Chemosphere 244 (2020) 125550

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Histopathological, genotoxic, and behavioral damages induced by manganese (II) in adult zebrafish

Gabriela Zimmermann Prado Rodrigues [a, *], Luciane Beatris Mentges Staudt [b], Marina Griebeler Moreira [a], Thainá Garbino dos Santos [c], Mateus Santos de Souza [d], Caio Júnior Lúcio [d], Jenifer Panizzon [d], Juliana Machado Kayser [e], Leonardo Airton Ressel Simões [d], Ana Luiza Ziulkoski [a], Carla Denise Bonan [f], Diogo Losch de Oliveira [c], Günther Gehlen [a]

[a] Post Graduation Program in Environmental Quality, Feevale University, Brazil
[b] Biological Science Student, Feevale University, Brazil
[c] Post Graduation Program in Biological Sciences, Biochemistry, Federal University of Rio Grande do Sul, Brazil
[d] Bacherol's Degree in Biological Science, Feevale University, Brazil
[e] Biomedicine Student, Feevale University, Brazil
[f] Post Graduation Program in Cellular and Molecular Biology, Pontifical Catholic University of Rio Grande do Sul, Brazil

HIGHLIGHTS

- Chronic exposure to $MnCl_2$ may cause behavioral anxiogenic type in zebrafish.
- Behavioral damages may be originated from the reduction of neurons in the Vd nucleus.
- $MnCl_2$ induces micronucleus and comet in erythrocyte after acute and chronic exposures.
- Liver histopathology shows vacuolization of hepatocytes after exposure to $MnCl_2$.

ARTICLE INFO

Article history:
Received 24 September 2019
Received in revised form
2 December 2019
Accepted 3 December 2019
Available online 5 December 2019

Handling Editor: David Volz

Keywords:
Ecotoxicology
Polluting metals
Biomarkers

ABSTRACT

Manganese is a metal often found as an environmental pollutant and very associated with neurological disorders when in high concentrations. However, little is known about the effects that this contaminant can cause when in environmentally relevant concentrations and occurrence, that is, much lower than those commonly studied. So, the aim of the study was to evaluate the effects that environmentally relevant concentrations of this metal would cause in different zebrafish organs (brain, liver, and blood). Acute 96-h and chronic 30-day exposures were performed using the manganese chloride salt as a pollutant. Behavioral alterations of anxiogenic type were observed in the animals after chronic exposures to 4.0 mg L^{-1} $MnCl_2$, which traveled a greater distance at the bottom of the aquarium. This may be associated with neuronal damages in the telencephalic region responsible for motor and cognitive activity of the fish, observed in animals from the same exposure. In addition, hepatic histopathological damage as vacuolization of hepatocytes and genotoxic damage, identified by comet assay and micronucleus test, was also observed after acute and chronic exposure, especially at the highest pollutant concentrations (8.0 and 16.0 mg L^{-1} in acute exposure, and 4.0 mg L^{-1} in chronic exposure. The study reinforces the risk that environmental pollutants pose to the ecosystem, even in low concentrations.

1. Introduction

Manganese (II) is an element of natural occurrence in some localities (Bouchard et al., 2018), mainly in the sites where igneous, sedimentary, and metamorphic rocks are found (Mena, 1980). However, it may occur from anthropic origin, being a fundamental

* Corresponding author. RS 239, 2755, 93352-000, Novo Hamburgo, RS, Brazil.
E-mail addresses: gabizpr@gmail.com (G. Zimmermann Prado Rodrigues), guntherg@feevale.br (G. Gehlen).

https://doi.org/10.1016/j.chemosphere.2019.125550
0045-6535/

raw material for the manufacture of fungicides, batteries, steel, metal welding, varnishes, animal supplementation, and many industrial products (HSDB, 2001; Olanow, 2004; Patil et al., 2016).

Under normal conditions, manganese has essential functions for the numerous physiological processes, including regulation of cellular energy homeostasis, amino acid metabolism, lipids, proteins, and carbohydrates (Erikson et al., 2005), immune system, and blood clotting (Erikson and Aschner, 2003). In addition, it acts as an important cofactor for enzymes such as superoxide dismutase (E.C. 1.15.1.1) (Hurley and Keen, 1987) and other enzymes involved in the synthesis and metabolism of neurotransmitters (Golub et al., 2005). However, several studies report that prolonged exposures or high concentrations of this metal are related to neurotoxicity in humans and animals (Huang et al., 2011; Yoon et al., 2011; O'neal et al., 2014; Altenhofen et al., 2017). It is noteworthy that among these studies, only a few used Mn^{+2} concentration ranges of possible environmental occurrence, that is, the majority used concentration bands well above the limit established by current legislation, whether Brazilian or worldwide (Huang et al., 2011; Sarkar and Shekhar, 2018; Altenhofen et al., 2017).

In Brazil, the concentrations of manganese commonly found in different water courses vary from 0.05 to 0.5 mg L^{-1} in stretches of the Sinos River (one of the most polluted river in the country) (IBGE, 2010; Nascimento et al., 2015; Dalzochio et al., 2017; Bianchi et al., 2019), to 19.3 mg L^{-1} in the Pampulha basin in Minas Gerais (Rietzler et al., 2001). Worldwide, it has been found in groundwater, superficial, and also in fish organs from contaminated rivers (Meyer et al., 2017; Superville et al., 2018; Subotic et al., 2015), becoming an environmental concern due to its long life and bioaccumulation capacity (Yousafzai et al., 2010).

In this context, in situ biomonitoring studies and also laboratory toxicity assays are essential for better characterization of the toxicity of this frequent environmental pollutant. The aim of this study was to evaluate the neurological, hepatic and genotoxic damages induced by environmentally relevant concentrations of manganese chloride ($MnCl_2$) in zebrafish, through histological analysis, MTT assay, micronucleus test, comet assay, and evaluation of the exploratory behavior of animals.

2. Materials and methods

2.1. Experimental procedures

All experimental procedures were performed according to the Brazilian Law for Laboratory Animal Care and Use (Law 11794/2008) and were previously approved by the Institutional Committee for Animal Care and Use from Feevale University (protocol number #02.16.046). A solid manganese chloride ($MnCl_2.4H_2O$.) (LabSynth®, purity 98%) was used to prepare the dilutions. Each 1 mg L^{-1} of $MnCl_2$ used corresponds to 0.28 mg L^{-1} of manganese, since manganese represents 27.8% of the mass of $MnCl_2 \cdot 4H_2O$. The concentrations ranged from 0.5 mg L^{-1} $MnCl_2$ (approximately 3.5 times less than the limit established for Class 3 waters, destined to supply human consumption after conventional or advanced treatment, by the National Council of the Environment (CONAMA) through Resolution n° 357 of 2005 (BRASIL, 2005), and 16.0 mg L^{-1}, still representing a dose of environmental occurrence.

Adult wild-type zebrafish (*Danio rerio;* 6–7 months old, males and females) were obtained from a local supplier. In laboratory, the water used for maintenance and exposure of animals were reconstituted water (ISO, 1996). During the period of acclimatization (seven days) and experimentation, the animals was maintained under the following environmental conditions: water temperature of 26 ± 2 °C, pH at 7.0–8.0, water hardness 75–100, 160 μS/cm of conductivity and with a maximum density of 5 animals per liter of water. The light/dark cycle was 14:10 h (lights on at 7:00 a.m.), in constant aeration (semi-static system), and they were fed thrice per day with commercial flake food (Alcon).

The study consisted of two exposure periods: acute (96 hours), where the animals (30 animals per group) were exposed from 0.5 mg L^{-1} to 16.0 mg L^{-1} of $MnCl_2$; and chronic (30 days of exposure) where animals (30 animals per group) were exposed to 0.5 and 4.0 mg L^{-1} of $MnCl_2$. In both experiments 50% of the tank water was renewed every 48 h.

The statistical analysis will be detailed in each topic of methodological procedure, but all analyses were performed in software GraphPad Prism 6. The data normality was assessed by the Kolmogorov-Smirnov test and $p < 0.05$ was considered significant.

2.2. Behavioral analyses

At the end of the exposures, the animals (20 animals per group) were submitted to the 'open tank' test. The apparatus consisted of a tank of 20 × 20 × 10 cm (height x length x width). The behavior of the animals was evaluated for 6 min and analyzed using ANY-maze software. The following parameters were evaluated: average speed; number of entries in the top, middle, and bottom zones of the apparatus; the length of stay; and the distance traveled in each zone. For statistical analysis, the one-way Anova test was applied, followed by the Tukey post-test for normal distribution data. All results were expressed as mean and standard error.

2.3. Genotoxicity assays

2.3.1. Micronucleus test

After filming, the animals were immediately sacrificed through a section of the spinal cord to obtain blood samples for blood smears (n = 10). Blood smears were fixed with methanol (10 minutes) and stained with 5% Giemsa solution (New Prov) for 10 minutes. The analysis was made by light microscopy (Olympus BX41), at 1000× magnification under an oil-immersion objective, which were numbered 3000 erythrocytes per fish and recorded the number of cells with micronuclei and nuclear abnormalities (binucleated cell, cellular invagination, and cell budding), according to the classification established by Fenech et al. (1999). Statistical analysis was performed using the Kruskal-Wallis test, followed by the Dunn post-test, and the data were expressed as mean and standard error.

2.3.2. Comet assay

To complement the genotoxicity assessment, the comet test was performed (5 animals per group). Briefly, in the absence of light, 5 μl of blood were mixed with fetal bovine serum and Low Melting agarose (Sigma). The contents were rapidly transferred to a slide already covered with regular agarose (LW Biotec), and the material was covered with coverslip. After material solidification (about 10 minutes at 8 °C), the coverslip was removed and the slides were dipped in lysis solution (2.5 M NaCl, 100 mM EDTA, 100 mM Tris) for 12 hours. After this period, the electrophoretic migration of DNA (25 V and 300 mA current for 10 minutes) into alkaline electrophoresis buffer was performed. After the run, slides were washed in reverse osmosis water, fixed in absolute ethanol and stored under refrigeration until the time of staining and analysis. DAPI fluorescence dye 1: 1000 (Sigma) was used, and the images were captured by a fluorescence microscope (Zeiss Axio Scope. A1) at a magnification of 100x, with the aid of a black and white CMOS camera (AXIOCAM ICM1 model) with 1.2 megapixel, coupled to a microcomputer with ZEN2 software. The analysis was performed by categorizing, on average, 100 cells per animal according to the comet tail size, ranging from 0 (no genetic damage, i.e. no tail) to IV

(maximum genetic damage) (Fig. 1). Data were expressed by calculating the genetic damage index, in which the total number of cells of each class is multiplied by the respective value of the class (Pitarque et al., 1999), so the index value can vary from 0 (all normal cells) to 400 (maximum damage in all cells). Samples that differed from the control group would be considered genotoxic. Statistical comparison was performed using the Kruskal-Wallis test, followed by the Dunn post-hoc test and data were expressed as mean and standard error.

2.4. Histological procedure

After blood collection, the livers and brains were removed. The samples were fixed in 10% Formol (Synth) for 12 hours. Slides preparation followed the protocol described by Dalzochio et al. (2016). Briefly, samples were dehydrated, embedded in paraffin, sectioned in a rotatory microtome (5 μm) (Leica®, Germany). Brains (about 5 animals per group) were stained with the Nissl technique (Horn and Rasia-Filho, 2018), and livers (10 animals per group) with the hematoxylin and eosin (H&E) technique for further analysis.

The livers were observed under optical microscopy (400x magnification), and 20 fields per animal were captured and recorded the degree of cellular alterations that varied from 0 (absent) to 6 (severe), as established by Bernet et al. (1999). We followed the classification of the same authors for the attribution of alterations found in the images, being circulatory (aneurysm and hyperemia), regressive (degeneration and vacuolization), progressive (hypertrophy), and inflammatory (infiltration). After assigning the degree of damage in each field analyzed (blinded to avoid any interference), the mean of these changes (value of degree of damage) was calculated per animal, and finally by group, for further statistical analysis. Statistical analysis was performed using the Kruskal-Wallis test, followed by the Dunn post-hoc test, and data were expressed as mean and standard deviation. The correlation coefficient for Spearman's rank between the tested concentrations and the degree of cell vacuolization was also determined (most frequent alteration).

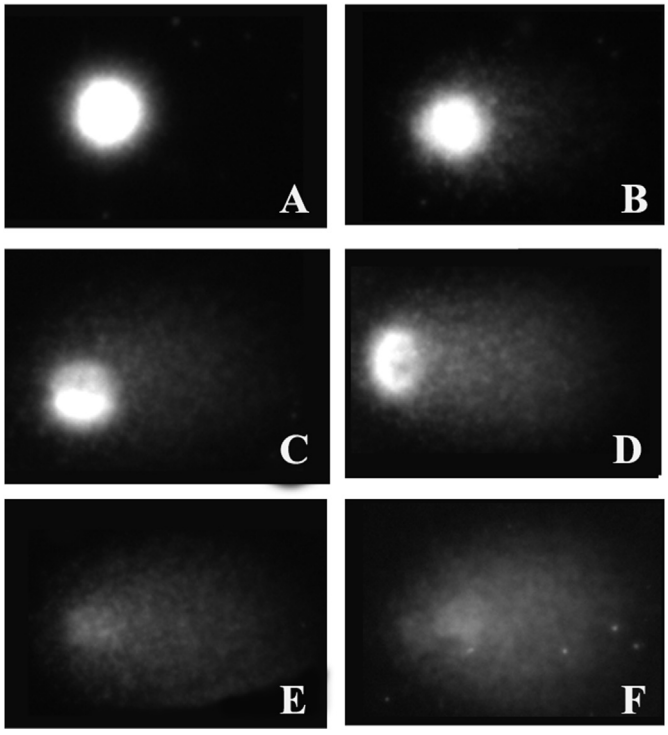

Fig. 1. Categorization used for the analysis of comets; normal (A), grade I (B), grade II (C), grade III (D) and grade IV (E and F) representing maximum genetic damage. (Captured and created by the authors).

For the histological analysis of the telencephalon, we were guided by the atlas of neuroanatomy of zebrafish elaborated by Wulliman et al. (1996) (Fig. 2). The analysis consisted of the observation, through optical microscopy (magnification of 400x), of 10 areas of interest in the size of 2 μm × 2 μm, located in the ventral region of the telencephalon, specifically in the periventricular area, in the dorsal nucleus (Vd) (specified in Fig. 2), considering only the quadrants away from the edges of the nucleus (region). In these quadrants, two inclusion borders (left and inferior) and two exclusion edges (right and superior) were defined, as described by Xavier et al. (2005) (Fig. 2C), where the cells were counted in the dorsal nuclei with well-colored nuclei, evident edges and without apparent overlap. Statistical analysis consisted of the Kruskal-Wallis test, followed by Dunn post-hoc test, and data were expressed as means and standard deviation. Spearman's correlation coefficient was also performed, due to the non-linearity of the data, between the concentrations tested and the number of cells counted in the dorsal nucleus.

2.5. MTT assay

The remaining animals (20 animals per group) were dissected for the removal of the telencephalon. The pool of samples for processing consisted of 2 telencephalons (2 animals), where half of the sample was directed to the protein dosage (Lowry's method), and the other half was directed to the MTT quantification (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). For MTT, the samples were washed in phosphate-saline buffer (PBS), hydrolysed in trypsin (Sigma) and incubated at 28 °C for 10 minutes. After that, fetal bovine serum was added to the samples, and they were centrifuged for 10 minutes (1500 rpm), the supernatant was discarded, and the samples resuspended in Leibowitz L-15 medium, for further MTT addition (Sigma). Samples were incubated at 28 °C for 2 h. Subsequently, the samples were centrifuged again (15 min - 1500 rpm), the supernatant was discarded, samples were resuspended in DMSO (dimethyl sulfoxide) and centrifuged for an additional 30 min (1500 rpm). The final contents were transferred to a microplate (96 wells) and absorbance readings were performed in a spectrophotometer (Molecular Devices® M3) at 540 nm. Statistical analysis was performed through Anova test followed by Tukey post-hoc test. Data were normalized and expressed in absorbance obtained by reading MTT in 50 μg protein (mean protein concentration).

3. Results

3.1. Behavior analysis

Animals that were both acutely and chronically exposed to $MnCl_2$ did not show significant behavioral changes, as can be seen in Fig. 3, except when observed the distance that the fish traveled in the bottom zone of the aquarium; Animals chronically exposed to 4.0 mg L^{-1} $MnCl_2$ showed a significant increase compared to animals in the control group ($p = 0.04$) (Fig. 3 C3). Consequently, animals exposed to 4.0 mg L^{-1} $MnCl_2$ spent less time within the top zone of the aquarium during the test, when compared to the control group ($p = 0.04$) (Fig. 3 D1). When evaluating the responses of animals from to the same concentrations, but comparing acute and chronic exposure, no relevant behavioral changes were observed either.

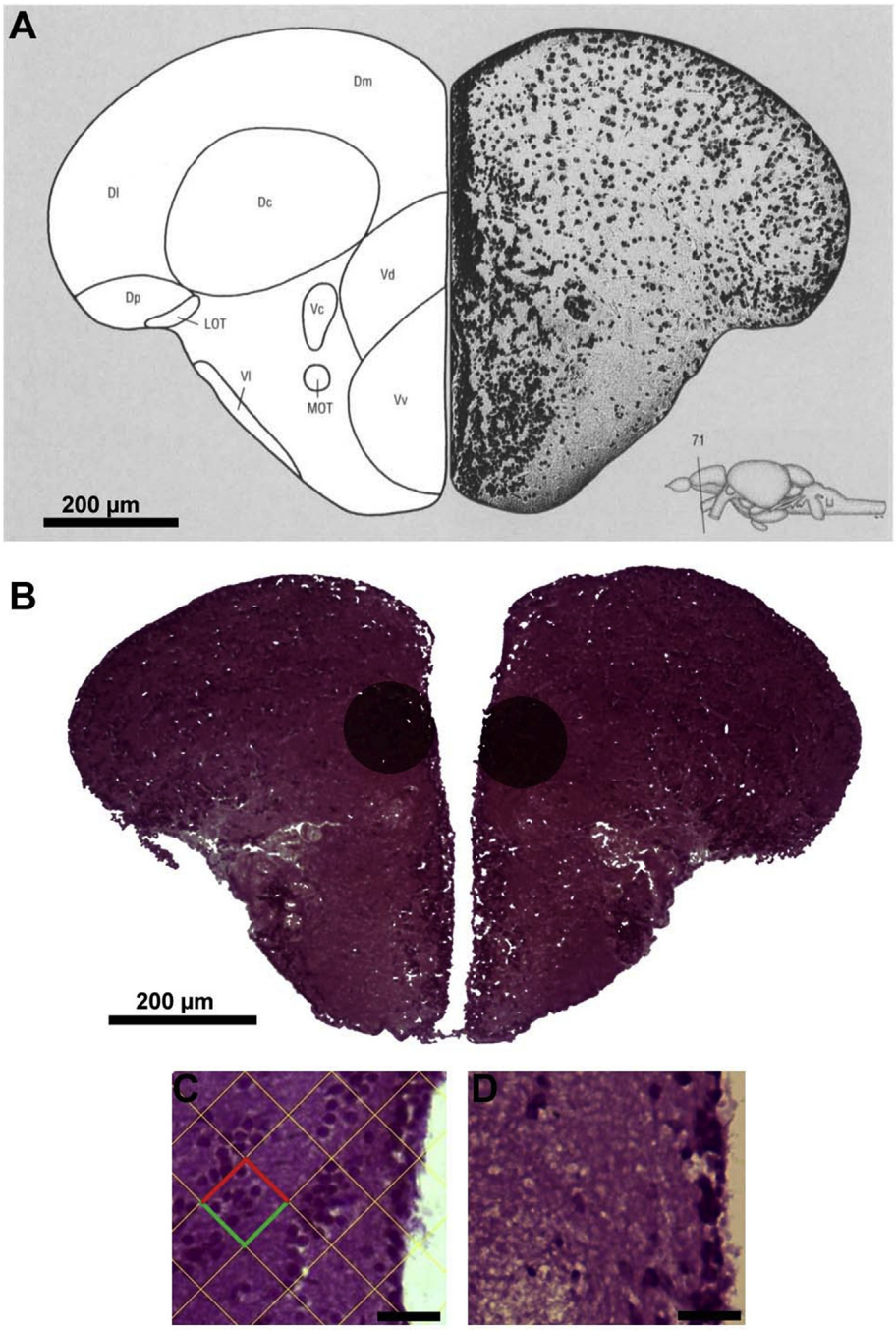

Fig. 2. A Image used as a reference for the localization and analysis of Vd (Wullimann et al., 1996). Fig. 2B: Histological section of the zebrafish telencephalon evidencing the studied region (processed by the author) (100x of magnification). Fig. 2C: Vd at higher magnification (400x), demonstrating the analysis quadrants for cell count, with red exclusion borders and green inclusion, representing an animal from control group. Fig. 2D: Vd at higher magnification (400x) with apparent reduction in the number of cell nuclei, representing an animal acutely exposed to 16 mg L^{-1}. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.2. Genotoxicity

Regarding to the comet assay, it was observed that fish exposed to 16.0 mg L^{-1} of $MnCl_2$ showed a higher DNA damage index ($p = 0.025$) in relation to the control group (Fig. 4A). However, no significant differences were observed for the DNA damage index of the animals from the chronic experimentation (Fig. 4A). In contrast the animals of the acute experiment did not show significant differences for the number of micronuclei (MN) and nuclear abnormalities (NA) (Fig. 4B and C). After chronic exposure, an increase of micronuclei in erythrocytes of fish exposed to 4.0 mg L^{-1} of $MnCl_2$ was observed in relation to the control animals ($p = 0.05$), as well as an increase in nuclear abnormalities erythrocytes of animals exposed to both concentrations (0.5 mg L^{-1} and 4.0 mg L^{-1}) when compared to the control group ($p = 0.003$).

3.3. Histological analysis and mitochondrial viability assay with MTT

The animals exposed to the highest concentrations of $MnCl_2$

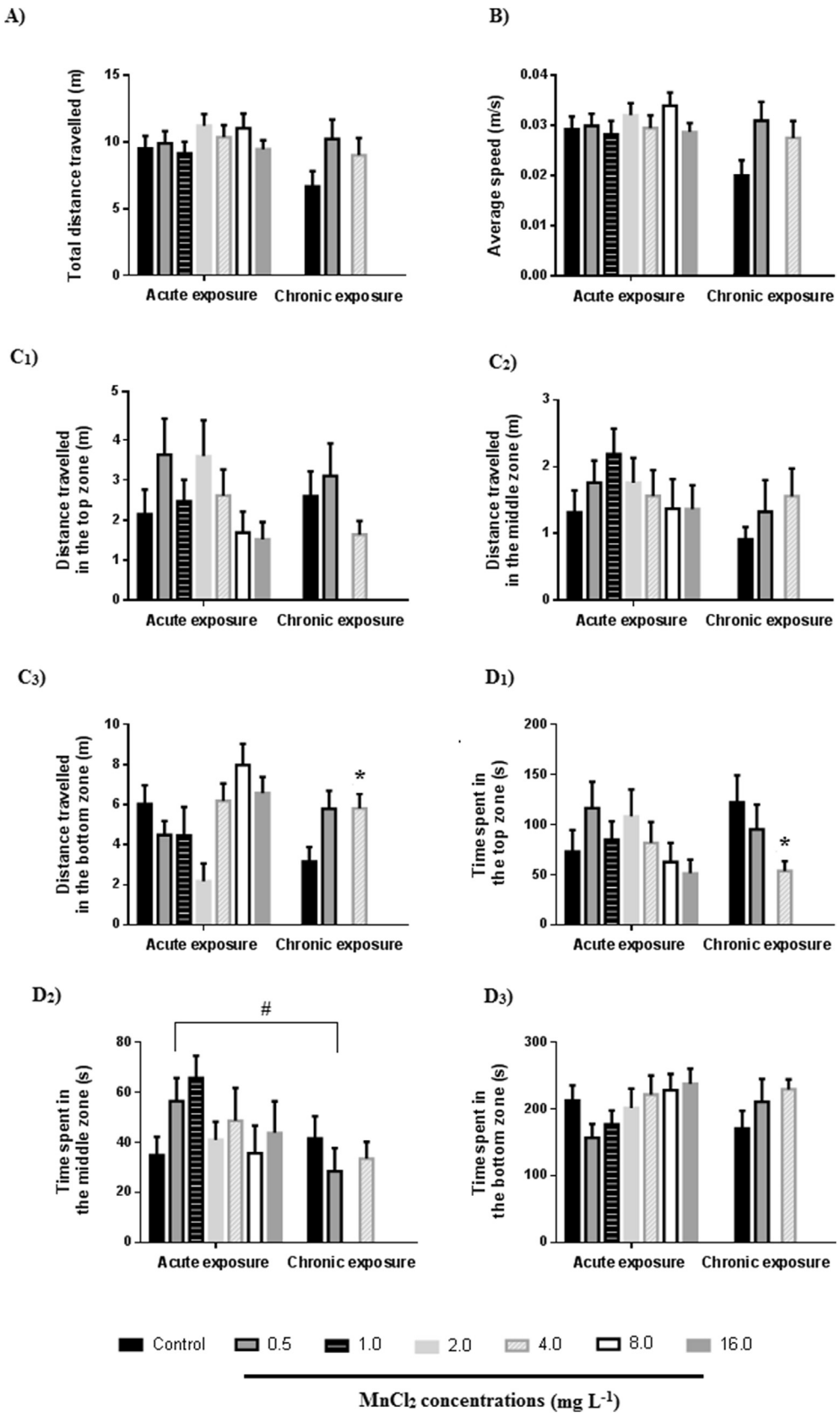

Fig. 3. Locomotor behavioral parameters evaluated in zebrafish exposed to $MnCl_2$. Data are expressed as mean ± standard error and asterisks represent a statistical difference compared to the control group within the same experimental period (acute or chronic), and the pound (#) represents a difference between the same concentrations but different exposures (acute and chronic).

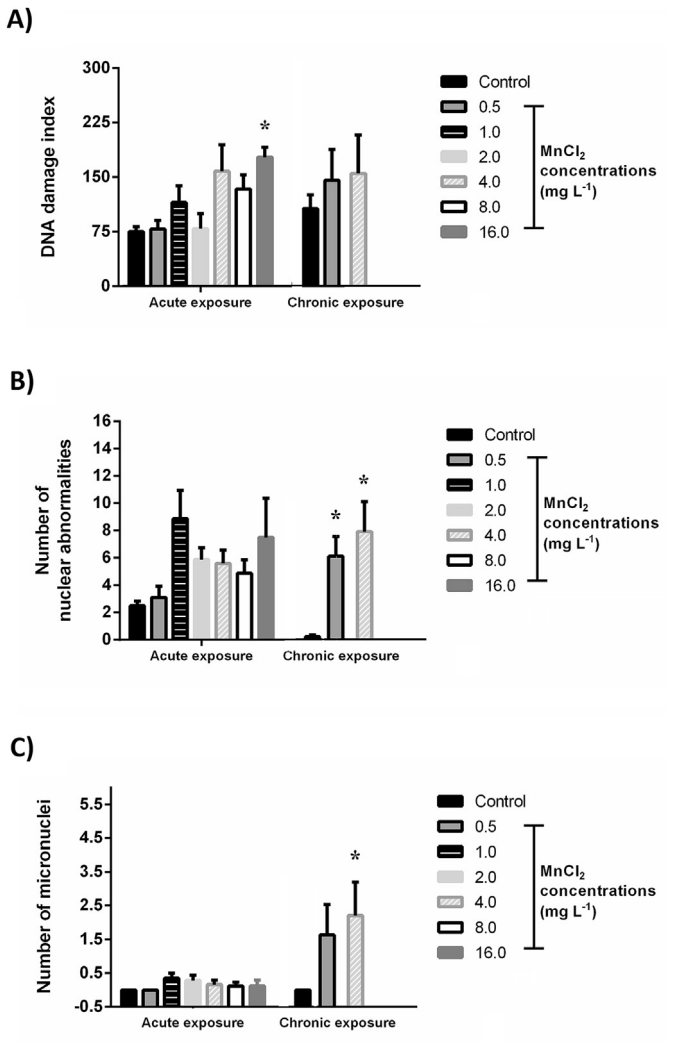

Fig. 4. Data of comet assay (A) and micronucleus test (B and C). Data are expressed as mean ± standard error and asterisks represent a statistical difference compared to the control group within the same experimental period (acute or chronic).

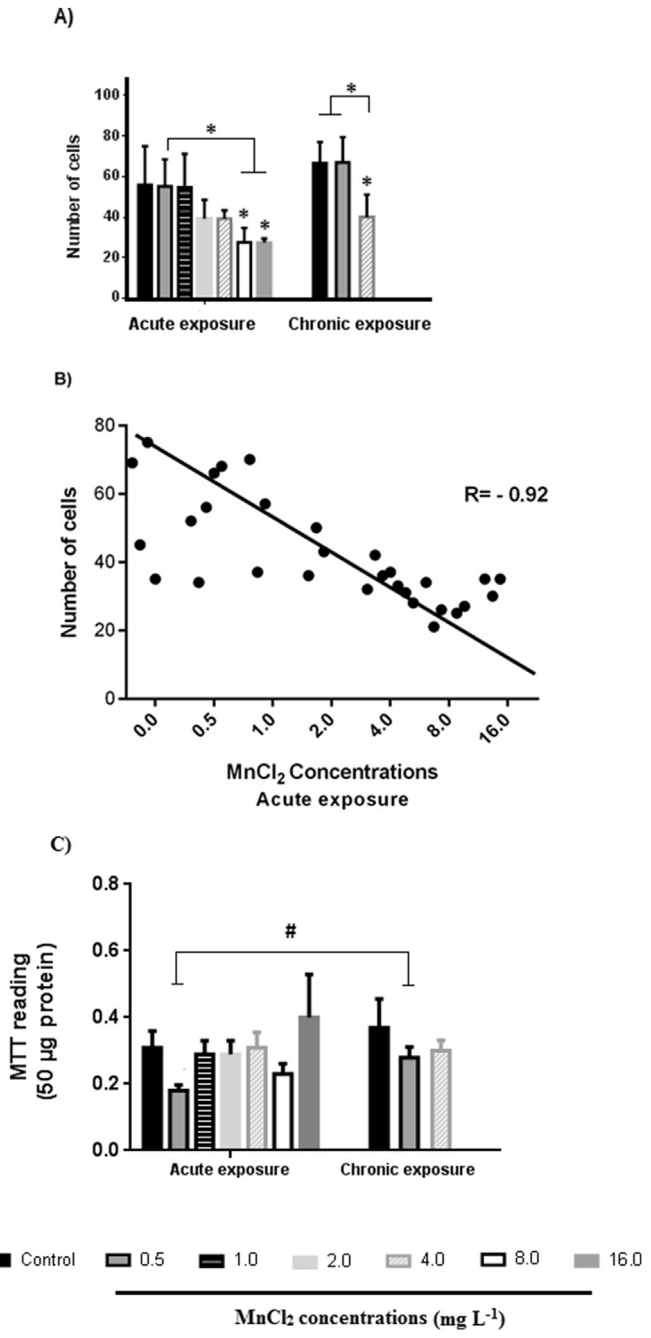

Fig. 5. A Number of cells recorded in Vd of zebrafish exposed to different $MnCl_2$ concentrations. Fig. 5B: Spearman correlation applied between $MnCl_2$ concentrations and the number of cells recorded in acute exposure animals (control n = 4; 0.5 mg L^{-1} n = 5; 1.0 and 2.0 mg L^{-1} n = 3; 4.0 and 8.0 mg L^{-1} n = 6; 16 mg L^{-1} n = 3. Fig. 5C: MTT reading (50 µg protein) of zebrafish after exposure to different concentrations of $MnCl_2$. All data expressed as mean and standard deviation. Asterisks represent statistical difference between groups within the same experiment (acute or chronic). And pound (#) represents statistical difference between animals of the same exposure concentration but from different experiments (acute and chronic).

(8.0 mg L^{-1} and 16.0 mg L^{-1}) demonstrated a significant reduction in the number of cells in Vd ($p = 0.004$) (Fig. 5A) when compared to the control group, or at animals exposed to $MnCl_2$ 0.5 mg L^{-1} ($p = 0.02$). There was a significant negative correlation ($R = -0.92$) ($p = 0.006$) between the concentrations tested and the number of cells counted (Fig. 5B), indicating that with the increase in concentration results in cell reduction. In relation to the chronic exposure, the animals exposed to 4.0 mg L^{-1} of $MnCl_2$ also differed significantly from the control group and those exposed to 0.5 mg L^{-1} of $MnCl_2$ ($p = 0.0006$ and 0.0012 respectively) (Fig. 5A). Fig. 5C details the results found in the mitochondrial telencephalic viability assay. There were no significant differences between the groups of animals from both exposures (acute and chronic) ($p = 0.13$ and 0.37 respectively). However, when comparing the animals exposed to 0.5 mg L^{-1} of $MnCl_2$ from the two experiments, a significant increase of the mitochondrial telencephalic activity was observed in the animals of the chronic experiment ($p = 0.002$).

In relation to the hepatic histological analysis, tissue damage was detected in both acute and chronic exposures in animals exposed to different concentrations of $MnCl_2$, especially cellular vacuolization, as observed in Fig. 6. Statistical analysis (Table 1) shows that the fish from the acute experiment exposed to 8.0 mg L^{-1} and 16.0 mg L^{-1} of $MnCl_2$ had a higher degree of cellular vacuolization than the control group ($p < 0.001$). However, the animals to the chronic experiment from the concentrations of 0.5 mg L^{-1} and 4.0 mg L^{-1} of $MnCl_2$ also presented a higher degree of cellular vacuolization when compared to the animals in the control group ($p = 0.0007$), confirming the sensitivity of the liver as the target organ of xenobiotics.

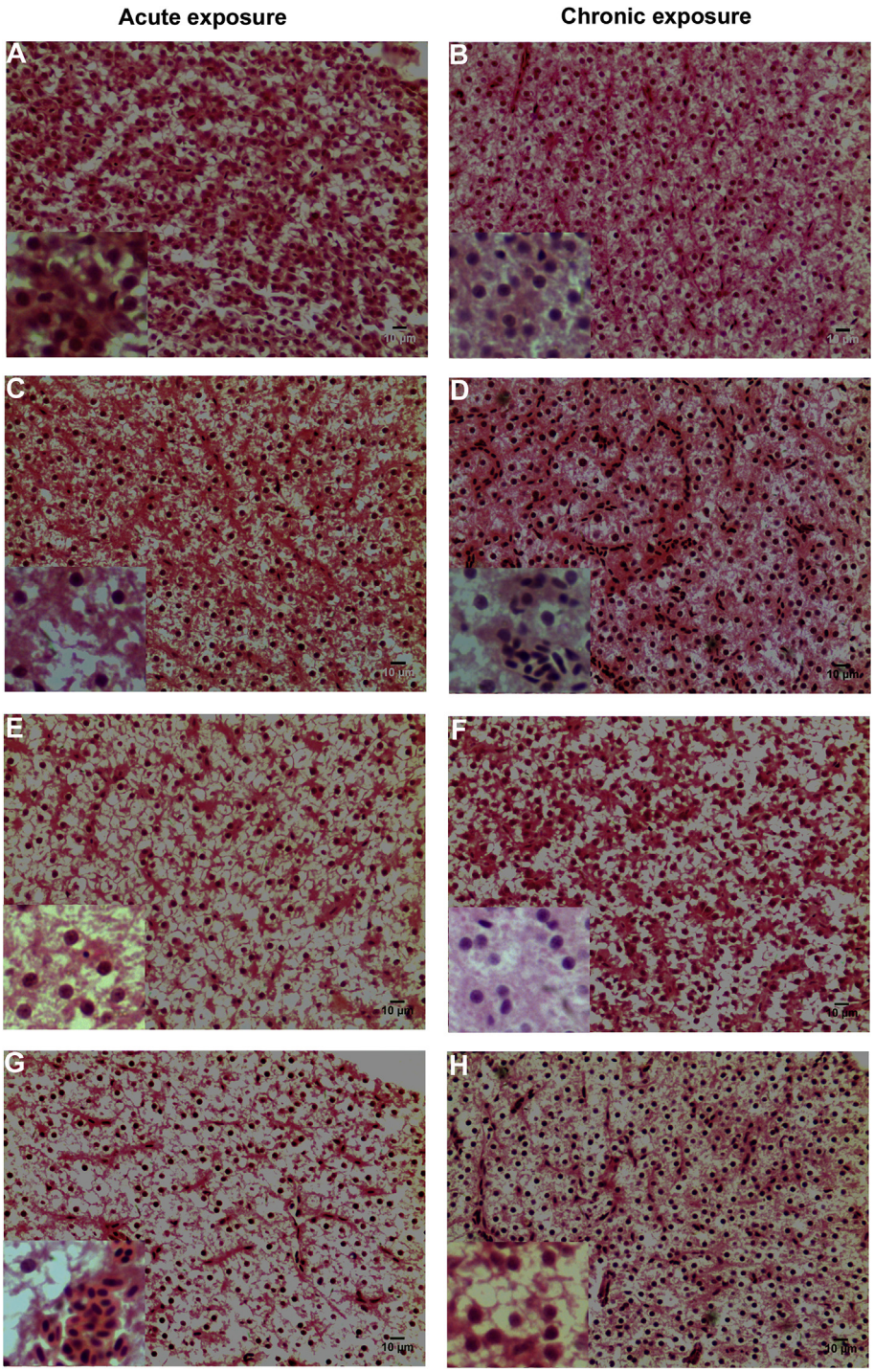

Fig. 6. Zebrafish hepatocytes exposed to different $MnCl_2$ concentrations; note comparison between acute and chronic exposures for control animals (A and B), exposed to 0.5 mg L^{-1} $MnCl_2$ (C and D) with mild degrees and severe cell vacuolation respectively, 4.0 mg L^{-1} $MnCl_2$ (E and F and H) with severe and moderate degrees of cell vacuolation respectively, and 16.0 mg L^{-1} $MnCl_2$ (G) with severe degree of vacuolization cell. In the inserts with greater maginification, we can visualize normal hepatocytes of the animals of the control group, but with some presence of vacuolization (A and B); cell degeneration (C), hyperemia (D); vacuolization-associated cell hypertrophy (F); aneurysm (G) and presence of extreme vacuolization in hepatocytes (H).

4. Discussion

This study investigated possible changes caused by concentrations and exposures that tried to simulate as much as possible the conditions found in the environment regarding manganese. In contrast to previous studies conducted with exposure to high manganese concentrations (O'neal et al., 2014; Altenhofen et al., 2017), the present study indicates that concentrations of relevance and environmental occurrence are incapable of altering motor behavior of adult fish, even after chronic exposures. However, animals chronically exposed to 4.0 mg L^{-1} of $MnCl_2$ spent less time in the top zone of the aquarium and traveled a longer distance in the bottom zone of the aquarium when compared to animals in the control group, indicating an anxiogenic behavior. Such findings

Table 1
Histological changes (value of degree) observed in zebrafish livers exposed to different concentrations of $MnCl_2$. (Data are expressed as mean ± standard deviation and different letters represent statistical difference, obtained through Kruskal-Wallis followed by Dunn's multiple comparison test).

		Control	0.5 mg L^{-1}	1.0 mg L^{-1}	2.0 mg L^{-1}	4.0 mg L^{-1}	8.0 mg L^{-1}	16.0 mg L^{-1}	p
Acute	Aneurysm	0.25 ± 0.28^{a}	0.10 ± 0.18^{a}	0.21 ± 0.32^{a}	0.15 ± 0.6^{a}	0.27 ± 0.33^{a}	0.31 ± 0.27^{a}	0.17 ± 0.20^{a}	0.94
	Hyperemia	0.38 ± 0.32^{a}	0.45 ± 0.31^{a}	0.44 ± 0.37^{a}	0.73 ± 0.06^{a}	0.24 ± 0.18^{a}	0.43 ± 0.34^{a}	0.39 ± 0.29^{a}	0.10
	Degeneration	0.18 ± 0.29^{a}	0,00^{a}	0,00^{a}	0.01 ± 0.27^{a}	0.00^{a}	0.07 ± 0.10^{a}	0.04 ± 0.05^{a}	0.06
	Vacuolization	1.36 ± 0.62^{a}	2.45 ± 0.89 ^{ab}	2.46 ± 1.16 ^{ab}	3.12 ± 0.03 ^{ab}	3.42 ± 1.51^{ab}	4.17 ± 1.08^{b}	4.16 ± 0.57 ^{b}	<0.0001
	Hypertrophy	0.42 ± 0.31^{a}	0.4 ± 0.89^{a}	0.86 ± 1.16^{a}	0.64 ± 0.03^{a}	0.48 ± 1.51^{a}	1.20 ± 1.08^{a}	0.70 ± 0.54^{a}	0.052
Chronic	Aneurysm	0,00^{a}	0.05 ± 0.12^{a}	–	–	0.06 ± 0.05^{a}	–	–	0.58
	Hyperemia	0.57 ± 0.72^{a}	2.04 ± 0.91 ^{b}	–	–	1.52 ± 0.37^{ab}	–	–	0.01
	Degeneration	0.65 ± 0.66^{a}	0.64 ± 1.05^{a}	–	–	0.08 ± 0.12^{a}	–	–	0.39
	Vacuolization	1.36 ± 0.94^{a}	4.35 ± 1.25^{b}	–	–	3.60 ± 2.28 ^{b}	–	–	0.007
	Hypertrophy	0.23 ± 0.55^{a}	0.38 ± 0.42^{a}	–	–	0.11 ± 0.19^{a}	–	–	0.53

corroborate with Altenhofen et al. (2017), where acute exposures of adult fish to high concentrations of $MnCl_2$ (90–270 mg L^{-1}) were also not sufficient to alter locomotor behavior.

Although the first available evidence in the literature relates the effects of manganese especially to locomotor deficits in humans (Couper, 1837; Mena et al., 1967; Kondakis et al., 1989), recently some studies have also shown cognitive alterations (Wasserman et al., 2011; Bjoklund et al., 2017; De Carvalho et al., 2018) in humans and rodents (Amos-Kroohs et al., 2017), which is in agreement with the main neurological pathway affected in manganese exposures; the dopaminergic (Aschner al., 2005). It is known that dopaminergic signaling regulates a wide range of physiological functions, including motor, cognitive, and memory activity (Jones and Miller, 2008; Altenhofen et al., 2017).

The same anxiogenic behavioral response was observed in studies performed with the same animal species exposed to other metals, such as nickel (Nabinger et al., 2017), arsenic (Baldissarelli et al., 2012), zinc (Sarasamma, 2018), and mercury (Pereira et al., 2016), suggesting that metal contaminants may, even at low concentrations, alter the fish behavior.

The behavioral damages mentioned above, however minor, may be a reflection of the reduction in the number of cells of the dorsal nucleus of the ventral telencephalic region, since the animals acutely exposed to 8.0 mg L^{-1} and 16.0 mg L^{-1} of $MnCl_2$ and chronically exposed to 4.0 mg L^{-1} of $MnCl_2$ showed a decrease in the number of cells in this region. In zebrafish, this region is where the tyrosine hydroxylase (TH) immuno-positive neurons are found, considered the equivalent region to the dopaminergic ones in mammals (Kaslin and Panula, 2001; Tay et al., 2011; Du et al., 2016).

TH exerts the function of metabolizing L-tyrosine in L-dopa, precursor of dopamine (Yamamoto et al., 2011), which plays a fundamental role in locomotion, cognition, emotion, and neuroendocrine function (Missale et al., 1998). Thus, changes in TH positive cells may reflect behavioral alterations. The reduction in the number of cells of the dorsal nucleus may derive from apoptosis-programmed cell death, since no evidence of necrosis was found on histological observation. In addition, studies report a significant increase in apoptotic markers induced by exposure to $MnCl_2$ in a neuronal cell line and in zebrafish (Yoon et al., 2011; Altenhofen et al., 2017).

The mechanism of cell death induced by $MnCl_2$ is still not well established, however there are hypotheses relating it to the stress of the endoplasmic reticulum and/or mitochondrial disorder (Yoon et al., 2011). In this context, cell viability was verified by the MTT assay, which in other studies (Yoon et al., 2011; Evren et al., 2015) was associated with the reduction of cell viability in neuronal cultures after acute $MnCl_2$ exposures, however, in the present study, no significant differences were observed between the groups of animals from both exposures (acute and chronic), indicating that the mechanism responsible for the neurotoxicity reported here may have no origin or connection with mitochondrial activity. However, it should be emphasized that this result can be also attributed to the use of the total telencephalon of the animals, being able to refer to other regions and cell types, masking possible changes in Vd.

In addition to the observed neurotoxicity, histopathological analysis of the livers also demonstrated damage. Cellular vacuolization in the hepatocytes was more frequently observed in the animals acutely and chronically exposed to the $MnCl_2$, which according to the protocol used for the analysis characterizes as a regressive alteration (Bernet et al., 1999), that is, that reduces the functional capacity of the organ. It may delay or decrease the $MnCl_2$ elimination and increase its relative amount in plasma (Ballatori, 2000).

Histopathological lesions in this organ have been studied by several authors as a way to evaluate the toxicity of different substances, due to its metabolizing function (Cáceres- Vélez et al., 2016; Souza et al., 2017). In other studies with zebrafish assessing toxicity of other substances, there are also reports of cell vacuolization as one of the most frequently encountered changes (Velasco-Santamaría et al., 2011; De Oliveira et al., 2015; Pereira et al., 2016). Hinton and Laurén (1990) indicate that this alteration is associated with inhibition of protein synthesis, energy depletion, microtubule disintegration, or changes in substrate utilization, and suggest the presence of regions with accumulation of lipid and glycogen inclusions, or the combination of toxic agents with intracytoplasmic lipids (Van Dyc et al., 2007).

In contrast, in mice, Huang et al. (2011) evaluated the toxicity of $MnCl_2$ (exposure by intraperitoneal injection of 6 mg L^{-1} of $MnCl_2$), and found changes in hepatic sinusoids and central veins, necrosis, and infiltration of mononuclear cells, but did not report the presence of cellular vacuolization. However, in the present study, the presence of necrosis or apoptosis in the hepatocytes, initially evaluated, is ruled out due to the absence of inflammatory cells, characteristic of necrosis, and morphologies traditionally found in apoptosis, such as cell shrinkage (Elmore, 2007). The fact is that due to liver metabolism and excretion of manganese, any hepatic dysfunction results in elevated plasma Mn levels, and consequent manganese deposition in the brain, characterizing neurotoxicity (Li et al., 2017).

In relation to the genotoxicity analysis, the present data indicate that the concentrations that are most similar to those found in the environment at present (0.5 and 4.0 mg L^{-1}) are sufficient to cause damage to zebrafish erythrocyte DNA after prolonged exposures due to the occurrence of micronuclei and nuclear abnormalities reported here, although the species has a basal rate of up to 1 micronucleus in 1000 cells (Thomé et al., 2016). Micronuclei are masses of cytoplasmic chromatin, originating from fragments or whole chromosomes left during anaphase, which reflect on structural problems or chromosomal changes during mitosis (Kampke

et al., 2018), thus detecting the clastogenic capacity of chemical substances (De Lima et al., 2016), warning the risk of prolonged exposure to manganese.

It is known that exposure time to xenobiotics is crucial to determine the frequency of genotoxicity in animals, i.e. it should be sufficient for the expression of genotoxic characteristics to occur, but not sufficient for the organism to develop a defensive mechanism (Lemos et al., 2001; Yadav et al., 2009). Therefore, 96 h of acute exposure may not have been sufficient for the occurrence of damage to the genetic material of the animals, when observing only the result of the micronucleus test. In contrast, the acute exposure of the animals to 16.0 mg L^{-1} of $MnCl_2$ resulted in an increase in DNA damage index expressed by the comet assay. This is corroborated by authors who argue that the assay is capable of detecting early and acute lesions in the DNA of target tissues, unlike the micronucleus test that corresponds to chronic cellular responses, so both tests are usually used in complementarity (Vasquez, 2009; Hariri et al., 2018).

5. Conclusions

The present study has shown that although scientific attention is usually focused on damage caused by exposures to high concentrations of pollutants or atypical situations where environmental contamination is extreme, such as in environmental disasters, contaminations caused by metals in low concentrations, should be studied and observed with greater care. Manganese (II), typically associated with neurodegenerative diseases, in humans living in regions with soil and water contaminated by high concentrations, as well as in animals, caused damage in all organs studied, reflecting even behavioral and DNA changes, especially after prolonged exposure to concentrations typically found in the environment. It should be noted that given the high toxicity of such metal, it is necessary to understand the mechanisms of action responsible for such threats, as well as whether these damages could affect subsequent generations or be reversed after possible decontamination or treatment of polluted environments. The study reinforces and encourages other authors to better describe the environmental toxicity caused by other metals as well as for further understanding of the effects caused by the synergism of these substances in the environment.

Declarations of interest

None.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This work received financial support from Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul – FAPERGS, Feevale University and Conselho Nacional de Desenvolvimento Científico e Tecnológico –CNPq through the granting of masters, doctorate and undergraduate scholarships. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - finance code 001.

Author contributions

Gabriela Zimmermann Prado Rodrigues: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Roles/Writing - original draft; Writing - review & editing; Luciane Beatris Mentges Staudt: Methodology; Marina Griebeler Moreira: Methodology.; Thainá Garbino dos Santos: Methodology; Data curation; Mateus Santos de Souza: Methodology; Data curation; Caio Júnior Lúcio: Methodology; Jenifer Panizzon: Methodology; Juliana Machado Kayser: Methodology; Leonardo Airton Ressel Simões: Methodology; Ana Luiza Ziulkoski: Investigation; Methodology; Data curation; Carla Denise Bonan: Investigation; Methodology; Data curation; Diogo Losch de Oliveira: Investigation; Methodology; Data curation; Günther Gehlen: Investigation; Methodology; Data curation; Project administration; Supervision; Validation.

References

Altenhofen, S., Wiprich, M.T., Nery, L.R., Leite, C.E., Vianna, M.R.M.R., Bonan, C.D., 2017. Manganese(II) chloride alters behavioral and neurochemical parameters in larvae and adult zebrafish. Aquat. Toxicol. 182, 172–183. https://doi.org/10.1016/j.aquatox.2016.11.013.

Amos-Kroohs, R.M., Davenport, L.L., Atanasova, N., Abdulla, Z.I., Skelton, M.R., Vorhees, C.V., Williams, M.T., 2017. Developmental manganese neurotoxicity in rats: cognitive deficits in allocentric and egocentric learning and memory. Neurotoxicol. Teratol. 59, 16–26. https://doi.org/10.1016/j.ntt.2016.10.005.

Aschner, M., Erikson, K.M., Dorman, D.C., 2005. Manganese dosimetry: species differences and implications for neurotoxicity. Crit. Rev. Toxicol. 35, 1–32.

Baldissarelli, L.A., Capiotti, K.M., Bogo, M.R., Ghisleni, G., Bonan, C.D., 2012. Arsenic alters behavioral parameters and brain ectonucleotidases activities in zebrafish (*Danio rerio*). Comp. Biochem. Physiol. 155, 556–572. https://doi.org/10.1016/j.cbpc.2012.01.006.

Ballatori, N., Zalups, R.K., Koropatnick, J., 2000. Molecular Mechanisms of hepatic metal transport. Molecular Biology and Toxicology of Metals. Taylor & Francis, Nova York, pp. 346–381.

Bernet, D., Schmidt, H., Meier, W., Burkhardt- Holm, P., Wahli, T., 1999. Differential modulation of cellular antioxidant status in zebrafish liver and kidney exposed to low dose arsenic trioxide. J. Fish Dis. 22, 25–34. https://doi.org/10.1016/j.ecoenv.2016.09.025.

Bianchi, et al., 2019. Ecohydrol. Hydrobiol. https://doi.org/10.1016/j.ecohyd.2019.05.002.

Bjorklund, G., Chartrand, M.S., Aaseth, J., 2017. Manganese exposure and neurotoxic effects in children. Environ. Res. 155, 380–384. https://doi.org/10.1016/j.envres.2017.03.003.

Bouchard, M.F., Surette, C., Cormier, P., Foucher, D., 2018. Low level exposure to manganese from drinking water and cognition in school-age children. Neurotoxicology (Little Rock) 64, 110–117. https://doi.org/10.1016/j.neuro.2017.07.024.

BRASIL. National Environment Council - CONAMA. Resolucao CONAMA nº 357, de 17 de marco de 2005. Dispoe sobre a classificacao dos corpos de agua e diretrizes ambientais para o seu enquadramento, bem como estabelece as condicoes e padroes de efluentes, e da outras providencias. Diario Oficial da Republica Federativa do Brasil, Brasilia, 18 mar. pp. 58-63. Disponivel em: <http://www.mma.gov.br/port/conama/res/res05/res35705.pdf> Acesso em: 05/05/2018.

Cáceres- Vélez, P.R., Fascineli, M.L., Grisolia, C.K., Lima, E.C. de O., Sousa, M.H., Morais, P.C. de, Azevedo, R.B. de, 2016. Genotoxic and histopathological biomarkers for assessing the effects of magnetic exfoliated vermiculite and exfoliated vermiculite in *Danio rerio*. Sci. Total Environ. 551–552, 228–237. https://doi.org/10.1016/j.scitotenv.2016.01.048.

Couper, J., 1837. On the effects of black oxide of manganese when inhaled in the lungs. British Annals of Medicine 1, 41–42.

Dalzochio, T., Goldoni, A., Rodrigues, G.Z.P., Petry, I.E., Silva, L.B., Gehlen, G., 2016. Gill histopathology and micronucleus test of *Astyanax jacuhiensis* (Cope, 1894) (Teleostei, Characidae) to evaluate effects caused by acute exposure to aluminum. Biotemas 29, 75–83. https://doi.org/10.5007/2175-7925.2016v29n1p75.

De Carvalho, C.F., Oulhote, Y., Martorelli, M., De Carvalho, C.O., Menezes- Filho, J.A., Argollo, N., Abreu, N., 2018. Environmental manganese exposure and associations with memory, executive functions, and hyperactivity in Brazilian children. Neurotoxicology (Little Rock) 69, 253–259. https://doi.org/10.1016/j.neuro.2018.02.002.

De Lima, F.F., Menegati, S.E.L.T., Traesel, G.K., De Araújo, F.H.S., Lescano, C.H., Peixoto, S.M., Silva, F.A.M., Vieira, S.C.H., Vieira, M.C., Oesterreich, S.A., 2016. Study on the cytotoxic, genotoxic and clastogenic potential of *Attalea phalerata mart*. Ex spreng. Oil pulp in vitro and in vivo experimental models. PLoS One 11, e0165258. https://doi.org/10.1371/journal.pone.0165258.

De Oliveira, L.B., Leonardo, A.S., De Lima, E.M.M., 2015. Macro- and microstructural descriptions of the zebrafish (*Danio rerio*) liver. Folia Morfologia 75, 382–387. https://doi.org/10.5603/FM.a2016.0009.

Du, Y., Guo, Q., Shan, M., Wu, M., Huang, S., Zhao, H., Hong, H., Yang, M., Yang, X., Ren, L., Peng, J., Zhou, H., Li, S., Su, B., 2016. Spatial and temporal distribution of dopaminergic neurons during development in zebrafish. Front. Neuroanat. 10, 1–7. https://doi.org/10.3389/fnana.2016.00115.

Elmore, S., 2007. Apoptosis: a review of programmed cell death. Toxicologic pathology. Toxicol. Pathol. 35, 495–516. https://doi.org/10.1080/01926230701320337.
Erikson, K.M., Aschner, M., 2003. Manganese neurotoxicity and glutamate-GABA interaction. Neurochem. Int. 43, 475–480. https://doi.org/10.1016/s0197-0186(03)00037-8.
Erikson, K.M., Syversen, T., Aschner, J.L., Aschner, M., 2005. Interactions between excessive manganese exposures and dietary iron-deficiency in neurodegeneration. Environ. Toxicol. Pharmacol. 19, 415–421. https://doi.org/10.1016/j.etap.2004.12.053.
Evren, V., Apaydin, M., Khalilnezhad, A., Erbaş, O., Taskiran, D., 2015. Protective effect of edaravone against manganese-induced toxicity in cultured rat astrocytes. Environ. Toxicol. Pharmacol. 40, 563–567. https://doi.org/10.1016/j.etap.2015.08.010.
Fenech, M., Crott, J., Turner, J., Brown, S., 1999. Necrosis, apoptosis, cytostasis and DNA damage in human lymphocytes measured sumultaneously whithin the cytokinesis- block micronucleus assay: description of the method and results for hydrogen peroxide. Mutagenesis 14, 605–612. https://doi.org/10.1093/mutage/14.6.605.
Golub, M.S., Hogrefe, C.E., Germann, S.L., Tran, T.T., Beard, J.L., Crinella, F.M., Lonnerdal, B., 2005. Neurobehavioral evaluation of rhesus monkey infants fed cow's milk formula, soy formula, or soy formula with added manganese. Neurotoxicol. Teratol. 27, 615–627. https://doi.org/10.1016/j.ntt.2005.04.003.
Hariri, M., Mirvaghefi, A., Farahmand, H., Taghavi, L., Shahabinia, A.R., 2018. In situ assessment of Karaj River genotoxic impact with the alkaline comet assay and micronucleus test, on feral brown trout (*Salmo trutta fario*). Environ. Toxicol. Pharmacol. 58, 59–69. https://doi.org/10.1016/j.etap.2017.12.024.
Hinton, D.E., Laurén, D.J., 1990. Liver structural alterations accompanying chronic toxicity in fishes: potential biomarkers of exposure. In: McCarthy, J.F., Shugart, L.R. (Eds.), Biomarkers of Environmental Contamination. Lewis Publishers, Boca Raton, pp. 51–65. https://doi.org/10.1201/9781351070263-4.
Horn, A.C.M., Rasia-Filho, A., 2018. The cytoarchitecture of theTelencephalon of *Betta splendens* Regan1910 (perciformes: anabantoidei) with a stereological approach to the supracommissural and postcommissural nuclei. Anat. Rec. 301, 88–110. https://doi.org/10.1002/ar.23699.
HSDB, 2001. Manganese Compounds. National Library of Medicine, Hazardous Substances Data Bank, Bethesda, MD. Available at: http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB.
Huang, P., Chen, C., Wang, H., Li, G., Jing, H., Han, Y., Liu, N., Xiao, Y., Yu, Q., Liu, Y., Wang, P., Shi, Z., Sun, Z., 2011. Manganese effects in the liver following subacute or subchronic manganese chloride exposure in rats. Ecotoxicol. Environ. Saf. 74, 615–622. https://doi.org/10.1016/j.ecoenv.2010.08.011.
Hurley, L.S., Keen, C.L., 1987. In: Underwood, Manganese E., Mertz, W. (Eds.), Trace Elements in Human Health and Animal Nutrition. Academic Press, New York, pp. 185–225.
Instituto Brasileiro de Geografia e Estatística – IBGE, 2010. Indicadores de Desenvolvimento Sustentável – Brasil. Disponível em: http://www.ibge.gov.br/home/geociencias/recursosnaturais/ids/default_2010.shtm Cited in Feb. 20th 2019.
ISO International Organization for Standardization, 1996. Water quality - determination of the acute lethal toxicity of substances to a freshwater fish [Brachydanio rerio Hamilton-Buchanan (Teleostei, Cyprinidae)]. ISO 7346-3: Flow-through method, Available: http://www.iso.org.
Jones, D.C., Miller, G.W., 2008. The effects of environmental neurotoxicants on the dopaminergic system: a possible role in drug addiction. Biochem. Pharmacol. 76, 569–581. https://doi.org/10.1016/j.bcp.2008.05.010.
Kampke, E.H., Barroso, M.E.S., Marques, F.M., Fronza, M., Scherer, R., Lemos, M.F., Campagnaro, B.P., Gomes, L.C., 2018. Genotoxic effect of *Lippia alba* (Mill.) N. E. Brown essential oil on fish (*Oreochromis niloticus*) and mammal (*Mus musculus*). Environ. Toxicol. Pharmacol. 59, 163–171. https://doi.org/10.1016/j.etap.2018.03.016.
Kaslin, J., Panula, P., 2001. Comparative anatomy of the histaminergic and other aminergic systems in zebrafish (*Danio rerio*). J. Comp. Neurol. 440, 342–377. https://doi.org/10.1002/cne.1390.
Kondakis, X.G., Makris, N., Leotsinidis, M., Prinou, M., Papapetropoulos, T., 1989. Possible health effects of high manganese concentrations in drinking water. Arch. Environ. Occup. Health 44, 175–178. https://doi.org/10.1080/00039896.1989.9935883.
Lemos, C.T. de, Rödel, P.M., Terra, N.R., Erdtmann, B., 2001. Evaluation of basal micronucleus frequency and hexavalent chromium effects in fish erythrocytes. Environ. Toxicol. 20, 1320–1324. https://doi.org/10.1897/1551-5028(2001)020<1320:eobmfa>2.0.co;2.
Li, Y., Mei, L.H., Qiang, J.W., Xue, C., Ju, S., 2017. Reduction of manganese intake improves neuropsychological manifestations in rats with minimal hepatic encephalopathy. Neuroscience 347, 148–155. https://doi.org/10.1016/j.neuroscience.2017.02.004.
Mena, I., 1980. Manganese. In: Waldron, H.A. (Ed.), Metals in the Environment, first ed. Academic Press, London, pp. 199–220.
Mena, I., Marin, O., Fuenzalida, S., Cotzias, G.C., 1967. Chronic manganese poisoning. Clinical picture and manganese turnover. Neurology 17, 128–136. https://doi.org/10.1212/wnl.17.2.128.
Meyer, C.M.C., Rodríguez, J.M., Carpio, E.A., García, P.A., Stengel, C., Berg, M., 2017. Arsenic, manganese and aluminum contamination in groundwater resources of Western Amazonia (Peru). Sci. Total Environ. 607–608, 1437–1450. https://doi.org/10.1016/j.scitotenv.2017.07.059.
Missale, C., Nash, S.R., Robinson, S.W., Jaber, M., Caron, M.G., 1998. Dopamine receptors: from structure to function. Physiol. Rev. 78, 189–225. https://doi.org/10.1152/physrev.1998.78.1.189.
Nabinger, D.D., Altenhofen, S., Bitencourt, P.E.R., Nery, L.R., Leite, C.E., Vianna, M.R.M.R., Bonan, C.D., 2017. Nickel exposure alters behavioral parameters in larval and adult zebrafish. Sci. Total Environ. 624, 1623–1633. https://doi.org/10.1016/j.scitotenv.2017.10.057.
Nascimento, C.A., Staggemeier, R., Bianchi, E., Rodrigues, M.T., Fabres, R., Soliman, M.C., Bortoluzzi, M., Luz, R.B., Heinzelmann, L.S., Santos, E.L., Fleck, J.D., Spilki, F.R., 2015. Monitoring of metals, organic compounds and coliforms in water catchment points from the Sinos River basin. Braz. J. Biol. 75, 50–56. https://doi.org/10.1590/1519-6984.1613.
Olanow, C.W., 2004. Manganese-induced parkinsonism and Parkinson's disease. Ann. N. Y. Acad. Sci. 1012, 209–223. https://doi.org/10.3390/ijerph120707519.
O'neal, S.L., Lee, J.W., Zheng, W., Canhão, J.R., 2014. Subacute manganese exposure in rats is a neurochemical model of early manganese toxicity. Neurotoxicology (Little Rock) 44, 303–313. https://doi.org/10.1016/j.neuro.2014.08.001.
Patil, D.S., Chavan, S.M., Oubagaranadin, J.U.K., 2016. 2016. A review of technologies for manganese removal from wastewaters. J. Environ. Chem. Eng. 468–487. https://doi.org/10.1016/j.jece.2015.11.028.
Pereira, P., Puga, S., Cardoso, V., Pinto-Ribeiro, F., Raimundo, J., Barata, M., Pousão-Ferreira, P., Pacheco, M., Almeida, A., 2016. Inorganic mercury accumulation in brain following waterborne exposure elicits a deficit on the number of brain cells and impairs swimming behavior in fish (white seabream—*Diplodus sargus*). Aquat. Toxicol. 400–412. https://doi.org/10.1016/j.aquatox.2015.11.031.
Pitarque, M., Creus, A., Marcos, R., 1999. Examination of various biomarkers measuring genotoxic endpoints from Barcelona airport personnel. Mutat. Res. 440, 195–204. https://doi.org/10.1016/S1383-5718(99)00026-1.
Rietzler, A.C., Fonseca, A.L., Lopes, G.P., 2001. Heavy metals in tributaries of Pampulha reservoir, Minas Gerais. Braz. J. Biol. 61, 363–370. https://doi.org/10.1590/S1519-69842001000300004.
Sarassama, S., 2018. Effects of zinc chloride on Zebrafish locomotion, circadian rhythm, social behavior and memory: correlation between toxicity and neurobehavior changes. Toxicol. Lett. 295, S266. https://doi.org/10.3390/ijms19103195.
Sarkar, A., Shekhar, S., 2018. Iron contamination in the waters of Upper Yamuna basin. Groundwater Sustain. Dev. 7, 421–429. https://doi.org/10.1016/j.gsd.2017.12.011.
Souza, J.P., Baretta, J.F., Santos, F., Paino, I.M.M., Zucolotto, V., 2017. Toxicological effects of graphene oxide on adult zebrafish (*Danio rerio*). Aquat. Toxicol. 186, 11–18. https://doi.org/10.1016/j.aquatox.2017.02.017.
Subotic, S., Visnjié-Jeftic, Z., Spasic, S., Hegedis, A., Krpo-Cetkovic, J., Lenhardt, M., 2015. Concentrations of 18 elements in muscle, liver, gills, and gonads of sichel (*Pelecus cultratus*), ruffe (*Gymnocephalus cernua*), and European perch (*Perca fluviatilis*) in the danube river near Belgrade (Serbia). Water Air Soil Pollut. 226, 1–11. https://doi.org/10.1007/s11270-015-2544-x.
Superville, P.J., Ivanovsky, A., Bhurtun, P., Prygiel, J., Billon, G., 2018. Diel cycles of reduced manganese and their seasonal variability in the Marque River (northern France). Sci. Total Environ. 624, 918–925. https://doi.org/10.1016/j.scitotenv.2017.12.189.
Tay, T.L., Ronneberger, O., Ryu, S., Nitschke, R., Driever, W., 2011. Comprehensive catecholaminergic projectome analysis reveals single-neuron integration of zebrafish ascending and descending dopaminergic systems. Nat. Commun. 171, 1–12. https://doi.org/10.1038/ncomms1171.
Thomé, R.G., Silva, P.M. da, Santos, H.B.dos, 2016. Avaliação de Genotoxidade da Água de um Rio Urbano Utilizando Estudo de Células Sanguíneas de *Danio rerio*. Conexão Ciência 11, 9–16. https://doi.org/10.24862/cco.v11i2.415.
Valesco- Santamaría, Y.M., Handy, R.D., Sloman, K.A., 2011. Endosulfan affects health variables in adult zebrafish (*Danio rerio*) and induces alterations in larvae development. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 153, 372–380. https://doi.org/10.1016/j.cbpc.2011.01.001.
Van Dyc, J.C., Pieterse, G.M., Van Vuren, J.H.J., 2007. Histological changes in the liver of *Oreochromis mossambicus* (Cichlidae) after exposure to cadmium and zinc. Ecotoxicol. Environ. Saf. 66, 432–440. https://doi.org/10.1016/j.ecoenv.2005.10.012.
Vasquez, M.Z., 2009. Combining the in vivo comet and micronucleus assays: a practical approach to genotoxicity testing and data interpretation. Mutagenesis 25, 187–199. https://doi.org/10.1093/mutage/gep060.
Wasserman, G.A., Liu, X., Parvez, F., Factor-Litvak, P., Ahsan, H., Levy, D., Kline, J., Van Geen, A., Mey, J., Slavkovich, V., Siddique, A.B., Islam, T., Graziano, J.H., 2011. Arsenic and manganese exposure and children's intellectual function. Neurotoxicology (Little Rock) 32, 450–457. https://doi.org/10.1016/j.neuro.2011.03.009.
Wullimann, M.F., Rupp, B., Reichert, H., 1996. Neuroanatomy of the Zebrafish Brain. Birkhäuser.
Xavier, L.L., Viola, G.G., Ferraz, A.C., Da Cunha, C., Deonizio, J.M.D., Netto, C.A., Achaval, M., 2005. A simple and fast densitometric method for the analysis of tyrosine hydroxylase immunoreactivity in the substantia nigra pars compacta and in the ventral tegmental area. Brain Res. 16, 58–64. https://doi.org/10.1016/j.brainresprot.2005.10.002.
Yadav, K., Trivedi, S.P., 2009. Sublethal exposure of heavy metals induces micronuclei in fish, *Channa punctate*. Chemosphere 77, 1495–1500. https://doi.org/10.1016/j.chemosphere.2009.10.022.
Yamamoto, K., Vernier, P., 2011. The evolution of dopamine systems in chordates. Institute of Neurobiology Alfred Fessard, CNRS, Gif-sur-Yvette, France. Front. Neuroanat. https://doi.org/10.3389/fnana.2011.00021.

Yoon, H., Kim, D., Lee, G., Kim, K., Kim, H., Chae, H., 2011. Apoptosis induced by manganese on neuronal SK-N-MC cell line: endoplasmic reticulum (ER) stress and mitochondria dysfunction. Environ. Health Toxicol. 26, 1–7. https://doi.org/10.5620/eht.2011.26.e2011017.

Yousafzai, A.M., Chivers, D.P., Khan, A.R., Ahmad, I., Siraj, M., 2010. Comparison of heavy metals burden in two freshwater fishes *Wallago attu* and *Labeo yocheilus* with regard to to their feeding habits in natural ecosystem. Pak. J. Zool. 42, 537–544.