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Cytotoxicity and Antioxidant Defences in *Euplotes aediculatus* Exposed to Single and Binary Mixtures of Heavy Metals and Nanoparticles

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Abstract: The aim of this study was to analyse the cytotoxicity of heavy metals (HMs) and nanoparticles (NPs) on populations of the ciliated protist *Euplotes aediculatus*. We used ecotoxicological tests, antioxidant assays, and the MixTOX tool in Microsoft® Excel to evaluate the toxic effect of HMs and NPs in single and binary mixtures on *E. aediculatus* and to detect the type of interaction between them. Based on our results, the order of toxicity was Cu > Cd >> Zn (1 h and 24 h) for HMs and ZnO > CuO >> TiO_2 >> SiO_2 (1 h) and CuO > ZnO >> TiO_2 >> SiO_2 (24 h) for NPs. The interaction between metals in binary mixtures was predominantly synergistic at low doses and antagonistic at high doses. The type of interaction depende on the metals present and their respective concentrations. Furthermore, both HMs and NPs were shown to trigger effective antioxidant responses in *E. aediculatus*. Our research highlights the importance of considering the combined effects of HMs and NP exposure and their potency in risk assessment.

Keywords: cytotoxicity; binary mixture; toxicology; total phenolic content; antioxidant; ciliated protists

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1. Introduction

Freshwater ecosystems contain a heterogeneous mixture of inorganic and organic pollutants [1], including heavy metals [2], nanoparticles [3], nitrates, phosphorus, domestic sewage, food processing waste, oil and oil dispersants, pesticides, pathogens, and emerging contaminants (endocrine disruptors, flame retardants, etc.), among others [4–8]. These pollutants reach freshwater ecosystems through various sources, such as industrial waste runoff, sewage treatment plants, feedlots, urban and agricultural runoff, septic systems, and illegal disposal of solid waste [1,7]. Heavy metals (HMs) and nanoparticles (NPs) are recognized as important toxic pollutants, with extensive literature describing their accumulation in different ecosystems. HMs and NPs pose a significant threat to a wide range of habitats, inducing harmful effects on organisms within polluted ecosystems. Some HMs are essential for cellular metabolism and growth at minimal concentrations [9], while others can cause harm even at very low concentrations [10]. Nanoparticles are introduced into freshwater ecosystems at various stages, from production to disposal, raising concerns about ecological risks, i.e., immediate responses and long-term damage to the environment [11,12]. NPs are extremely small particles, typically ranging from 1 to 100 nanometers in size. Due to their small size, NPs have unique physical and chemical properties that are different from larger particles, including a high surface area-to-volume ratio and increased reactivity. These

properties make them valuable in various applications, such as medicine for drug delivery, electronics for developing advanced materials, environmental science for pollution remediation, and potential antimicrobial applications in food packaging [13]. However, their increased use has raised concerns about potential environmental and health impacts, particularly their behaviour and effects in natural ecosystems. In aquatic environments, NPs can interact with biological organisms in complex ways, potentially causing toxicity. Research focuses on understanding how NPs affect aquatic microorganisms and examining factors such as bioaccumulation, oxidative stress, and disruption of biological processes. Aquatic ecotoxicity research on NPs has increased in recent years, with a greater focus on freshwater compared to other ecosystems, such as salt water and soil [14–18]. Despite the unknown concentrations of most HMs and NPs in the environment, exposure modelling suggests that freshwater and soil could act as significant sinks for these pollutants, accumulating at higher levels than in the air [19–21]. Ciliated protists, an important group of primary consumers, are unicellular eukaryotic microorganisms well adapted to life in freshwater, soil, and many other ecosystems, from the subterranean groundwater ecosystem (i.e., caves) to the Antarctic coastal seawater [22–24]. Due to their widespread presence in different ecosystems, these microorganisms are a good model for monitoring the effects of various physical and chemical factors on biological communities [25–27]. Additionally, their rapid response to various toxic chemicals, coupled with a short generation time and a delicate cell membrane without external covering, makes them valuable for such studies [28–32]. Studies and reviews have explored ciliate tolerance to HMs and NPs [33–37]. In recent years, the research focus has shifted to the interactions between HMs and NPs with microorganisms, particularly freshwater ciliates. Generally, ciliates demonstrate the ability to endure long-term exposure to areas polluted by HMs and NPs, suggesting the existence of mechanisms for tolerance, resistance, or detoxification [36–39]. *Euplotes aediculatus* is a freshwater ciliate belonging to the class Hypotrichea and the family Euplotidae. Although this and other congeneric species have been extensively studied in various biological aspects, including toxicity assessment [40–43], to the best of our knowledge, only one recent study has attempted to analyse the toxic effects induced by NPs, specifically Cu and CuO, on *E. aediculatus* [44]. In this regard, our study further extends this analysis by increasing the number of NPs tested (i.e., ZnO, CuO, TiO_2, and SiO_2) and for the first time, assessing their acute and chronic effects in combination with heavy metals (i.e., Cd, Zn, and Cu) on *E. aediculatus*. In addition, the present study also analyses the antioxidant defences of *E. aediculatus* in response to both single and binary mixtures of these pollutants by using three different types of antioxidant assays, namely total phenol content (TPC), α,α-diphenyl-β-picrylhydrazyl (DPPH), and hydroxyl radical scavenging assay (HRSA). The ultimate goal is to confirm *E. aediculatus* as a suitable eukaryotic model organism, to demonstrate its potential as a bioindicator of HMs and NPs in polluted habitats, and, ultimately, to establish its effectiveness as a biomarker for robust environmental monitoring initiatives. Overall, this study will provide novel information about the antioxidant defence responses elicited by HMs and NPs in ciliates and their prospective application as biomarkers in risk assessment (RA) studies.

2. Materials and Methods

2.1. Ciliate Strain and Culture Conditions

For our study, the freshwater ciliate *Euplotes aediculatus* was employed, which was isolated from the Frasassi cave complex (Genga, AN, Italy) [20] and kindly provided by Prof. Claudio Ortenzi and Prof. Federico Buonanno, University of Macerata (Italy). *Euplotes aediculatus* was cultivated in a salt basal medium (SMB) [45] at a temperature of 18 ± 1 °C. The cells were fed with the green algae *Chlorogonium elongatum*. Concurrently, the algae *C. elongatum* was cultured in Jaworski's medium (JM) [46] at a temperature of 18 ± 1 °C. The experiments were conducted using cells of *E. aediculatus* in the logarithmic growth phase.

2.2. Metal Salts (Chemicals)

High-grade analytical chemicals with a purity of ≥99% were employed for the ecotoxicological assays as sources of metal ions. Specifically, cadmium chloride (anhydrous $CdCl_2$), zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$), copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), zinc oxide (ZnO) (nanopowder with a particle size < 100 nm), titanium (IV) oxide (TiO_2) (a mixture of rutile and anatase nanopowder, < 100 nm particle size as per BET, 99.5% trace metals basis), silicon dioxide (SiO_2) (nanopowder with a particle size of 10–20 nm as per BET, 99.5% trace metals basis), and copper (II) oxide (CuO) (nanopowder with a particle size < 50 nm as per TEM) were procured from Sigma-Aldrich (Milan, Italy).

2.3. Single HM and NP Toxicity Tests at 1 h and 24 h

To determine the toxicity of HMs and NPs to *E. aediculatus*, we conducted preliminary toxicity range-finding tests using different concentrations of HMs and NPs. These tests aimed to establish the concentration range for the final assays. Each test included 5–7 different concentrations, ranging from the lowest concentration with no observed effect on cell viability to the highest concentration, resulting in 100% cell death. We prepared 0.1 M stock solutions for HMs and 1 M stock solutions for NPs by dissolving each chemical in a salt basal medium (SMB) with a pH of 7. Daily working solutions were then prepared by diluting the stock solutions to nominal concentrations as follows: 10–100 mg Cd/L, 0–2 mg Cu/L, 50–250 mg Zn/L, 10–150 mg ZnO/L, 1–10 mg CuO/L, 500–8000 mg TiO_2/L, and 1000–15,000 mg SiO_2/L. We conducted ecotoxicity assays at 1 h and 24 h using specific 3-well depression glass slides. One hundred cells from an exponentially growing culture were inoculated into each well, resulting in a final volume of 1 mL of ciliate culture medium containing the specified chemical concentration. The setup was covered to prevent evaporation and incubated for 1 h and 24 h in a humid chamber at 18 ± 1 °C in the dark, without feeding the ciliates during the test period. After the 1 h and 24 h incubation periods, *Euplotes* cells were examined under a stereoscopic microscope (20–40× magnification) to determine mortality and survival at different test concentrations. The ciliates were considered dead if they were missing due to a cell burst or if they remained stationary at the bottom of the well, unresponsive to gentle mechanical stimulation with the tip of the micropipette. The control wells contained the same number of ciliates but lacked HMs and NP solutions. Replicates (n = 3) were averaged for each concentration treatment. Cytotoxicity assessment endpoints included median lethal concentration (LC) LC_{20} (concentration causing 20% mortality) and LC_{50} (concentration causing 50% mortality) at 1 h and 24 h. Mean mortality was determined using a regression equation. Due to the number of exposed organisms, each analysis required a larger amount of working solution, resulting in minimal absorption of HMs and NPs due to bioaccumulation. To minimize chemical adsorption/release levels, we used 3-well depression glass slides, aiming to reduce these levels compared to conventional toxicity test setups.

2.4. Bimetallic Mixture (Cd + Zn, Cd + ZnO) Toxicity Tests at 1 h and 24 h

Toxicity assessment in binary mixtures followed the same procedure as in single-metal treatment assays. We structured the data in a full factorial design, including all possible combinations of binary mixtures to ensure comprehensive coverage. For this toxicity test involving binary mixtures, we included a control (medium only) and examined 16 binary mixtures of Cd + Zn, and Cd + ZnO based on the total concentration of the two chemicals in the mixture. The concentration of each chemical was expressed using the toxic unit (TU) approach according to Sprague [47]. In this study, the binary mixtures were prepared by combining the toxicants according to their individual LC_{50} values, where 1TU corresponds to the LC_{50} values of single-metal toxicities. Seven total concentrations were tested: 0.5, 0.75, 1, 1.25, 1.5, 1.75, and 2TUs. The total concentrations were calculated as the sum of the TUs of the single chemicals constituting the mixture. Expected mortality rates were determined as the sum of the single-metal toxicities obtained for each metal in the mixture at the same concentration. An interaction was considered synergistic if the observed mortality rate was

higher than the expected mortality rate, and antagonistic if the observed mortality rate was lower than the expected mortality rate at the same concentration.

2.5. Total Phenolic Content and Antioxidant Activity Assays

To evaluate the presence of antioxidant activity in the ciliate exposed to different concentrations of the single and binary mixture of HMs and NPs, three different types of antioxidant assays, namely total phenol content (TPC), α,α-diphenyl-β-picrylhydrazyl (DPPH), and hydroxyl radical scavenging assay (HRSA), were applied. To analyse the antioxidant activity of these ciliate cells, 2000 cells/mL were taken from an exponentially growing culture flask and transferred to Petri plates in a volume of 10 mL and exposed to different concentrations of single and binary mixtures of HMs and NPs for 1 h and 24 h. For single-metal exposure, concentrations 0.25, 0.5, 0.75, and 1 TUs of Cd, Zn, and ZnO, respectively, were selected and used to analyse the antioxidant activity and enzyme assays. In the binary mixture, minimal cut-off concentrations (less than or equal to 50% mortality) were selected. Based on this minimal cut-off, we selected seven concentrations in the binary mixture of Cd + Zn (0.25 + 0.25, 0.5 + 0.25, 0.25 + 0.5, 0.75 + 0.25, 0.25 + 0.75, 0.75 + 0.5, and 0.75 + 0.75) at 1 h, nine concentrations in the binary mixture of Cd + Zn (0.25 + 0.25, 0.5 + 0.25, 0.25 + 0.5, 0.75 + 0.25, 0.5 + 0.5, 0.25 + 0.75, 1 + 0.75, 0.5 + 1, and 0.75 + 0.75) at 24 h, and six concentrations in the binary mixture of Cd + ZnO (0.25 + 0.25, 0.5 + 0.25, 0.25 + 0.5, 0.75 + 0.25, 0.5 + 0.5, and 1 + 0.25) at 24 h and used them to analyse the antioxidant activity. In the binary mixture of Cd + ZnO at 1 h toxicity test, we could not select any concentrations for the analysis of antioxidant activity assays, because in this test, all the concentrations were above the minimal cut-off concentration. Nanoparticles were not conditioned before antioxidant activity. Antioxidant activity assays were measured in the supernatant of the cell extract (intracellular) according to Ravindran et al. [48].

The analysis of TPC was based on the Folin assay of Vattem and Shetty [49] with minor modifications. In this method, 100 µLof cell extract was added to a test tube, mixed with 2 mL of sodium bicarbonate, and incubated for 2 min at 18 ± 2 °C. Then, 100 µLof the Folin–Ciocalteu reagent was added and incubated in the dark at 18 ± 2 °C for 30 min. After the incubation period, the absorbance was measured at λ = 725 nm using a spectrophotometer. Gallic acid 1 mg/mL was used as standard, and standard curves were obtained using different concentrations of gallic acid. We also used the culture medium (without cells) as a control, as no phenols were observed. We used the DPPH scavenging assay to determine the antioxidant capacity of the *Euplotes* extracts. Antioxidant activity was determined by its ability to scavenge α,α-diphenyl-β-picrylhydrazyl (DPPH) radicals. This analysis is based on the procedure of Yıldırım et al. [50] with minor changes. Briefly, 1 mM DPPH stock solution was prepared in 95% ethanol. Then, 800 µLof 1 mM DPPH solution was added to 200 µLof sample extract. The samples were mixed well and incubated for 30 min in the dark at room temperature. After incubation, the samples were transferred to centrifuge tubes and centrifuged at 14,000 rpm for 5 min. The supernatant was collected and the absorbance measured at λ = 517 nm using a spectrophotometer. In total, 200 µLof 95% ethanol was used as a control. Butylated hydroxy anisole (BHA) was used as a reference compound. Antioxidant activity is expressed as a percentage (%). The percentage of DPPH scavenging was calculated using the formula below:

$$DPPH\ scavenging\ (\%) = \frac{control\ absorbance - extract\ absorbance}{control\ absorbance} \times 100$$

The HRSA assay is based on the Fenton reaction of Kunchandy and Rao [51] and follows the procedure of Ravindran et al. [48] with slight modifications. The hydroxyl radical is generated by the Fe^{3+} ascorbate EDTA H_2O_2 system i.e., Fenton reaction. To analyse this reaction, the chemicals of 2–deoxyribose (2.8 mM), $FeCl_3$ (100 µM), H_2O_2 (1 mM), and EDTA (100 µM) were dissolved in 20 mM phosphate buffer at pH 7.4. From this mixture, 800 µL was then transferred to another test tube. Then, 10 µL of ascorbic acid (10 mM) and 100 µL sample extract were added. Finally, ascorbic acid was added

and incubated at 37 °C for 1 h. After the incubation, 1 mL of 2.8% TCA and 1 mL of 1% aqueous TBA were added, and the mixture was heated at 90 °C for 15 min to develop colour. After cooling, the absorbance was measured at 532 nm against a suitable blank solution. Mannitol was used as a reference compound. The percentage of scavenging was determined according to the formula below:

$$Hydroxyl\ radical\ scavenging\ (\%) = 1 - \frac{Sample\ absorbance}{Blank\ absorbance} \times 100$$

2.6. Statistical Analysis

Statistical analysis was performed using InfoStat software ver. 2012 and Microsoft® Excel v.2010 spreadsheet; the difference between control and test groups was evaluated using one-way ANOVA with Bonferroni corrected post-hoc *t*-test and correlation coefficient (R). And $p < 0.05$ was considered statistically significant. All experiments were performed at least in triplicate ($n = 3$), and the results were presented as mean ± standard error (SE) values.

Toxicity data from binary mixtures of the different compounds were analysed using the Microsoft® Excel spreadsheet MixTOX tool [52]. Concentration addition (CA) and independent action (IA) models were used to analyse the bimetallic mixtures. This tool assesses if and how the observed data deviate from both models and a better significance of the observed data could be realized using deviation functions such as synergism or antagonism (S/A), dose level-dependent deviation (DL), and doseratio-dependent deviation (DR). The strict concentration addition model occurs when the toxic unit value of a mixture equals 1 [52,53]. In the independent action model, the dose–response relationship of chemical mixtures can be expressed by multiplying the non-response of each chemical in the mixture at a given exposure concentration [52,53]. The deviation arrays of the 1 h Cd + Zn (1a), 24 h Cd + Zn (1b), 1 h Cd + ZnO (1c), and 24 h Cd +ZnO (1d) mixtures were analysed using the MixTOX tool. The Solver program and Visual Basic functions in Microsoft Excel v.2010 were used to guide the software design. The different types of toxicity data of the binary mixtures (1a, 1b, 1c, and 1d) were fitted to the deviation functions (S/A, DL, and DR) using the maximum likelihood method, while minimizing the sum of squared residuals (SS). Chi-square ($\chi 2$) tests were used to calculate the statistical significance of the improved model fit from additional parameters. In this study, a $p(\chi 2)$ value < 5% it was considered statistically significant. A detailed description of these mixture models can be found in Jonker et al. [52].

3. Results

3.1. Acute and Chronic Cytotoxicity of Single HMs (Cd, Cu, and Zn) and NPs (CuO, ZnO, TiO_2, and SiO_2)

The LC_{20} and LC_{50} values were determined from the mortality curve based on the regression equation for 1 h and 24 h exposures of HMs and NPs to *E. aediculatus*. In the case of single-metal exposures (HMs and NPs), *E. aediculatus* showed higher resistance to HM Zn at both 1 h and 24 h. *E. aediculatus* cells also showed greater resistance to NP SiO_2 at both time points, with no mortality observed at 1 h and 24 h in the control group. Analysing the mean lethal concentration values after 1 h exposure (1 h LC_{20} and LC_{50}) and 24 h exposure (24 h LC_{20} and LC_{50}), Cu emerged as the most toxic metal to *E. aediculatus*, with LC_{20} and LC_{50} values of 0.18 and 0.43 mg/L, respectively (Table 1). At the same time, the cellular toxicity of TiO_2, SiO_2, and CuO NPs was lower than that of ZnO at 1 h LC_{20} and LC_{50} values, while CuO exhibited greater toxicity compared to all other NPs at 24 h LC_{20} and LC_{50} values. The 1 h LC_{20} and LC_{50} values for *E. aediculatus* were 282.41 and 283.95 mg/L for Zn, and 7.05 and 7.36 g/L for TiO_2, respectively. The 24 h LC_{20} and LC_{50} values for Zn were 76.47 and 137.65 mg/L, and for TiO_2they were 2.61 and 4.44 g/L, respectively (Table 1). The order of toxicity of HMs was Cu > Cd > Zn at both 1 h and 24 h. For NPs, the toxicity order was ZnO > CuO > TiO_2 > SiO_2 at 1 h and CuO > ZnO > TiO_2 > SiO_2 at 24 h.

Table 1. LC_{20} and LC_{50} values of *E.aediculatus* for HMs and NPs at 1 h and 24 h.

HMs	1 h—*Euplotes*		24 h—*Euplotes*	
	LC_{20} (mg L^{-1})	LC_{50} (mg L^{-1})	LC_{20} (mg L^{-1})	LC_{50} (mg L^{-1})
$CdCl_2$	42.53	91.80	19.85	53.72
$CuSo_4$	34.78	76.41	0.18	0.43
$ZnSo_4$	282.41	283.95	76.47	137.65
NPs	**1 h—*Euplotes***		**24 h—*Euplotes***	
	LC_{20} (mg L^{-1})	LC_{50} (mg L^{-1})	LC_{20} (mg L^{-1})	LC_{50} (mg L^{-1})
CuO	5922.50	6535.03	3.10	4.93
ZnO	77.32	139.99	30.93	56.16
TiO_2	7042.94	7361.62	2608.55	4439.97
SiO_2	Up to 60,080 mg L^{-1}, there is no effects to the cells			

3.2. 1 h Binary Mixture of Cd + Zn—MixTOX Analysis

The simplest CA module does not appear to adequately capture the overall toxicity data; in fact, the squared R^2 value is only 34%, indicating that a significant proportion of the variance in the data is not explained by the model. This limitation is not only due to the binary mixture toxicity data but also extends to the data for Zn, which shows a threshold effect above 280 mg/L, which the CA model is unable to predict. Attempts to fit the slope with a value lower than −39 resulted in an error. This is not the case for the SA, DR, and DL subsystems (as well as with the IA model). For cadmium toxicity, a good fit can be achieved. Consequently, we relied on modules that take into account deviations from additivity, which have much better predictive capabilities. The most conservative (lower L) among them, the DL module, with its positive and negative *a* and *b* values, respectively (Table 2), suggests antagonism, indicating a decrease in toxic effects with increasing doses. In addition, positive *a* and *b* parameters for the DR model suggest antagonism in scenarios where the toxicity of the mixture is primarily due to one component (e.g., high Cd with low zinc, and vice versa).

3.3. 24 h Binary Mixture of Cd + Zn—MixTOX Analysis

In the 24 h dataset for Cd + Zn exposure in *E. aediculatus*, the concentration addition (CA) module is statistically significant, although it explains a relatively small proportion of the data variance. The inclusion of different sub-models improves the explanation of the data variance up to 77%. However, these models generally struggle to accurately predict mixture toxicity (Table 2). Specifically, the S/A model and the DR models both yield positive values for their parameters (*a* and *b*), which typically indicate antagonism in CA systems. However, the DR model shows synergism at low doses and antagonism at high doses. Notably, a strong synergistic effect was observed at the lowest concentrations of chemical 1 (Cd) and chemical 2 (Zn) (0.25 TU), followed by antagonism in the central dose range and again synergism at the highest tested concentrations tested. This non-linear dynamic could be attributed to hormesis, as observed in previous studies [54–56].

Table 2. Fitting results of the toxicity data of the bimetallic mixture (Cd + Zn) to the four models describing the deviations from concentration addition (CA) and independent action (IA) of *Euplotes aediculatus* using the MixTOX tool.

$CdCl_2$ + $ZnSo_4$ Exposure	Parameter	The Concentration Addition (CA)-Based Module			
		CA	S/A	DR	DL
1 h	*a*	–	1.405	0.917	0.106
	b	–	–	1.018	−7.852
	R^2	0.34	0.85	0.85	0.87
	$p(\chi 2)$ CA vs.	–	1.8×10^{-232} *	2.3×10^{-232} *	2.98×10^{-238} *
	S/A vs.	–	–	0.008 *	5.247×10^{-09} *
24 h	*a*	–	1.54	0.724	−0.362
	b	–	–	0.414	2.058
	R^2	0.72	0.77	0.77	0.77
	$p(\chi 2)$ CA vs.	–	7.04×10^{-21} *	4.86×10^{-08} *	3.61×10^{-18} *
	S/A vs.	–	–	0.486×10^{-08} *	3.206×10^{-05} *

Table 2. *Cont.*

	Parameter	The Independent Action (IA)-Based Module			
		IA	S/A	DR	DL
1 h	*a*	–	−1.282	−4.864	−3.020
	b	–	–	6.742	1.371
	R^2	0.82	0.86	0.88	0.87
	$p(\chi 2)$ IA vs.	–	7.42×10^{-17} *	6.38×10^{-25} *	2.65×10^{-24} *
	S/A vs.	–	–	9.78×10^{-11} *	4.2×10^{-10} *
24 h	*a*	–	−0.378	−0.744	−1.871
	b	–	–	−0.680	1.482
	R^2	0.77	0.77	0.77	0.78
	$p(\chi 2)$ IA vs.	–	0.17	0.331	0.003 *
	S/A vs.	–	–	0.533	0.002 *

1 h: 1 h exposure period; 24 h: 24 h exposure period; CA: the concentration addition model; IA: the independent addition model; a and b: interaction parameters; S/A: the model describing synergy or antagonism with respect to the reference model has one interaction parameter (*a*); DR: the model describing dose ratio-dependent deviations from the reference model has two interaction parameters (*a* and *b*); DL: the model describing dose level-dependent deviations from the reference model has two interaction parameters (*a* and *b*); R2: the coefficient of determination; $p(\chi 2)$: the outcome of the likelihood ratio test; vs.: versus, which was used to show the comparison between two models; –: not applicable. *: Significant at the 5% significance level.

3.4. 1 h and 24 h Binary Mixture of Cd + ZnO—MixTOX Analysis

The dataset for Cd + ZnO conspicuously shows synergistic effects, as predicted by both the CA and IA sub-modules (Table 3) at 1 h.

Table 3. Fitting results of the toxicity data of the bimetallic mixture (Cd + ZnO) to the four models describing deviations from concentration addition (CA) and independent action (IA) of *Euplotes aediculatus* using the MixTOX tool.

$CdCl_2$ + ZnO Exposure	Parameter	The Concentration Addition (CA)-Based Module			
		CA	S/A	DR	DL
1 h	*a*	–	−5.325	−3.654	−15.34
	b	–	–	−5.365	−8.324
	R^2	0.47	0.97	0.98	0.99
	$p(\chi 2)$ CA vs.	–	0.000 *	0.000 *	0.000 *
	S/A vs.	–	–	7.4×10^{-11} *	5.17×10^{-14} *
24 h	*a*	–	−0.043	−2.805	−0.205
	b	–	–	5.643	0.362
	R^2	0.87	0.87	0.91	0.87
	$p(\chi 2)$ CA vs.	–	0.587	5.49×10^{-14} *	0.768
	S/A vs.	–	–	6.42×10^{-15} *	0.631
	Parameter	**The Independent Action (IA)-Based Module**			
		IA	**S/A**	**DR**	**DL**
1 h	*a*	–	−5.325	−3.654	−15.34
	b	–	–	−5.365	−8.324
	R^2	0.35	0.78	0.85	0.99
	$p(\chi 2)$ IA vs.	–	1.5×10^{-282} *	0.000 *	0.000 *
	S/A vs.	–	–	1.72×10^{-45} *	1.52×10^{-138} *
24 h	*a*	–	−1.478	−7.544	−1.812
	b	–	–	10.366	0.312
	R^2	0.86	0.97	0.92	0.87
	$p(\chi 2)$ IA vs.	–	1.1×10^{-07} *	3.89×10^{-23} *	6.48×10^{-7} *
	S/A vs.	–	–	4.66×10^{-18} *	0.574

1 h: 1 h exposure period; 24 h: 24 h exposure period; CA: the concentration addition model; IA: the independent addition model; a and b: interaction parameters; S/A: the model describing synergy or antagonism with respect to the reference model has one interaction parameter (*a*); DR: the model describing dose ratio-dependent deviations from the reference model has two interaction parameters (*a* and *b*); DL: the model describing dose level-dependent deviations from the reference model has two interaction parameters (*a* and *b*); R^2: the coefficient of determination; $p(\chi 2)$: the outcome of the likelihood ratio test; vs.: versus, which was used to show the comparison between two models; –: not applicable. *: Significant at the 5% significance level.

The concentration addition (CA) module is statistically significant at 24 h, but the dose ratio (DR) dependence module outperforms it. Notably, this module explains 91% of the

variance in the data. A negative "*a*" parameter indicates synergism, while a positive "*b*" parameter indicates antagonism at specific dose ratios. When the data were examined, strong antagonism was observed when Cd toxicity was particularly high. These results are consistent with the results of the IA sub-modules (Table 3).

3.5. Antioxidant Properties of E. aediculatus Extracts Treated with Single and Binary Mixture at 1 h and 24 h

The total phenolic content has been identified as the major naturally occurring antioxidant component in the extracts of the freshwater ciliate *E. aediculatus*. In this study, the total phenolic content (TPC) of ciliate extracts treated with single metals (Cd, Zn, and ZnO) and bimetallic mixtures (Cd + Zn and Cd + ZnO) at 1 h and 24 h was evaluated. The Folin–Ciocalteu Method was used for the measurement, revealing concentrations ranging from 95.44 to 148.65 µg/L. *E. aediculatus* showed the lowest phenolic value at concentrations of 1 TU ZnO, while the maximum was observed at 0.5 TU Cd and 0.5 TU Zn at 1 h and 24 h, respectively, with a significant level of $p < 0.001$. In the case of single-metal treatments, the TPC of *E. aediculatus* was higher after 24 h of exposure compared to 1 h. In the bimetallic mixture treatments, the TPC was lower in the binary mixture of Cd + ZnO compared to the binary mixture of Cd + Zn at 24 h (Figure 1).

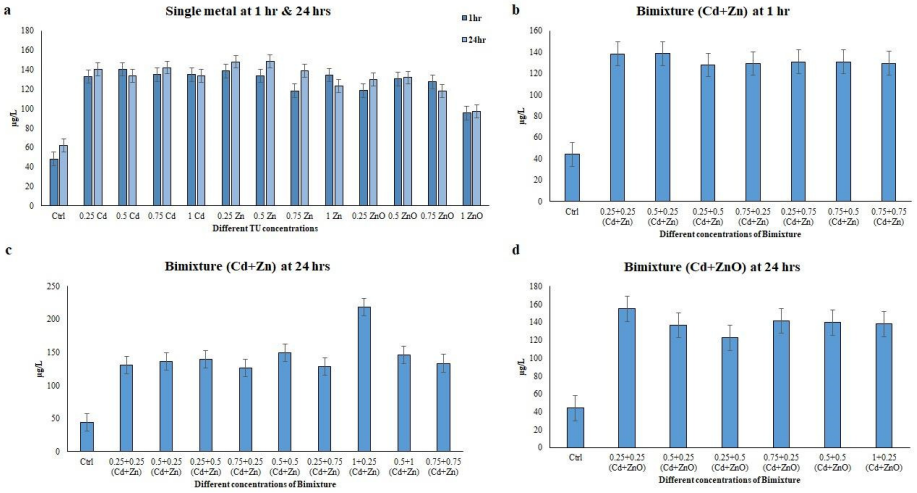

Figure 1. Total phenolic content measured in the *Euplotes aediculatus* cell extracts at single-metal (Cd, Zn, and ZnO) and bimetallic mixture concentrations. (**a**) Single metal, (**b**) bimetallic mixture of Cd + Zn at 1 h exposure period, (**c**) bimetallic mixture of Cd + Zn at 24 h exposure period, and (**d**) bimetallic mixture of Cd + ZnO at 24 h exposure time. The results are presented as means of three replicate experiments.

In the binary mixture, the TPC ranged from 122.58 to 218.54 µg/L. The highest TPC was observed in the binary mixtures of 1 + 0.25 TU (Cd + Zn) and 0.25 + 0.25 TU (Cd + ZnO) ciliate extracts after a 24 h exposure period, respectively ($p < 0.001$) (Figure 1). Importantly, the TPC of the ciliate extract, an indicator of antioxidant capacity, was significantly increased in cells exposed to different single heavy metal conditions and bimetallic mixtures compared to untreated cells ($p < 0.001$). The free radical scavenging activity of the ciliate extracts were evaluated using the DPPH assay, with BHT employed as a standard. Extracts obtained from *E. aediculatus* showed a maximum scavenging activity of 34.90% at the concentration of 0.75 TU Cd ($p < 0.001$) and a minimum activity of 26.97% at the concentration of 0.25 TU ZnO ($p < 0.001$) after 1 h. After 24 h, the maximum activity was found at 0.25 TU Cd, while the minimum activity was found at 0.5 TU Cd, with values of 48.97% and 33.04% ($p < 0.001$), respectively (Figure 2).

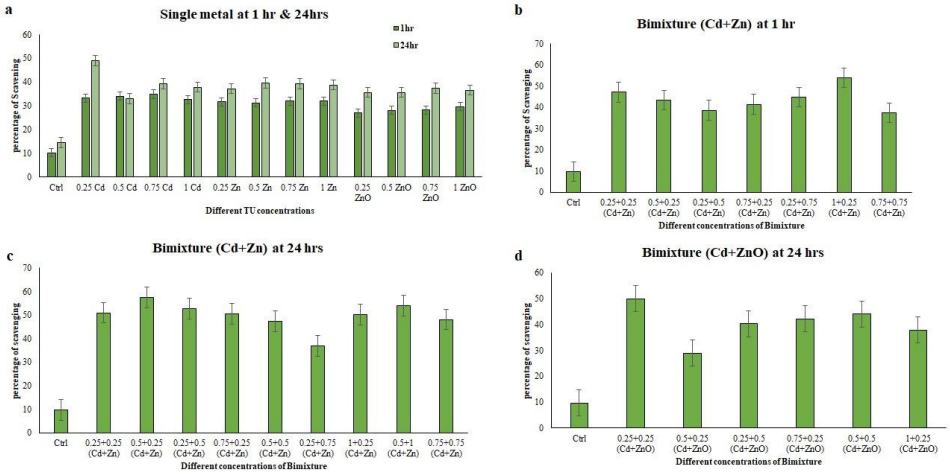

Figure 2. DPPH scavenging activity measured in *Euplotes aediculatus* cell extracts at single-metal (Cd, Zn, and ZnO) and bimetallic mixture concentrations. (**a**) Single metal, (**b**) bimetallic mixture of Cd + Zn at 1 h exposure time, (**c**) bimetallic mixture of Cd +Zn at 24 h exposure time, and (**d**) bimetallic mixture of Cd + ZnO at 24 h exposure time. The results are ± SD of three parallel experiments.

The DPPH scavenging activity ranged from 25.69% to 48.97%. In the single-metal treatments, concentrations of 0.75 TU and 0.25 TU Cd showed higher DPPH scavenging activity, with significant values of $p < 0.001$ at 1 h and 24 h, respectively (Figure 2). In the bimetallic mixtures, Cd + Zn showed higher DPPH activity during chronic exposure compared to other bimetallic mixture exposure periods. The maximum activity was observed with 0.5 + 0.25 TU (Cd + Zn) ciliate extracts at 24 h ($p < 0.001$), while the minimum value was found at the concentration of 0.5 + 0.25 TU (Cd + ZnO) at 24 h ($p < 0.001$) (Figure 2). Among the concentrations of the bimetallic mixture, the values ranged from 28.87% to 57.56%. To establish correlations between the results obtained by different methods, a regression analysis was conducted, and the correlation coefficients (R) are presented in Table 4.

Table 4. Correlation coefficient (R) between single and binary mixture metal exposure of different antioxidant assays on *Euplotes aediculatus*.

	Single Metal Treated 1 h				**Single Metal Treated 24 h**					
	DPPH	**HRSA**	**TPC**		**DPPH**	**HRSA**	**TPC**			
DPPH	1			**DPPH**	1					
HRSA	0.86	1		**HRSA**	0.86	1				
TPC	0.90	0.85	1	**TPC**	0.80	0.86	1			
	Bimixture (Cd + Zn) 1 h				**Bimixture (Cd + Zn) 24 h**				**Bimixture (Cd + ZnO) 24 h**	
	DPPH	**HRSA**	**TPC**		**DPPH**	**HRSA**	**TPC**		**DPPH**	**HRSA**	**TPC**
DPPH	1			**DPPH**	1			**DPPH**	1		
HRSA	0.93	1		**HRSA**	0.7	1		**HRSA**	0.91	1	
TPC	0.93	0.74	1	**TPC**	0.77	0.89	1	**TPC**	0.95	0.81	1

Significant correlations were observed between different methods used to determine the antioxidant potential, with notable associations between total phenolic content (TPC) and DPPH assays showing positive correlations of $R = 0.90$ and $R = 0.80$ at 1 h and 24 h, respectively. For the bimetallic mixture of Cd + Zn at acute exposure, a robust correlation of $R = 0.93$ was observed between the TPC and DPPH assays at 24 h. Similarly, Cd + ZnO showed very strong correlations in TPC and DPPH assays, with $R = 0.95$ at 24 h (Table 4). A significant positive correlation was observed between DPPH and TPC, as well as DPPH and HRSA ($R = 0.90$; $R = 0.86$; $p < 0.001$), reflecting the antioxidant capacity of the single-metal treatments at both 1 h and 24 h (Table 4). In the acute exposure of the bimetallic mixture

Cd + Zn, strong correlations (R = 0.93) were found between DPPH and HRSA, as well as between DPPH and TPC. However, lower correlations were identified between the antioxidant assays in the chronic condition of this mixture (Table 4). At the same time, in the bimetallic mixture of Cd + ZnO during chronic exposure, DPPH and HRSA as well as DPPH and TPC showed a strong correlation R = 0.91 and R = 0.95 at 24 h, respectively (Table 4). The hydroxyl radical is a highly important and reactive free radical formed in biological systems, capable of interacting with all molecules within living cells and causing severe damage. The ability of *E. aediculatus* cell extracts to scavenge hydroxyl radicals is expressed as the percentage of scavenged hydroxyl radicals, using ascorbic acid used as a standard. As shown in Figure 3, the cell extracts showed varying levels of radical scavenging ability in single-metal treatments, ranging from 31.20% to 68.98% at 1 h and from 48.83% to 71.92% at 24 h.

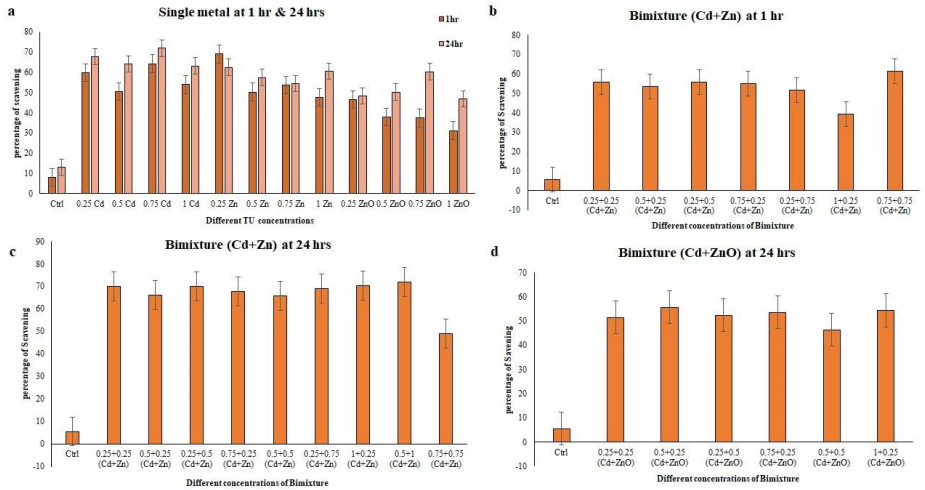

Figure 3. Hydroxyl radical scavenging activity measured in *Euplotes aediculatus* cell extracts at single -metal (Cd, Zn, and ZnO) and bimetallic mixture concentrations. (**a**) Single metal, (**b**) bimetallic mixture of Cd + Zn at 1 h exposure time, (**c**) bimetallic mixture of Cd +Zn at 24 h exposure time, and (**d**) bimetallic mixture of Cd + ZnO at 24 h exposure time. The results are ± SD of three parallel experiments.

In the case of binary mixtures, the hydroxyl radical scavenging activity ranged from 39.39% to 71.88% (Figure 3). The minimum and maximum hydroxyl radical scavenging activity was observed in the 0.5 + 1 mixture at 1 h and 24 h of Cd + Zn, respectively. In the binary mixture of Cd + ZnO, higher activity was observed in the mixture of 0.5 + 0.25 (55.74%), and in Cd + Zn at 24 h, higher scavenging activity was observed in the binary mixture of 0.5 + 1 (71.88%) (Figure 3) with a significant p-value of < 0.001. Generally, Cd + Zn at 1 h and Cd + ZnO at 24 h showed a wide range of scavenging abilities (39.39–61.38% and 46.31–55.74%, respectively), with Cd + Zn at 24 h consistently showing higher scavenging activity (49–71.88%). In the present study, a significant and positive correlation was observed between HRSA and TPC assays in single-metal treatments at both 1 h (R = 0.85) and 24 h (R = 0.86). The correlation was stronger in the bimetallic mixture compared to single-metal exposure, with the lowest correlations found between the antioxidant assays of HRSA and TPC at 1 h in the binary mixture of Cd + Zn (Table 4). However, strong correlations were found in Cd + Zn and Cd + ZnO at 24 h, with R values of 0.89 and 0.81, respectively.

4. Discussion

Anthropogenic activities contribute significantly to ecosystem pollution, a global challenge, with heavy metals (HMs) and nanoparticles (NPs) emerging as key pollutants [2,3]. Research on the interaction between HMs/NPs and ciliated protists is limited [29,31,36,41,57], especially compared to studies on other organisms [37,58–64]. Cell biomass plays a pivotal role in bioassay treatments, modifying pollutant bioavailability and enhancing data accu-

racy [65]. The order of toxicity of HMs is Cu > Cd > Zn at 1 h and 24 h, a pattern consistent with the findings of Pudpong and Chantangsi [66] and Martín-González et al. [29] for HMs. Martín-González et al. [29] used *Euplotes* sp. in their study and found concentrations of 0.7, 4.8, and 110.2 mg/1 for Cd, Cu, and Zn, respectively, after a 24 h exposure. Notably, these concentrations are lower than those observed in our study, except for the Cu LC_{50} value. The ciliate *Bresslauides* sp. showed lower LC_{50} values for Cd and Zn compared to our study [66]. Díaz et al. [67] and Rico et al. [68] observed higher LC_{50} values for Cu and Zn in *Colpoda* genus isolates compared to our study, except for the Cd LC_{50} value. Our NP LC_{50} values exceed those reported by Blinova et al. [69] for the ciliate *Tetrahymena thermophila*. Mortimer et al. [38] found ZnO values lower and CuO values higher than our results for the ciliated protozoa *T. thermophila* at 24 h exposure. According to a study by Zhao et al. [44], the LC_{50} value of CuO on *E. aediculatus* was found to be 1.24 mg/L after a 24 h exposure. However, our study showed a higher 24 h LC_{50} value of CuO compared to the study by Zhao et al. [44]. These different sensitivities may be due to the different origins of the *E. aediculatus* strains used in the experiments. Overall, Zn shows fewer toxic effects compared to other HMs, as ciliated protists adapt quickly to excess Zn without adverse effects. In contrast, the *E. aediculatus* cells used in this study showed lower resistance to Cu compared to previous studies [29]. In general, the mixtures of Cd + ZnO showed predominantly synergistic effects at both 1 h and 24 h. This particular interaction (Cd + Zn) has been studied and documented in various organisms, with consistent outcomes [37,67,70,71]. On the other hand, the differing responses of *E. aediculatus* to heavy metals and nanoparticles can be attributed to the distinct physical and chemical properties of these substances, their differing mechanisms of uptake and bioavailability, and the unique ways in which they interact with biological systems and the environment. Moreover, the toxicity of nanoparticles (NPs) can vary depending on the duration of exposure. Some nanoparticles dissolve slowly, releasing toxic ions over time, which can lead to a delayed onset of toxicity. The level of toxicity may increase or decrease based on the duration of exposure. These differences highlight the complexity of nanoparticle toxicity and the need for further research to fully understand their impacts on soil microorganisms and ecosystems.

In our analyses, the independent action (IA) module outperformed the concentration addition (CA) module, as evidenced by the R2 value, which explains 82% of the data variance. Modules taking into account deviations from the norm performed even better. The synergism/antagonism (S/A) sub-module provides a broad indication of synergisms, while the dose ratio-dependent deviation (DR) and the dose level-dependent deviation (DL) sub-modules show a more complex pattern, moving from synergism to antagonism. DR has the most conservative score, yet it is very similar to DL. Specifically, in the case of DR, a negative "*a*" and a positive "*b*" indicate the presence of antagonism, where the toxicity of the mixture is primarily due to one component (either high Cd or high Zn). The DL sub-module suggests a shift from synergism to antagonism at toxic values higher than LC_{50}. It is noteworthy that the responses identified by the two systems (CA, IA, and their derived models) are not identical. However, it should be pointed out that they account for additivity in markedly different ways. In particular, CA tends to overestimate mixture toxicity, making it relatively simpler to highlight an antagonistic effect from its derived sub-modules. In the IA module, each sub-module explains a very similar amount of data variance, approximately 77%. Among these sub-modules, DL stands out as the best performer, significantly outperforming both IA and SA. The parameters of the DL sub-module, labelled as "*a*" and "*b*," indicate synergism at low dose levels and antagonism at high doses. It is noteworthy that although the results of the CA and IA modules are not identical, their significant DL modules are similar in their predictions. In particular, they both indicate synergism at very low doses, such as the no observed adverse effect levels (NOAELs) for either Cd or Zn. This study represents a novel exploration, marking the first analysis of the MixTOX tool in the context of bimetallic mixtures involving Cd, Zn, and ZnO in the freshwater ciliate *E. aediculatus*. The data analysis shows no substantial differences in the predictability of joint effects using CA and IA for the tested bimetallic

system. Both models explain between 34% and 88% of the variance in the 1a–1b bimetallic mixture, and between 35% and 99% in the 1c–1d binary mixture. The MixTOX tool emerged as the superior and predominant method, demonstrating statistically significant deviations in bimetallic mixtures by incorporating additional parameters to tailor the dataset. For instance, with the exception of the Cd + Zn and Cd + ZnO mixtures, the inclusion of S/A, DR, and DL deviation parameters in the reference models results in a modest increase of less than 5% in the goodness of fit, as measured by R2. While CA and IA predictions are nearly identical in most cases, there are subtle differences. For instance, in the case of the Cd + Zn mixture, positive "*a*" values at 1 h and 24 h are observed in all deviation patterns of the CA module, except DL, while the opposite is true for the IA module. Conversely, in the case of Cd + ZnO, both modules give similar results at 1 h and 24 h. In general, the CA models explain the mixture toxicity of the 1a–1d mixtures based on free ion activity. For Cd + Zn mixtures, the CA model explains the variation, showing an increase from 34% to 72% at 24 h, while the IA model shows a decrease from 82% to 77% over the same period. For the bimetallic mixture of Cd + ZnO, the CA model shows an increase from 47% to 87%, while the IA module shows an increase from 35% to 86%. Notably, IA predictions are consistently lower than CA predictions, as exemplified by the mixture of Cd + ZnO at 1 h, where IA accounts for 35% of the model predictability compared to 47% for CA. The lower predictability of IA has been studied in various organisms, with particular emphasis on chemicals known to interact with xenobiotic metabolism [72–74]. We observed that the presence of one metal does not significantly affect the free ion activity of another metal in the mixed solution. Our results indicate that the joint toxicity effect of Cd, Zn, and ZnO, after different exposure times, is predominantly antagonistic at chronic levels, whereas it shows synergism at acute levels. This behaviour can be attributed to the consideration of free ion activity and is significantly enhanced in the MixTOX tool when incorporating deviations from both models. Our findings are in agreement with the observations of Jonker et al. [52], who reported similar joint toxic effects of Cd and Zn mixtures in their reference model. Furthermore, He et al. [75] investigated mixtures of Ni and Co in the soil-dwelling annelid *Enchytraeus crypticus* and reported a shift from synergism at low concentrations to antagonism at high concentrations, indicating that the effects depend on metal uptake and elimination processes [76]. We encountered difficulty in distinguishing between the CA- and IA-based models, as both models performed comparably well in assessing the overall toxicity of 1a, 1b, 1c, and 1d mixtures. This observation is consistent with the findings of Farley and Meyer [77] and Cedergreen et al. [73], who reported similar results, indicating that neither the CA nor the IA model showed significant superiority over the other. As a result, researchers are often faced with the challenge of decisively selecting or favouring one reference model for mixture toxicity over the other. Their toxicity often follows a dose–response curve, where low concentrations may be tolerable or even essential (as trace nutrients), while higher concentrations become toxic. Additionally, biological systems have the capacity to detoxify and excrete metals up to a certain limit. Once this threshold is exceeded, toxic effects become more pronounced. The type of interaction can shift from being tolerable or beneficial at low concentrations to being harmful at high concentrations. In our test model system, the response closely matched that of the CA model, making it difficult to distinguish the joint toxicity results from those predicted by the IA model. The difficulty arises from the unknown action of the chemicals; they may have a similar mode of action or different specific modes of action, contributing to the challenge of model selection. No prior total phenolic content (TPC) studies have been conducted on ciliate species. Before this study, numerous phenolic compounds demonstrated effective antioxidant properties in various organisms, including fungi, bacteria, various plant species, marine algae, and natural products, among others. Previous research has highlighted the antioxidant activity of marine algae extracts, particularly their polyphenols [78]. Additionally, phenolic content plays a crucial role in an organism's ability to withstand various environmental factors or stresses by acting as a component of the cell's antioxidant defence system. This aspect is particularly important for plants, fungi, and protozoa due to their hydroxyl radical

scavenging activity. The presence of HMs and NPs can lead to the generation of reactive oxygen species (ROS) in *E. aediculatus*, either directly or indirectly. This oxidative stress causes damage to the cells, prompting a strong antioxidant response. The response includes the activation of transcription factors, increased production of antioxidant and antioxidant enzymes, synthesis of metal-binding proteins (metallothioneins and phytochelatins), and the activation of repair mechanisms to counteract oxidative damage. These defence mechanisms collectively assist the organism in managing and neutralizing the toxic effects of HMs and NPs. Thus, the defence mechanisms help the ciliate *E. aediculatus* cope with an increase in the production of antioxidants. Therefore, in our experiment, we evaluated the total phenolic content of *E. aediculatus* cell extracts to understand their antioxidant activity against different concentrations of HM and NP stresses, using a series of antioxidant assays. DPPH scavenging activity was assessed for both single and bimetallic mixtures at acute and chronic exposures. Remarkably, the bimetallic mixture at 24 h showed robust DPPH scavenging activity, surpassing other bimetallic mixtures such as Cd + Zn at 1 h and Cd + ZnO at 24 h. Additionally, while the DPPH scavenging activity of single metals showed lower activity at both 1 h and 24 h, the combination of single metals with other metals resulted in higher activity. This phenomenon can be attributed to the joint toxicity of the chemical mixture ($p < 0.001$). In this study, the bimetallic mixtures showed potent antioxidant capacity. Consistent with our findings, a bimetallic mixture of Cd + Zn at chronic exposure has previously demonstrated effective antioxidant activity using the DPPH radical scavenging method [48]. Although no data are available for the analysis of DPPH scavenging activity in ciliated protists, DPPH has been extensively used as a radical to evaluate reducing substances in food materials [79]. Sanaye et al. [80] analysed the antioxidant activity in alligator pipefish and showed lower DPPH scavenging activity compared to our values. It is worth noting that DPPH is a stable free radical that becomes a diamagnetic molecule when it accepts an electron or hydrogen radical.

As previously stated, there are no available data on hydroxyl radical scavenging activity (HRSA) in ciliated protists, but numerous results are available for other organisms. For instance, Sanaye et al. [80] demonstrated HRSA ranging from 84.89% to 91.02%, and these levels of activity are relatively higher than those observed in our results. The robust correlation observed between TPC and HRSA strongly suggests that phenolic compounds play a crucial role in donating electrons to hydroxyl radicals, effectively neutralizing them into water [81]. The efficiency of *E. aediculatus* cell extract is demonstrated by the significant positive correlation ($p < 0.001$) between HRSA and other kinds of antioxidant activity. This positive correlation suggests that the ciliate cell extract stabilizes lipid peroxidation through its hydrogen-donating potential, contributing to its overall antioxidant capacity. This study has some limitations. First, the findings are based on a single ciliate species, which may not be representative of other aquatic organisms. Additionally, the experiments were conducted in controlled environments, which may not fully replicate natural conditions, potentially limiting the applicability of results to real-world scenarios. Furthermore, the study tested only specific combinations of heavy metals and nanoparticles, leaving out numerous possible mixtures and interactions that could occur in natural settings. The study primarily focused on acute toxicity (1 h) and chronic toxicity (24 h), as well as immediate antioxidant responses, potentially overlooking long-term ecological impacts such as effects over 2 days, 4 days, 6 days, and one week for long-term chronic exposure. Moreover, the study may have used concentrations that do not accurately reflect environmental levels, either being too high or too low compared to what is typically found in polluted habitats. Addressing these limitations in future research could provide a more comprehensive understanding of the ecological risks posed by heavy metals and nanoparticles.

5. Conclusions

Our results indicate that both HMs and NPs have statistically significant toxic effects on the selected ciliate species. In particular:

- *E. aediculatus* shows different sensitivities to the two types of pollutants, with a higher resistance to HMs than to NPs. Furthermore, both HMs and NPs can induce effective antioxidant responses in *E. aediculatus*, as evidenced by an increase in antioxidant properties and activity.
- Despite being exposed to oxidative stress, *E. aediculatus* exhibits a strong response with greater effectiveness.
- Various antioxidant assays reveal a significantly increased level of antioxidant activity and free radical scavenging activity in *E. aediculatus* when exposed to both single and bimetallic mixtures.
- In conclusion, our results suggest that *E. aediculatus* can be used as a bioindicator organism for the detection of the bioavailable fraction of various toxic pollutants, such as HMs and NPs, in real environmental samples. The observed differential sensitivity of this species to the toxic effects of HMs and NPs suggests that they can be used in the development of rapid, simple, and cost-effective ciliate test arrays for the detection of specific pollutants in different environmental matrices.

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