more sensitive molecular indicators. In particular, the responsiveness of LMS, LF, and AOX demonstrates their potential as early-warning tools in soil quality monitoring, enhancing the capacity of regulatory frameworks to detect sublethal stress before population-level declines occur.

5. Conclusion

This study evaluated seven biomarkers and three life-cycle endpoints in *E. andrei* to determine whether short-term physiological responses can serve as early warning indicators of soil contamination. Cellular and biochemical biomarkers showed lower EC50 values than life-cycle endpoints for both Cu and Cr, confirming their higher sensitivity. Reproductive traits were the most responsive life-cycle endpoints, with Cr exerting stronger toxic effects than Cu. Among biomarkers, LMS, LF, and CaATP were the most sensitive indicators of Cu exposure, while AOX emerged as the most responsive to Cr. These biomarkers reacted at concentrations below those affecting reproduction, demonstrating their value as early-warning tools for ecological risk assessment.

Overall, the integration of biomarkers such as LMS, LF, CaATP, and AOX with traditional endpoints enhances the capacity to detect sublethal stress before population-level effects arise. The results provide a robust framework for linking molecular responses to ecological outcomes and support the incorporation of biomarker-based approaches into soil quality monitoring and regulatory assessment schemes. Considering the recent EU Soil Monitoring and Resilience Directive (2025), these findings highlight the potential role of earthworm biomarkers as practical tools for inclusion in future EU-wide soil health monitoring frameworks.

CRediT authorship contribution statement

Nicolas Greggio: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Andrea Pasteris:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Enrico Dinelli:** Writing – review & editing, Validation, Supervision, Resources, Conceptualization. **Alessandro Buscaroli:** Writing – review & editing, Supervision, Formal analysis. **Paola Valbonesi:** Writing – review & editing, Investigation, Formal analysis. **Beatrice M.S. Giambastiani:** Writing – review & editing, Investigation. **Elena Fabbri:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2025.119628.

Data availability

Data will be made available on request.

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