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Research Article

Acute toxicity of lead on the survival of *Macrobiotus hufelandi* (Eutardigrada: Parachela: Macrobiotidae)

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Abstract

Aquatic ecosystems are constantly exposed to heavy metals and various chemicals that cause toxic effects on living organisms with increasing human activity. The tardigrades can survive in extreme conditions, in the present study, their ability to survive in various concentrations of toxic metal (lead) was evaluated. The acute mean lethal toxicity (LC_{50}) indicates toxic effect assessment on organisms after exposure to heavy metals. This study assessed for the first time the LC_{50} values of lead toxicity after 24, 48, 72, and 96 hours of exposure in the tardigrade species *Macrobiotus hufelandi* are presented, based on animal mortality. The tolerance of *Mac. hufelandi* to lead is quite high in the 24 hours (LC_{50}: 94.651 mg/L). The LC_{50} was estimated as 43,540 mg/L after 48 hours of exposure, and the mortality rates increased depending on time and concentration, LC_{50} was estimated as 22,344 mg/L after 72 hours, and calculated as 8,048 mg/L after the 96th hour. In addition, for the differences in the number of tardigrade deaths over time between groups (pairwise) Friedman's test findings were found between 24h-96h, 48h-96h and 24h-72h, respectively. The results demonstrate that tardigrades can be appropriate invertebrate models to provide insights into heavy metal tolerance research.

Keywords: Acute toxicity, Tardigrada, Lead, LC_{50}, *Macrobiotus*

Introduction

The concentration of pollutants in aquatic environments has increased in recent years, leading to a global environmental crisis. These pollutants, such as petroleum products, microorganisms, detergents, pesticides, radioactive particles, and heavy metals, are primarily anthropogenic. Although organisms require some metals even in small amounts, heavy metals, recognized as

toxic pollutants capable of causing considerable damage, refers to metals and metalloids such as chromium, lead, cadmium, arsenic, and zinc that have a specific density of 5 g/cm^3 and can have a toxic effect at low concentrations (Nassouhi et al., 2018; Rajkumar et al., 2020). Some heavy metals, such as Pb (lead), do not have any biological function and are harmful to the environment and organisms, even in trace amounts (Santos et al., 2014). Among these elements, lead, which is found naturally in the earth's crust, is one of the heavy metal pollutants with the highest anthropogenic release due to its use as an additive in building materials, pigments, piping systems, and especially fuels (Rehman et al., 2008). Due to anthropogenic activities, lead is released into the environment, particularly in aquatic ecosystems. Much research has been done to measure lead concentration in sediment and water in different water bodies (Table 1).

Table 1. Detected lead concentrations in freshwaters and marine environments

Regions	Lead concentration	References
Freshwater		
Taihu Lake, China	0.01–10.5 µg/L	(Li et al., 2021)
Korotoa River, Bangladesh	27-35 µg/L	(Islam et al., 2015)
Tundzha River, Bulgaria	126 µg/L	(Valkova et al., 2016)
Yangtze River, China	2.03–15.03 µg/L	(Li et al., 2020)
Acheloos River, Greece	0.1-2.9 µg/L	(Scoullos & Botsou, 2018)
Dalyan River, Turkey	14.14-169.4 µg/L	(Arslan & Avşar, 2020)
Sapanca Lake, Turkey	38.19-0.59 µg/L	(Kaymak et al., 2021)
Lower Pearl River, USA	8.6-47.2 µg/L	(Paul et al., 2021)
Freshwater Sediment		
Nile River, Egypt	14.82–30.00 µg/L	(El-Amier & El-Gawad, 2016)
Acheloos River, Greece	3.7-34.9 mg.kg-^{1}	(Scoullos & Botsou, 2018)
Korotoa River, Bangladesh	54-63 mg.kg^{-1}	(Islam et al., 2015)
Tundzha River, Bulgaria	42.96 mg.kg^{-1}	(Valkova et al., 2016)
Yangtze River, China	16.89–41.76 mg.kg^{-1}	(Li et al., 2020)
Turnasuyu River, Turkey	41.4-72.7 mg.kg^{-1}	(Tepe et al., 2022)
Melet River, Turkey	18.1 mg.kg^{-1}	(Kontaş & Bostancı 2020)
Beyşehir lake, Turkey	15.22-0,09 mg.kg^{-1}	(Şener et al., 2023)
Marine Water		
Weeks Bay	11.8 µg/L	(Paul et al., 2021)
Malaga Bay, Spain	1.09 µg/L	(Castilo et al., 2013)
Black Sea - Bosphorus	150–2702 ng/L	(Yiğiterhan et al., 2011)
Eastern Black Sea	17.5–39.0 mg/L^{-1}	(Cevik et al., 2008)
Giresun Bay, Turkey	7.92 ± 0.76 mg/kg	(Baltas et al., 2017)
Marine Sediment		
Malaga Bay, Spain	19.05 mg/kg	(Castilo et al., 2013)
Eastern Black Sea, Turkey	208.2 mg.kg^{-1}	(Alkan et al., 2020)
Gorgan Bay, Southeast Caspian Sea	7.4 mg/L	(Gholizadeh & Patimar, 2018)
Red Sea, Saudi Arabia	11.43 µg/g	(Nour et al., 2019)
Izmit Bay, Turkey	10.0-33.8 mg.kg^{-1}	(Tan & Aslan, 2020)
British Columbia, Canada	14 mg.kg^{-1}	(Kim et al., 2023)

Black Sea, Turkey	13-83 $mg.kg^{-1}$	(Apaydın et al., 2022)

Studies have shown that the toxic effects of lead can cause changes in water's chemical, physical, and biological structure, which can affect aquatic organisms, including a variety of sublethal consequences and lethal ones, including inhibited growth, behavioural changes and hindered reproduction (Chen et al., 2017; Miao et al., 2020; Zhang & Yu, 2020). Lead is known to disrupt cell signaling and damage the central nervous system, kidney, and hematopoietic systems in vertebrates (Flora et al., 2006; Mitra et al., 2017; Iqubal et al., 2020; Deidda et al., 2021). Furthermore, in marine invertebrates (annelids, some crustaceans, echinoderms, molluscs, and cnidarians), lead exposure is known to cause osmotic stress by disrupting sodium ion regulation, disorders in different life stages, reproductive disruptions, and death (Kim et al., 2016; Botté et al., 2022).

Invertebrates are the most diverse group of animals, comprising numerous eukaryotic species that are found globally in complex living habitats and they interact with many trophic groups, from primary producers to top predators, and will thus likely have significant indirect impacts on ecosystem services (Brusca & Brusca, 2002; Traill et al., 2010). This makes them essential organisms to study of heavy metal ecotoxicity (van den Brink et al., 2019; da Silva et al., 2022). Toxicity studies can use crustaceans, some zooplankton, fish, bacteria and fungi as biological models to identify and assess the potential environmental risks of different contaminants (Ribeiro et al., 2023; de Melo et al., 2019). Numerous species have been studied for the impacts of lead belongs including Echinodermata, Mollusca, Annelida, and Crustacea (Maddock & Taylor, 1980; Fernández & Beiras, 2001; Beiras & Albentosa, 2004, Gopalakrishnan et al., 2008; Santos et al., 2014) but tardigrades have not been the subject of any studies.

Tardigrades, also known as water bears, are micrometazoans that require a thin film of water to be active. These organisms possess a remarkable tolerance to extreme environments, including vacuum, extreme drying, ionizing radiation, space conditions, and exposure to extremely high and low temperatures and heavy metals (Cabral et al., 2015; Nelson et al., 2015; Erdmann & Kaczmarek, 2017; Glime, 2017). Tardigrades enter a state of cryptobiosis, by slowing their metabolism. These animals achieve this by decreasing their surface area; becoming a “tun” state; and losing up to 95% of their body water content. At the beginning of the century, it was established that tardigrades are sensitive to polluted environments (Iharos, 1937). This sensitivity makes them ideal candidates for biomonitoring, as their survival can indicate clean or polluted conditions. These species play a role in the food chain and can be found permanently

or temporarily in moss habitats. Organisms living in moss are effective bioindicators for assessing heavy metal pollution. Research suggests that acute tests with invertebrates like tardigrades can be used as initial monitoring to estimate the potential lethal toxicity of some chemicals on mammals and humans (Guilhermino et al., 2000). Acute toxicity testing aims to reveal the harmful effects of chemicals on test organisms. The most prevalent form of toxicity testing in aquatic animals is the acute mortality test, typically performed to gather information on a specific median lethal concentration (LC_{50}) (Stephan, 1997).

This study aims to utilize *Macrobiotus hufelandi* C.A.S. Schultze, 1834 as model organisms in biomonitoring research to investigate the effects of lead exposure on tardigrade communities. Our primary objective is to address the knowledge gap in research on the effects of heavy metals on tardigrades by assessing the acute toxicity of lead (measured as LC_{50}) on the semi-terrestrial tardigrade species *Mac. hufelandi* after 24, 48, 72, and 96 hours of exposure. The obtained LC_{50} values from this study are compared to published LC_{50} values of lead in other invertebrate species (Table 5). A box plot was generated during the statistical analysis to manage the variations in death numbers with various lead exposure levels and detect any outlier data points.

Material and methods

Collection of the Tardigrades

The moss samples were collected in Lake Abant Nature Park, Bolu (Turkey). The collected samples were dried and stored in paper envelopes in the laboratory. 20 grams of moss were rehydrated in beakers filled with distilled water for 6 hours by the method used by Dastych (1980) to collect tardigrades. Individuals of *Mac. hufelandi* were isolated from sieved samples using a 400-600 µm sieve and then collected with a micropipette. Active tardigrades were carefully transferred to Petri dishes. All abbreviations of the generic names were appropriate to the International Code of Zoological Nomenclature (ICZN), according to Perry et al. (2019)

Acute Toxicity Testing

Lead (II) nitrate ($Pb(NO_3)_2$ (MERCK, Germany)) was dissolved in distilled water (GFL Gesellschaft für Labortechnik, Germany) in required amounts to prepare 1000 mg/L lead (Pb) stock solution. The chemicals and reagents used throughout the experiments were analytical grade, and all the glassware was acid-washed and rinsed twice with distilled water before use. The methods determined by Hygum et al. (2017) were adapted and used in this study. For the LC_{50} experiments, adults within the size range between 400 and 600 µm were selected. Tardigrades were not fed for 24 hours before testing and were kept in petri dishes filled with distilled water at 20°C. Afterwards, the specimens were assigned to 10 groups of varying

concentrations of lead (0mg/L (control), 0.4mg/L, 0.8mg/L, 1.6mg/L, 3.2mg/L, 6.4mg/L, 12.8mg/L, 25.6mg/L, 51.2mg/L, 102.4mg/L and 204.8 mg/L). The experiments were performed in 15 ml petri dishes under static conditions. Petri dishes were supplied with designated concentrations and 10 tardigrades per group. Each group was exposed to lead for 24, 48, 72 and 96 hours, and the acute toxicity tests were conducted in 3 replicates. The mortality of the tardigrades was observed under a stereo microscope (Leica EZ4) every 12 hours, and when they ceased responding to tactile stimuli, tardigrades were considered dead and removed.

Statistical Analysis

To determine the LC_{50} value, the mortality rates of *Mac. hufelandi* during the acute test phase was examined using the probit analysis. In addition, the conformity of the data to the normal distribution was tested with Shapiro-Wilk W (Royston, 1982). The Kruskal Wallis-H Test, which is a nonparametric method, was used to test the statistical significance of the differences in the mean tardigrade mortality numbers according to different heavy metal concentrations (Kruskal & Wallis, 1952). In addition, Friedman's test (Tables 3, and 4) was applied to test the statistical significance of the differences between the average tardigrade mortality numbers of the exposure times (24h, 48h, 72h, 96h) and the differences in the form of pairwise comparisons (Friedman, 1937). Finally, simple regression analysis graphs were created to determine the linear relationships in the number of tardigrade mortalities depending on the duration of different heavy metal concentrations (Chambers, 1992). All statistical analyses were performed using the R Studio software and the statistical package program IBM SPSS (Version 23.0), with the level of statistical significance set at $p < 0.05$.

Results

The LC_{50} values for *Mac. hufelandi* were estimated to be 94.651 mg/L, 43.540 mg/L, 22.344 mg/L, and 8.048 mg/L, respectively, after 24, 48, 72, and 96 hours of acute exposure (Table 2). No mortality was observed in the control groups. None of the tardigrades that were deemed to be dead showed any activity after removal from lead solutions and signs of decomposition were evident.

Therefore, acute exposure to high lead concentrations is proven to be lethal in these animals as LC_{50} values are frequently used as a common indicator of a substance's acute toxicity.

Table 2. LC values (mg /L), 95% confidence limits of lead for 24, 48, 72 and 96 h on tardigrades.

LC	24h	48 h	72 h	96 h
LC 10	14.965	11.734	5.977	2.330
LC 25	35.853	21.837	11.162	4.192

LC 50	**94.651**	**43.540**	**22.344**	**8.048**
LC 75	249.871	86.813	44.728	15.452
LC 90	598.637	161.55	83.536	27.795

Initially, as part of the statistical analysis process, a box plot graph (Fig. 1) was created to control the variations in the number of deaths with different concentrations of lead exposure and to control the presence of outlier data.

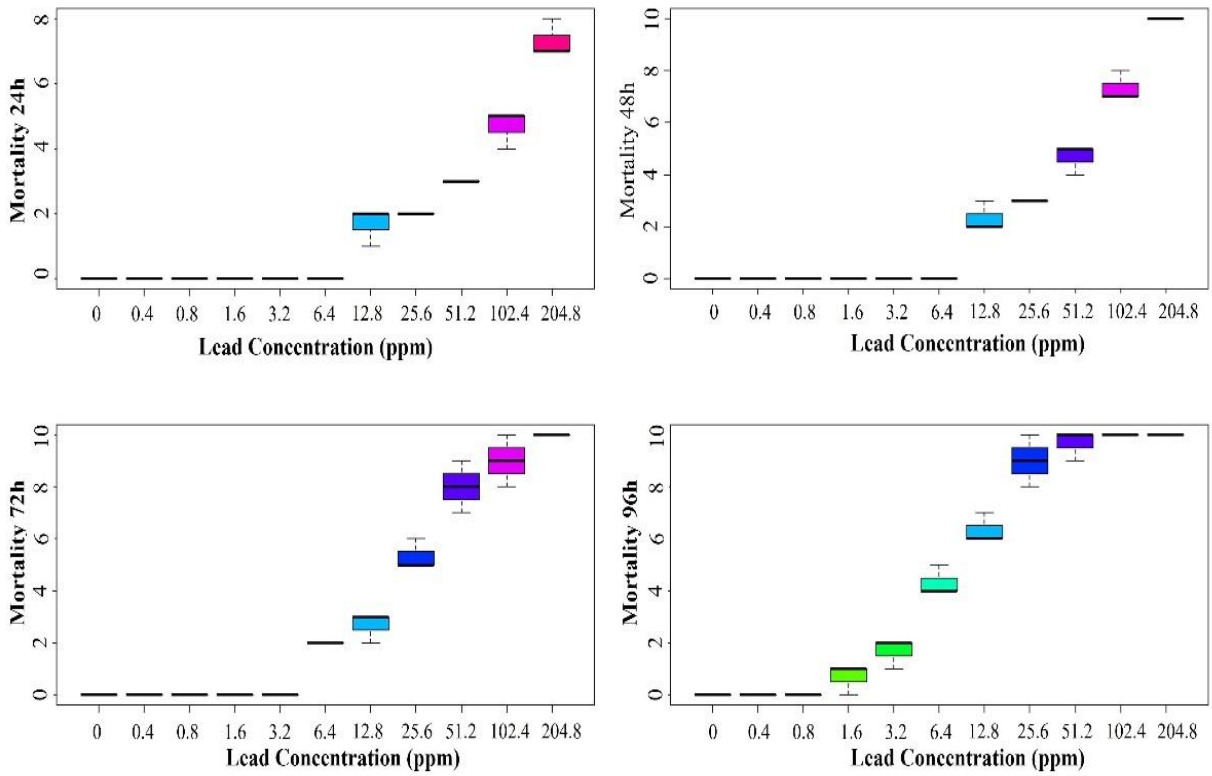

Figure 1. The effects of different heavy metal concentrations on mortality in tardigrades

As seen in the box plot graph, while no mortality was observed in tardigrades exposed to concentrations up to 12.8 ppm for 24 hours, an increase in mortality was observed as concentrations increased starting from 12.8 mg/L. At the end of 24 hours, it was observed that 80% of the tardigrades exposed to the highest concentration (204.8 ppm) died. Although a similar trend in 48 h groups was observed, in 48 h groups all tardigrades were dead at the highest concentration (204.8 ppm). On the other hand, while mortality was first observed at 6.4 ppm in 72 h groups, this value decreased to 1.6 ppm in 96 h groups. It was observed that all individuals in the group were exposed to 204.8 ppm after 72 hours of exposure. In addition, it was observed that all individuals died in the groups exposed to 204.8 ppm and 102.4 ppm after 96 hours. Through the utilization of the Shapiro-Wilk (W) Test, it was determined that the average

mortality rates based on concentration and exposure time, were not normally distributed. Therefore, the Kruskal Wallis H Test, which is a nonparametric method, was applied to examine the statistical significance of variations in the concentration-related mortalities in each exposure period.

Table 3. Friedman test findings of Tardigrade mortality (LC_{50}) Over Time (N: 33)

	N	Mean	Std. Deviation	Minimum	Maximum	Kruskal-Wallis H	Sig.
24h	33	1.70	2.378	0	8	31.885	0.000
48h	33	2.48	3.392	0	10	31.885	0.000
72h	33	3.36	3.896	0	10	31.688	0.000
96h	33	4.70	4.283	0	10	31.070	0.001

Findings of Kruskal Wallis H Test applied to test the significance of variations between mean mortalities according to heavy metal concentrations and durations of exposure

As a result of this analysis, it was found that the variations in lead concentrations across various durations of exposure affect tardigrade mortalities in a statistically significant manner ($p< 0.05$).

Table 4. Pairwise Friedman's test findings of differences in the number of tardigrade mortality (LC_{50}) over time (N: 33)

Mortality (LC_{50})	Test Statistic	Std. Test Statistic	p
24h - 48h	-0.530	-1.669	0.571
24h - 72h	-0.985	-3.099	0.012
24h - 96h	-1.758	-5.530	0.000
48h - 72h	-0.455	-1.430	0.916
48h - 96h	-1.227	-3.862	0.001
72h - 96h	-0.773	-2.431	0.090

According to Friedman's test results, it was determined that there was a statistically significant difference in all exposure durations that affected mortality (Fig. 2). In pairwise comparisons, the highest statistically significant ($p<0.05$) differences were found between 24h-96h, 48h-96h and 24h-72h, respectively. No statistically significant difference was found between the paired comparisons of other groups.

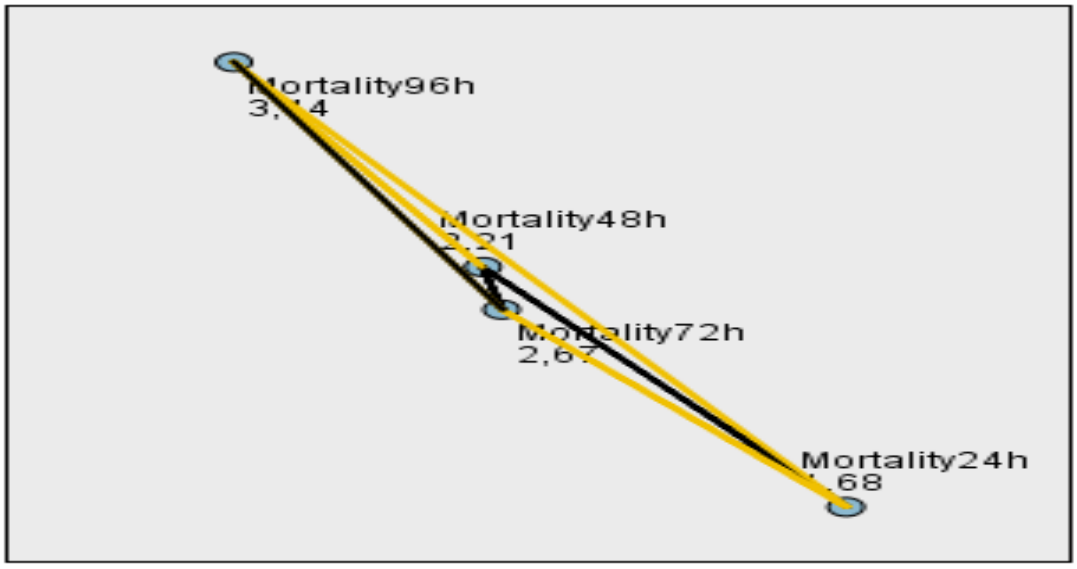

Figure 2. Friedman's test results of Tardigrade mortality difference in all exposure durations

The results of linear regression analysis applied to determine the relationships between heavy metal concentrations and total mortality of tardigrades of each exposure duration are given in Fig. 3. According to our findings, the linear relationship between the number of mortalities and lead concentration varies depending on exposure duration. The linear relationship between mortality and concentration was found to be most significant (r^2 = 0.941) at 24 hours whereas the increase in deaths at 96 hours was seen more likely due to the prolonged exposure time rather than the increase in concentration.

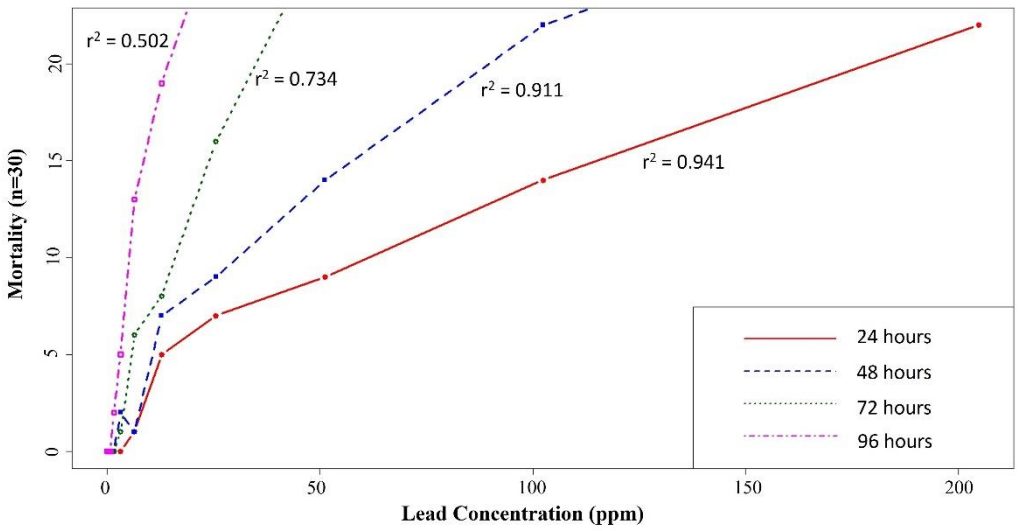

Figure 3. Relationships between lead Concentration and Mortality numbers at different times

Discussion

Due to their small size, tardigrades can be expected to be relatively sensitive to Pb exposure. Our findings indicate that tardigrades have a higher tolerance to lead than anticipated based on their body size owing to their exceptional adaptability to environmental challenges (Nelson et al., 2015; Glime, 2017; Schill, 2018; Rebecchi et al., 2019). We present lead tolerance levels in tardigrade species based on activity assessments as a measure of survival. The present study is the first to investigate tardigrades' tolerance to lead. The acute toxicity, morphological effects of lead on Tardigrada species *Mac. hufelandi* were calculated at 24, 48, 72, and 96 h, the LC_{50} values for lead were 94.651 mg/L, 43.540 mg/L, 22.344 mg/L, and 8.048 mg/L, respectively. According to our results, high lead concentrations negatively affect on tardigrades. The mortality rate of tardigrades was found to increase with both the concentration and duration of exposure to lead.

In the experiment, tardigrades showed no mortality when exposed to concentrations up to 12.8 ppm in 24 hours. On the other hand, the peak concentration (204.8 ppm) at the end of the 24 hours led to mortalities. After 48 hours, dose and duration combined affected mortality rates. As the exposure time increased, the mortality rate decreased to 1.6 ppm in the 96-hour group. Prolongation of exposure time had an impact on mortality rates. Although the LC_{50} value of Pb for *Mac. hufelandi* is higher than most other animals tested so far in various studies. A limitation of this study is the absence of direct Pb exposure experiments on other tardigrade species. However, a study by Vargha et al. (2002) analyzing heavy metal content in moss samples reported a significant difference in the abundance of *Macrobiotus richtersi* and *Mac. hufelandi* in habitats with high lead concentrations (19.7 $\mu g.g^{-1}$). They observed a lower abundance of *Mac. richtersi* compared to *Mac. hufelandi*, suggesting a potential difference in lead tolerance between these two species. Further research directly comparing the Pb-tolerance of various tardigrade species is warranted to elucidate interspecific variability and establish *Mac. hufelandi* as a potential biomonitoring tool for lead pollution.

Given that different heavy metals have various harmful effects on organisms, the effects of lead on tardigrades have not yet been thoroughly elucidated. In our study, *Mac. hufelandi* has shown a greater degree of tolerance to lead compared to invertebrates in the Arthropoda phylum. For example, *Artemia nauplii* exposed to lead for 48 hours had a lethal concentration (LC_{50}) estimate of 1.4 (mg/L) (Gajbhiye & Hirota,1990) and *D. magna* exposed to lead had an LC_{50} estimate of 0.441 mg/L after 24 hours of exposure (Altındağ et al., 2008). This study estimated the 24 h LC_{50} value of lead exposure for *Mac. hufelandi* as 94.651 mg/L. Several reports have shown the effects of lead exposure on aquatic invertebrates (Table 5).

Table 5. Lead LC_{50} values of various invertebrates

Species	Form of Pb	Exposure time	LC_{50} Concentration (mg/L)	Source
Macrobiotus hufelandi C.A.S. Schultze 1834 (Tardigrada; Eutardigrada; Parachela)	$Pb(NO_3)_2$	24 h 48 h 72 h 96 h	94.651 43.540 22.344 8.048	Current study
Daphnia sp. (Arthropoda; Crustacea; Branchiopoda)	$Pb(NO_3)_2$	24 h 48 h 96 h	2.51 1.88 1.65	Offem & Ayotunde (2008)
Cyclop sp. (Arthropoda; Crustacea; Copepoda)	$Pb(NO_3)_2$	24 h 48 h 96 h	3.11 2.97 2.61	
Ceriodaphnia dubia Richard 1894 (Arthropoda; Crustacea; Branchiopoda)	$Pb(NO_3)_2$	48 h	0.2088	Cooper et al. (2009)
Daphnia carinata King 1853 (Arthropoda; Crustacea; Branchiopoda)	$Pb(NO_3)_2$	48 h	0.444	
Fenneropenaeus indicus H. Milne Edwards 1837 (Arthropoda; Crustacea; Decapoda)	$Pb(CH_3COO)_2$ $3H_2O$	96 h	7.223	Chinni et al. (2002)
Crangon crangon Linnaeus 1758 (Arthropoda; Crustacea; Decapoda)	Tetramethyllead	96 h	0.270 0.100	Maddock & Taylor (1980)
Palaemon adspersus Rathke 1836 (Arthropoda; Crustacea; Decapoda)	Tetramethyllead	96 h	68.00	Bat et al. (2001)
Aiptasia pulchella Carlgren 1943 (Cnidaria; Actiniaria)	$PbCO_3$	96 h	8.06	Howe et al. (2014)
Babylonia areolata Link 1807 (Mollusca; Gastropoda)	$Pb(NO_3)_2$	24 h 48 h 72 h 96 h	29.31 14.64 12.44 10.50	Supanopas et al. (2005)
Mytilus edulis Linnaeus 1758 (Mollusca; Bivalvia)	Tetramethyllead Tetraethyllead	96 h	0.110 0.020	Maddock & Taylor (1980)
Heliodiaptomus viduus Gurney 1916 (Arthropoda; Crustacea; Copepoda)	Not stated	48 h	3.1	Chishty et al. (2012)
Thermocyclops hyalinus Rehberg, 1880 (Arthropoda; Crustacea; Copepoda)			8.0	
Heterocypris sp. (Arthropoda; Crustacea; Ostracoda)			5.5	
Daphnia lumholtzi Sars 1885 (Arthropoda; Crustacea; Branchiopoda)			5.5	
Ceriodaphnia sp. (Arthropoda; Crustacea; Branchiopoda)			2.3	
Moina sp. (Arthropoda; Crustacea; Branchiopoda)			0.92	

Brachionus sp. (Rotifera; Monogononta)			0.11	
Monostyla sp. (Rotifera; Monogononta)			0.05	
Filinia sp. (Rotifera; Monogononta)			0.1	
Euchlanis dilatata Ehrenberg, 1832 (Rotifera; Monogononta)	$Pb(NO_3)_2$	48 h	0.0353	Arias-Almeida & Rico-Martínez (2011)
Tisbe holothuriae Humes 1957 (Arthropoda; Crustacea; Copepoda)	$Pb(CH3COO)_2$ $3H_2O$	48 h	6.34	Verriopoulos & Dimas (1988)
Cerithidea cingulata Gmelin 1791 (Mollusca; Gastropoda)	$Pb(NO_3)_2$	24 h 48 h 72 h 96 h	64.839 36.380 23.972 15.507	Ramakritinan et al. (2012)
Modiolus philippinarum Hanley 1843 (Mollusca; Bivalvia)	$Pb(NO_3)_2$	24 h 48 h 72 h 96 h	13.545 11.071 4.929 2.876	
Meretrix meretrix Linnaeus 1758 (larva) (Mollusca; Bivalvia)	$Pb(NO_3)_2$	48 h 96 h	>7.1 0.353	Wang et al. (2009)
Penaeus monodon Fabricius 1798 (Arthropoda; Crustacea; Decapoda)	$Pb(NO_3)_2$	96 h	0.41	Rajkumar et al. (2011)
Hydroides elegans Haswell 1883 (Annelida; Sedentaria)	$PbCl_2$	24 h 96 h	25.017 0.946	Gopalakrishnan et al. (2008)
Perna viridis Linnaeus 1758 (Mollusca; Bivalvia)	$PbCl_2$	96 h	8.820	Chan (1988)
Artemia franciscana (San Francisco Bay Strain) Kellogg 1906 (nauplii) (Arthropoda; Crustacea; Branchiopoda)	$Pb(NO_3)_2$	24 h 48 h	1.7 1.4	Gajbhiye & Hirota (1990)
Daphnia magna Straus 1820 (Arthropoda; Crustacea; Branchiopoda)	$Pb(NO_3)_2$	24 h	0.441	Altındağ et al. (2008)

We hypothesise that tardigrades have a high tolerance to toxicants due to their unique adaptations. In particular, Olatoregun (2021) demonstrated that the tardigrade species *Hypsibius exemplaris* revealed great tolerance to cadmium during a 24-hour exposure period, with a concentration of 7.5 mg/L. The observed high resistance of tardigrades to heavy metals is due to increased *hsp70* expression, which allows cell survival and functioning against cadmium (Olatoregun, 2021). Hygum et al. (2017) evaluated the copper tolerance of four species of tardigrades, and it was discovered that *Echiniscus testudo* and *Halobiotus crispae* showed better tolerance than *Echiniscoides sigismundi* and *Ramazzottius oberhaeuseri*. It is thought that the existence of a second cuticle in the P1 phase may be the cause of the high copper tolerance of *H. crispae*. Additionally, they emphasized the capacity of tardigrades to defend themselves against high copper concentrations regardless of their size through cryptobiosis (Hygum et al., 2017).

Many organisms have developed protective mechanisms to cope with metal exposure by reducing intake, promoting removal, and activating stress response (Olatoregun, 2021). As invertebrates, tardigrades exhibit limited immunity as they lack adaptive immune systems and instead rely solely on innate immunity, which is characterized by defined receptors, signalling cascades, and effector cells. The modulation of immune responses can be mediated by various factors such as viruses, bacteria, toxins, and chemicals (Iwanaga & Lee, 2005).

In the current study, we focused on lead, a most common heavy metal contaminant, particularly in surface waters of many aquatic environments as a consequence of anthropogenic activities (Roig et al., 2015). Lead disrupts interactions between different ions (Na^{+}, Ca^{2+}, Mg^{2+} and $Fe^{2+)}$ involved in cellular metabolism, leading to dysfunctions in important cellular activities (Carocci et al., 2015). Due to this, vital cellular processes like cell signaling, cellular adhesion, apoptosis, enzyme control, nervous system and nucleotide metabolism are affected (Luckey & Venugopal 1979; Antonio et al., 2002). The effects of lead have been widely investigated in many invertebrates such as *Drosophila melanogaster* and aquatic invertebrates such as *Daphnia magna* (Liu et al., 2020; Botté et al., 2022). Kim et al. (2017) showed that lead promoted oxidative stress in *Daphnia magna*, and as a consequence of 24 hours of exposure to Pb, the expression of all genes decreased whereas Mn-SOD and CAT mRNA levels significantly increased in a concentration-dependent manner.

Conclusion

It has been shown that duration is more effective in mortality rates in long-term lead exposure. This indicates that increasing the level of stress in the organism and the transition of the defense mechanism to an irreversible state may contribute to mortality. We believe that further research at the enzyme level after exposure may lead to a better understanding of defense mechanisms. Such findings will provide crucial insights into the adaptability and resilience of invertebrates in the face of adverse environmental conditions.

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