Assessment of heavy metal toxicity in four species of freshwater ciliates (Spirotrichea: Ciliophora) from Delhi, India

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In vitro **laboratory experiments were conducted to determine the toxicity (per cent survival and LC_{50}) of essential and non-essential heavy metals (cadmium, copper, nickel, lead and zinc) in four spirotrich ciliates: *Euplotes* sp., *Notohymena* sp., *Pseudourostyla* sp. and *Tetmemena* sp. isolated from three different freshwater ecosystems in the Delhi region, India. The toxicity of the heavy metals was found to vary among the different ciliates. Copper was most toxic (24 h-LC_{50} value ranged between 0.125 and 0.74 mg/l) and zinc was least toxic (24 h LC_{50} value ranged between 46.98 and 144.32 mg/l) to each of the ciliates. Of the four ciliates, *Notohymena* sp. had the highest tolerance limit to three heavy metals (Cu, Cd and Pb) out of the five tested. This study shows the high potentiality of using freshwater ciliates for monitoring the intensity and potency of ecological damage caused by heavy metals in aquatic ecosystems.**

Keywords: Ciliates, freshwater, heavy metals, toxicity.

THERE is a global increase in the concentration of heavy metals in the environment mainly due to anthropogenic activities and India is no exception to this[1]. Although some heavy metals are essential micronutrients, all may be toxic if present in sufficiently high concentration in a bioavailable form, mainly as a result of metabolic interference and mutagenesis. The presence of heavy metals in aquatic environments is a major concern because of their threat to plant and animal life, thus disturbing the natural ecological balance[2]. Many freshwater ecosystems, including lakes, ponds, rivers and reservoirs are exposed to heavy metal contamination from a range of sources, primarily wastewater discharges from industry and households[3,4]. Most of the heavy metals have a long half-life and cannot be degraded, but may instead bio-accumulate throughout the food chain leading to physiological stress causing ecological disturbance[5–7].

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Toxicity of various heavy metals can be studied using ciliated protists[8–10]. These eukaryotic microorganisms are found in a variety of trophic niches, have generation time of 3–7 h and many are easy to culture *in vitro*[11]. Ciliates share a higher degree of functional and genetic similarities with humans than bacteria or yeast (microbial eukaryotic model organism)[12–14]. All these properties make them suitable candidates both for eco-toxicological studies and for monitoring water quality[15–17].

In the present study, we assess the toxicity of essential (Cu and Zn) and non-essential (Cd, Ni and Pb) heavy metals on ciliated protists isolated from three different freshwater ecosystems (river, lake and pond) in the Delhi region, India. The diversity of free-living ciliates in the study sites was observed for a period of one year. The most frequently encountered ciliate species were from four genera, namely *Euplotes*, *Notohymena*, *Pseudourostyla* and *Tetmemena* (Figure 1) and all were easily cultured under laboratory conditions. Toxicity assays were carried out *in vitro* in order to determine the sensitivity and survival of *Euplotes* sp., *Notohymena* sp., *Pseudourostyla* sp. and *Tetmemena* sp. to different doses of heavy metals.

Materials and methods

Study area

Delhi is located in northern India. It is bordered by the states of Haryana to the north, west and south, and Uttar Pradesh (UP) to the east. Prominent features of the geography of Delhi include the floodplains of River Yamuna. In the present study three sites were selected in different ecological regions of Delhi.

Site I: Okhla Bird Sanctuary (28.5700°N, 77.3023°E): This is a bird sanctuary at the Okhla Barrage over the Yamuna. The site is located at the point where the river exits Delhi and enters UP. The most prominent feature of the sanctuary is the large lake created by a dam over the

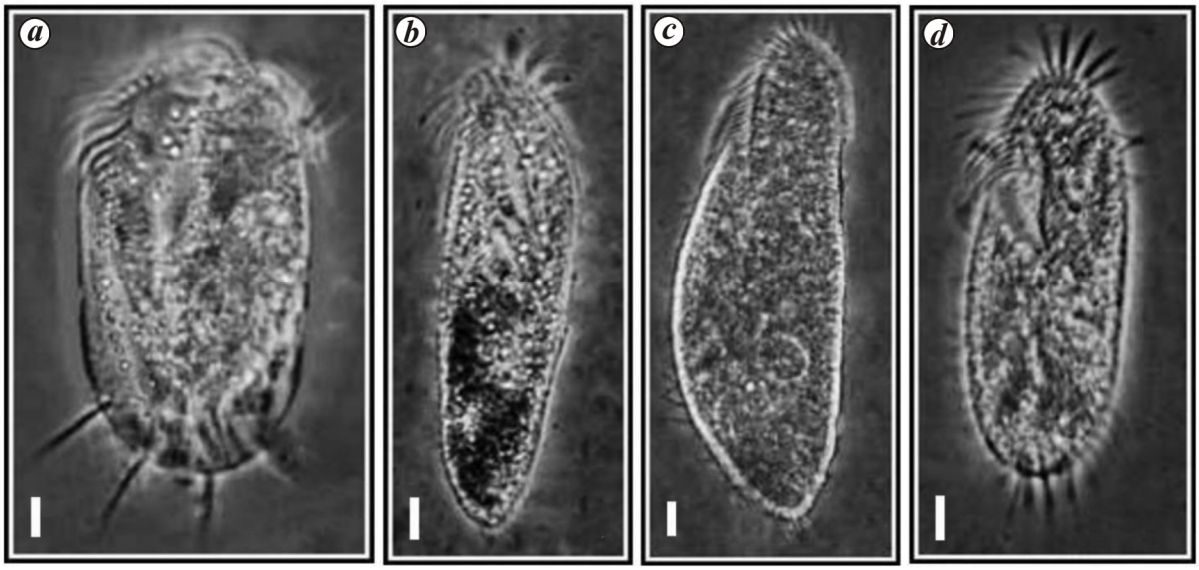

Figure 1. Phase contrast photomicrographs from life: ***a***, *Euplotes* sp.; ***b***, *Notohymena* sp.; ***c***, *Pseudourostyla* sp.; ***d***, *Tetmemena* sp. (scale bar represents 20 μm).

river between Okhla village to the west and Gautam Budh Nagar to the east. Spread over 4 sq. km, the vegetation in the areas around the barrage is mainly thorny scrub, grassland and a wetland that was formed as a result of the Okhla Barrage. The sediment in the wetland consists of organic debris and fine sand. There is extensive growth of water hyacinths as well.

Site II: Sanjay Lake (28.6142°N, 77.3039°E): This is an artificial lake developed by the Delhi Development Authority (DDA) in Trilokpuri, East Delhi. Its surface area is about 1 sq. km and it has extensive growth of water hyacinth. It is mainly fed by rainwater, but also receives inputs of sewage.

Site III: Raj Ghat (28.6406°N, 77.2494°E): This is a memorial to Mahatma Gandhi. A man-made pond is situated in the vicinity; it has a maximum depth of about 4 m, an average depth of about 2 m and a surface area of about 0.01 sq. km.

Collection, isolation and cultivation of ciliates

Water samples, each 1000 ml, were collected from all the sampling sites at a depth of approx. 1 m using wide-mouthed plastic bottles. Nytex nets of decreasing mesh sizes were used in succession to filter out large crustaceans, debris and other unwanted materials. Several liters of water samples were strained through a mesh of size <50 μm and the concentrate containing ciliates was transferred to large troughs in the laboratory. Mixed planktonic cultures were initially grown at room temperature with addition of fresh boiled cabbage pieces to promote the growth of bacteria which serve as the food organism. Such water samples were subjected to periodic microscopic examination for about 20 days. Identification of ciliates was carried out *in vivo* under bright field and phase contrast microscopy at 10× and 40× magnification. Genus-level identifications were made according to Berger[18]. For each ciliate species, clonal cultures were established and maintained at 22° ± 1°C in Pringsheim's medium[19]. Also, boiled cabbage was added to the medium.

Determination of heavy metal toxicity

Toxicity assays for heavy metals (Cd, Cu, Ni, Pb and Zn) were carried out in order to determine the tolerance degree limits (i.e. 0% to 100% survivability) of each of the four ciliate species. Stock solutions (1000 mg/l) of $CuSO_4$, $CdCl_2$, $NiCl_2 \cdot 6H_2O$, $Pb(NO_3)_2$ and $ZnCl_2$ were prepared in Pringsheim's medium and verified analytically by Atomic Absorption Spectroscopy (AAS-6300 Shimadzu Corp. 00740, University Science Instrumentation Centre (USIC), Delhi). For each species, 20 ciliate cells from the clonal cultures were exposed to a range of concentrations – Cu (0.1–2.0 mg/l), Cd (1–10 mg/l), Ni (1–10 mg/l), Pb (1–40 mg/l) and Zn (20–280 mg/l). Each treatment was carried out in triplicate and was performed without adding fresh food to the medium. Appropriate control experiments (without heavy metal additions) with the same number of cells were performed simultaneously. The time period for the toxicity treatment was selected as 24 h to allow the cells to undergo 2–3 divisions in the presence of various heavy metals. After 24 h, the cells were counted under a Magnüs stereoscopic microscope at 20–40× magnification to determine per cent survival and

Table 1. The 24 h LC_{50} mean values of four freshwater ciliates tested with heavy metals (n = 60)

	Species			
	24 h LC_{50} (mg/l) (95% confidence limit)			
Metals	*Euplotes* sp.	*Notohymena* sp.	*Pseudourostyla* sp.	*Tetmemena* sp.
Cd	2.2 (1.6–2.8)	5.1 (4.0–6.9)	2.3 (1.3–3.4)	2.4 (1.7–3.2)
Cu	0.2 (0.1–0.2)	0.7 (0.4–1.2)	0.1 (0.1–0.2)	0.1 (0.1–0.2)
Ni	4.5 (3.9–5.1)	4.3 (3.6–5.0)	5.0 (3.6–6.6)	1.9 (1.6–2.4)
Pb	5.5 (4.7–7.7)	22.9 (17.7–29.5)	5.4 (4.6–7.6)	7.7 (6.1–12.5)
Zn	124.2 (92.7–158.7)	46.9 (30.5–54.7)	74.6 (49.1–113.1)	144.3 (111.4–216.6)

Figures in brackets represent the range of LC_{50} values.

mortality rate under different concentrations of test solutions. Non-motile and missing cells were considered as dead; mean value of viable cells in triplicates was calculated[10,20,21]. Per cent survival was determined as

Total cells – Dead cells/Total cells × 100.

Statistical analysis

The LC_{50} values and 95% confidence limits were determined by the Probit method[22,23]. The significance of difference of LC_{50} among species was compared by multiple comparison T3 Dunnett test[24]. These analyses were performed using the program IBM SPSS Statistics 19.

Results

Heavy metal toxicity

Table 1 shows the 24 h LC_{50} values and associated 95% confidence limits of the four ciliate species exposed to the heavy metals (copper, cadmium, nickel, lead and zinc). The order of toxicity to heavy metals is as follows:

Euplotes sp.: Cu > Cd > Ni > Pb > Zn;
Notohymena sp.: Cu > Ni > Cd > Pb > Zn;
Pseudourostyla sp.: Cu > Cd > Ni > Pb > Zn;
Tetmemena sp.: Cu > Ni > Cd > Pb > Zn.

Figures 2–6 show the mean survival value (±SD) of the cells to different concentrations of heavy metals. The relative sensitivity of the ciliates to each metal is as follows:

Cd: *Euplotes* sp. > *Pseudourostyla* sp. > *Tetmemena* sp. > *Notohymena* sp.
Cu: *Pseudourostyla* sp. > *Tetmemena* sp. > *Euplotes* sp. > *Notohymena* sp.
Ni: *Tetmemena* sp. > *Notohymena* sp. > *Euplotes* sp. > *Pseudourostyla* sp.
Pb: *Pseudourostyla* sp. > *Euplotes* sp. > *Tetmemena* sp. > *Notohymena* sp.
Zn: *Notohymena* sp. > *Pseudourostyla* sp. > *Euplotes* sp. > *Tetmemena* sp.

Statistical analysis

Significant differences ($P < 0.001$) were observed in the LC_{50} mean values for the four ciliates to each heavy metal (Table 1). The relative sensitivity of these ciliates to each metal was also found to be significantly different as analysed by T3 Dunnett test (Table 2).

Discussion

Toxicity data collated from various studies show that different protists exhibit variable sensitivity to different heavy metals (Table 3). Heavy metals influence the survival of ciliates by affecting certain physiological and ecological processes such as reduction in food uptake, inhibition of growth and reduction in the rate of endocytosis as observed in *Tetrahymena*[25,26]. The present study also shows that copper is the most toxic heavy metal and zinc the least toxic in all the four studied species of ciliates. Earlier studies on *Uronema* sp., *Drepanonomus revoluta* and *Euplotes* sp. also illustrated that cellular toxicity of zinc is low[9]. Zinc is an essential metal which constitutes a catalytic and structural compound for many enzymes. A study conducted by Nilsson[27] on *T. pyriformis* also concluded that this ciliates adapt quickly to excess amount of zinc. The toxicity of Cu may be more than Zn as suggested in *in silico* studies on *Tetrahymena* that zinc-binding proteome constitutes up to 9% of the total proteome, whereas Cu-binding proteome constitutes only 0.07% of the entire proteome[28].

Among the four ciliates, *Notohymena* sp. had the highest tolerance to three of the tested heavy metals (Table 2). The high tolerance in *Notohymena* sp. may be attributed to the presence of a large number of cytoplasmic granules within the cell. It has been demonstrated that in other protists such as the ciliate *Tetrahymena*, cytoplasmic granules play a major role in compartmentalization of metals, thereby increasing tolerance[29,30]. Exposure of ciliates to heavy metals also led to the formation of cytoplasmic vacuoles that contain electron-dense particles. The phenomenon of metal accumulation in granules and membrane-enclosed vesicles is widespread in numerous phyla

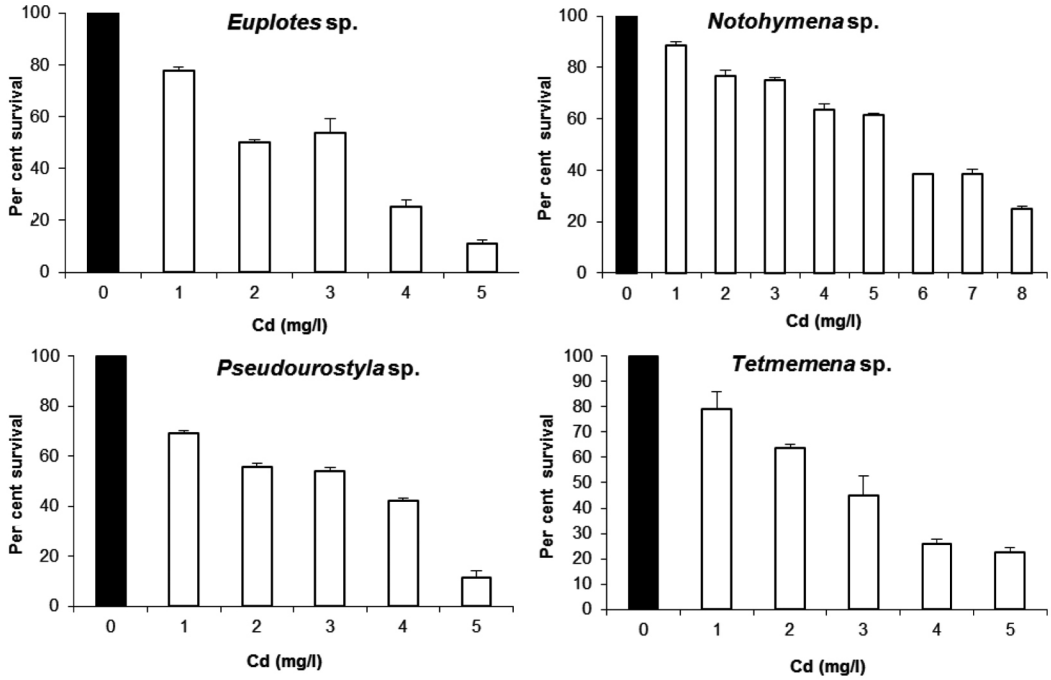

Figure 2. Mean survival values (±SD) of the four freshwater ciliate species after 24 h exposure to cadmium.

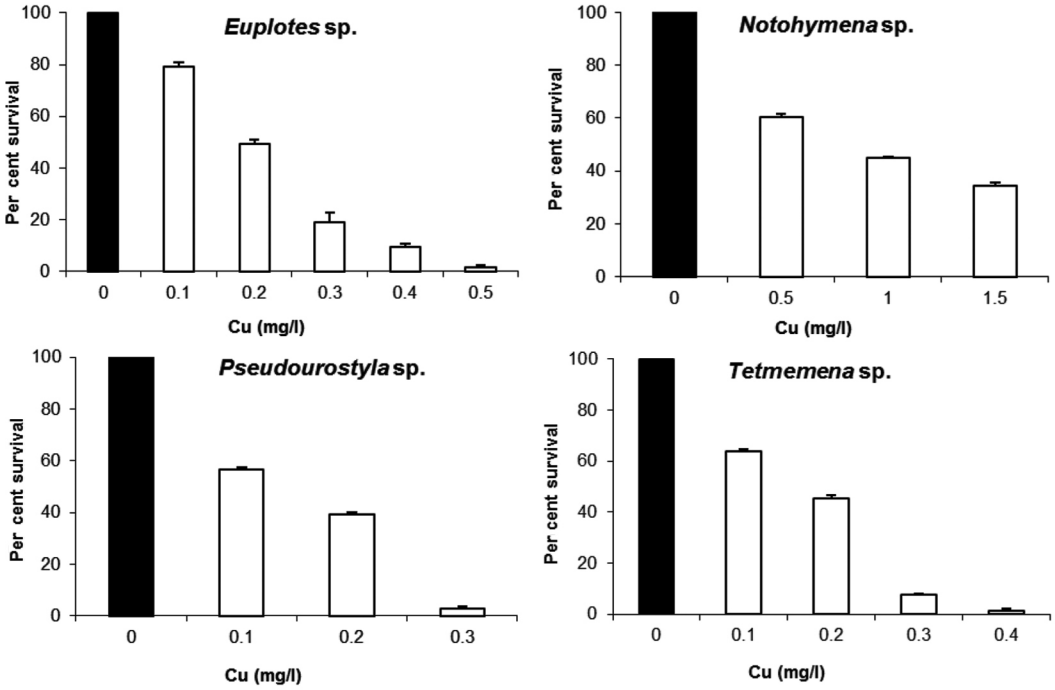

Figure 3. Mean survival values (±SD) of the four freshwater ciliate species after 24 h exposure to copper.

from protozoa to mammals. Similar cytoplasmic electron-dense accumulations have also been found in *Stylonchia lemnae*[31]. It is possible that these cytoplasmic granules consist of complexes formed by metallic cations (Cd^{2+}, Zn^{2+}, etc.) and metallothioneins (MTs) as revealed by the use of metal fluorophores for the first time in ciliates[21]. The expression of MT genes increases in the presence of heavy metals, thus helping detoxification[32–37]. The high tolerance of ciliates to Zn may be explained by the fact that Cd–MT can also be induced by Zn[21,38]. The order of bioaccumulation of metals in ciliates is Zn > Cd > Cu[21]. Higher bioaccumulation of zinc compared to cadmium

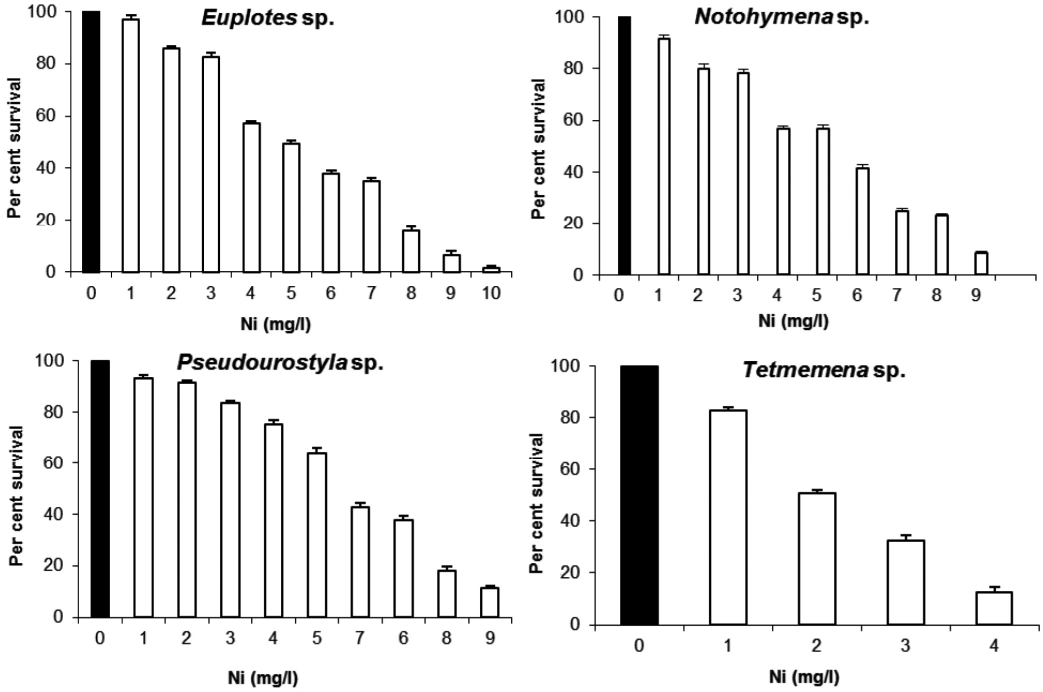

Figure 4. Mean survival values (±SD) of the four freshwater ciliate species after 24 h exposure to nickel.

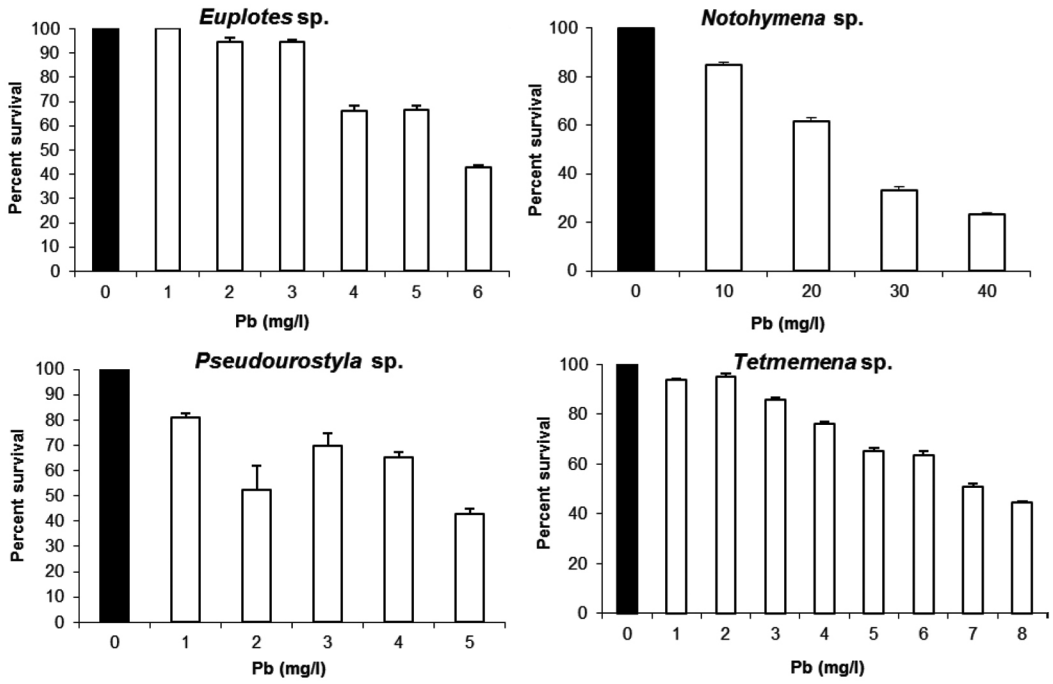

Figure 5. Mean survival values (±SD) of the four freshwater ciliate species after 24 h exposure to lead.

and copper may also be attributed to low toxicity of the former compared to cadmium and copper. Other reported studies show that ciliates are more sensitive to Cu and its bioaccumulation seems to be less efficient than in other microorganisms, particularly microalgae such as *Chlorella*[21,39,40].

In the present study, variation in tolerance range to various metals in different genera was observed. The apparent difference in toxicity of any given metal might, however, be due to many factors, one of which could be the diversity of experimental conditions: certain factors such as pH and composition of the surrounding medium

Table 3. Comparison of toxicity level of five heavy metals to protists and metazoans ($LC_{50}/EC_{50}/IC_{50}$)

Organism	Metal (mg/l)							
Protozoans	Cd	Cu	Ni	Pb	Zn	Time (h)	Source of experimental organisms	Reference
Acanthamoeba polyphaga (CCAP1501/3A; IC_{50})	3.6			8.6	42	72	Not mentioned	44
Acanthamoeba sp. (SW isolate; IC_{50})	7			9	36	72	Setiu wetlands, Terengganu, Malaysia	44
Aspidisca cicada	0.31	0.02			2.4	24	Activated sludge, Reggio Emilia, Italy	45
Aspidisca cicada				1.261		24	Activated sludge, Reggio Emilia, Italy	41
Blepharisma americanum	1.4	0.001			1.05	24	Activated sludge, Reggio Emilia, Italy	45
Colpoda elongata (FM2)	4.4	13.3			132.3	24	Compost sample of the enterprise 'Fertilizantes Martin', Madrid, Spain	21
Colpoda inflate (AZ2)	1.8	7.1			94.7	24	Heavy metal-polluted superficial soil sample, Aznalcóllar, Seville, Spain	21
Colpoda steinii (010A)	4.2	8.1			33.9	24	Garden soil sample, Rome, Italy	21
Colpoda steinii (AZ1)	5	5.4			147.4	24	Heavy metal-polluted superficial soil sample, Aznalcóllar, Seville, Spain	21
Colpoda steinii (FM1)	0.5	5.5			78.7	24	Compost sample of the enterprise 'Fertilizantes Martin', Madrid, Spain	21
Colpoda steinii (RT1)	2.5	5.5			78.7	24	Tinto river, Huelva, Spain	46
Colpidium colpoda	0.89	0.05	1.19	0.23		24	Garda lake, Italy	9
Dexiostoma campylum	0.205	0.012			1.85	24	Activated sludge, Reggio Emilia, Italy	45
Dexiostoma campylum				1.099		24	Activated sludge, Reggio Emilia, Italy	41
Dexiostoma campylum			1.05			24	Stirone stream, Italy	8
Dexiostoma granulosa	0.3	0.17	1	0.12		24	Garda lake, Italy	9
Drepanomonas revoluta	0.19	0.002		0.875	0.254	24	Activated sludge, Reggio Emilia, Italy	41
Drepanomonas revoluta (BQ1)	7.7	6.7			52.3	24	Activated sludge plant, Butarque, Madrid, Spain	32
Glaucoma scintillans			1.4			24	Stirone stream, Italy	8
Halteria grandinella	0.07	0.01	0.61	0.12		24	Garda lake, Italy	9
Holosticha kessleri			1.1			24	Stirone stream, Italy	8
Loxodes striatus			1.67			24	Stirone stream, Italy	8
Paramecium bursaria			0.36			24	Stirone stream, Italy	8
Paramecium bursaria	0.64					24	Furnas lake, Minas Gerais, Brazil	10
Paramecium caudatum	0.18	0.01			2.5	24	Activated sludge, Reggio Emilia, Italy	45
Paramecium caudatam				2.26		24	Activated sludge, Reggio Emilia, Italy	41
Paramecium caudatam			0.49			24	Stirone stream, Italy	8
Paramecium putrinum			1.3			24	Stirone stream, Italy	8
Spirostomum teres	0.557	0.0035		1.083	0.672	24	Activated sludge, Reggio Emilia, Italy	41
Spirostomum teres			0.17			24	Stirone stream, Italy	8
Spirostomum teres	1.95	0.037		10.78	8.93	24	Pond, Puy-de-Dôme, France	11
Tetrahymena pyriformis		>200			45	24	Not mentioned	47
Tetrahymena sp. (RT1)		60				120	Tannery effluents of Kasur	48
Tetrahymena sp. (RT2)	0.52	3.3			196	24	Tinto river, Huelva, Spain	46
Tetrahymena thermophila (SB1969)	0.195	0.47			3.58	24	University of California, Santa Bárbara, USA	49
Uronema marinum				60	400	24	Robin Hood's bay, Yorkshire, England	50

(*Contd*)

Table 3. (*Contd.*)

Organism	Metal (mg/l)							
Protozoans	Cd	Cu	Ni	Pb	Zn	Time (h)	Source of experimental organisms	Reference
Uronema nigricans	0.62	0.014			2.9	24	Activated sludge, Reggio Emilia, Italy	45
Uronemanigricans				1.616		24	Activated sludge, Reggio Emilia, Italy	41
Uronema nigricans (BQ2)	0.9	4.5			135.1	24	Activated sludge plant, Butarque, Madrid, Spain	32
Euplotes aediculatus	0.59	0.01	0.03	0.5		24	Garda lake, Italy	9
Euplotes affinis	0.4	0.064			3.1	24	Activated sludge, Reggio Emilia, Italy	45
Euplotes affinis				2.323		24	Activated sludge, Reggio Emilia, Italy	41
Euplotes crassus (EC_{50})		1.58		4.13	4.97	48	Korea Ocean Research and Development Institution	51
Euplotes moebiusi			1.28			24	Stirone stream, Italy	8
Euplotes patella	2.65	0.01			50	24	Activated sludge, Reggio Emilia, Italy	45
Euplotes patella				2.177		24	Activated sludge, Reggio Emilia, Italy	41
Euplotes patella			7.7			24	Stirone stream, Italy	8
Euplotes vannus (IC_{50})		0.41				24	Palude Della Rosa, Lagoon of Venice	52
Euplotes sp. (BQ3)	0.7	4.8			110.2	24	Activated sludge plant, Butarque, Madrid, Spain	32
Euplotes (RE-1)		48				192	Tannery effluents of Kasur, Pakistan	48
Euplotes (RE-2)		49				192	Industrial effluents of Sialkot, Pakistan	48
Euplotes sp.	2.24	0.177	4.52	5.54	124.17	24	Pond, Rajghat, Delhi	Present study
Notohymena sp.	5.06	0.74	4.31	22.9	46.98	24	Sanjay lake, Delhi	Present study
Pseudourostyla sp.	2.32	0.125	5.03	5.42	74.58	24	Sanjay lake, Delhi	Present study
Tetmemena sp.	2.44	0.14	1.95	7.75	144.32	24	Yamuna river, Okhla, Delhi	Present study
Stylonychia pustulata			1.02			24	Stirone stream, Italy	8

Table 2. Multiple comparisons of the mean LC_{50} values by T3 Dunnett test

Metal	Species	LC_{50} (mg/l)	SD	Significance of the difference between mean LC_{50} values			
				a	b	c	d
Cd	a	2.44	0.54	–	ns	*	ns
	b	2.32	0.66	ns	–	*	ns
	c	5.06	0.45	*	*	–	**
	d	2.24	0.43	ns	ns	**	–
Cu	a	0.14	0.01	–	ns	*	ns
	b	0.12	0.005	ns	–	*	ns
	c	0.74	0.12	*	*	–	*
	d	0.18	0.02	ns	ns	*	–
Ni	a	1.95	0.26	–	**	*	**
	b	5.03	0.37	**	–	ns	ns
	c	4.31	0.51	*	ns	–	ns
	d	4.52	0.35	**	ns	ns	–
Pb	a	7.75	0.56	–	ns	*	ns
	b	5.42	2.82	ns	–	**	ns
	c	22.97	2.97	*	**	–	*
	d	5.54	0.80	ns	ns	*	–
Zn	a	144.32	1	–	*	*	*
	b	74.58	1	*	–	*	*
	c	46.98	0.5	*	*	–	*
	d	124.17	1.01	*	*	*	–

*$P < 0.05$; **$P < 0.01$; ns, Nonsignificant.

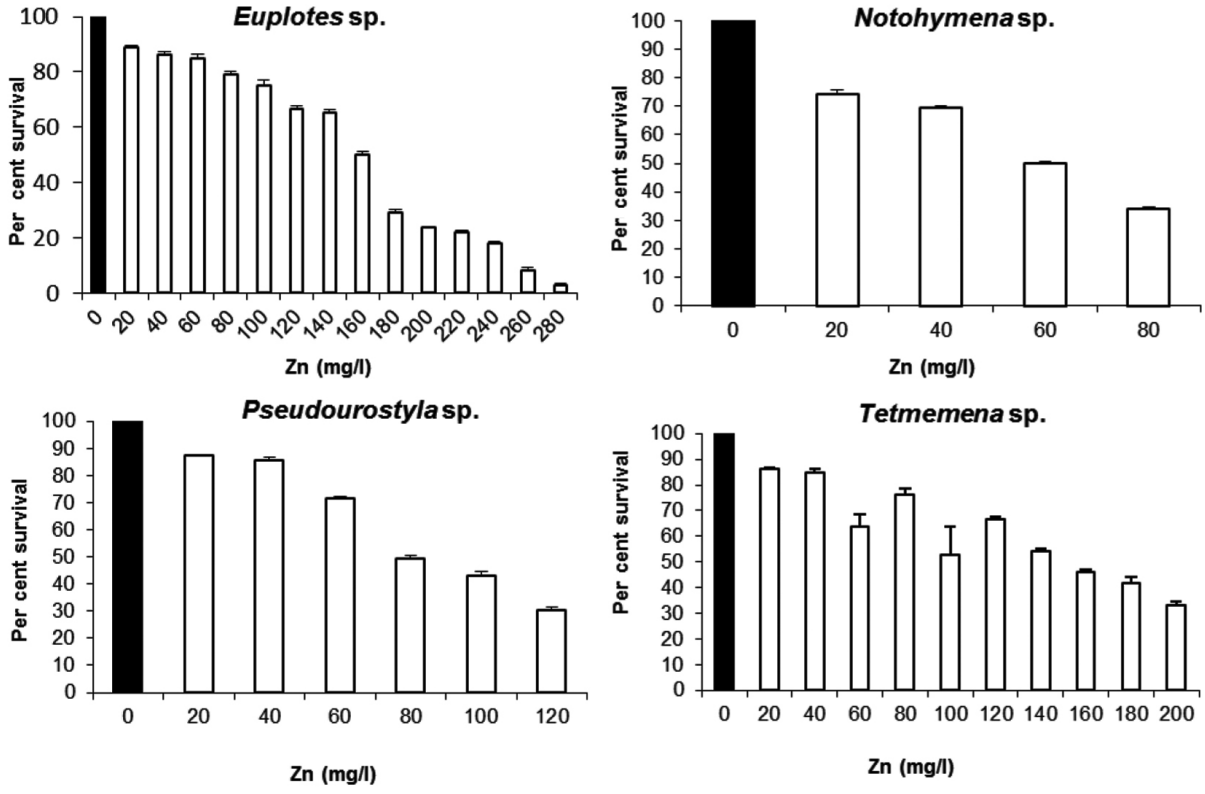

Figure 6. Mean survival values (±SD) of the four freshwater ciliate species after 24 h exposure to zinc.

are known to influence the bioavailability of metallic ions and therefore their potential toxicity[16]. Another important factor is the cell concentration used during treatment. Some tests have used 8–12 cells per bioassay, whereas in others 10^3–10^5 cells/ml have been used[16,41,42]. Cell concentration modifies metallic bioavailability and thereby changes the cytotoxicity.

The results reported herein showed the potential utility of ciliates in ecotoxicological studies because of their varied sensitivity to different heavy metals. Many studies on the dynamics of protozoan communities in heavy metal-polluted waters, activated sludge and wastewater treatment plants have shown the potentiality of using ciliates in ecotoxicological bioassays[9,21,43]. Also, this approach is economical and simple, and may provide a benchmark for monitoring the intensity and potency of ecological damage caused by anthropogenic activities. Future work will focus on the mechanism of heavy metal action on ciliates and to elucidate the cellular and molecular mechanisms that ciliates adopt to combat heavy metal stress. The possibility of using ciliates as whole-cell biosensors to assess heavy metal pollution in the freshwater bodies of Delhi region appears an achievable goal.

Conflict of interest. The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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