BEHAVIOR AS AN EARLY SIGN OF TOXICITY AND RESPIRATORY SYSTEM INJURY INDUCED BY CADMIUM IN ZEBRAFISH

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Abstract

The aim of the present study was to determine the validity of behavior as an early sign of toxicity and respiratory injuries induced by acute exposure to cadmium in adult zebrafish *(Danio rerio).* The effects of three cadmium concentrations (35, 45, and 55 µg/L) on zebrafish behavior (i.e., general activity, exploratory/motor behavior, climbing to the water surface, tremors, and erratic movements) and gill histology after 1 h of exposure was assessed. Compared with controls, cadmium exposure increased the number of climbs to

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the water surface and total time spent at the water surface, increased the percentage and intensity of tremors, and increased erratic movements. Cadmium exposure also caused stage I injury to the gills, with the presence of chloride cells in secondary lamellae, dilation of the capillaries, hyperplasia of the gill epithelium, and fusion of the secondary gill lamellae. These effects were observed mainly at concentrations of 45 and 55 µg/L. Our results indicate that 45 and 55 µg/L cadmium induces behavioral dysfunction and 55 µg/L significant gill injury, even with only 1 h exposure, revealing respiratory system impairments. The present model may be an interesting tool for analyzing early toxicity and respiratory injuries in fish.
Keywords: metals poisoning; *Danio rerio;* behavior; histopathology; neurotoxicity.

O COMPORTAMENTO COMO UM SINAL PRECOCE DE TOXICIDADE E LESÕES NO SISTEMA RESPIRATÓRIO INDUZIDAS POR CÁDMIO EM ZEBRAFISH

Resumo

O objetivo do presente estudo foi determinar a validade do comportamento como sinal precoce de toxicidade e as lesões respiratórias induzidas pela exposição aguda de cádmio em peixe-zebra adulto (*Danio rerio*). Foram avaliados os efeitos de três concentrações de cádmio (35, 45 e 55 µg / L) no comportamento do peixe-zebra (atividade geral, comportamento exploratório / motor, subida à superfície da água, tremores e movimentos erráticos) e a histologia das brânquias após 1 h de exposição. Comparados aos grupos controle, a exposição dos peixes ao cádmio aumentou o número de subidas para a superfície da água, o tempo total gasto na superfície da água, a porcentagem e a intensidade dos tremores e de movimentos erráticos. A exposição ao cádmio também causou lesão de estágio I das brânquias, com presença de células de cloreto em lamelas secundárias, dilatação dos capilares, hiperplasia do epitélio branquial e fusão das lamelas de brânquias secundárias. Esses efeitos foram observados principalmente em concentrações de 45 e 55 µg / L. Estes resultados indicam que as concentrações de 45 e 55 µg / L de cádmio induzem disfunção comportamental e 55 ug/L lesão branquial, mesmo com apenas 1 hora de exposição, revelando comprometimento do sistema respiratório. O presente modelo pode ser uma ferramenta interessante para analisar toxicidade precoce e lesões respiratórias em peixes.

Palavras-chave: intoxicação por metais; *Danio rerio;* comportamento; histopatologia; neurotoxicidade.

1. INTRODUCTION

Cadmium (Cd) is a biologically nonessential heavy metal that has gained great importance from toxicological (Rana, 2014, Kozlowski et al., 2014) and ecotoxicological (WHO -World Health Organization, 2007) perspectives. The natural presence of Cd in abiotic processes in water does not necessarily indicate pollution. However, anthropogenic activity may cause elevated concentrations of Cd that exceed natural background levels. The maximum limit of Cd in fresh water that was established by the CONAMA 357/2005 resolution (CONAMA, 2005) is 1 µg/L.

Cadmium is a well-described environmental pollutant that is known to have adverse effects in several fish species. Previous studies have reported hepatic toxicity (Chen et al., 2013), branchial cellular damage (Xuan et al., 2014), metabolic changes (e.g., increases in lactate, protein, amino acid, and ammonia levels and a decrease in glucose levels (Pretto et al., 2014), and behavioral impairments (Eissa et al., 2010).

Changes in fish behavior appear to be among the most sensitive and early indicators of the toxicity of several substances (Eissa et al., 2010, Hill et al., 2005,Teraoka et al., 2003). Roch; Maly (1979) reported that Cd exerts its toxic effects in aquatic organisms by blocking the uptake of calcium (Ca^{2+}) from water. Calcium is an essential element that is taken up from water by organisms via specialized Ca^{2+} channels. However, when Cd^{2+} is present in water, this metal competes with Ca^{2+} for binding sites, thus inhibiting Ca^{2+} uptake and resulting in hypocalcemia.

Zebrafish (*Danio rerio*, Hamilton 1822) is a member of the genus Danio of the family Cyprinidae. It was referred to in the scientific literature as Brachydanio rerio for many years until its redesignation as the genus Danio (Westerfield, 2000). Zebrafish are a highly valued model organism in developmental biology, genetic studies, and drug screening (Hill et al., 2005, Zon; Peterson, 2005). Adult and larval zebrafish offer many perspectives in neuroscientific studies because they are a vertebrate species with high physiological and genetic homology to humans

(Kalueff et al., 2014,Joshua;Lisberger, 2014). Zebrafish are considered a useful species for investigating central drug effects (Rihel; Schier, 2012,Tan; Zon, 2011), psychiatric diseases (Brennan, 2011,Kabashi et al., 2011,Jones; Norton, 2014) the immune system (Mulligan; Weinstein, 2014), behavioral effects (Xu et al., 2007, Spence et al., 2008, Bernardi et al., 2013) and neurotoxicity (Bailey et al., 2013,Nishimura et al., 2015).

Adult zebrafish were evaluated for general activity, exploratory/motor behavior, climbing to the water surface, tremors, and erratic movements. The gills were evaluated because they are a metabolically active and readily available organ that is commonly used for bio-monitoring analyses in fish (Yeslbudak; Erdem, 2014), and such analyses can reveal respiratory system impairments (Hwang; Chou, 2013).

The aim of the present study was to determine the validity of behavior as early sign of toxicity and respiratory injuries induced by cadmium acute exposure in adult zebrafish *(Danio rerio).* In addition, correlations between behavioral and morphological effects in fishes may open protocols for respiratory system studies that are related to toxicology.

2. MATERIAL AND METHODS

Adult zebrafish (4-5 cm length, 8-9 months of age) were obtained from a commercial breeder (Izael BaHi, Indaiatuba, São Paulo, Brazil) and brought to the laboratory within 30 min in plastic bags with sufficient air. The plastic bags were placed in an 80 L maintenance aquarium for 30-35 min for acclimation, after which time the bags were opened to release the fish into the aquarium. They were maintained in the laboratory for 15 days for acclimation before the experimental procedures. Dechlorinated water from São Paulo was used and maintained at a temperature of 23 ± 2ºC by heaters. The water hardness was 42 mg/L $CaCO_3$, pH 7.0 ± 0.2. The luminous intensity was 600 lux, with a natural light/dark photoperiod. With the exception of during the experiments, the aquaria were aerated using air compressors and connected to water filtration systems with acrylic wool and active charcoal to improve water quality. Every 7 days, 25% of the total water volume was changed. We fed the zebrafish with Tetramin (Spectrum Brands,Inc., Blacksburg,VA, U.S.A.) as recommended by

the manufacturer and in accordance with CETESB 1990 guidelines. Before the tests the fish were fed normally. Only during testing, the fish were not fed, and pH, dissolved oxygen, and conductivity were analyzed at the beginning and end of each test. All of the animal procedures were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (CEUA-UNIP- permit 09/17).

Forty zebrafish were divided into four groups (*n*= 10/group): one control group and three experimental groups that were exposed to Cd chloride ($CdCl_2$) at concentrations of 35, 45, and 55 µg/L (Sigma-Aldrich, catalog no. 202908, São Paulo, Brazil), dissolved in aquarium water immediately before tests. The control group was observed in an aquarium that was maintained similarly to the experimental aquarium but was not exposed to Cd.

General activity was observed as previously reported in our laboratory (Bernardi *et al.*, 2013). Briefly, an aquarium (15 cm length × 15 cm width × 10 cm height) was used. The front wall of the aquarium was divided into six equal 5-cm parts. Because adult zebrafish have a maximum size of 5 cm, the counts of areas crossed were sufficiently sensitive to detect zebrafish movements. The fish were individually introduced into the aquarium that contained 0 (control), 35, 45, or 55 µg/L Cd. Behavior was observed 4-5, 14-15, 29-30, 44-45, and 59-60 min after introducing the fish to the aquarium. The following parameters were observed: (*i*) number of times the zebrafish presented tremors (one tremor was counted each time the zebrafish moved and stopped rapidly, with progressive contractions of the whole body from head to tail; the data are expressed as a percentage; (*ii*) intensity of tremors (0 tremors, 1 tremor, 2-3 tremors (few tremors], 4-6 tremors [moderate tremors], > 6 tremors [many tremors]), (*iii*) run frequency (i.e., the number of times that the fish swam in any direction, except when climbing to the surface; one run was counted each time the fish started and stopped a run; the total run frequency was obtained by summing the frequencies of runs), (*iv*) erratic movements (i.e., movement in a stereotyped zigzagging pattern; 0 = no erratic movements, 1 = 1-3 erratic movements, 2 = 4-6 erratic movements, 3 = > 6 erratic movements; (*v*) frequency of climbs to the water surface (i.e., the number of times the zebrafish climbed to the water surface; the total frequency of climbs to the water surface was calculated as the sum of frequencies of climbing to the water surface), and (*vi*) time (in seconds) the zebrafish remained at the water surface

(i.e., in the upper quadrant of the aquarium very near the surface; the total time at the water surface was obtained by summing the time at the water surface). Behavior was video recorded for later analysis by a blinded observer. The presence of tremors and runs were analyzed by two-way ANOVA followed by the Bonferroni test. Total runs were analyzed by one-way ANOVA followed by the Tukey multiple-comparison test. Tremor intensity and erratic movement scores were analyzed by the Kruskal-Wallis test followed by Dunn's multiple-comparison test. The number of climbs to the water surface and frequency of climbs to the water surface were analyzed by two-way ANOVA followed by the Bonferroni *post hoc* test. The total time at the water surface and total frequency of climbs to the water surface were analyzed by one-way ANOVA followed by the Tukey multiple-comparison test.

Immediately after the behavioral observations, the zebrafish were euthanized with benzocaine hydrochloride (250 mg/ml), and the spinal cord was sectioned transversely to remove the gills. The material was fixed in cold McDowell solution (1% glutaraldehyde and 4% formaldehyde in phosphate buffer, pH 7.4) (McDowell;Trump, 1976). Tissues were passed through alcoholic dehydration, embedded in Leica historesin (glycol methacrylate), cut with a microtome (Jung) and stained with toluidine blue basic fuchsin for histopathology. The gills were analyzed according to parameters that were established by Poleksic and Mitrovic-Tutundzic modified (1994). This method classifies gill alterations into three stages: I (slight damage), II (moderate damage), and III (severe damage). The presence of histopathological alterations in the gills was semi-quantitatively determined by the degree of tissue alterations, based on the Histopathologic Alterations Index (HAI). The HAI was calculated for each animal using the following formula: $HAI = (1 \times SI) + (10 \times SII) + (100 \times SIII)$, where *I*, *II,* and *III* correspond to the number of stages of alterations I, II, and III, and *S* represents the sum of the number of alterations for each particular stage.

The results are expressed as mean ± SEM, medians (min-max limits), or percentages. Homoscedasticity was verified using the F test or Bartlett's test. Normality was verified using the Kolmogorov-Smirnov test. Erratic movement data were analyzed using one- or two-way analysis of variance (ANOVA) followed by the Tukey or Bonferroni *post hoc* test. The HAI was analyzed using Kruskal-Wallis (KW) ANOVA. Percentages were analyzed using the Fisher test. The level of statistical significance was $p < 0.05$.

3. RESULTS

Figure 1 shows the effects of acute Cd exposure on tremors, runs, and erratic movements. The presence ($p < 0.05$; Figure 1A) and intensity (KW = 0.02; Figure 1B) of tremors increased only after 55 µg/L Cd exposure compared with the control group. There were no significant differences in the frequency of runs between treatment ($F_{3,180} = 1.50$, $p = 0.215$; Figure 1C), but the analysis revealed an effect of time of observation ($F_{4,180} = 3.05$, $p = 0.01$), with no interaction between factors ($F_{12,180} = 0.72$, $p = 0.73$). The total number of runs was not affected by treatment ($F_{3,39} = 1.059$, $p = 0.378$, Figure 1D). Erratic movements increased after 45 µg/L Cd exposure and increased further after 55 µg/L Cd exposure compared with the control group (KW = 16.49, $p = 0.0009$; Figure 1E). Thus, only 55 µg/L induced tremors, whereas both 45 and 55 µg/L induced erratic movements, revealing a neurotoxic effect of Cd. None of the Cd concentrations influenced the run parameters.

Figure 2 shows the effects of acute Cd exposure on the time at the water surface and frequency of climbs to the water surface. The analysis revealed a significant effect of treatment on time at the water surface ($F_{3,180} = 5.22$, $p = 0.002$; Figure 2A) but no effect of time of observation ($F_{4,180} = 1.833$, $p = 0.125$) and no interaction between factors ($F_{12,180} = 0.76$, $p = 0.691$). The *post hoc* test indicated an increase in time at the water surface at a Cd concentration of 45 µg/L at intervals of 44-45 and 59-60 min compared with the control group. Similarly, the total time at the water surface differed between groups ($F_{3,36} = 4.07$; p = 0.014; Figure 2B), with an increase only after 45 µg/L Cd exposure. No significant differences were detected between groups in the frequency of climbs to the water surface (Figure 2C) or total frequency of climbs to the water surface (Figure 2D). These data suggest that Cd did not modify these motor parameters, although a decrease in respiratory function was observed. Importantly, no zebrafish died during the experiments at any of the Cd concentrations tested.

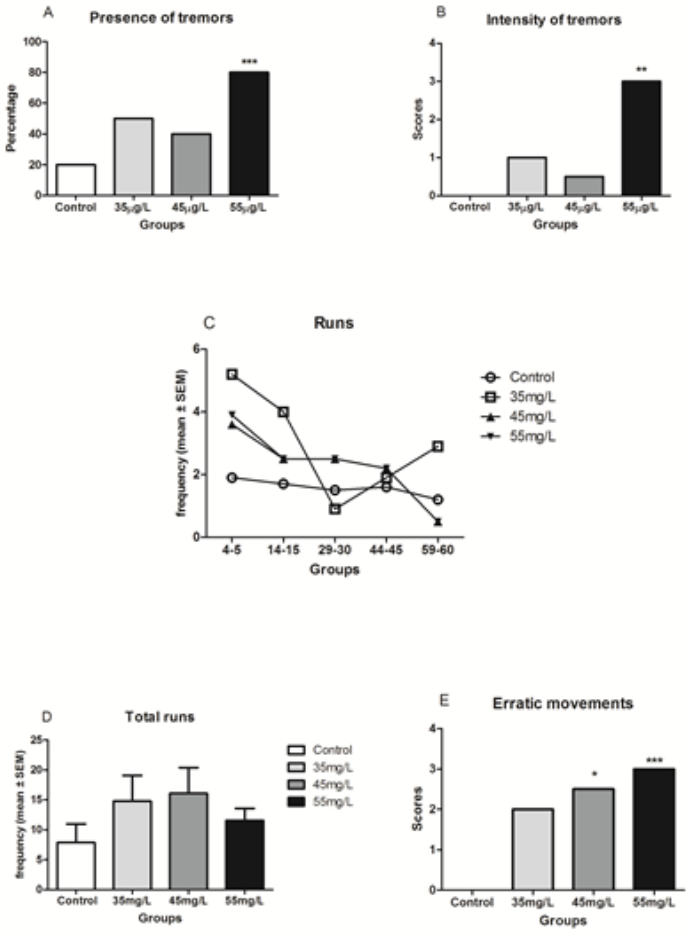

Figure 1. The presence of tremors (A), intensity of tremors (B), runs (C), total runs (D), and erratic movements (E) in zebrafish after exposure to 35, 45, and 55 µg/L Cd for 1 h.. *$p <$ 0.05, **$p <$ 0.01, ***$p <$ 0.001, compared with control group.

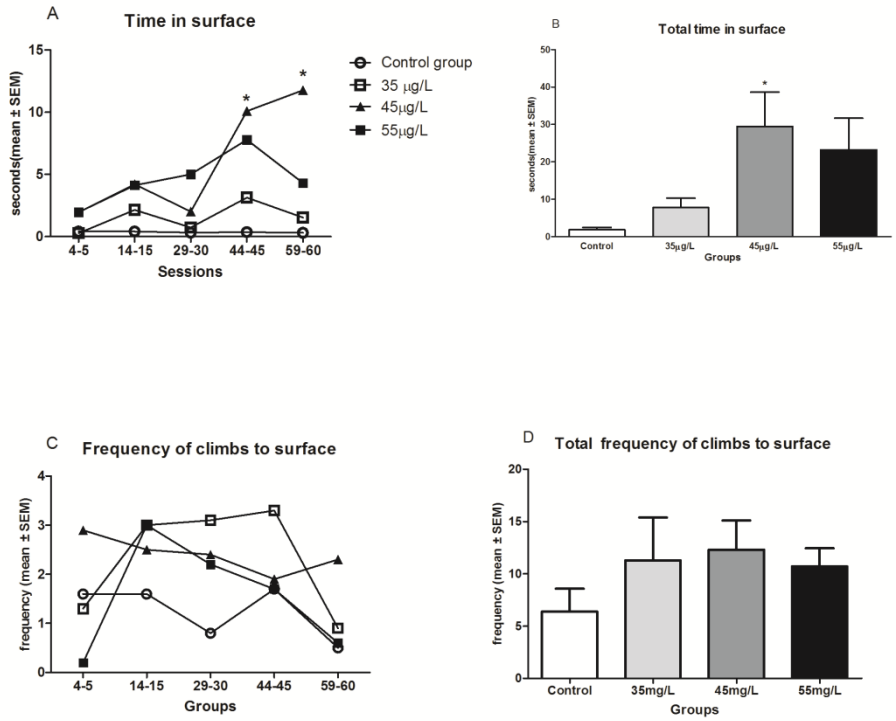

Figure 2. Number of climbs to the water surface (A), total time at the water surface (B), frequency of climbs to the water surface (C), and total frequency of climbs to the water surface (D) in zebrafish exposed to 35, 45, and 55 μg/L Cd for 1 h*$p <$ 0.05, compared with control group.

Figure 3 shows the histopathological analysis of the gills in the control group (Figure 3A) and zebrafish exposed to Cd concentrations of 45 μg/L (Figure 3B) and 55 μg/L (Figure 3C) for 1 h. The 35 μ/L concentration data are not presented because no differences were observed between the experimental and control groups. Chloride cells were detected in the secondary lamellae, with a greater frequency of capillary dilation (Figure 3B). Hyperplasia of the gill epithelium and partial or complete fusion of the secondary gill lamellae were observed (Figure 3C). These alterations were observed in both the 45 and 55 μg/L groups, and both could be classified as stage I. Aneurysms were found in two zebrafish in the control group, one zebrafish in the 45 μg/L group, and four zebrafish in the 55 μg/L group (Table 1 and figure.4). The Histopathologic index (HAI) was 3.7 in the 35 μg/L group, 5.5 in the 45 μg/L group, 8.2 in the 55 μg/L group, and 3.75 in the control group. The Kruskal-Wallis analysis reveal differences in the HAI between groups (KW = 8.43, p = 0.0379). The Dunn's test indicates an increased HAI in 55 μg/L group. Thus, zebrafish that were exposed to 35 and 45

µg/L Cd and the control group both had normally functioning gills, whereas zebrafish that were exposed to 55 µg/L Cd presented gill damage (stage I).

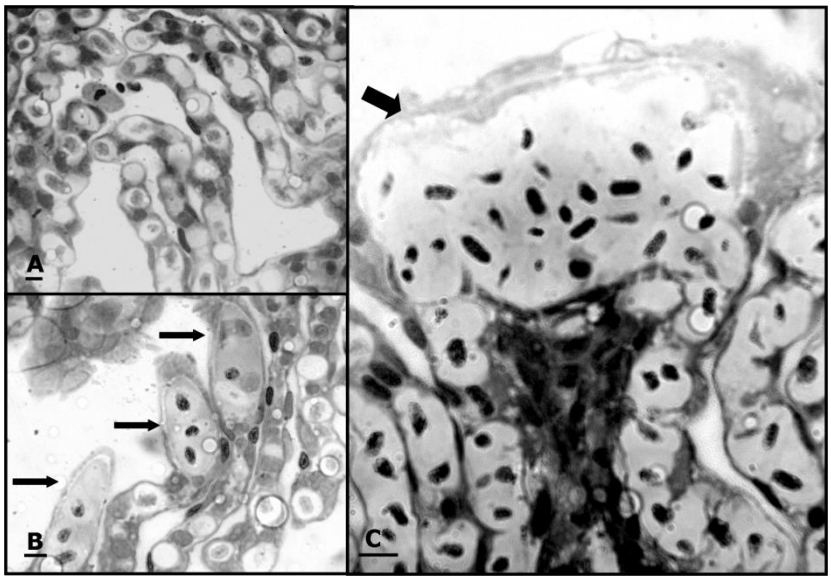

Figure 3. Photomicrography of zebrafish secondary lamellae. (A) Integrity of lamellae in the control group. (B) Small aneurysms (arrows). (C) Large aneurysm and lamellar fusion (arrow) with Cd exposure for 1 h. Scale bar = 10 µm. Staining: toluidine blue/fuchsin. Santos et al, 2017.

Table 1: Histopathologic index of lesions in gills of *D. rerio* exposed to different concentrations to Cd observed during 1h (N=8-10 fishes per group). Kruskall-Wallis analysis of variance. São Paulo, 2015.

Histopathologic Alterations Index (HAI)				
N	**0µg/l**	**35µg/l**	**45µg/l**	**55µg/l**
1	4.00	4.00	5.00	17.00
2	4.00	4.00	3.00	4.00
3	5.00	4.00	4.00	4.00
4	4.00	3.00	5.00	4.00
5	3.00	4.00	3.00	4.00
6	3.00	5.00	4.00	5.00
7	4.00	3.00	15.00	13.00
8	3.00	3.00	8.00	4.00
9	-	4.00	5.00	13.00
10	-	3.00	3.00	14.00
Mean	**3.75**	**3.70**	**5.50**	**8.20***
SEM	**0.25**	**0.21**	**1.16**	**1.69**

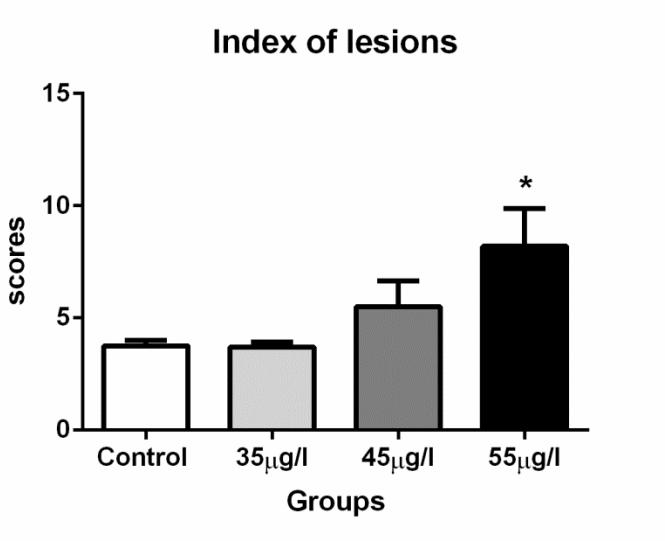

Figure 4. Histopathological Alterations Index of lesions in the gills of zebrafish exposed to different concentrations of Cd for 1 h (*n* = 8-10 fish per group). The data were analyzed using Kruskal-Wallis ANOVA followed by the Dunn's test.

4. DISCUSSION

We evaluated the effects of acute exposure to three low Cd concentrations (35, 45, and 55 µg/L) on zebrafish behavior and gill histology after 1 h of exposure. The highest Cd concentration (55 µg/L) was approximately 10-times lower than the 96 h short-term benchmark concentration for zebrafish (603 µg/L) (Wang; Du ,2013). Short-term exposure benchmark concentrations are derived using severe-effects data (such as lethality) for defined short-term exposure periods (24-96 h). These benchmarks identify estimators of severe effects on aquatic ecosystems and are intended to provide guidance on the impacts of severe, but transient, situations (e.g., spill events in aquatic receiving environments and the infrequent release of short-lived/non persistent substances). Short-term benchmark concentrations do not provide guidance on protective levels of substances in aquatic environments, in which short-term benchmarks are levels that do not protect against adverse effects.

Alterations in neurological function are generally expressed behaviorally. Many of the behavioral models that have been established for mammals can be translated to zebrafish (Hill et al. 2005). For example, certain tests can observe alterations in motor function, changes associated with exteroceptive and interoceptive sensory cues, and alterations in learning and memory performance (Tierney, 2011,' Bernardi et al. 2013).

The present results indicated that 55 µg/L Cd significantly increased both the percentage and intensity of tremors. Tremors can reflect an increase in nervous system excitability either centrally or peripherally and may appear prior to seizures (Brito, 1994)· Cadmium exposure can also severely affect nervous system function (López et al. 2003,' (Wang; Du, 2013). This metal has been shown to produce free radicals in the brain in primary oligodendrocyte cultures, which may potentially damage neurons and oligodendrocytes and lead to myelin injury (Almazan et al. 2000). Additionally, Cd-induced white matter damage was also reported in an isolated rat optic nerve preparation (Fern et al. 1996). Cadmium can also be a potent neurotoxicant in the peripheral nervous system, and long-term exposure can result in peripheral polyneuropathy (Goedee et al. 2013). The main nervous system symptoms of Cd toxicity in mammals include headache, vertigo, olfactory dysfunction, parkinsonian-like symptoms, a slowing of vasomotor functioning, peripheral neuropathy, a decrease in equilibrium, an inability to concentrate, and learning disabilities (Wang ;Du, 2013). In rats, Cd toxicity has been reported to dose-dependently produce biochemical and behavioral dysfunctions that may cause adverse effects on several organs, including the central nervous system (Haider et al. 2015).

In fish, Cd neurotoxicity can result in such behavioral alterations as surfacing, erratic swimming, and restlessness, indicating avoidance behavior (Kasherwani et al., 2009). In the present study, we found that erratic movements increased after 45 and 55 µl/L Cd exposure, corroborating the neurotoxic effects of exposure to Cd. The lack of effects of Cd exposure on the frequency of climbs to the water surface may indicate that motor impairment was not induced by Cd. Additionally, Cd crosses the blood-brain barrier, enters the brain and neurons (Nishimura et al. 2006), and produces neurological changes in both humans (Godt *et al.*, 2006) and mice (Łukawski et al. 2005). Cadmium also activates the hypothalamic-hypophyseal-adrenal axis to release corticosterone (Lafuente, 2013). When fish are exposed to a predator, they present anxiety-like behavior and an increase in erratic movements(Collier et al. 2017). In zebrafish, "anxiety" induces not only erratic movements but also freezing behavior (Blaser et al. 2010). This may explain the lack of effects of 55 µg/L Cd exposure on total time at the water surface in the present study. Thus, the increases in tremors and erratic movements suggest that Cd induced neurotoxicity at both 45 and 55 µg/L.

The number of climbs to and total time at the water surface were increased by 45 µg/L Cd, mainly at the end of the behavioral observations. Thus, 45 µg/L Cd exposures appeared to impair the respiratory system. The phenomenon of increased air gulping reflects an attempt by the fish to extract more oxygen to meet energy demands, and this action may also be correlated with the formation of an hypoxic condition that is attributable to interference with gaseous exchange that is caused by the accumulation of mucous on the gill epithelium (Kasherwani et al. 2009). Cadmium sulfate has been reported to cause irritation of the respiratory track and liver and kidney dysfunction in humans (Andujar et al., 2010).

Toxic substances that are present in the environment can cause morphologic impairments in several organs, including vital functions in the gills (Poleksic ; Mitrovic-Tutundzic, 1994). Exposure to low levels of Cd in Oncorhynchus mykiss (rainbow trout) affects the social behavior of this fish through accumulation in the olfactory apparatus (Sloman et al. 2003). In addition, dominant fish accumulate more Cd in the gills than subordinate fish during chronic water-borne exposure (Sloman et al. 2003). Thus, histopathological analyses of fish gills have been used to evaluate the quality of aquatic ecosystems. In the present study, the presence of chloride cells, dilation of the capillaries, and hyperplasia were observed after Cd exposure. Similar alterations in the gills have also been reported in other fish species (Gill et al. 1990), and after exposure to other metals (Palaniappan et al. 2008). Hyperplasia of the cell epithelium and lamellar fusion can interfere with the efficiency of the gills, resulting in a reduction of gas exchange (Macdonald et al. 2002, Shaw et al. 2012).

5. CONCLUSIONS

Our results indicate that 45 and 55 µg/L cadmium induces behavioral dysfunction and 55 µg/L significant gill injury, even with only 1 h exposure, revealing respiratory system impairments. The present model may be an interesting tool for analyzing early toxicity and respiratory injuries in fish.

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