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Ecotoxicological effect of heavy metals in free-living ciliate protozoa of Lake Maracaibo, Venezuela

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Abstract: Multiple anthropogenic agents have turned Lake Maracaibo into a hypereutrophic environment. Heavy metals resulting from the steel and oil industry augment pollution in the lake. There is a lack of research on the ecotoxicological effect of heavy metals in protozoa. To evaluate the ecotoxicological effect of Cr^{3+}, Cr^{6+}, Cd^{2+}, Pb^{2+} and Ni^{2+} on free-living ciliated protozoa and to identify suitable ciliated protozoa candidates for bioindicators of water quality; we estimated the lethal concentration for 50% of the protozoa population (LC_{50}) in samples from two stations (S_1: narrow of Maracaibo and S_2: South of the lake) using ecotoxicological tests in the Sedgewick–Rafter chamber and Probit analysis. The general toxicity patterns obtained for S_1 protozoa (*Euplotes* sp. and *Oxytricha* sp.) were $Cr^{3+} > Cd^{2+} > Pb^{2+} > Cr^{6+} > Ni^{2+}$; and those corresponding to S_2 (*Coleps* sp. and *Chilodonella* sp.) were $Cr^{6+} > Cr^{3+} > Cd^{2+} > Pb^{2+} > Ni^{2+}$. We found statistically significant difference ($p < 0.05$) in the LC_{50} of protozoa exposed to Cr^{3+}, Cr^{6+}, Ni^{2+} and Pb^{2+} when comparing the two sampling stations. The differences observed in toxicity patterns are probably the result of various kinds of protozoa adaptation, possibly induced by various sources, levels and incidents of exposure to heavy metals contamination of the protozoa studied and to the physicochemical conditions prevailing in the two selected stations. The levels of tolerance observed in the present study, allow us to infer that S_2 ciliates are the most susceptible to the contaminants studied and can be used as possible microbiological indicators that provide early warning in studies of contamination by heavy metals in Lake Maracaibo.

Keywords: contamination, ecotoxicological tests, lethal concentration LC_{50}, microbiological indicators of early warning, toxicity

INTRODUCTION

Widespread presence, persistence and toxicity of heavy metals in environmental systems is a cause for concern. The accumulation of heavy metals in different environmental compartments, such as urban soil, sediments, road dust and bodies of water should be considered chemical time bombs waiting to be detonated by environmental triggers [Kumar *et al.* 2017]. The heavy metal contamination of water is a threat to living organisms because most of these metals are toxic to humans and aquatic life [Sall *et al.* 2020].

Free-living ciliated protozoa are biotic components of an ecosystem. They are eukaryotic microorganisms distributed in diverse habitats around the world. Around 30% of these species are endemic in their environments [Lynn 2008]. Endemism is influenced by their remarkable tolerance and adaptability to different intervals of physicochemical conditions in the environment [Dopheide *et al.* 2009]. Free-living ciliated protozoa are abundant and widely distributed not only in aquatic environments but in different geographic and climatic habitats. They maintain the balance of the ecosystem and play a crucial role in the regulation of microbial food webs, as predators of bacteria, small protists and even microscopic animals [Abraham *et al.* 2019]. Ciliates in aquatic ecosystems greatly contribute to organic matter decomposition and energy transfer to higher trophic levels. Free-living ciliated protozoa are considered good biological indicators of chemical pollution as they are relatively sensitive to heavy metal contamination [Villas-Boas *et al.* 2020a; Weisse 2017] and environmental changes. They are more sensitive to toxicity than bacteria [Metcalf, Eddy 2003]; thus, they have been identified as effective bioindicators of water quality and environmental contamination [Kim *et al.* 2012].

Ciliates can be used as simple models to study metal toxicity in complexes of organisms and biological mechanisms involved in detoxification. Eukaryotic microorganisms use two main processes to resist heavy metals, i.e. bioabsorption and bioaccumulation [Martín-González *et al.* 2006; Mortuza *et al.* 2009]. Intracellular detoxification of heavy metals involves the participation of a group of low molecular weight proteins called metallothioneins (MTs). Metallothioneins participate in storage, transport and metal binding mechanisms [Chatterjee *et al.* 2020]. Metallothioneins are induced by oxidative stress inducers [Díaz *et al.* 2006]. Some enzymatically biosynthesized molecules are also involved in the detoxification of cellular metals, such as glutathione and phytochelatins. All of them contain numerous free -SH groups (from cysteine residues) in their molecules because they constitute reactive groups for heavy metal chelation [Gutiérrez *et al.* 2008]. Ciliated protozoa also respond to heavy metal toxicity through different biochemical mechanisms, such as immobilization, exclusion, chelation, and compartmentalization of metal ions [Clemens 2001].

Ciliates have many characteristics that make them suitable for the evaluation of environmental toxicity. Therefore, they can potentially contribute to the establishing of more accurate guidelines and risk management programs. The also represent a robust system that can be used to study how environmental contaminants impact normal cell biological functions [Vilas-Boas *et al.* 2020b].

This study aims to evaluate the ecotoxicological effect of heavy metals (Cr^{3+}, Cr^{6+}, Cd^{2+}, Pb^{2+} and Ni^{2+}) on free-living protozoa from two sampling stations in Lake Maracaibo, to estimate the lethal concentration for 50% of the test population (LC_{50}), which constitutes the first report for the genera *Oxytricha* sp., *Coleps* sp. and *Chilodonella* sp., isolated from this body of water.

MATERIALS AND METHODS

STUDY AREA AND SAMPLING STATIONS

The Lake Maracaibo system (Sp. Lago de Maracaibo), the Northwest of Venezuela, covers the states of Zulia, Falcón, Trujillo, Lara and Mérida, as well as the Republic of Colombia (70°30' and 73°24' W longitude and 8°22' and 11°51' N latitude). The hydrographic basin of the system has an extension of 89,756 km^2 [Ávila *et al.* 2010], with 13,820 km^2 corresponding to Lake Maracaibo itself [Gutiérrez-Peña *et al.* 2018], with a maximum depth of 47 m, and 1,090 km^2 in the strait and bay [Ávila *et al.* 2010]. The Lake's Maracaibo basin is one of the largest petroleum centres in the world [Gutiérrez-Peña *et al.* 2018]. Various sources of heavy metals, including pesticides, domestic and industrial discharge and oil industry, contaminate the basin of the lake [Rodríguez (ed.) 2000]. This environmental problem has generated an ecological imbalance of the biotic and abiotic components in the largest estuary in America.

Two sampling stations were chosen based on their accessibility and diversity of anthropogenic activities in their areas of influence (Fig. 1).

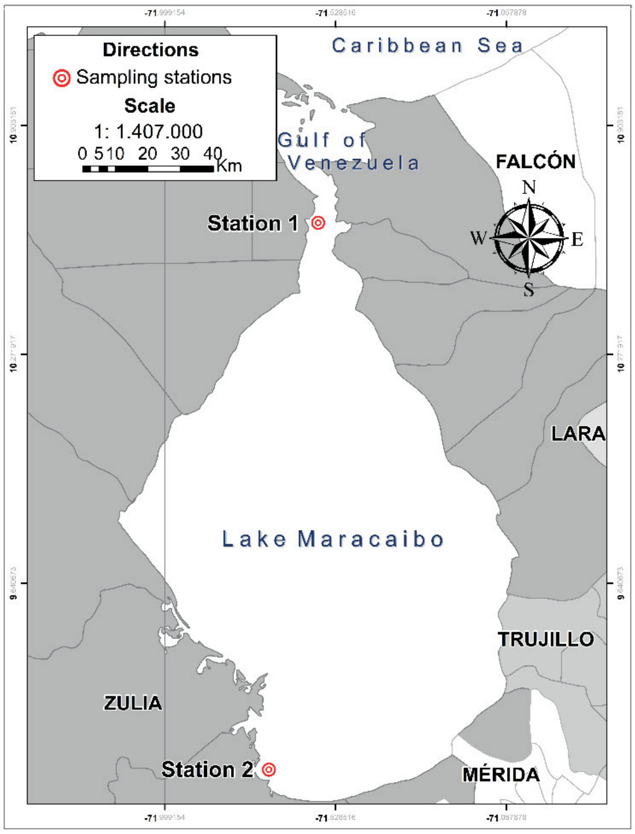

Fig. 1. Location of the sampling stations in the Lake Maracaibo system, Zulia state, Venezuela; source: own elaboration

- **Station 1 (S_1):** Lake sidewalk in the city of Maracaibo, located at 10°39'29.69" N latitude and 71°35'22.02" W longitude. Main sources of contamination with heavy metal in this station originate from indiscriminate discharge of domestic and industrial sewage, as well as oil spills from oil exploitation on the western shore of the lake.
- **Station 2 (S_2):** Puerto Concha, located south of the lake at 9° 5'31.90" N latitude and 71°42'23.81" W longitude, is an agricultural area that constantly receives oil spills derived from oil pipeline sabotage in the Catatumbo River, Colombia.

SAMPLING AND ANALYSIS OF SAMPLES

Water, sediment, and free-living protozoa were collected in four sampling sessions from the two stations, during February 2013, 2014, and April and June 2015. Sampling frequency depended on the growth dynamics of microorganisms in the laboratory and the evolution of toxicity bioassays. Water samples were collected near the shore using a manual method. Samples were stored in polyethylene containers. Some samples of approximately 600 cm^3 each, were used to test for heavy metals, while others were stored in 5 dm^3 containers for future use in ciliated protozoa initial cultures and maintenance. Ciliated protozoa were obtained from surface zooplankton using a 55 μm conical mesh and stored in sterile glass containers. The samples were transferred to the laboratory in a styrofoam cellar with ice. Superficial sediment samples (0–0.6 m) were collected in triplicate using an Ekman dredger (approximately 250 g per sample), placed in black plastic bags with hermetic closure. Water samples used for the determination of metals, were acidified to pH < 2 with concentrated nitric acid (69%) (Merck). In general, 1.5 cm^3 $HNO_3 \cdot dm^{-3}$ was sufficient to reach the desired acidity.

All samples were transported to the Department of Sanitary and Environmental Engineering (Sp. Departamento de Ingeniería Sanitaria y Ambiental – DISA) of the University of Zulia, in plastic coolers with ice. There, samples were tested for physicochemical parameters *ex situ* and protozoan culture assembly, as well as storage of samples at 4°C to undergo the heavy metals analysis. Water samples were used to measure physicochemical parameters *in situ*: pH, redox potential (*ORP*), dissolved oxygen (*DO*), percentage of oxygen saturation (% sat. O_2), temperature, salinity (PSU) and electrical conductivity (*EC*), while other parameters were measured *ex situ*, including total alkalinity, total hardness and heavy metals. Testing was done in triplicate using standardized methods [APHA *et al.* 2012]. We analysed total Cr, Cd, Ni and Pb contents in water and sediments using atomic absorption spectrometry (Perkin–Elmer model 3100 equipment) with graphite furnace (Perkin–Elmer model AS60 equipment) after acid digestion in a Milestone microwave oven Ethos model 1. For water sample digestion, we used 50 cm^3 of water and 5 cm^3 of concentrated HNO_3 (Riedel-de Haën, Germany). We used 5 g of lyophilized sample (Labconco Freezone 6 freeze dryer) and 5 cm^3 of HCl-HNO_3 mixture (4:1) for the digestion of a sediment sample. We validated our method for heavy metals analysis using a recovery study on the following certified reference standard materials: NIST (National Institute of Standards and Technology, Gaithersburg, MD, USA) 1646a sediment and trace elements in natural water 1640a from the NIST. The recovery percentages were within the accepted range (100 ±5%) which indicated accuracy of the method. Precision was expressed as a relative standard deviation below 5%, which showed an acceptable degree of variability in the replicas [RUBINSON, RUBINSON 2000].

CULTURE, ISOLATION AND IDENTIFICATION OF FREE-LIVING CILIATED PROTOZOA

Protozoa were isolated from zooplankton present in fresh water samples from Lake Maracaibo. Samples of 230 cm^3 were placed in 250-cm^3 glass bottles. Air pumps were used (Power Life brand – P-500) to aerate bottles and bacterial growth was enhanced by adding crushed oatmeal flakes as mentioned in FRIED *et al.* [2002] protocol. Weekly renewal of 50% of the media culture was provided using filtered and autoclaved lake water (0.2 μm pore size Whatman membrane filters) with subsequent additions of oat flakes to maintain the "stock" media standard recipe.

Serial dilutions with Pasteur pipettes and standard culture recipe ("stock") were used to isolate protozoa, which grew and better adapted to lab conditions [RAVVA *et al.* 2010]. Each protozoa specie was massified by culturing in filtered water from Lake Maracaibo using the same standard recipe and conditions indicated for the initial cultures. Species were identified by comparing microscope observations with illustrated taxonomic verification keys [JAHN *et al.* 1980; LYNN 2008; PATTERSON 1996]. Motion was controlled with 0.5 mM ethylenediaminetetracetic acid (EDTA) [LINDHOLM 1982] and silver staining with protargol (0.3% w/v) [SKIBBE 1994] revealed cytological details necessary for the final genus level identification [APHA *et al.* 2012].

ECOTOXICOLOGICAL TESTS WITH PROTOZOA

Acute toxicity studies were performed with heavy metal solutions (Cr^{3+}, Cr^{6+}, Pb^{2+}, Ni^{2+} and Cd^{2+}) prepared from salts for the analysis, such as $CrCl_3 \cdot 6H_2O$ (Riedel-de Haën, Germany), $K_2Cr_2O_7$ (Merck, Germany), $Pb(NO_3)_2$ (Merck, Germany), Ni_2SO_4 (Merck, Germany) and $CdCl_2$ (Merck, Germany). Two pure genera of protozoa per station were exposed to heavy metal solutions to determine LC_{50}. Aliquots of 100 μdm^3 of culture containing an average population of 46 ±12 protozoa using 900 μdm^3 of water from Lake Maracaibo were previously filtered and sterilized.

For the ecotoxicological bioassays, two series were established: a control group without the addition of metals, but containing water from Lake Maracaibo; and a test group to study the effect of metals on the protozoa. Absence of ciliary movement was considered a positive test as defined by [MEINELT *et al.* 2009]. Each ion was tested using triplicates of five concentrations dissolved in Lake Maracaibo water and subjected to experimental conditions described in Table 1. Concentrations of heavy metals were adjusted according to the tolerance level displayed by each genus studied. Differences in tolerance levels were possibly due to the diverse nature of activities carried out on the stations. Control and test bioassays were performed in separate Sedgewick–Rafter chambers.

The effect of metals on protozoa was monitored with a binocular light microscope (Óptima brand, model XSZ-207) at

Table 1. Conditions used for acute toxicity tests with ciliated protozoa isolated from surface waters of Lake Maracaibo

Type of test	Static, without renewal of the test solution
Duration	1 h (definitive test)
Test containers	Sedgewick–Rafter chamber
Test volume	1 cm^3
Culture age at inoculum	3–5 days
Inocula cell density	$cell \cdot cm^{-3}$, variable
Tested concentrations	5 plus negative control (definitive test)
Replications by concentration	3
Measured effect	absence of motility
Periodicity of observations	every 10 min
Test acceptance criterion	cell density in the control, at the end of the test, must be the same as at the beginning

Source: own elaboration.

100 × magnification, every 10 min for 1 h for each of the metal concentrations [Esteban, Téllez 1990]. Protozoa showed higher mortality rates at longer exposure times.

STATISTICAL DATA ANALYSIS

Descriptive statistics with 95% confidence interval, as well as analysis of variance (ANOVA) were calculated with the statistical package of IBM SPSS Statistics ver. 22 for Windows. Ecotoxicological indices were determined through the PriProbit 1.63 program (Masayuki Sakuma 1996–2000). We performed one-way ANOVA to determine statistically significant differences in the mean concentrations of physicochemical parameters, metals in water and sediments between the stations, as well as between the ecotoxicological indices (LC_{50}) corresponding to the protozoa of the stations under study; the significance level $p < 0.05$. One-way ANOVA with Dunnett's T3 test was used to compare the number of microorganisms from each station based on metals tested and to establish differences in the level of adaptation to each of the metal ions used in the ecotoxicological tests. Prior to ANOVA, we checked the homogeneity of variances and the distribution of residuals.

RESULTS

PHYSICOCHEMICAL CHARACTERIZATION AND CONTENT OF HEAVY METALS IN LAKE MARACAIBO

Table 2 shows arithmetic means for the concentrations of physicochemical parameters.

Figure 2a shows the average concentrations, with confidence levels of 95%, of the metallic content in waters of S_1 and S_2, respectively, while those corresponding to the sediments of the said stations are illustrated in Figure 2b.

ISOLATED FREE-LIVING CILIATED PROTOZOA

Four representatives of four genera were isolated and identified from the group of free-living ciliated protozoa that grew in the Lake's Maracaibo water samples. *Euplotes* sp. and *Oxytricha* sp. were selected as test species for S_1, while *Coleps* sp. and *Chilodonella* sp. for S_2 (Fig. 3).

ECOTOXICOLOGICAL EFFECT OF HEAVY METALS ON FREE-LIVING CILIATED PROTOZOA

Below are detailed mortality percentages (%) and the LC_{50} identified per concentrations of each metal ion used in the ecotoxicological tests at different exposure times.

Chromium (VI). Results of the ecotoxicological tests carried out with the ciliated protozoa exposed to Cr^{6+}, expressed as percentage of mortality, are illustrated in Figure 4, while the LC_{50} are presented in Table 3.

Chromium (III). Mortality presented by the ciliated protozoa during different times of exposure to Cr^{3+} are shown in Figure 5 and the LC_{50} in Table 4.

Cadmium (II). Exposure of the protozoa to different concentrations of the Cd^{2+} ion generated the % mortality shown in Figure 6 and the different LC_{50} (Tab. 5).

Table 2. Descriptive statistics for the physicochemical parameters measured in situ in surface waters of Lake Maracaibo

Parameter	Station	Number of samplings	Arithmetical mean	Standard deviation	Confidence interval for the average at 95%	
					lower	higher
Temperature (°C)	S_1	4	29.68	1.17	28.93	30.42
	S_2	4	30.77	0.75	30.29	31.24
pH	S_1	4	8.08	0.33	7.87	8.28
	S_2	4	8.38	0.17	8.28	8.49
Oxidation-reduction potential (mV)	S_1	4	–61.74	14.01	–70.64	–52.84
	S_2	4	–80.22	3.49	–82.43	–78.00
Dissolved oxygen (mg·dm^{-3})	S_1	4	3.82	0.48	3.52	4.12
	S_2	4	4.01	0.49	3.70	4.32
Oxygen saturation (%)	S_1	4	50.29	6.31	46.28	54.30
	S_2	4	54.15	7.90	49.13	59.17
Electrical conductivity (mS·cm^{-1})	S_1	4	7.64	0.07	7.60	7.69
	S_2	4	6.03	0.19	5.91	6.16
Salinity (PSU)	S_1	4	4.23	0.42	3.96	4.50
	S_2	4	3.31	0.14	3.22	3.40
Total dissolved solids (mg·dm^{-3})	S_1	4	3 745	35	3 723	3 767
	S_2	4	2 901	50	2 869	2 933
Total alkalinity (mg $CaCO_3$·dm^{-3})	S_1	4	48	13	39	56
	S_2	4	42	6	38	45
Total hardness (mg $CaCO_3$·dm^{-3})	S_1	4	786	105	720	853
	S_2	4	546	41	520	573

Source: own study.

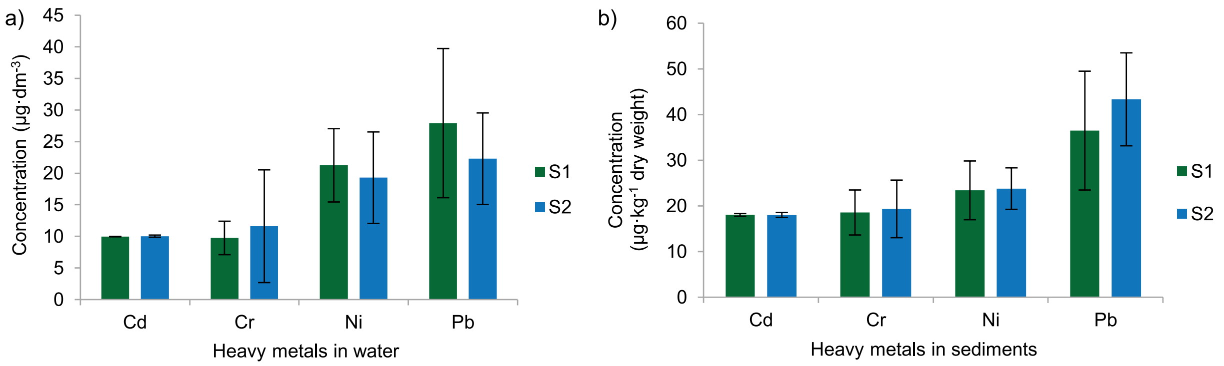

Fig. 2. Average concentration of heavy metals and standard deviation in the two stations on Lake Maracaibo during sampling periods: a) in water,
b) in superficial sediments; vertical bars indicate the arithmetic mean ±standard deviation for n = 12; source: own study

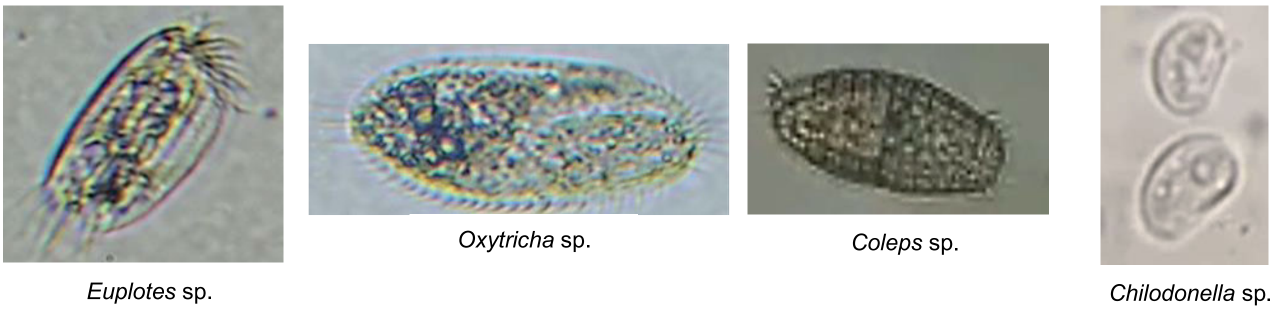

Fig. 3. Free-living ciliated protozoa used for ecotoxicological tests; source: own study

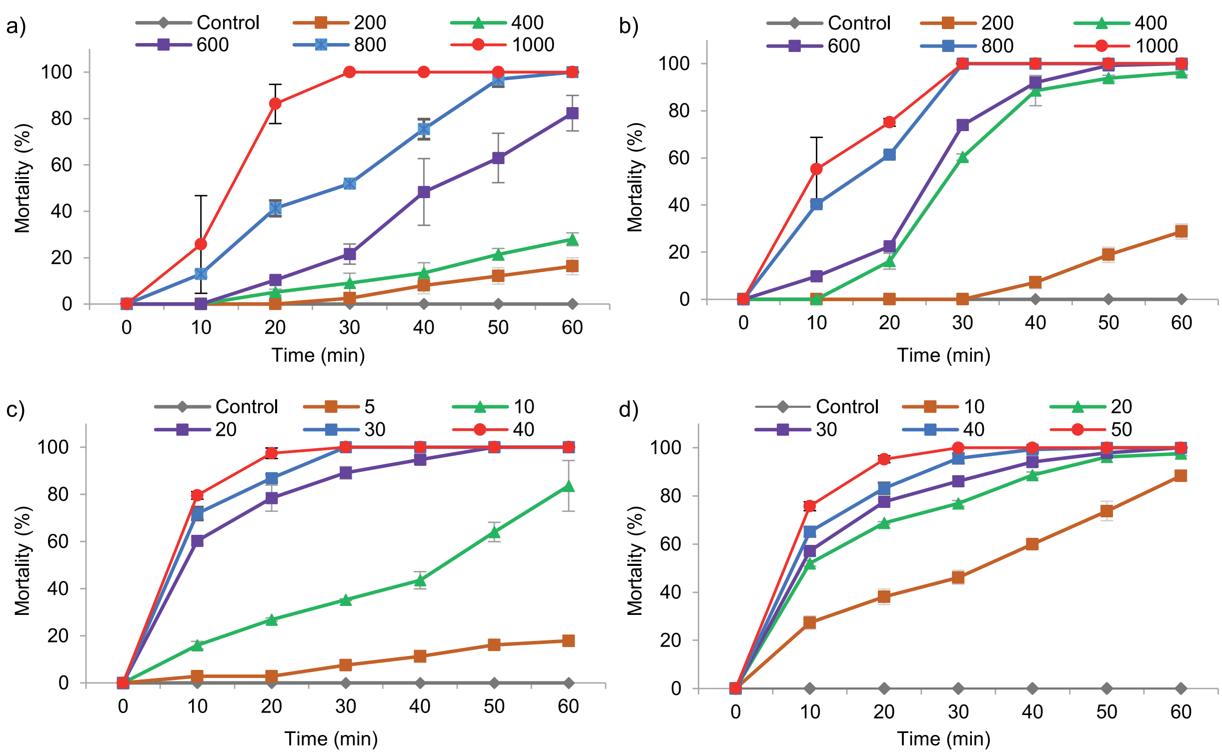

Fig. 4. Percentages of mortality observed for ciliated protozoa from Lake Maracaibo affected by different concentrations of Cr^{6+}; a) *Euplotes* sp., b) *Oxytricha* sp., c) *Chilodonella* sp., d) *Coleps* sp.; source: own study

Nickel(II). The percentage of mortality presented by the ciliated protozoa during different times of exposure to Ni^{2+} and those of the control, are shown in Figure 7 and the LC_{50} in Table 6.

Lead(II). The arithmetic means of the mortality recorded for the four ciliated protozoa of Lake Maracaibo, which were exposed to different concentrations of Pb^{2+}, are illustrated in Figure 8, while the LC_{50} are presented in Table 7.

Table 3. Lethal concentrations for 50% of the ciliated protozoan population from Lake Maracaibo that are affected by Cr^{6+}

Station	Ciliated protozoa	Concentration range ($mg \cdot dm^{-3}$)	Exposure time (min)	LC_{50} ($mg \cdot dm^{-3}$)	Limits ($mg \cdot dm^{-3}$)		NOLC ($mg \cdot dm^{-3}$)
					LL	HL	
S_1	*Euplotes* sp.	200–1000	10	1 164.2	1 058.9	1 408.3	800
			20	805.6	748.4	884.5	–
			30	712.2	603.0	916.7	–
			40	571.5	497.7	659.6	–
			50	482.7	420.9	546.3	–
			60	417.1	360.9	470.9	–
	Oxytricha sp.	200–1000	10	915.6	881.5	957.6	600
			20	738.2	706.9	773.0	–
			30	411.4	372.4	446.7	–
			40	310.5	281.2	338.8	–
			50	259.7	247.8	271.8	–
			60	236.0	225.3	247.1	–
S_2	*Chilodonella* sp.	5–40	10	19.3	10.3	13.2	5
			20	13.9	12.8	15.2	–
			30	11.1	10.3	12.1	–
			40	9.9	9.1	10.7	–
			50	8.1	7.5	8.7	–
			60	7.0	6.5	7.5	–
	Coleps sp.	10–50	10	21.4	17.9	24.8	<10
			20	13.3	10.9	15.4	–
			30	11.2	9.3	12.8	–
			40	8.4	6.6	10.0	–
			50	6.5	4.6	8.1	–
			60	4.5	1.9	6.5	–

Explanations: LC_{50} = lethal concentration for 50%, LL = lower limit, HL = higher limit, NOLC = no observed lethal concentration.
Source: own study.

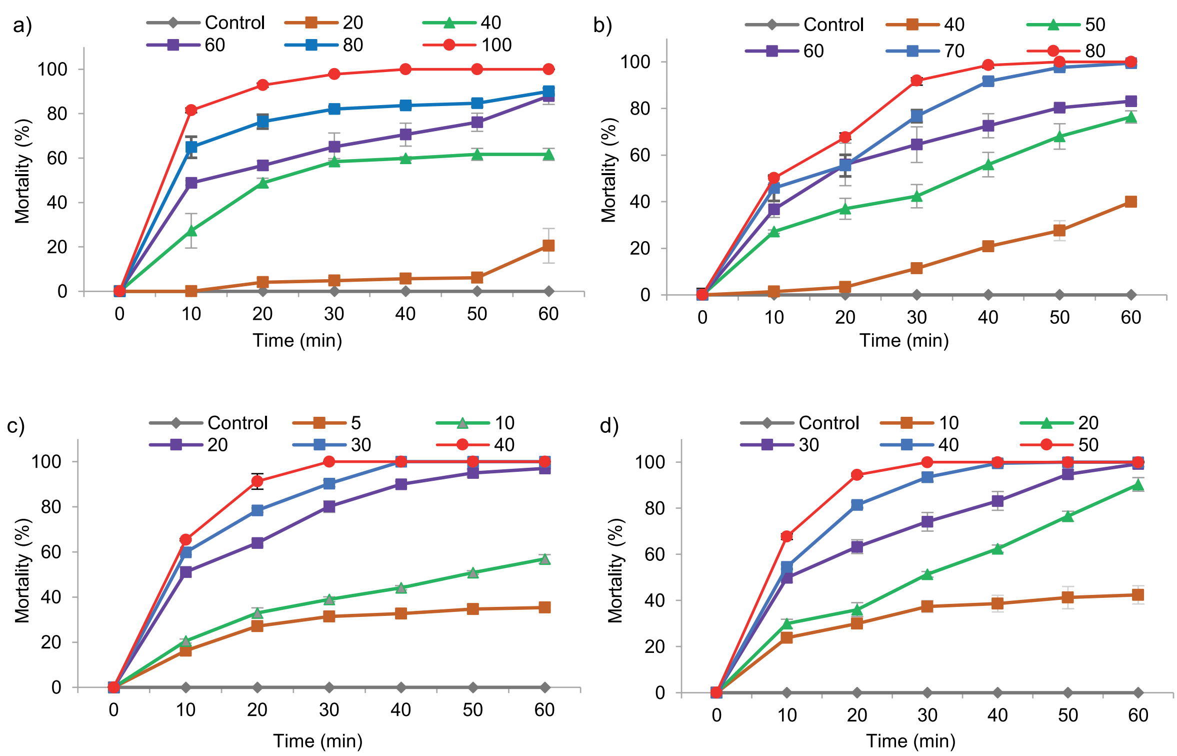

Fig. 5. Observed mortality percentages for ciliated protozoans from Lake Maracaibo affected by different concentrations of Cr^{3+}: a) *Euplotes* sp., b) *Oxytricha* sp., c) *Chilodonella* sp., d) *Coleps* sp.; source: own study

Table 4. Lethal concentrations for 50% of the ciliated protozoan population of Lake Maracaibo that are affected by Cr^{3+}

Station	Ciliated protozoa	Concentration range ($mg \cdot dm^{-3}$)	Exposure time (min)	LC_{50} ($mg \cdot dm^{-3}$)	Limits ($mg \cdot dm^{-3}$)		NOLC ($mg \cdot dm^{-3}$)
					LL	HL	
S_1	*Euplotes* sp.	20–100	10	61.4	58.3	64.7	20
			20	48.1	45.5	50.8	–
			30	42.6	38.8	46.3	–
			40	40.8	37.3	44.3	–
			50	39.4	36.1	42.7	–
			60	32.7	30.7	34.8	–
	Oxytricha sp.	40–80	10	73.5	70.2	78.0	40
			20	63.2	59.5	67.5	–
			30	54.6	53.0	56.2	–
			40	49.1	47.6	50.6	–
			50	45.9	44.4	47.2	–
			60	9.2	0.2	20.4	–
S_2	*Chilodonella* sp.	5–40	10	22.9	19.9	26.9	<5
			20	12.7	11.3	14.2	–
			30	9.9	2.6	3.9	–
			40	8.7	7.5	9.9	–
			50	7.9	6.9	8.9	–
			60	7.5	6.8	8.1	–
	Coleps sp.	10–50	10	31.5	27.3	37.3	<10
			20	19.3	16.6	22.0	–
			30	14.9	12.6	17.1	–
			40	13.3	12.0	14.6	–
			50	11.8	10.7	12.8	–
			60	10.9	10.1	11.7	–

Explanations: as in Tab. 3.
Source: own study.

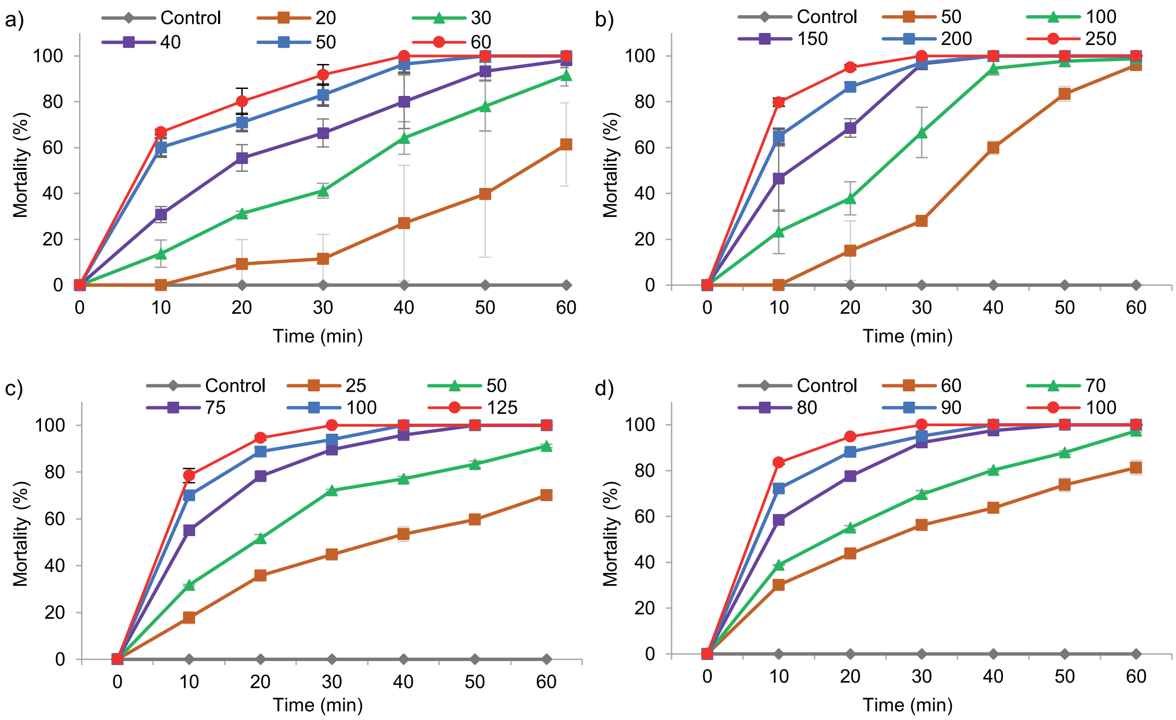

Fig. 6. Percentages of mortality observed for ciliated protozoans from Lake Maracaibo affected by different concentrations of Cd^{2+}: a) *Euplotes* sp., b) *Oxytricha* sp., c) *Chilodonella* sp., d) *Coleps* sp.; source: own study

Table 5. Lethal concentrations for 50% of the ciliated protozoan population of Lake Maracaibo that are affected by Cd^{2+}

Station	Ciliated protozoa	Concentration range (mg·dm^{-3})	Exposure time (min)	LC_{50} (mg·dm^{-3})	Limits (mg·dm^{-3})		NOLC (mg·dm^{-3})
					LL	HL	
S_1	*Euplotes* sp.	20–60	10	47.8	45.2	51.1	20
			20	38.3	35.8	40.9	–
			30	33.3	31.2	35.3	–
			40	26.3	23.5	28.8	–
			50	22.7	20.2	24.7	–
			60	18.5	16.2	20.1	–
	Oxytricha sp.	50–250	10	160.4	150.8	171.4	50
			20	107.6	95.8	120.7	–
			30	70.8	66.0	75.6	–
			40	44.6	39.7	48.6	–
			50	27.6	18.0	34.3	–
			60	9.2	15.8	20.4	–
S_2	*Chilodonella* sp.	25–125	10	64.9	57.7	71.8	<25
			20	39.1	33.0	44.4	–
			30	29.7	24.4	34.3	–
			40	26.0	21.4	30.0	–
			50	23.4	18.9	27.1	–
			60	19.2	14.0	23.2	–
	Coleps sp.	60–100	10	74.2	71.1	77.0	<60
			20	64.9	61.4	67.7	–
			30	59.7	55.8	62.5	–
			40	57.7	54.0	60.3	–
			50	54.9	49.9	57.9	–
			60	53.3	46.3	56.6	–

Explanations: as in Tab. 3.
Source: own study.

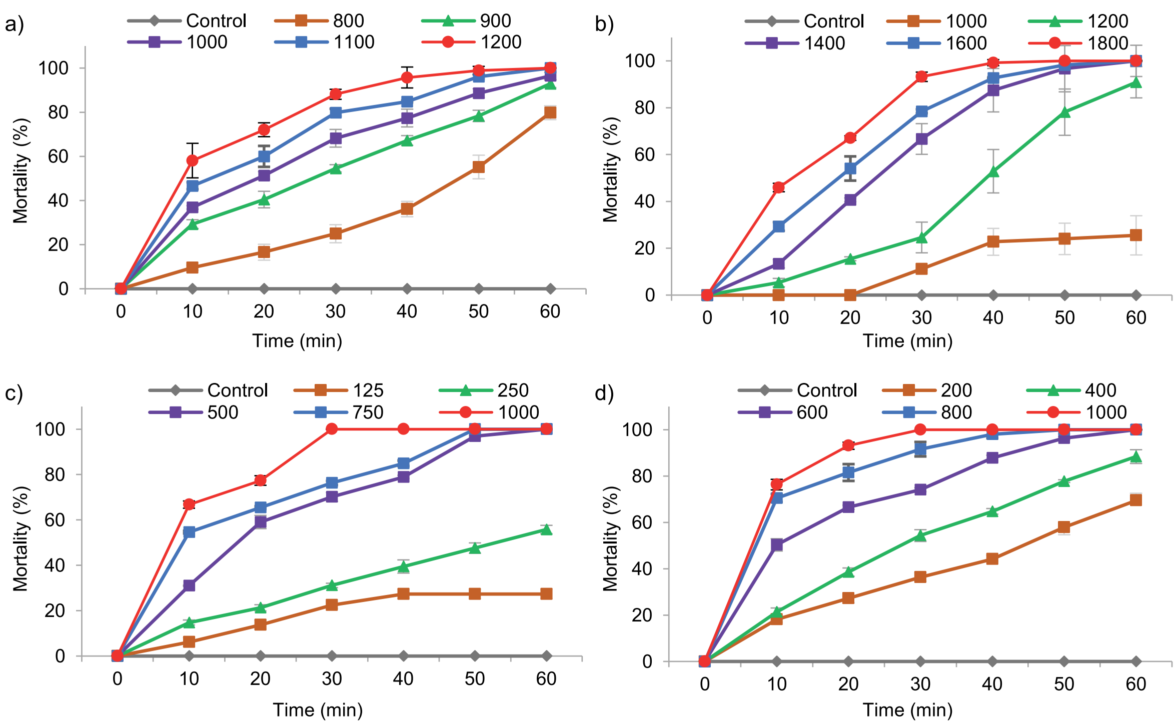

Fig. 7. Percentage of mortality observed for ciliated protozoa from Lake Maracaibo affected by different concentrations of Ni^{2+}: a) *Euplotes* sp., b) *Oxytricha* sp., c) *Chilodonella* sp., d) *Coleps* sp.; source: own study

Table 6. Lethal concentrations for 50% of the ciliated protozoan population from Lake Maracaibo that are affected by Ni^{2+}

Station	Ciliated protozoa	Concentration range ($mg \cdot dm^{-3}$)	Exposure time (min)	LC_{50} ($mg \cdot dm^{-3}$)	Limits ($mg \cdot dm^{-3}$)		NOLC ($mg \cdot dm^{-3}$)
					LL	HL	
S_1	*Euplotes* sp.	800–1200	10	1 116.9	1 073.8	1 180.1	<800
			20	1 008.0	972.8	1 044.7	–
			30	905.2	870.8	933.6	–
			40	849.7	810.0	880.1	–
			50	779.1	729.1	814.2	–
			60	689.2	592.5	742.3	–
	Oxytricha sp.	1000–1800	10	1 825.0	1 759.5	1 915.4	1000
			20	1 543.5	1 511.4	1 580.1	–
			30	1 320.5	1 296.3	1 345.0	–
			40	1 156.9	1 134.4	1 178.4	–
			50	1 094.1	1 076.1	1 111.5	–
			60	1 059.7	1 047.1	1 072.6	–
S_2	*Chilodonella* sp.	125–1000	10	702.3	612.0	829.7	<125
			20	453.7	392.5	522.5	–
			30	309.2	265.9	352.4	–
			40	250.5	212.7	287.3	–
			50	199.4	177.3	221.7	–
			60	187.1	167.4	207.2	–
	Coleps sp.	200–1000	10	571.9	513.7	641.2	<200
			20	398.4	253.6	442.2	–
			30	306.2	265.9	342.9	–
			40	248.6	213.5	279.9	–
			50	189.2	154.2	218.9	–
			60	153.1	116.5	182.4	–

Explanations: as in Tab. 3.
Source: own study.

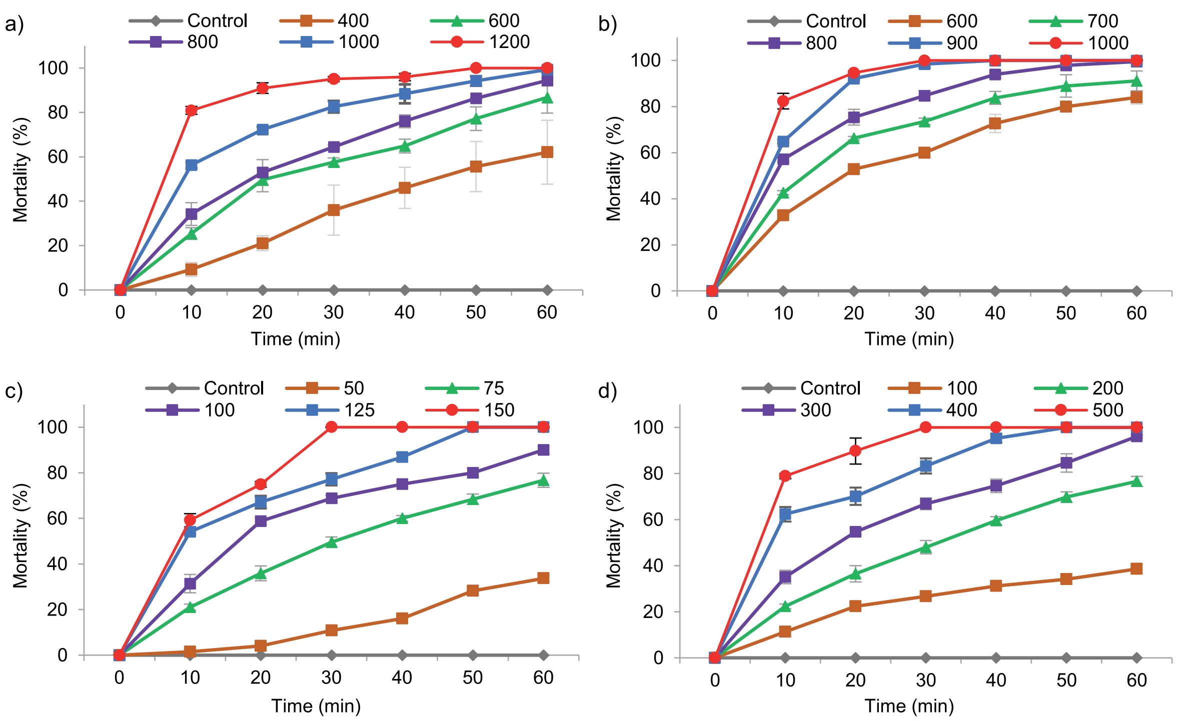

Fig. 8. Percentage of mortality observed for ciliated protozoa from Lake Maracaibo affected by different concentrations of Pb^{2+}: a) *Euplotes* sp.; b) *Oxytricha* sp., c) *Chilodonella* sp., d) *Coleps* sp.; source: own study

Table 7. Lethal concentrations for 50% of the ciliated protozoan population from Lake Maracaibo exposed to Pb^{2+}

Station	Ciliated protozoa	Concentration range ($mg \cdot dm^{-3}$)	Exposure time (min)	LC_{50} ($mg \cdot dm^{-3}$)	Limits ($mg \cdot dm^{-3}$)		NOLC ($mg \cdot dm^{-3}$)
					LL	HL	
S_1	*Euplotes* sp.	400–1200	10	887.0	828.4	958.3	<400
			20	663.4	611.7	714.3	–
			30	534.1	475.7	584.8	–
			40	446.4	381.8	498.8	–
			50	378.5	316.8	427.5	–
			60	349.1	294.8	391.4	–
	Oxytricha sp.	600–1000	10	745.9	706.6	778.4	<600
			20	612.6	558.4	649.0	–
			30	593.2	546.2	625.6	–
			40	543.5	480.7	583.7	–
			50	516.1	436.2	562.7	–
			60	511.3	424.3	558.8	–
S_2	*Chilodonella* sp.	50–150	10	124.3	114.9	137.4	50
			20	97.7	91.8	104.7	–
			30	80.3	75.9	84.9	–
			40	72.6	68.5	76.7	–
			50	63.2	59.5	66.9	–
			60	58.3	54.8	61.6	–
	Coleps sp.	100–500	10	328.3	299.1	364.1	<100
			20	239.1	213.9	265.1	–
			30	186.0	165.8	205.1	–
			40	156.2	138.6	172.4	–
			50	136.7	121.3	150.7	–
			60	122.6	108.8	135.0	–

Explanations as in Tab. 3.
Source: own study.

MULTIPLE COMPARISONS OF MEAN LC_{50} VALUES AND ESTABLISHMENT OF GENERAL TOXICITY PATTERNS

Multiple comparisons of mean LC_{50} values were made using Dunnett's T3 test. Their results are shown in Table 8, while overall LC_{50-1h} values are illustrated in Figure 9.

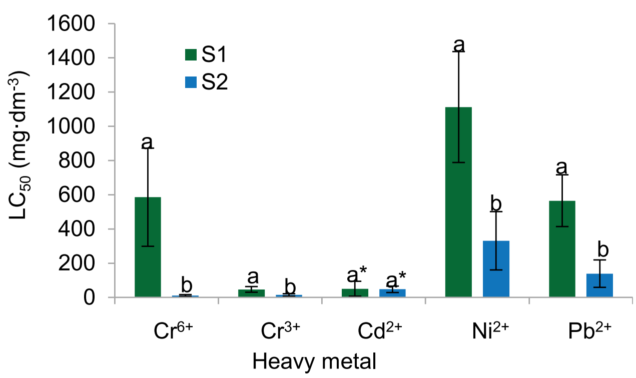

Fig. 9. General values of lethal concentration 50% (LC_{50-1h}) of heavy metals on protozoa from Lake Maracaibo; vertical bars indicate the arithmetic mean ±standard deviation for $n = 12$; equal letters with an asterisk for the same metal indicate that there are no significant differences between the values ($p > 0.05$): source: own study

Table 8. Multiple comparisons of mean LC_{50} values for all exposure times with heavy metals by Dunnett's T3 test in ciliated protozoa from Lake Maracaibo

Metal	Station	Protozoa		Significance level
Cd^{2+}	S_1	*Euplotes* sp.	*Oxytricha* sp.	0.6119
	S_2	*Chilodonella* sp.	*Coleps* sp.	0.0468*
Cr^{3+}	S_1	*Euplotes* sp.	*Oxytricha* sp.	1.0000
	S_2	*Chilodonella* sp.	*Coleps* sp.	0.5950
Cr^{6+}	S_1	*Euplotes* sp.	*Oxytricha* sp.	0.7035
	S_2	*Chilodonella* sp.	*Coleps* sp.	1.0000
Ni^{2+}	S_1	*Euplotes* sp.	*Oxytricha* sp.	0.0671
	S_2	*Chilodonella* sp.	*Coleps* sp.	0.9989
Pb^{2+}	S_1	*Euplotes* sp.	*Oxytricha* sp.	0.9954
	S_2	*Chilodonella* sp.	*Coleps* sp.	0.0694

Explanations: * = significant difference at the 0.05 level.
Source: own study.

DISCUSSION

PHYSICOCHEMICAL CHARACTERISTICS OF SURFACE WATER

Significant differences ($p > 0.05$) in the *DO*, % sat. O_2 and total alkalinity obtained in the sampling stations are not observed using one-way ANOVA. Significant differences ($p < 0.05$) were obtained for temperature, pH, redox potential (*ORP*), electrical conductivity (*EC*), salinity, total dissolved solids and total hardness. Results in Table 3 shows these values were comparable to previously reported data from Lake Maracaibo [Bracho *et al.* 2016; Marín *et al.* 2017; Marín-Leal *et al.* 2014; Polo 2012; Rojas 2012]. We also found average temperatures oscillating from 31.40 and 31.55°C, pH close to 8.0, *ORP* close to 80.0 mV and dissolved oxygen concentrations close to 4.0 mg·dm^{-3}, among other determined parameters.

METAL CONTENT IN WATER AND SEDIMENTS

ANOVA results showed no significate differences ($p > 0.05$) in the concentration for each of heavy metal (Cr, Ni, Pb and Cd) in water and sediments per sampling station. Heavy metal presence is potentially associated with industrial discharges, accidental oil spills and agricultural activities, domestic waste and the burning of fossil fuels [Ogoyi *et al.* 2011; Rodríguez (ed.) 2000], as well as the uncontrolled use of agrochemicals and the discharge of wastewater sludge [Kapahi, Sachdeva 2019], which contributes to aquatic ecosystems contamination. In water, the order of magnitude was Pb > Ni > Cd > Cr for the average metal content in S_1, while for S_2 this order was Pb > Ni > Cr > Cd (Fig. 2a). Meanwhile, the sediments corresponding to the stations under study showed little variability for the average metal content; with order of magnitude Pb > Ni > Cr > Cd in both stations (Fig. 2b).

Heavy metal concentrations detected in water from S_1 were as follows Pb: 27.93 ±11.81; Ni: 21.24 ±5.80; Cd: 97 ±0.03 and Cr: 9.75 ±2.65 μg·dm^{-3}, while for S_2 were: Pb: 22.29 ±7.24; Ni: 19.28 ±7.25; Cr: 11.61 ±8.92 and Cd: 9.97 ±0.03 μg·dm^{-3}. Cr, Pb and Cd levels were higher than those reported for lake Maracaibo by other researchers. Rojas [2012] and Bracho *et al.* [2016] found concentrations lower than 10 μg Cr·dm^{-3} [Bracho *et al.* 2016; Rojas 2012], 7.0 μg Pb·dm^{-3} [Bracho *et al.* 2016] and 0.054 ±0.109 μg Cd·dm^{-3}. The Ministry of Environment and Natural Resources (Decree N° 883) established levels of metals and other toxic substances should not be detectable in marine waters or coastal environments intended for the breeding and exploitation of molluscs consumed raw (type 3) and for waters destined for spas, aquatic sports, sport, commercial and subsistence fishing (type 4) [Ministerio … 1995]. Our results showed evidence of non-compliance with Venezuelan standards. However, both stations met water quality criteria established by USEPA [2016] for the content of metals in water that allows to protect aquatic life in terms of the maximum concentration criterion, except for Cd, as well as for the continuous concentration criterion for Cr and Ni.

The heavy metal content in S_1 sediments included: Pb: 35.65 ±11.14; Ni: 23.40 ±6.43; Cr: 18.56 ±4.92 and Cd: 18.03 ±0.29 mg·kg^{-1} for dry. S_2 levels were as follows: Pb: 43.33 ±10.18; Ni: 23.79 ±4.54; Cr: 19.34 ±6.30; Cd: 18.02 ±0.53 mg·kg^{-1} for dry weight. Cr and Pb concentrations determined by our study in sediments from Lake Maracaibo were comparable with those obtained by Ávila *et al.* [2014]. They reported 14.53 mg Cr·kg^{-1} and 34.57 mg Pb·kg^{-1}. However, Ni concentrations were lower than those reported by Ávila *et al.* [2014], which were 53.06 mg Ni·kg^{-1}. Bracho *et al.* [2016] reported higher levels of Cd (10.57 ±6,548 mg Cd·kg^{-1}) compared to our data. Both Cr and Ni levels in sediments complied with current regulations established by the Canadian Council of Ministers of the Environment [CCME 2001] for the protection of aquatic life as for level of a probable effect. Lead and cadmium results did not comply with the regulations.

RESISTANCE OF CILIATED PROTOZOA TO HEAVY METALS

Our data demonstrated that at longer exposure to Cr^{3+}, Cr^{6+}, Cd^{2+}, Ni^{2+} and Pb^{2+} ions, lower concentrations were required to reach LC_{50} compared to shorter exposure times. These results showed that S_1 protozoa were more tolerant to Cr^{6+} than those corresponding to S_2, which can be attributed to biological differences between species, as they are ciliates with contrasting morphologies, motility and feeding strategies. Madoni [2000] indicated that this behaviour has been also observed for other ciliate species. When comparing the LC_{50} obtained for the protozoa exposed to Cr^{6+} (Fig. 4, Tab. 3) with those obtained by the exposure to Cr^{3+} (Fig. 5 and Tab. 4), it was shown that *Euplotes* sp. and *Oxytricha* sp. from S_1 were more tolerant to Cr^{6+} and more susceptible to Cr^{3+}, whereas for S_2, the organisms of *Chilodonella* sp. presented practically the same tolerance to both chromium ions, with *Coleps* sp. the most tolerant to Cr^{3+} and the most susceptible to Cr^{6+}.

Findings of Cr^{3+} being more toxic than Cr^{6+} were not well documented in the scientific literature. However, in studies involving microalgae in modified ISO medium,Vignati *et al.* [2010] also observed Cr^{3+} being more toxic than Cr^{6+}.

Susceptibility to Cr^{3+} and tolerance to Cr^{6+} displayed by ciliates of S_1 (*Euplotes* sp. and *Oxytricha* sp.) compared with susceptibility to Cr^{3+} and tolerance to Cr^{6+} of S_2 *Coleps* sp. could be explained by these protozoa being crawler ciliates. Crawler ciliated feed on particles or bacteria found in substrates, while *Coleps* sp. is a free swimmer that feeds on bacteria present in the water column.

Recent studies regarding the effects of Cd^{2+} on ciliated protozoa [Mera *et al.* 2016] describe it as the culprit for disturbances that cause cell death when protozoa reach their threshold of tolerance. Cell death could be the result of respiratory inhibition after decoupling of oxidative phosphorylation in the mitochondrial respiratory chain [Benlaifa *et al.* 2016]. Mitochondrial degeneration and/or disintegration of cristae are the most important modifications in ciliates, especially those treated with higher concentration of non-essential heavy metals, such as cadmium [Iftode *et al.* 1985]. Protozoa are competent producers of reactive oxygen species (ROS) [Pinot *et al.* 2000], such as superoxide radicals, hydrogen peroxides, and hydroxyl ions, which are involved in several abnormal processes, including lipid peroxidation, protein oxidation, and nucleic acid damage and finally inducing cell death by apoptosis [Pulido, Parrish 2003]. Mortality rates from the exposure of protozoa to Cd^{2+} obtained in the present study can be explained based on these arguments. They showed a certain degree of tolerance, which according to Benlaifa *et al.* [2016] was due to their high metabolic rate, small cell volume, and a relatively high surface contact with their environment. Ciliates can respond very quickly to chemical stress. Furthermore, Liao *et al.* [2002] indicated that, Cd is normally chelated by cytoplasmic proteins and transported to lysosomes at the cellular level where it is stored and finally expelled from the cell.

The LC_{50-1h} results obtained for protozoa exposed to Cd^{2+} in this study (Fig. 6, Tab. 5), were higher than those reported by AL-RASHEID and SLEIGH [1994] for *Euplotes mutabilis* in Lepe (Spain), where it was 0.48 mg·dm^{-3}. The differences are possibly due to the fact that the protozoan were collected from a beach area where sediments contained lower amounts of heavy metals, including Cd, compared to our results in Lake Maracaibo. Thus, Lepe´s study protozoa were found less resistant to Cd^{2+} compared to the protozoa in our research, which lived in Cd^{2+} levels that for some of them were more decisive in generating tolerance mechanisms against the metal.

Different LC_{50-1h} obtained show that ciliated protozoa from S_1 were more tolerant to Ni^{2+} than those of S_2 (Fig. 7, Tab. 6). The cytotoxicity observed is related to the gradual inhibition of ciliary movement in protozoa exposed to Ni^{2+}, which prevents it from developing its vital functions [BENEDETTI *et al.* 2011]. In this regard, LARSEN and NILSSON [1983] demonstrated that after one hour exposure of the genus *Tetrahymena* to various concentrations of Ni^{2+} in protease peptone medium, the endocytosis rate is completely suppressed, and no food vacuoles are formed. Furthermore, the impact of Ni^{2+} on general metabolism, reflected in ATP production, has been demonstrated [LIBRI 2010].

The results of LC_{50-1h} derived from Ni^{2+} bioassays for the protozoa in the present study were superior to the LC_{50-1h} obtained for *E. mutabilis* by AL-RASHEID and SLEIGH [1994] in Lepe (Spain), where they were reported to be 3.90 mg·dm^{-3}.

Some factors that can explain the intrinsic variability of their chemical susceptibility among the species include ecological origin, morphology, behaviour, and ecological niche of the diverse ciliates [VILAS-BOAS *et al.* 2020a]. Heavy metal resistance mechanisms include detoxification, i.e. active export, which is based on the existence of ATP-dependent membrane efflux pumps that export the metal from the inside to the outside of the cell [GUTIÉRREZ *et al.* 2008]. Numerous membrane transporters of inorganic cations have been detected in the genomes of two model ciliates, *Tetrahymena thermophila* and *Paramecium tetraurelia* [EISEN *et al.* 2006].

In the case of Pb^{2+}, the LC_{50-1h} results obtained for the protozoa of the present investigation (Fig. 8, Tab. 7) were superior to the LC_{50-1h} obtained by AL-RASHEID and SLEIGH [1994] for *E. mutabilis* in another study, where 0.37 mg·dm^{-3} was reported. Tolerance levels to Pb^{2+} may be related to epigenetic mechanisms that ciliated protozoa have developed due to the contamination of Lake Maracaibo, as SOMASUNDARAM *et al.* [2019] indicated the mechanisms were to combat heavy metal toxicity. Ciliates have developed many defence mechanisms, e.g. increased production of several antioxidant enzymes and stress-induced genes, namely metallothionein (MT) and heat shock proteins (HSPs) for their survival.

No reports were found in the literature of LC_{50-1h} being determined for ciliated protozoa exposed to Cr^{6+} and Cr^{3+}, which allowed for the comparison with the present study. However, short test duration periods, such as the one used (1 h), are appropriate for microorganisms that have difficulty adapting to laboratory conditions. Furthermore, they allow us to infer that our values of LC_{50} are due to the action of the metal on the protozoa and not the changes in physicochemical conditions of the culture medium.

Both abiotic and biotic environmental stressors can modify gene activities via epigenetic mechanisms, representing a connection between environmental change and genome response. Several epigenetic control events (opening or closing gene expression) have been reported in organisms undergoing environmental stress [MEYER 2015]. The continuous or regular exposure to a specific stressor involves a cell acclimatization to an environmental stressor. This adaptive change can reverse to the non-acclimatized cellular stage after the stressor is removed or disappears from the environment. When the stressor agent appears in the environment, a cell recognition mechanism carries out a chemical transduction by specific or unspecific receptors, indicating presence of that stressor in the cell. From this point, a complex signalling network connects the initial receptor with the molecular mechanism involved in the cell response against that specific stressor [SLAVEYKOVA *et al.* 2016].

COMPARISON OF GENERAL ECOTOXICOLOGICAL INDICES

Dunnett's T3 test results indicated there were only statistically significant differences ($p < 0.05$) between *Chilodonella* sp. and *Coleps* sp. of S_2 in their level of response to Cd^{2+} ion. This shows that one of the two genera managed to adapt and respond better to the different concentrations of Cd^{2+} used in ecotoxicological tests (Tab. 8), being *Coleps* sp. the best adapted because it showed the highest LC_{50} for the different exposure times. According to MARTINS *et al.* [2008], in contaminated environments, the response of microbial communities to heavy metals depends on the concentration, availability, and actions of complex processes controlled by factors such as: type of metal, nature of the medium, and microbial species.

Table 8 shows that S_1 protozoa display no statistically significant differences in their response to the corresponding metal exposure ($p > 0.05$) Cd^{2+}, Cr^{3+}, Cr^{6+}, Ni^{2+} and Pb^{2+}, while S_2 shows only differences ($p < 0.05$) between the LC_{50} derived from exposure to Cd^{2+}, which indicates possibly the same levels of adaptation and response to each metal ion for which they do not experience significant differences. General toxicity patterns obtained were: $Cr^{3+} > Cd^{2+} > Pb^{2+} > Cr^{6+} > Ni^{2+}$ and $Cr^{6+} > Cr^{3+} > Cd^{2+} > Pb^{2+} > Ni^{2+}$, for S_1 and S_2 protozoa, respectively; However, significant differences ($p < 0.05$) between the sites for LC_{50} derived from exposure to Cr^{3+}, Cr^{6+}, Ni^{2+} and Pb^{2+} (Fig. 9) are due to the dissimilarity in the adaptations experienced by the microorganisms as a result of the exposure to various sources of heavy metals and prevailing physicochemical conditions at each site, as well as complex reactions. These reactions included absorption and flocculation that take place in the sediments and that could be impacting the water column [DE BAUTISTA *et al.* 1999].

The protozoa with the best adaptation to contamination show the best response levels, and achieve greater tolerance to metal ions in ecotoxicological tests [MADONI, ROMEO 2006]. Untreated domestic and industrial wastewater discharges that impact on S_1, and the navigation channel maintenance dredging in the lake strait produce dispersion of pollutants from the sediment [DE BAUTISTA *et al.* 1999]. They could explain the greater tolerance shown by S_1 protozoa compared to S_2 (Fig. 9). However, under certain concentrations and/or exposure times, heavy metals can affect their survival in diverse ways, because they can concentrate on cell membranes and destroy their integrity, causing lysis. It is because most metals rapidly affect enzymes and inactivate them by binding to sulfhydryl, amino and imino groups of the enzyme [Albergoni, Piccinni 1983]. Some physiological and ecological processes affected by metals are the reduction of food absorption, growth inhibition, and the reduction of endocytosis, which influence survival [Benedetti *et al.* 2011].

Metal tolerant protozoa have been reported in wastewater and contaminated environments. Survival in media containing relatively high concentrations of metal ions shows that the organisms studies have developed strategies to tolerate or detoxify organic substances and heavy metals [Madoni, Romeo 2006]. Furthermore, the eukaryotic genome of protozoa is similar to metazoan genome. Their reactions to environmental changes can thus be related to higher organisms more convincingly than those of the prokaryotes [Foissner 2004].

CONCLUSIONS

Ecotoxicological indices (LC_{50}) determined for the protozoa studied were high. However, the reported values indicate that they exhibit various levels of tolerance to heavy metal ions they were exposed to in the bioassays. The $LC_{50\text{-}1h}$ obtained for S_1 protozoa were: for *Euplotes* sp. 417.1, 32.7, 18.5, 689.2 and 349.1 mg·dm^{-3} for Cr^{6+}, Cr^{3+}, Pb^{2+}, Ni^{2+} and Cd^{2+} ions, respectively; while for *Oxytricha* sp. the $LC_{50\text{-}1h}$ obtained were 236.0, 9.2, 1059.7 and 511.3 mg·dm^{-3} for the same ions in the order indicated. In the case of S_2 protozoa, the $LC_{50\text{-}1h}$ obtained for *Chilodonella* sp. were 7.0, 7.5, 19.2, 187.1 and 58.3 mg·dm^{-3} for Cr^{6+}, Cr^{3+}, Pb^{2+}, Ni^{2+} and Cd^{2+} ions, respectively; while for *Coleps* sp. the $LC_{50\text{-}1h}$ were 4.5, 10.9, 53.3, 153.1 and 122.6 mg·dm^{-3} for the ions used in the specified order, which shows the existence of tolerance and/or detoxification mechanisms that allow them to maintain homeostasis. Furthermore, only S_2 members (*Coleps* sp. and *Chilodonella* sp.), were the most susceptible and could be used as possible early warning microbiological indicators in studies of contamination by heavy metals in Lake Maracaibo. However, it is necessary to standardise test conditions for these organisms to use them in reference studies.

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