Environmentally relevant lead (Pb) water concentration induce toxicity in zebrafish (*Danio rerio*) larvae

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Abstract

Early developmental stages of aquatic organisms including fish are inherently vulnerable to lead (Pb) and other water metal contaminants. However, reports on the deleterious effects of environmentally relevant Pb levels are limited. To this end, we exposed 2.5 hours post fertilization (hpf) old zebrafish (*Danio rerio*) embryos to a range of Pb concentrations encompassing environmentally relevant levels (1, 10, 25, 50 and 100 µg/L Pb) until 96 hpf. Exposure negatively impacted the development and survival of zebrafish embryos by inducing embryo coagulation related mortalities in a concentration-dependent manner. At 24 hpf, the highest level of exposure (100 µg/L Pb) had impaired embryo activity characterized by reduced burst activity and the number of movements per minute made by embryos. At 72 hpf, newly hatched larvae exhibited adverse cardiovascular effects (100 µg/L Pb group) and neuromuscular effects (50 and 100 µg/L Pb groups). The antioxidant system dysregulation evidenced by downregulation of catalase, and upregulation of mRNA expression of glutathione S-transferase and cytochrome oxidase subunit I were observed. The pro-apoptotic tumor protein P53 (*TP53*) and the anti-apoptotic B cell lymphoma -2 (*Bcl-2*) mRNA expression levels were also affected. The former was downregulated across exposed groups and the latter was upregulated and downregulated in the groups with Pb concentrations less than 50 µg/L Pb and downregulated in 50 µg/L Pb, respectively. These findings suggest that Pb within environmentally relevant levels may be deleterious to developing zebrafish.

Keywords: zebrafish; lead; cardiovascular; apoptosis; twitching

1. Introduction

Lead (Pb) is a metal that naturally occurs in small amounts in the environment (Hailegnaw et al., 2015). High quantities of Pb in the environment are traceable to anthropogenic activities such as mining, Pb products production processes and their use (Komárek et al., 2008). An example of a heavily Pb polluted town with mining activities as a source is Kabwe, Zambia following 9 decades of unregulated waste management at the now closed lead-zinc mine (Yabe et al., 2018). The extensive Pb pollution of the Kabwe town has plunged the majority of its residents into chronic Pb poisoning especially children having blood Pb levels above the minimum Pb reference value of 5 µg/dL (Yabe et al., 2020, 2015). Domesticated animals, free-range chickens including free-roaming dogs around the region of the closed mine have been found with remarkable Pb levels in blood (Nakayama et al., 2011; Toyomaki et al., 2020; Yabe et al., 2013, 2011). Moreover, water sampled from natural water bodies and boreholes inside the region of the closed mine was found to have a Pb concentration range of 0.1- 94 µg/L against the country's regulatory limit of 50 µg/L Pb in water (Nachiyunde et al., 2013).

Lead exposure to developing fish embryos has been linked to undesirable effects including delayed hatching, premature hatching, and malformations of larvae, which leads to mortalities (Jezierska et al., 2009). In addition, Pb poisoning causes an imbalance of the antioxidants and eventual dysregulation of the antioxidant system (Kim and Kang, 2017). Studies have demonstrated that the antioxidant system dysregulation through increased generation of reactive oxygen species (ROS) is the major cause of oxidative-induced damage in fish exposed to Pb (Kim and Kang, 2017). A review paper citing Pb exposure studies revealed that early developmental stages of fish are more sensitive to

Pb-induced toxicity (Sfakianakis et al., 2015). However, the Pb exposure concentrations in most of the cited studies ranged from 100 to 10000 µg/L (Sfakianakis et al., 2015), which may not reflect the real prevailing environmental Pb levels.

Altered swimming behaviour accompanied by an increased oxidative stress response in larval zebrafish following acute exposure to water Pb concentrations in Kabwe, Zambia has been reported (Kataba et al., 2020). However, a dearth of data on the impacts of these Pb levels on the cardiovascular, neuromuscular and ROS-induced toxicity on the early developmental stages of fish exists. Moreover, to the best of our knowledge, the environmentally relevant Pb-induced neuromuscular toxicity in hatched embryos from the 50 and 100 µg/L Pb level of exposure has never been reported before. To bridge this information gap, we investigated the undesirable effects of the Kabwe water Pb levels on fish health by means of the zebrafish embryo toxicity testing (FET) protocol. Zebrafish (*Danio rerio*) has been known to be an ideal model for toxicological investigations owing to its morphological, biochemical and physiological data that is obtainable in early life stages such as embryos (Hill et al., 2005). Furthermore, the zebrafish lifecycle that can be managed in a laboratory environment, the embryo's ability to absorb compounds in water (Yin et al., 2017) and the shared sensitivity range with other fish species endemic to Africa (Botha et al., 2015) made the zebrafish embryos a choice model for the present study. The FET test was employed to investigate: 1) how safe are the water Pb levels reported from Kabwe, Zambia (range 0.1 - 94 µg/L Pb) on early developing stages of fish?; and 2) is the country's regulatory limit for Pb in water (50 µg/L) as reported by Nachiyunde et al., (2013) conducive to support early developmental stages of fish?

99 ## 2. Materials and Methods

100 ### 2.1. Fish husbandry and embryo collection

101 Wild-type zebrafish breeding stock kept at 26 – 28ºC on a 14-hr light and 10-hr dark cycle
102 in a ZebTec (Tecniplast, Italy) system at the North-West University's National Aquatic
103 Bioassay Facility (NABF), South Africa were used to breed fertilized embryos. All the
104 experiments conducted following and in strict adherence to research guidelines mandated
105 by the North-West University AnimCare Ethics Committee (Approval number NWU-
106 00269-16-A5). The breeding process and the collection of embryos were carried out as
107 previously described by Kataba et al. (2020).

108 ### 2.2. Lead (Pb) stock solution preparation and concentrations selection

109 A 10 mg/L Pb stock solution was prepared using pure grade (99.5%) lead acetate
110 trihydrate in lead-free ultrapure water. From the Pb stock solution, five dilutions (1, 10,
111 25, 50, and 100 µg/L) were prepared using the embryo development medium (each litre
112 contains 0.875 g NaCl, 0.038 g KCl, 0.120 g $MgSO_4$, 0.021 g KH_2PO_4, and 0.006 g
113 Na_2HPO_4) constituted in deionized water (pH 8.2). The selections of the first four
114 exposure concentrations (1-50 µg/L) were chosen to reflect the range reported in water
115 samples from Kabwe, Zambia and the permissible water Pb level by the national authority
116 body (Nachiyunde et al., 2013). The 100 µg/L Pb concentration was included in
117 adherence with the Fish Embryo toxicity (FET) test protocol recommended by the
118 Organisation for Economic Co-operation and Development (OECD) test guidelines (TG
119 236) for the testing of Chemicals (Busquet et al., 2014).

120

121 ### 2.3 Fish Embryo Toxicity (FET) test and embryo activity analysis

122 Fertilized zebrafish embryos (2.5 hpf old) within an early developmental stage,
123 following sorting under a Zeiss stemi microscope, were assigned to 5 concentrations of
124 Pb at selected concentrations (0, 1, 10, 25, 50, and 100 µg/L) diluted with the embryo
125 media in plastic 6-well plates (total volume 5000 µL/ well). A positive control with 3,4-
126 dichloroaniline test solution was prepared. Six well plate replicates (n = 5 embryos per
127 well plate) for the control with 30 total embryos, five replicates of total 25 embryos per
128 treatment (1-100µg/L) were performed without renewal of the treatment solutions. The
129 plates were covered with self-adhesive, oxygen-permeable sealing film (BRAND®,
130 Sigma Alrich) to prevent evaporation of the test solution. The embryos were incubated at
131 28 °C for 96 h and their morphological condition was monitored every 24 h interval.
132 Normal embryo morphology referencing was as described by Kimmel et al., (1995) and
133 any dead or coagulated embryos were recorded and removed from the test plate. The six
134 control replicates numbers (30 embryos) used were in line with the OECD guidelines (TG
135 236) for the testing of chemicals (Busquet et al., 2014).

136 ### 2.4 Sub-lethal embryo activity, cardiology and twitching

137 A non-invasive video recording technique of assessing embryos within their chorions or
138 hatched larvae were used. To assess embryo activity at 24 hpf, movements of embryos
139 within test solutions were recorded for 1 min using a remote-controlled stereomicroscope
140 (Zeiss, Germany) connected to a camera. Videos from 8 randomly selected individual
141 embryos per replicates were assessed using DanioScope V1 software (Zeiss, Germany).

142 The burst activity and the burst count/min were computed as a representation of embryo
143 activity. The mean burst activity represents the percentage of time (from total

144 measurement duration) the embryo was moving, and the burst count/per minute
145 represents the number of movements per minute.

146 At 72 hpf, blood flow and heart rate were assessed using 6 newly hatched larvae per group
147 (n = 6) that were randomly selected using video recording followed by analysis.
148 Individual zebrafish larvae were picked with a pipette and placed in a drop of the exposure
149 media on a glass slide and videos were taken using a stereomicroscope (Zeiss, Germany)
150 using a remote-controlled microscope camera. Heart rate videos were taken for 30
151 seconds with the heart in view while the larvae lay in lateral recumbence in the exposure
152 media in a temperature-controlled room at the same used during the exposure **period**. The
153 videos were imported in DanioScope and automatically the numbers of beats per minute
154 (BPM) were calculated. The blood flow analysis video recordings were taken by focusing
155 on the caudal artery caudal to the anal pore in view for 30 seconds. The blood flow was
156 presented as an activity percentage.

157 The muscular activity of the larvae was assessed using video recordings which were later
158 analyzed as an indicator of twitching movements. The full video was used, and
159 movements were confirmed by watching the video as well as action potential output
160 graphs. A peak of the action potential indicated a twitch count, and the action potential
161 peak width represented the duration of the twitch over the 60 second time interval. The
162 raw data was exported and used for time scale determinations (DanioScope V1 Software,
163 Noldus Information Technology, Wageningen, Netherlands). In the present study, only
164 action potentials with an amplitude of 5% and above were classified as muscular twitche**s**
165 (Fig. 3 C - H). Normal swimming or pectoral fin movement of larval zebrafish was not

166 considered as twitch activity**.** Fig. SV1 shows the representative larvae video recordings
167 for each Pb exposure level that were included in the current study.

168 **2.5. RNA extraction and real-time PCR analysis**

169 At the end of the exposure period (96 hpf), hatched embryos (larvae) from the
170 different groups were sacrificed using ice-cold embryo media and 5-10 larvae were
171 collected as a pooled sample and immediately preserved in RNA Later solution (SIGMA
172 Life Science, St. Louis, MO, USA) at -80°C prior to their transportation to Hokkaido
173 University, Japan. Following the sample transportation using a cold chain system on dry
174 ice to the Laboratory of Toxicology, Faculty of Veterinary Medicine, Hokkaido
175 University the samples were stored at -80°C until RNA extraction. The total RNA was
176 extracted from 5-10 pooled zebrafish hatched embryos and the cDNA synthesis was done
177 using the TOYOBO cDNA kit (TOYOBO Co., Ltd., Life Science Department, Osaka**,**
178 Japan**)**. The quantitative reverse transcription polymerase chain reaction conditions used
179 **were** as previously described by Kataba et al. (2020). The primer sets used shown in
180 Table S1 and all primers underwent validation and were obtained as described **by** Kataba
181 et al. (2020). The normalization and the mRNA expression levels calculations was done
182 using the comparative ($2^{-\Delta\Delta Ct}$) method.

183 **2.6. Verification of Pb in exposure concentrations**

184 The nominal exposure concentrations that were prepared (0, 1, 10, 25, 50, and 100
185 µg/L) and used in the current study were verified using the inductively coupled plasma
186 mass spectrometry (ICP-MS). Aliquots of freshly prepared Pb exposure concentrations in
187 which zebrafish embryos and larvae were reared during the exposure period were used
188 for verification by two independent analysts. The sample treatment and analysis were

done as previously described by Kataba et al. (2020). Recoveries from freshly prepared Pb solutions ranged from 97 to 110 % with actual concentrations of 0, 1.1, 9.7, 25.2, 49.6, and 100.7 µg/L Pb, respectively.

2.7. Data Analysis

The FET test data statistics were generated from the TOXRAT® software. GraphPad Prism software (Prism 7 for Windows; Version 5.02, California USA) was used to perform the rest of the data analysis including embryo activity, blood flow, heart rate, twitching and gene expression. The data were first tested for normality using Kolmogorov–Smirnov test. For normally distributed data, an analysis of variance (one-way ANOVA) and the differences among test groups were assessed using Tukey's test. A non-parametric Kruskal–Wallis test followed by a Mann–Whitney test (all other comparisons) was applied for non-normally distributed data were used. The data were reported as mean and standard error of the mean (SEM). The hatching and overall survival rates proportions differences between the exposed and control groups were analysed by MedCalc® which uses the "N-1" Chi-squared test (Campel, 2007). The difference between groups was considered at two levels of significance and was marked at $p < 0.05$ (*) and at $p < 0.01$ (**). The graphical presentation of data was done using GraphPad Prism software (Prism 7 for Windows; Version 5.02, California, USA).

211 ## 3. Results

212 ### 3.1. Lead induced developmental toxicity in zebrafish embryo toxicity

213 The overall survival in the present study is defined as the total number of embryos
214 that survived prior to hatching and those that hatched at 72 hpf. All the embryos that died
215 during the exposure period were removed from testing plates. Hatching rates in the
216 control and exposed groups are shown in Table 1. Lead exposure reduced the overall
217 survival rate of exposed zebrafish embryos in a concentration-dependent manner; from
218 84% in the 1.0 µg/L to 52% in the 100 µg/L Pb. Lead-induced coagulation embryo
219 mortalities that increased with the increase in Pb exposure concentration **(Table 1)** were
220 recorded between 24 and 72 hpf. All surviving embryos hatched in all treatments by 72
221 hpf. No mortalities were observed in hatched larvae between 72 and 96 hpf periods.

222

Table 1. Overall survival and hatching rates of embryos

Treatment (µg/L)	**Total introduced**	**Mortality 24 - 72 hpf**	**Mortality (%)**	**Hatched 72 hpf**	**Hatch 72 hpf (%)**	**Overall survival rate (%)**
Control	30	2	6.7	28	93.3	93.3
1.0	25	4	16	21	84.0	84.0
10	25	13	52	12	48.0**	48.0
25	25	8	32	17	68.0*	68.0
50	25	8	32	17	68.0*	68.0
100	25	12	48	13	52.0**	52.0

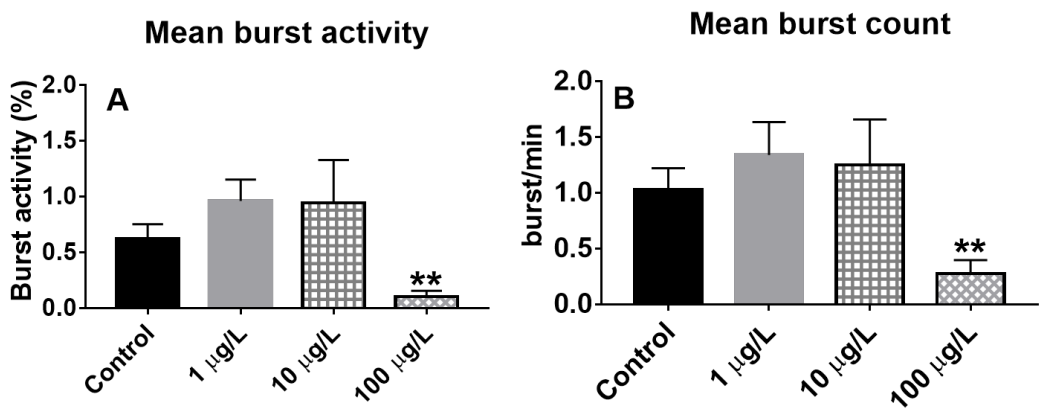
223 (* $p < 0.05$ and ** $p < 0.01$ between exposure groups and the control group).

224

225 ### 3.2. Lead exposure attenuated zebrafish embryo activity

226 Lead exposure affected the burst activity (Fig. 1A) and the burst count per minute
227 (Fig. 1B) of zebrafish embryos at 24 hpf. Only the 100 µg/L Pb group (0.10 ± 0.05%)
228 recorded significant ($p < 0.01$) lower burst activity compared to the control group (0.62

229 ± 0.13 %). The burst count per minute was significantly lower ($p < 0.01$) in the 100 µg/L
230 Pb group (0.24 ± 0.16 burst/min) relative to the control group (1.00 ± 0.19 burst/min).
231 The embryo activity data retrieved for two concentrations namely; 25 and 50 µg/L Pb at
232 24 hpf from the video recordings were not sufficient for data analysis.



238

239 **3.3. Lead exposure induced cardiovascular dysfunction**

240 Lead exposure caused changes in cardiovascular responses of 72 hpf zebrafish for
241 heart rate (Fig.2A) and blood flow (Fig.2B). The heart rate was significantly lower in the
242 100 µg/L Pb compared to the control ($p < 0.05$). No differences were observed in heart
243 rate in exposed groups with Pb concentrations less than 100 µg/L. The blood flow at the
244 100 µg/L Pb exposure concentration was significantly elevated when compared to the
245 control groups ($p < 0.05$).

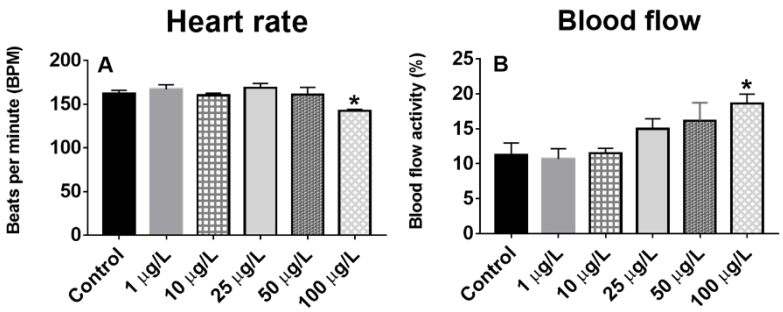


Fig. 2 Effects of Pb on the cardiovascular system of larvae (72 hpf). (A) Heart rate in beats per minute (n = 6). (B). Blood flow (n = 6 for all groups). Values are presented as mean ± SEM. The asterisk represents significant differences from the control using Mann-Whitney test (*$p < 0.05$).

3.4. Lead exposure induced muscular twitching

In the present study, muscular twitches were observed in zebrafish larvae in the 50 and 100 µg/L Pb exposed groups. The muscul**ar** twitching effects were absent in zebrafish larvae in exposed groups with less than 50 µg/L Pb concentration and the control group (Fig. 3). The twitching increased from 3.5 ± 1.4 twitches per min in the 50 µg/L exposure group to 17.0 ± 3.3 twitches per minute in the 100 µg/L Pb exposed group (Fig. 3A). The muscular twitch durations were 0.55 ± 0.16 and 0.51 ± 0.06 seconds in the 50 µg/L Pb and the 100 µg/L Pb groups, respectively (Fig. 3B).

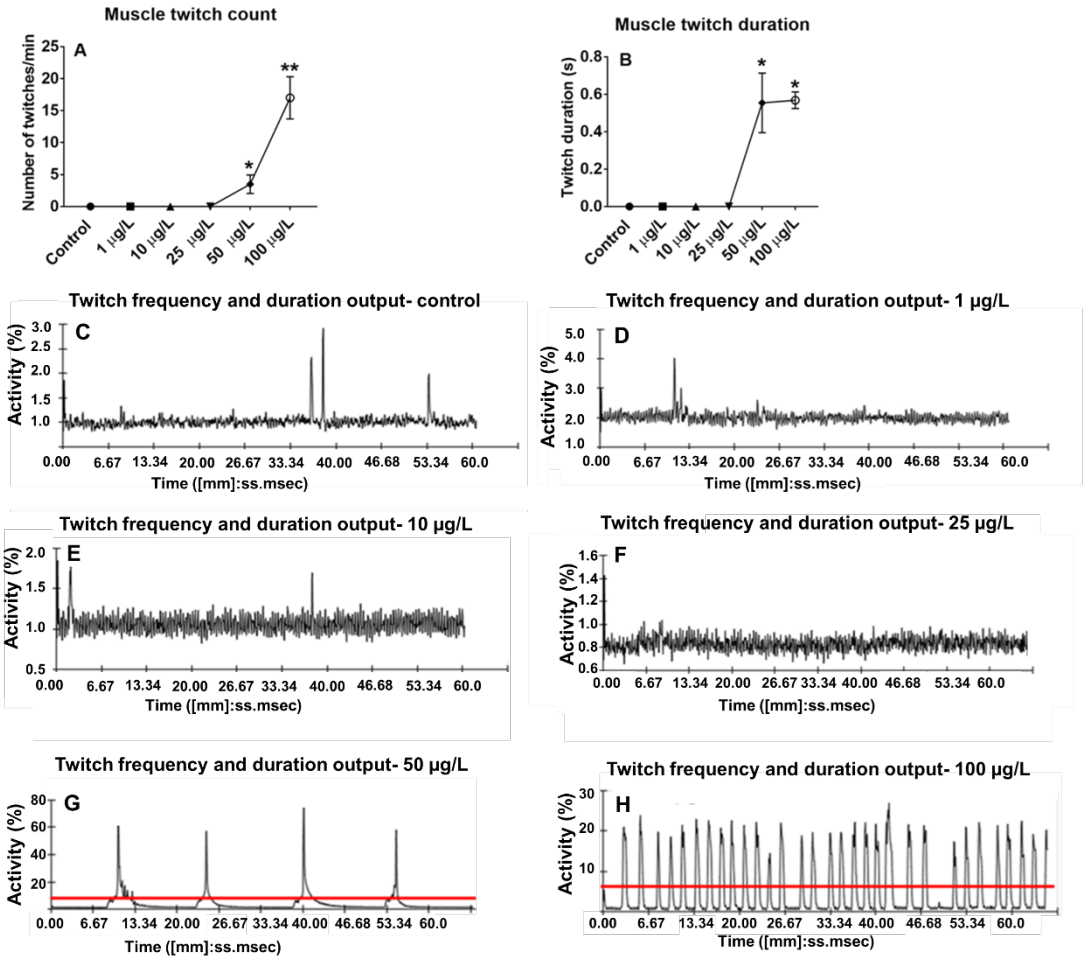


Fig. 3. Lead induced involuntary muscular twitching (n = 6): **A**. Muscle twitching (number of twitches/min); **B**. Twitch durations (s) Values are presented as mean ± SEM. The asterisk represents significant difference from the control using Mann-Whitney test (*$p < 0.05$; **$p < 0.01$); **C, D, E, F, G and H**. Representative outputs files indicating twitch frequency and duration. The red line represents the 5% activity used as a cut-off point mark. Activity of less than 5% in a larva fish was considered a normal muscular activity and above 5% represented muscular twitching.

3.5. Lead exposure affected mRNA expression

Changes in the mRNA expression levels of antioxidant enzymes (CAT, GPX, SOD, GST, HO-1 and Nrf2) following exposure to different Pb concentrations are shown in Fig. 4. Lead induced significant down regulation of CAT mRNA expression in 50 µg/L Pb group (0.6-fold change) in relation to the control (Fig. 4A). The mRNA expression levels

275 of GPX, SOD and Nrf2 enzymes across the exposed groups remained unchanged when
276 compared with the control (Fig. 4 B, C and F). The mRNA levels of GST were
277 upregulated significantly in comparison with the control. The GST expression levels
278 followed a concentration-dependent pattern, i.e. 1 µg/L Pb (2.5-fold change), 10 µg/L Pb
279 (4.9-fold change), 25 µg/L Pb (8.0-fold change), 50 µg/L Pb (7.9-fold change) and 100
280 µg/L Pb (7.6-fold change), respectively (Fig. 4D). Furthermore, Pb exposure induced
281 significant upregulation of HO-1 mRNA levels at 100 µg/L Pb with 2.2-fold change (Fig.
282 4E).
283

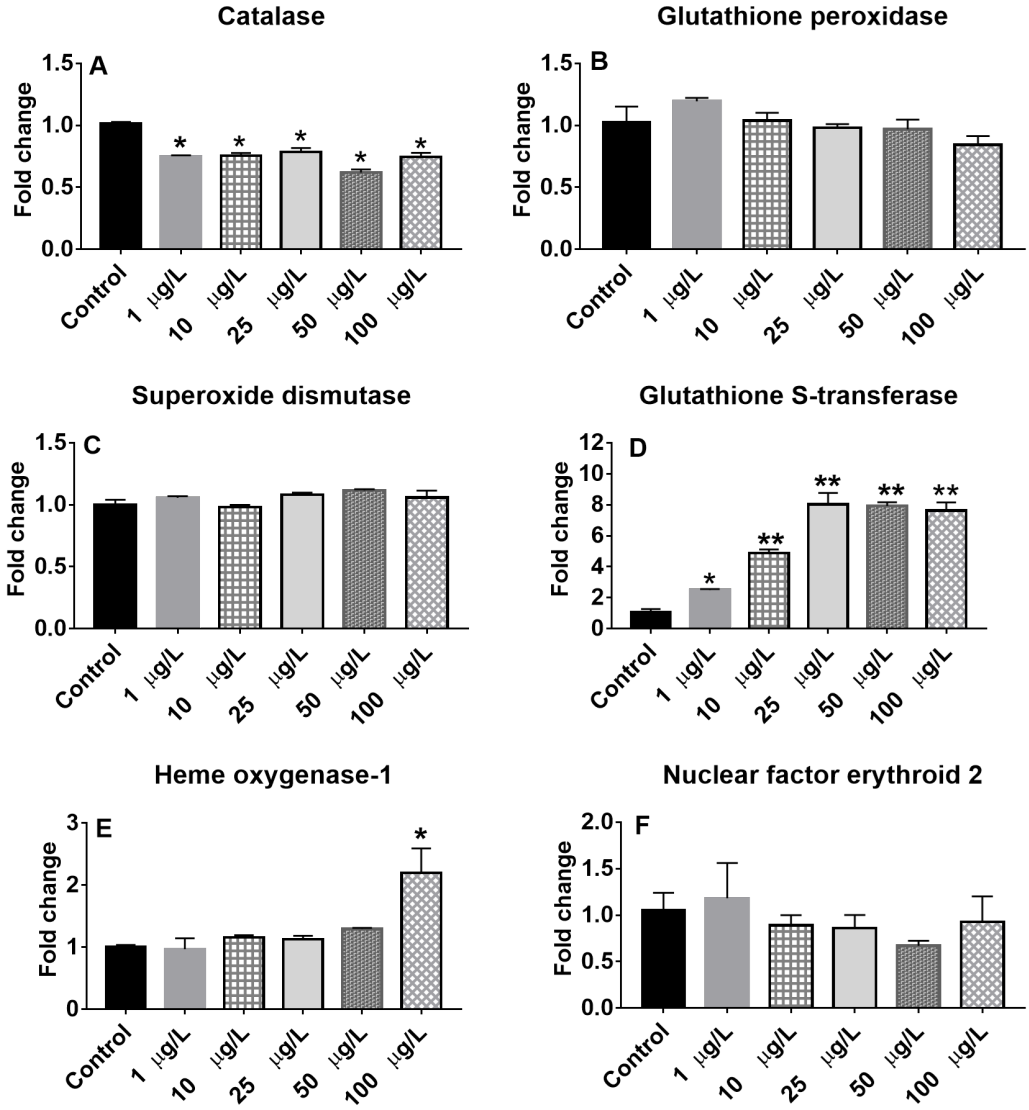


Fig. 4. Expression of **mRNA** in the pooled samples (n = 4). Values were normalized against Tubulin alpha-1A (used as house-keeping gene) and represent the mean mRNA expression value ± SEM relative to those of the controls. The asterisk represents significant difference when compared with the controls (*$p < 0.05$ and **$p < 0.01$; Mann-Whitney test)

3.6. Lead exposure affected mRNA expression of pro-apoptotic and anti-apoptotic enzymes

Mitochondrial related electron transport reactive oxygen species (ROS) associated oxidative stress response enzymes namely uncoupling protein-2 (*Ucp-2*) and cytochrome c oxidase subunit I (*CoxI*) mRNA levels were investigated. Lead exposure did not induce

295 *Ucp-2* mRNA expression changes across the exposed groups (Fig. 5A). On the other hand,
296 *CoxI* mRNA levels were significantly upregulated across all the exposed groups with 7.8-
297 fold change (1 µg/L Pb), 5.9-fold change (10 µg/L Pb), 5.9-fold change (25 µg/L Pb),
298 5.4-fold change (50 µg/L Pb) and 7.3-fold change (100 µg/L Pb) compared to the control
299 (Fig. 5B.)

300 The expression of the mRNA levels of the pro-apoptotic encoding protein tumour
301 protein p53 (*TP53*) was significantly downregulated across all the exposure groups except
302 in the 100 µg/L treatment when compared to the control (Fig. 5C). The B cell lymphoma-
303 2 (*Bcl-2*) mRNA expression was significantly upregulated with 2-fold change (1 µg/L
304 Pb), 1.8-fold change (10 µg/L Pb) and 1.7-fold change (25 µg/L Pb) when compared to
305 the control. A significant downregulation of *TP53* mRNA expression with 0.8-fold at 50
306 µg/L Pb concentration was observed (Fig. 5D).

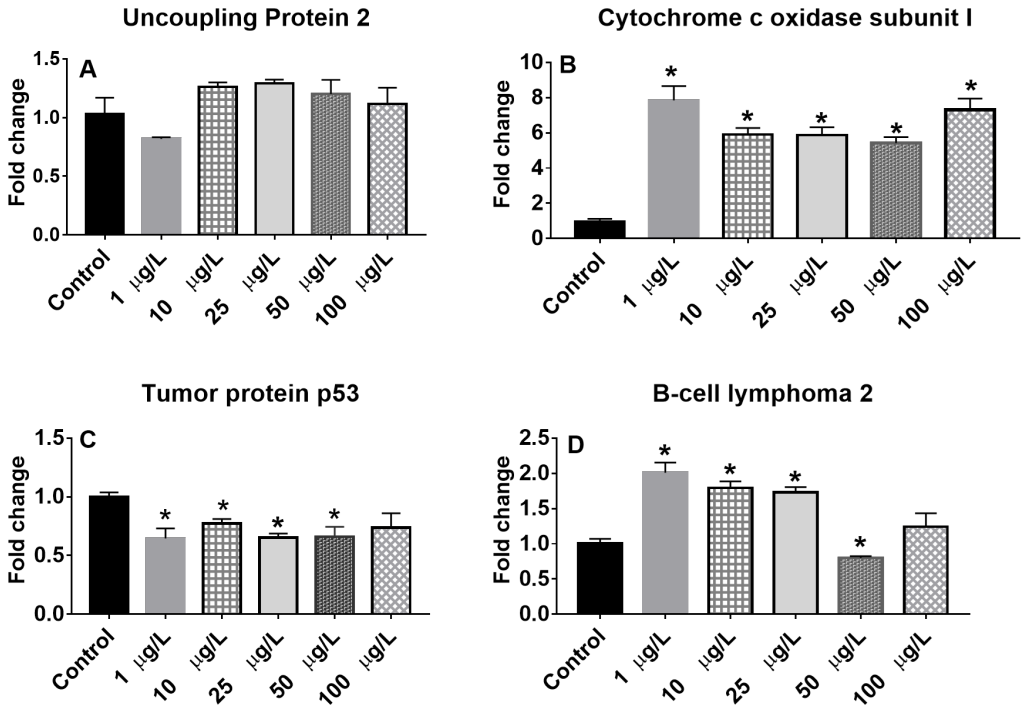


Fig. 5. Expression of mRNA of mitochondrial related enzymes (A, B, C and D) in the pooled samples (n = 4). Values normalized against Tubulin alpha-1A (used as house-keeping gene) and represent the mean mRNA expression value ± SEM (n = 4 pooled samples) relative to those of the controls. The asterisk represents a statistically significant difference when compared with the controls (*$p < 0.05$; Mann-Whitney test).

4. Discussion

The current study sheds light on the negative effects of the environmental Pb water levels including the permissible value of 50 µg/L that were reported by Nachiyunde et al. (2013) in Kabwe, Zambia on aquatic life. The permissible dissolved Pb in water Kabwe was comparable to the acute toxic criteria limit for dissolved Pb of 54.1 µg/L at a hardness of 85 mg/L (as $CaCO_3$) as set by the United States Environmental Protection Agency (USEPA) as recently reported by DeForest et al., (2017). However, regulatory institutions around the globe seem not to have common criteria for dissolved Pb in water to ensure good water quality to support aquatic life (Li et al., 2019). For instance, the action level for dissolved Pb in water delivered to users for public consumption according to the Comprehensive Environmental Response Compensation and Liability Act (CERCLA), USA is 15 µg/L (DeForest et al., 2017).

The present study showed that varying Pb concentrations induced toxicity in zebrafish embryos between 1-100 µg/L Pb. The deleterious effects of Pb exposure in the development and survival of zebrafish embryos were observed within environmentally relevant and regulatory Pb concentrations. Neuromuscular, cardiovascular and antioxidant system effects due to Pb exposure with similar to what has been reported in studies which had higher Pb concentrations (Chen et al., 2012; Zhao et al., 2019) are discussed.

333 In developmental toxicity studies involving zebrafish embryos, mortality and
334 hatching of embryos are widely regarded as endpoints that influence the overall survival
335 rate of zebrafish (Hallare et al., 2006). In this study, none of the surviving embryos failed
336 to hatch at 72 hpf across all the exposed groups, pre-hatched embryo mortalities were
337 observed with highest concentration having the highest percentage of the mortalties. All
338 the mortalities were recorded between 24 and 72 hpf, a feature that was consistent with
339 reports that indicated that the early embryonic stages after fertilization are more
340 vulnerable to metal intoxication (Jezierska et al., 2009). The high permeability of the
341 embryo membrane to metal ions and rapid organogenesis accounts for the high sensitivity
342 and vulnerability of embryos during the early stages (Fraysse et al., 2006). The
343 permeability of the embryo membrane or chorion imply that the concentration Pb in the
344 exposure solution influences the level Pb ion that accumulates in the body of the embryo
345 to cause residual toxicity. Although the mechanisms of accumulated Pb-induced toxicity
346 in embryos may be complex, an indirect connection between survival rates and
347 neuromuscular effects was observed in the present study suggesting Pb cumulative
348 toxicity. For instance 50 and 100 µg/L Pb concentrations with larvae that had
349 neuromuscular toxicity, the former that exhibited mild effects had 68% survival rate, and
350 the latter with much pronounced effects had 52% survival rate.

351 Muscular twitching has been suggested as neuromuscular toxic effects of Pb (Van
352 Den Avyle et al., 1989). In the present study, the 100 µg/L Pb group which had decreased
353 embryo activity at 24 hpf had pronounced muscular twitching at 72 hpf suggestive of
354 early onset of Pb-induced neuromuscular toxicity. Although altered spontaneous
355 movements in zebrafish larvae have been reported at 1000 µg/L Pb (Chen et al., 2012),
356 the neuromuscular toxicity being reported in our study at 50 µg/L Pb suggest that

357 zebrafish embryos may be vulnerable to Pb-induced toxicity even in low Pb
358 concentrations. The lack of the mature functional blood-brain barrier and rapid growth
359 that incorporates Pb into cellular processes due to the high affinity of Pb^{2+} for Ca^{2+}
360 dependent processes could be among the reasons low Pb concentrations in the present
361 study elicited similar effects with studies that employed high Pb concentrations.

362 Lead induced cardiovascular toxicity was observed at 100 µg/L Pb level of exposure
363 characterized by reduced heart rate and increased blood flow in the present study. These
364 findings were in tandem with previously reported cardiovascular toxicity in zebrafish
365 embryos exposed to much higher concentrations of Pb (3000, 6000 and 12 000 µg/L Pb)
366 than our study (Yin et al., 2017). The reduced heart rate may be linked to the antagonism
367 between Pb and calcium and the ability of Pb^{2+} ions to block calcium channels causing
368 impaired calcium availability for optimum heart function (Mattos et al., 2017). The
369 increase in blood flow observed in the 100 µg/L Pb exposed group corroborated evidence
370 indicating that Pb may induce increased blood flow. Although the mechanisms that
371 accounting for Pb-induced blood flow increase are complex, impaired nitric oxide system,
372 inhibition of endothelial cell growth, oxidative stress and altered cellular Ca^{2+} tracking
373 have been implicated in Pb-induced hypertension (ATSDR, 2019). Moreover, the Pb
374 concentration at which the cardiovascular toxicity observed in the present study was close
375 to the upper limit of the environmentally reported water Pb concentration (94 µg/L Pb) in
376 Kabwe, Zambia (Nachiyunde, 2013).

377 Lead induced oxidative stress is one of the mechanisms by which Pb exposure induces
378 toxicity in animals including fish (Kim and Kang, 2017). Therefore, mRNA expression
379 of the antioxidant system and related genes were analyzed in the present study. The major

380 antioxidant gene coding enzymes were dysregulated in the Pb exposed zebrafish larvae
381 with the groups that showed cardiovascular and neuromuscular toxicity having enhanced
382 dysregulation in some cases. For instance, catalase enzyme mRNA expression was
383 downregulated in all exposed groups. Catalase enzyme gene downregulation is associated
384 with the inhibition of catalase enzyme activity (Craig et al., 2007). Catalase offers cellular
385 protection against oxidative damage due to its involvement in the detoxification and
386 elimination of hydrogen peroxide generated from reactive oxygen species (Stancová et
387 al., 2015). Hence the downregulation of the catalase transcripts observed in the current
388 study could be a reflection of an overwhelmed antioxidant response due to excessive
389 generation of non-radical hydrogen peroxide molecules. The other two key antioxidant
390 encoding genes, heme oxygenase- 1 (*HO-1*) and glutathione-S-transferase (*GST*) mRNA
391 expression levels were upregulated with the latter following a Pb concentration response.
392 The *GST* enzyme is a vital metabolic and antioxidant enzyme whose gene expression
393 pattern suggests an enhanced response in an attempt to decrease Pb toxicity through
394 conjugation of Pb to glutathione to facilitate elimination (He et al., 2011).

395 It has been elucidated that ROS generation is linked to the activity of the mitochondria
396 through the electron transport chain (Flora et al., 2012). Thus, we analyzed vital
397 mitochondrial cytochrome c oxidase I (*CoxI*) and uncoupling protein-2 (*Ucp-2*) enzymes
398 gene expression levels. *CoxI* is a terminal electron acceptor of the mitochondrial
399 respiratory chain related to the generation of superoxide anion and *Ucp-2* mitigates
400 against the impact of mitochondrial superoxide anion (Sohal et al., 2008). In the present
401 study, *CoxI* was upregulated across all exposed groups without an accompanying
402 upregulation of *Ucp-2* mRNA expression. This was in contrast to previous findings in
403 larval zebrafish exposed to acute environmentally relevant Pb levels (Kataba et al., 2020).

404 The difference observed in the expression of the *CoxI* and *Ucp-2* mRNA expression in
405 Kataba et al.(2020) and the current study may be due to the differences in the Pb exposure
406 duration as well as the age of the zebrafish at the beginning of the exposure. The younger,
407 the fish, the more susceptible it is to waterborne toxicants (Jezierska et al., 2009). This
408 upregulation of *CoxI* without the concurrent upregulation of *Ucp-2* may explain the Pb-
409 induced toxic effects observed in zebrafish embryos as *Ucp-2* activity tend to neutralize
410 the impact of *CoxI* (Kataba et al., 2020). Moreover, increased superoxide anion and
411 disruption of the *Ucp-2* levels have been linked to neuronal oxidative damages and
412 sustained neuronal oxidative damage causes neuronal apoptosis (Wu et al., 2010). The
413 neuronal apoptosis may manifest as neurotoxicity a probable mechanism behind the
414 neuromuscular toxicity observed in the present study.

415 In aquatic organisms, oxidative stress has been linked to enhanced apoptosis
416 (Livingstone, 2001). In the current study, we used the B-cell lymphoma 2 (*Bcl-2*) and
417 tumour protein p53 (*TP53*) genes as transcriptional markers related to the apoptosis
418 signalling processes (Jin et al., 2011). The *Bcl-2* mRNA expression levels in Pb exposure
419 concentrations less than 50 µg/L (1-25 µg/L Pb) were **upregulated** and downregulated
420 in Pb exposure concentrations equal to or greater than 50 µg/L. The upregulation of the
421 Bcl-2 mRNA expression in Pb concentrations less than 50 µg/L Pb could be an indirect
422 compensatory and protective response against Pb-induced apoptotic elements (Bonneau
423 et al., 2013). Whereas, the downregulation of *Bcl-2* mRNA expression levels at 50 µg/L
424 Pb could be a reflection of an exhausted Bcl-2 protein compensatory mechanism due to
425 the high presence of Pb (Jin et al., 2010). On the other hand, Pb exposure seemed to have
426 triggered the downregulation of *TP53* mRNA expression across exposed groups. The *Bcl-*
427 *2* gene encodes for protein that is a member of the Bcl-2 family that regulates, suppresses

428 and prevents aberrant apoptosis (Jin et al., 2010). The prevention is achieved by
429 neutralization of the pro-apoptotic proteins (p53) by Bcl-2 proteins (Pyati et al., 2007).
430 Notwithstanding the striking non-Pb concentration-dependent mRNA expression of the
431 antiapoptosis and proapoptosis genes, the mRNA expression pattern of these genes
432 suggested that apoptosis could have been among the possible contributors to the
433 neuromuscular twitching observed. Moreover, the significant downregulation of anti-
434 apoptotic and pro-apoptotic gene transcripts at 50 µg/L Pb and non-significant
435 downregulation at 100 µg/L Pb reflects a hormetic response, a feature that has been
436 reported in larval zebrafish **(Kataba et al., 2020).** Overall, the study has demonstrated
437 the deleterious effects of the water Pb levels in Kabwe on zebrafish embryos **and larvae**
438 including cardiovascular and neuromuscular toxicity.

439 There are limitations to our study. The lack of accompanying enzymatic assays and
440 non-enzymatic such as lipid peroxidase or protein carbonyl compound analyses and
441 antioxidant tissue levels analyses are among the major limitations to support the gene
442 expression **effects** thus observed (Mccarthy and Smyth, 2009). The non-retrieval of the
443 embryo activity data at 24 hpf for 25 and 50 µg/L Pb poses another limitation on the
444 comparison of the embryo activity and neuromuscular toxicity at these levels of exposure.
445 Furthermore, in the light of anti-apoptotic and pro-apoptotic genes, mRNA dysregulation
446 observed without accompanying apoptosis assays such as acridine orange staining limits
447 the interpretation of our results. Notwithstanding, the present study has demonstrated that
448 environmentally relevant Pb levels could affect the overall survival rates of zebrafish
449 embryos through or accompanied by cardiovascular, neuromuscular, and antioxidant
450 system aberrations.

5. Conclusions

Lead dissolved in water poses a threat to aquatic life even **in** lowest quantifiable amounts. Water Pb concentrations that are below and within the "permissible limit" (10 to 50 µg/L Pb) could be detrimental to zebrafish life especially at early developmental stages **as** evidenced by embryonic coagulation linked mortalities. Furthermore, our FET test concentrations provided additional insights on the Kabwe Pb water concentrations. Lead water concentrations of 50 to above 100 µg/L could even be more detrimental to developing fish embryos with a myriad Pb linked toxicities. Embryonic activity aberrations, cardiovascular toxicity (reduced heart rate, increased blood flow activity), oxidative stress system imbalance, antiapoptotic and proapoptotic balance and the neuromuscular toxicity (muscle twitching) are among the deleterious effects of environmentally relevant Pb levels. Further investigations on the impact of environmentally relevant water Pb concentrations and the permissible (regulatory) water Pb levels on reproduction, development and health of locally available fish species are needed.

6. Ethical statement

All experimental procedures were done with the due approval by the AnimCare animal research ethics committee (ethics approval number: NWU-00269-16-A5) at the North-West University. All animals were maintained, and procedures carried out in adherence with the code of ethics in research, training and testing of drugs in South Africa and complied with national legislation (NHREC reg. number AREC-130913-015).

7. Authors contribution

Andrew Kataba: Conceptualization; Data curation; Formal analysis; Methodology; Writing - original draft; Writing - review & editing. **Tarryn Lee Botha, Yared B. Yohannes**: Validation, Methodology, Software, Writing - review & editing. **Shouta M.M. Nakayama** and **Yoshinori Ikenaka**: Funding acquisition; Resources; Writing - review & editing. **Victor Wepener**, **Mayumi Ishizuka**: Funding acquisition Resources; Supervision; Writing - review & editing**.**

8. Declaration of Competing Interest

The authors declare that they have no conflict of interest relating to the work presented in this manuscript.

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