

Reduced metabolic rate and increased heart beat as early signs of sub lethal Copper toxicity in developing Zebrafish

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Abstract

The current study looks at acute and sub-lethal Copper toxicity on developing zebrafish embryos up to 96 hours post fertilisation (PSF). We allocated four-hour PSF zebrafish embryos in four groups with duplicates, and 500 mg, 50 mg, and 0.05 mg l⁻¹ and a control group for 96 hours PSF. All fish exposed to 500 mg l⁻¹ copper died within the first 6 hours PSF. Significant sub-lethal copper toxic effects on developing Zebrafish were manifested in the 0.05 and the 50 mg groups, by increased heartbeat rate within the first 25 hours PSF in the 0.05 and 50 mg group. The sub-lethal toxic effect was also manifested in the metabolism of developing embryos, with a reduced rate of utilisation of yolk and lower growth rate and anatomical malformations within the first 25 hours in the 50 mg group and after 50 hours PSF in both the 0.05 and 50 mg group. The results indicate that reduced metabolic rate and increased heartbeat rate are potential early signs of sub-lethal Cu toxicity in developing Zebrafish well before other developmental defects are visually evident.

Keywords: Toxicity, Development, Fish, Metabolism

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Introduction

Aquatic ecosystems may exhibit a range of pollutants with an anthropogenic origin, including heavy metals. Even at concentrations not alarming for humans, heavy metals may have a significant effect on aquatic life (Milanov et al., 2016). In the early stages of their life cycle, fish are, especially susceptible to heavy metals (Linbo et al., 2006). Copper (Cu) is found in nature and is a major trace element for vertebrates (Mount, 1968). However, in higher concentration, Cu

becomes toxic (Flemming and Trevors, 1989), with effects on fish organs (Bashir et al., 2018). Waterborne Cu decreases the swimming potential and metabolism considerably, even at small levels (De Boeck et al., 2006). Dave and Xiu (1991) in their experimental study, concluded that Cu and nickel are major inhibitors of zebrafish embryonic hatching compared to cobalt, lead, and mercury. Also, the literature asserts that adult fish are less susceptible to heavy metal toxicity in comparison with the larvae (Von Westernhagen, 1988) resulting in significant



effects on larval health (Hernandez et al., 2011; Finn, 2007).

The effect of toxic compounds may vary according to different stages of development (Jeziarska et al., 2009). For example, in early life stages, the most vulnerable and crucial periods for larvae are the first stages of embryonic development and subsequently, the hatching stage (Gellert and Heinrichsdorff, 2001). Heavy metals of environment can cause acute toxicity to zebrafish, whose effects associated varies according to different embryonic stages of development (Yang et al., 2016).

The use of a range of anatomical, biochemical, and viability indicators are useful in assessing the toxic effect of enhanced concentrations of Cu on developing embryos (Parichy et al., 2009). Heartbeat rate is one of the parameters affected by heavy metals and has the potential to be widely and easily used for toxicological research on developing zebrafish embryos (Kang et al., 2018). It is a reliable method for piloting experimental work required for designing toxicological experiments, such as selecting a potentially toxic agent and its concentration for further research. Also, the heartbeat rate is a reliable indicator of the functional status of this organ (Rubinstein, 2006; Yang et al., 2016). Heavy metals like Cu can affect heartbeat rate of fish embryos (Mu et al., 2013) with a variable magnitude according to the concentration of the toxic compound tested (Suvarchala and Philip, 2016; Yang et al., 2016). Sighting or dose-ranging studies are widely used as pilots for experimental work in toxicological research providing a rapid assessment of the experimental dosage for usage in the main experimental work (Stallard et al., 2003; Mielke et al., 2017).

Copper, as a trace element has an essential role in the physiology of embryonic development (Lee et al., 2001; Mackenzie et al., 2004) but in excess quantities, it has negative effects on the metabolism and development of fish (Johnson et al., 2007). The present experimental work aimed to assess the potential use of heartbeat rate for the initial evaluation of waterborne Cu exposure on zebrafish embryonic development and survival. For this reason, we monitored the survival rate and a range of anatomical malformations and heartbeat rate in developing zebrafish embryos exposed to different levels of Cu.

Material and Methods

We conducted experiments between March 2018 and

January 2019 at the Department of Agricultural Sciences, University of Ioannina. The experiments observed the fish welfare recommendations of the Faculty of Veterinary Medicine, University of Thessaly.

Experimental animals and reproduction of zebrafish

Adult zebrafish were acclimated and maintained in 10 liter aquaria at 28.5°C. All parent zebrafish were aged about 3-month old. We fed the fish *ad libitum* and under a photoperiod of 12 hours with male and female (ratio 30♂:15♀) broodstock fish kept in separately before the breeding day. The eggs were collected the next day early in the morning, within 1 hour after turning on the lights. We placed the viable eggs in standard zebrafish hatchery water (Brand et al., 2002). Water parameters that were monitored daily included temperature ($29 \pm 1^\circ\text{C}$), NO_x , with NO_2 and NO_3 remained at low levels ($<0.01 \text{ mg l}^{-1}$ and $<100 \text{ mg l}^{-1}$, respectively), NH_x , with NH_3 and NH_4 remained low levels ($<0.009 \text{ mg l}^{-1}$ and $<0.5 \text{ mg l}^{-1}$, respectively). General hardness ranged between 7° and 14° , and Carbonate hardness was 6° and, pH ranged between 7.2 and 7.3.

Using a stereoscope randomly selected fertilised eggs (2 or 4 cell-stage zebrafish) were placed in glass Petri dishes floating in experimental tanks. Subsequently, the eggs were divided in the different experimental groups and replicated ($n = 15$ per replicate) and placed in plate Corning® wells (24-well) containing the different experimental dilutions of Cu sulphate.

Copper exposures

In a pilot experiment carried out in our laboratory, the LC_{50} value of Cu for embryonic stages of zebrafish, the results showed 0.075 mg l^{-1} (Gouva et al., 2019). In the present work, test solution preparation included as needed with a series of dilutions using Copper sulphate- CuSO_4 . The current work includes duplicate control and experimental groups.

Monitored parameters up 96 hours after hatching

In each location, we used a Corning® plate (24-well) initially placed with successive diluted solutions of Cu were fertilised eggs were immersed. We consequently monitored all fertilised eggs (duplicates of 15 fertilised eggs for each treatment) during the following stages of exposure for observable sub-lethal effect and mortality. The duration of Cu exposure was 96 hours. The Corning® plates (24-well) were semi



immersed/floating in aquarium tanks where the temperature remained constant at 30°C using a temperature-controlled system with automatic thermostats. In the first 48 hours, observations and recordings took place every 6 hours, while in the next 48 hours they took place every 12 hours using a digital camera (Motic B₁ series, Motic China Group Co.) and a stereoscope (Motic SMZ-168 series, Motic China Group Co) with a built-in camera. We measured the anatomical features to the nearest 1 mm. Total length (TL) and estimated the yolk sack volume from samples obtained at 25, 50, and 75 hours after fertilisation ($n = 6$ from each group). We calculated the yolk sack volume (V) in accordance to the ellipsis equation: $V = 4/3 * \pi * (a/2) * (b/2)^2$ (a : max Length of yolk, b : max Width of yolk).

Heartbeat rate

For the needs of measuring the heart rate, we selected three larvae at random for each experimental group to which we carried out repeated measurements. The heart rate measurement was performed 6 hours after hatching for each experimental group, according to the methodology described by Barrionuevo et al. (2010). During the measurement, water temperature was kept at 28°C using a room temperature control unit. For greater accuracy in recording the measurement of heart rate of the selected larvae, we used a digital camera. We determined the heart rate by calculating the number of heartbeats from 20 to 30 seconds periods and subsequently we converted these values to heart rate in beats per minute, based on data from triplicate measurements for each sample of each duplicate.

Statistical analysis

To assess the effects of different exposure dilutions, we used Kruskal–Wallis non-parametric test after failing normality assumptions based on the Shapiro–Wilk test. Non-parametric *post hoc* tests followed to determine significance among the different groups. Cox regression analysis was applied to examine differences in hatching time and survival times among the different Cu dilution groups. All presented results have 95% confidence intervals (CI) and statistical

significance is set at $p < 0.05$. All analyses carried out using SPSS v23.0.

Results

Anatomical malformations

Exposure to Cu resulted in anatomical malformations which included abnormal formation of spinal curvature, pericardial edema, undeveloped lower jaws, and uninflated swim bladder. At 52 and 75 hours PSF, embryos of zebrafish larva of the 0.05 mg l⁻¹ Cu exposure, exhibited posterior body scoliosis, dysplastic yolk sac, abnormal fattening of their posterior part, and severe pericardial edema. We did not observe these manifestations in the previous sample obtained at 36 hours PSF. At 60 hours post fertilisation (PSF) larvae of the 50 mg l⁻¹ Cu exposure exhibited severe scoliotic middle skeletal portion, a curvature of caudal part and caudal tip malformation, full spine curvature, and caudal crus shortening. In particular, in the experimental group of 50 mg l⁻¹ Cu we observed one larva with skeletal problems, while in the experimental group of 0.05 mg l⁻¹ Cu we observed two larvae with skeletal problems.

Survival curves for time to hatching after Cu exposure and Hazard to death

Table 1 presents the hatching curves after Cu exposure. The survival curve regarding hatching showed that Cu affects hatching time. Cox regression analysis (Table 1) indicates that the greater the Cu concentration, the greater the hazard of non-hatching. More particularly, the analysis showed that the hazard ratio of non-hatching is 4.01 times higher (95% C.I.:1.97–8.14) in the 50 mg l⁻¹ group and 1.96 times higher (95% C.I.:1.12–3.43) in the 0.05 mg l⁻¹ group compared to the control group. These two groups also differ from one another with a hazard ratio of 2.04 (95% C.I.:1.04–4.01) higher hazard of non-hatching in the 50 mg l⁻¹ group compared to the 0.05 mg l⁻¹ group. Figure 1 presents the survival curves for time to hatching by type of Cu dilution for the control group and dissolved Cu group.



Table-1: Survival curves for time to hatching after copper exposure and Cox regression analysis on types of copper dilution for hatching time.

Treatment	Case Processing Summary				Means and Medians for Survival Time				Overall Comparisons					
	Total N	N of Events	Censored		Mean ^a				Log Rank (Mantel-Cox)	Chi-Square	df	Sig.		
			N	Percent	Estimate	Std. Error	95% Confidence Interval							
								Lower Bound	Upper Bound					
Control Group	28	28	0	.0%	32.464	1.076		30.356	34.573	Test of equality of survival distributions for the different levels of type of copper dilution.	26.697	2	.000	
Copper 50 mg l ⁻¹	16	16	0	.0%	48.188	3.280		41.758	54.617					
Copper 0.05 mg l ⁻¹	26	26	0	.0%	37.808	1.432		35.002	40.613					
Overall	70	70	0	.0%	38.043	1.230		35.632	40.454					
Treatment					p		HR		95,0% CI for HR					
Copper 50 mg l ⁻¹ vs Control Group					.000		4.01		1.97		8.14			
Copper 0.05 mg l ⁻¹ vs Control Group					.018		1.96		1.12		3.43			
Copper 50 mg l ⁻¹ vs Copper 0.05 mg l ⁻¹					.038		2.04		1.04		4.01			

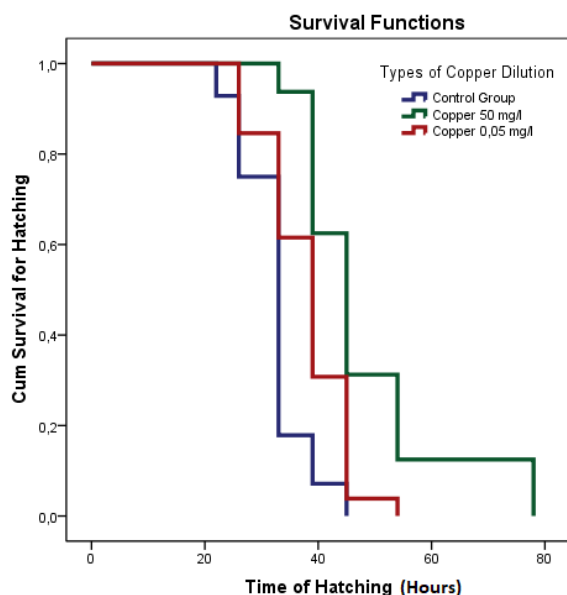


Figure-1: Post-hatching survival curves of zebrafish exposed to copper.

Survival of embryos and larvae and hazard to death up to 96 hours PSF

Table 2 presents the viability of fertilised eggs during embryonic and larval development. In the 500 mg l⁻¹ group, we observed total mortality of the fertilised eggs. At the same time, we observed 36.7% and 10.0% mortality in 0.05 and 50 mg l⁻¹ groups, respectively. Over the entire period of 96 hours PSF, the control and 0.05 mg l⁻¹ fish had similar viability, but the embryos

exposed to 50 mg l⁻¹ copper showed significantly lower viability compared to them.

Hazard analysis of the results provided some important information for the hazard of embryos exposed to Cu. Survival curves (Table 2) show that Cu is affected in developing embryos and larval viability after each exposure time. Cox regression analysis (Table 2) showed that the greater the Cu concentration, the greater the hazard of death. In particular, the analysis showed that compared to the control, the hazard ratio was 21.34 times higher (95% C.I.:6.11–74.50) when we exposed the fish to 500 mg l⁻¹; 6.20 times higher (95% C.I.:2.76–13.96) when we exposed the fish to 0.05; and 3.07 times higher (95% C.I.:1.57–6.02) in the 50 mg l⁻¹ fish group. The 50 mg l⁻¹ group also has a significantly higher hazard ratio compared to the control group 6.94 (95% C.I.:2.01–23.94). Differences between the Cu 0.05 mg l⁻¹ group and the control group as well as differences between the Cu 50 mg l⁻¹ and the Cu 0.05 mg l⁻¹ group were borderline non-significant ($p = 0.061$ and 0.084 , respectively) and 1.96 times higher (95% C.I.:1.12–3.43) in the 0.05 mg l⁻¹ group compared to the control group. The two groups further differ from one another with a hazard ratio of 2.04 (95% C.I.:1.04–4.01) higher hazard of non-hatching in the 50 mg l⁻¹ group compared to the 0.05 mg l⁻¹ group. The control group of survival times and dissolved Cu groups at 500, 50, and 0.05 mg l⁻¹ are significantly different.



Table-2: Survival curves for time to death after copper exposure and Cox regression analysis for lifetime after copper exposure.

Treatment	Case Processing Summary				Means and Medians for Survival Time				Overall Comparisons			
	Total N	N of Events	Censored		Mean ^a				Log Rank (Mantel-Cox)	Chi-Square	df	Sig.
			N	Percent	Estimate	Std. Error	95% Confidence Interval					
							Lower Bound	Upper Bound				
Control Group	30	3	27	90.0%	89.467	4.321	80.997	97.936	Test of equality of survival distributions for the different levels of type of copper dilution.	74.709	3	.000
Copper 500 mg l ⁻¹	30	30	0	.0%	1.000	.000	1.000	1.000				
Copper 50 mg l ⁻¹	30	16	14	46.7%	52.967	8.123	37.046	68.887				
Copper 0.05 mg l ⁻¹	30	10	20	66.7%	83.833	5.248	73.547	94.119				
Overall	120	59	61	50.8%	56.817	4.156	48.670	64.963				
Treatment					p		HR		95,0% CI for HR			
Copper 500 mg l ⁻¹ vs Control Group					.000		21.34		Lower		Upper	
Copper 50 mg l ⁻¹ vs Control Group					.002		6.94		6.11		74.50	
Copper 0.05 mg l ⁻¹ vs Control Group					.061		3.44		2.01		23.94	
Copper 500 mg l ⁻¹ vs Copper 0.05 mg l ⁻¹					.000		6.20		0.95		12.50	
Copper 50 mg l ⁻¹ vs Copper 0.05 mg l ⁻¹					.084		2.02		2.76		13.96	
Copper 500 mg l ⁻¹ vs Copper 50 mg l ⁻¹					.001		3.07		0.91		4.47	
									1.57		6.02	

Growth and metabolism

Figure 2 presents the growth in length of the developing larvae. Growth and development of the 0.05 and the 50 mg l⁻¹ groups were affected by Cu. Growth of the control fish was significantly higher compared to the 0.05 and the 50 mg l⁻¹ group.

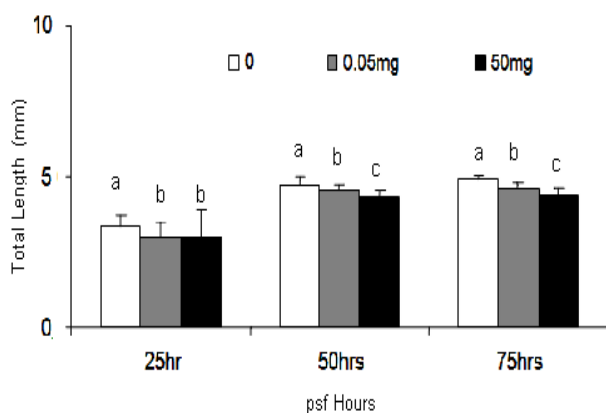


Figure-2. Effect of copper on the growth rate of zebrafish embryonic and larval stages of development.

At 25, 50 and 75 hours, TL of the controlled growth was significantly longer than the 0.05 and the 50 mg l⁻¹ fish group ($p < 0.05$), but there was no significant difference between 0.05 and 50 mg groups at 25 hours PSF.

Cu affected the metabolism of the developing larvae. Gradually after hatching, the fish of the control group utilised at a higher rate, the reserves of their yolk sack. The difference between the control and the Cu exposed group was more pronounced in the 50 mg l⁻¹ group (Fig. 3).

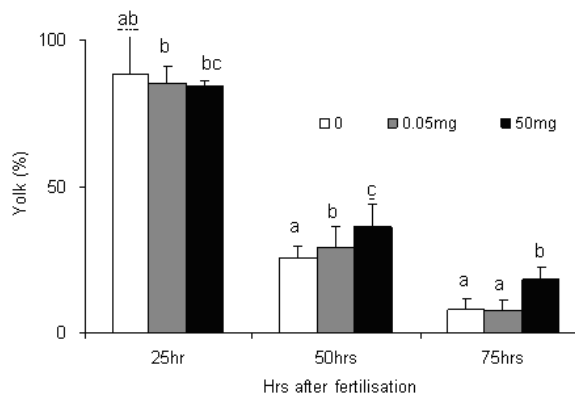


Figure-3: Cu effects on the embryonic metabolism as indicated by the yolk absorption rate

(Y axis indicates the amount of remaining yolk reserves as %) during embryonic and larval development of Zebrafish. All monitored stages (25, 50 and 75 hours) exhibited significant difference ($p < 0.05$) between the 50 mg l⁻¹ and the other two groups. There are significant differences between points that do not share the same letter.

Heart rate assessment

Figure 4 presents the comparative boxplot of heart rate by Cu dilution for the control group and treated groups. We observed that the levels of Cu concentration resulted in significantly higher heart rates in embryos exposed to 50 and 0.05 mg l⁻¹, showing higher heart rates at 6 hours after hatching for each experimental group compared to the non-exposed to Cu embryos of the control group. Kruskal–Wallis non-parametric *post hoc* analysis among heart rate and types of Cu dilution showed that Cu induced higher levels of heart rate. According to the above, the control group's heart rate was substantially lower than 50 mg l⁻¹ ($p = 0.002$) and 0.05 mg l⁻¹ ($p = 0.008$) group (Table 3). The two groups containing Cu did not significantly differ from one another ($p = 0.795$).

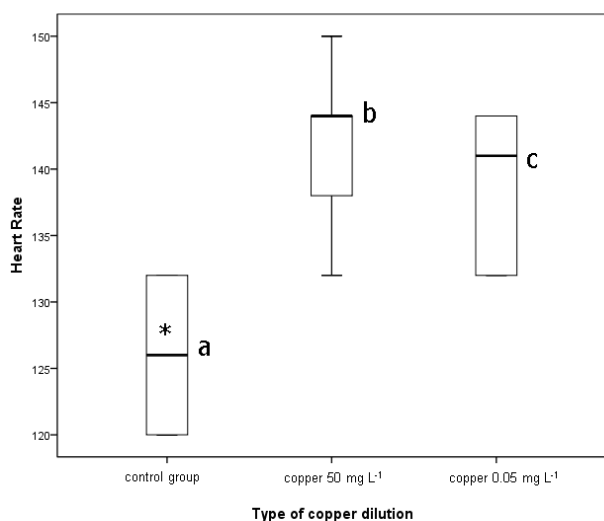


Figure-4: Comparative Boxplot of Heart Rate by Copper Dilution. Different letters indicate significant differences ($p < 0.05$; Kruskal–Wallis non-parametric *post hoc* test).

Table-3: Multiple Comparisons of heart rate among different treatments.

Treatment (Median Range)	Heart Rate Mean Rank	P value vs Control Group
Control Group (126.00 , 12)	4.00	-
Copper sulphate CuSO ₄ , 50 mg l ⁻¹ (144.00, 18)	13.17	.002
Copper sulphate CuSO ₄ , 0.05 mg l ⁻¹ (141.00, 12)	11.33	.008
Kruskal Wallis non parametric <i>post hoc</i> analysis.		

Discussion

There was a noticeable effect of Cu on all the monitored parameters in the present study. Survival, pathology, metabolism, and growth were affected by increasing concentrations of Cu. Mortality varied according to developmental stage and Cu concentration. Within the first 6 hours after fertilisation, we observed high mortality of fertilised eggs at the highest tested concentration of Cu (500 mg l⁻¹). Also, compared to the control group, we observed delayed embryonic development of embryos exposed to concentrations of 50 mg l⁻¹.

These results are in agreement with previous studies reporting that Cu inhibits pre-hatch embryonic development (Bai et al., 2010; Almond and Trombetta, 2016; Mohammadbakir, 2016). Cu can inhibit hatching (Johnson et al., 2007), and delay hatching in various species of fish (Mohammadbakir, 2016) and fish embryos exposed to Cu (Jeziarska et al., 2009). Moreover, according to Almond and Trombetta (2016), there is a direct correlation between hatching ability, time, and exposure and the ability of hatching is dose-dependent. Furthermore, the data of viability for developing embryos support proportional hazards assumption for the use of the Cox Proportional Hazards model, with not crossing curves for each treatment (McLaskey et al., 2016).

Similar results were observed even after exposure at lower Cu concentrations and in most cases, the highest mortality levels are exhibited within the first 24 hours PSF (Hernández et al., 2006; Hen Chow and Cheng, 2003), but the survival and magnitude of the effect vary in fish species and Cu concentration. According to Johnson et al. (2007), exposure to Cu during the first 24-hour PSF (hpf) in zebrafish (*Danio rerio*) resulted in delayed embryonic development and high mortality.

The results of the current study supports previous studies showing that exposure to Cu significantly decreased the survival of zebrafish embryos, particularly during the stages of gastrulation and segmentation (Johnson et al., 2007), with lethal and sub-lethal effects of environmental chemicals varying between the pre-hatch and post-hatch stages in zebrafish (Horie et al., 2017) and the concentration of the heavy metals (Ganesan et al., 2016).

In agreement with previous studies (Cheng et al., 2000; Villeneuve et al., 2014; Horie et al., 2017), the results of the present work indicate that exposure of embryos to Cu led in embryonic and larval anatomical

and functional defects. Pathological effects of Cu were present in all the experimental groups exposed to Cu, with skeletal malformations and pericardial edema as well as elevated heart rate. A significant observed effect of Cu was the morphological abnormalities, severe pericardial edema, spine curvature, posterior body scoliosis, caudal tip malformation, and abnormal fattening of the posterior part in zebrafish embryos. These results are in agreement with reported pathological problems after exposure to Cu, such as teratogenicity of Pb^{2+} and Cu^{2+} on zebrafish embryos, including bent spine, double tail spine, pericardial edema, and yolk sac edema, while several kinds of malformations often appeared together in one larva (Li et al., 2015; Sfakianakis et al., 2015; Zhang et al., 2015; Almond and Trombetta, 2016; Ganesan et al., 2016).

Exposure to Cu significantly affected embryonic development, with groups exposed to Cu, exhibiting lower rates of utilisation of yolk sac nutrient reserves. Embryonic metabolism relies on the utilisation of yolk reserves and can be adequately supported by oxygen diffusion during the pre-hatch stage via inward oxygen diffusion and outward carbon dioxide diffusion through the zona radiata (Nathanailides, 1996). After hatching, diffusion of respiratory gasses is facilitated via the skin and can adequately support the post-hatching metabolic requirements of developing zebrafish embryos (Pelster and Burggren, 1996). The rate of yolk absorption is an indication of the embryonic metabolism of developing fish. Yolk converts to body mass, and fast conversion rate indicates an elevated metabolic rate (Rombough, 1988). In other words, under optimal conditions, fish will efficiently and rapidly convert yolk reserved to body weight and environmental variables such as pollutants can impair this process (Peterson et al., 1983).

The results of the present work indicate that pre-hatch embryonic development indicates a significant effect of each tested Cu concentration with significant differences between each group. In embryos exposed to levels of 50 mg l^{-1} and above, we noted decreased hatching success and reduced rate of embryonic growth. Compared to the control and 0.05 mg l^{-1} , the 50 mg l^{-1} exhibited significantly delayed hatching. In the same manner, we observed the effects of Cu exposure on the metabolism of developing zebrafish in post-hatching stages. For example, groups exposed to Cu exhibited lower rates of utilisation of yolk sac nutrient reserves at Cu concentration as low as 0.05 mg l^{-1} .

In assessing the toxicity of Cu on developing zebrafish embryos, we used a range of different parameters. The copper-induced alterations of heartbeat rate observed in the present study are in agreement with previously published results for zebrafish embryos and larvae affected by toxic compounds, reporting that larvae exposed to Cu exhibited significantly increased heart rates compared to the control groups (D'amico et al., 2012; Mohammadbakir, 2016; Horie et al., 2017). The cellular mechanism of this may involve a stress response in the larvae (Barton, 2011), such as impaired aerobic metabolism with consequent effect on the cardiovascular system, resulting in an increased heart rate and, possibly, as the present results indicate, subsequent pericardial edema (Barrionuevo et al., 2010). Cardiovascular, respiratory and metabolic functions may exhibit developmental plasticity according to environmental parameters, including temperature, oxygen, and heavy metals (Nathanailides, 1996; Jezierska et al., 2009). Consequently, the toxic effect of Cu on zebrafish may involve a wider range of physiological processes with varying sensitivity to Cu according to different stages of development.

Interestingly sub-lethal toxic effects of Cu were manifested in heartbeat rate well before other monitored parameters of sub-lethal effects become significantly different in all the groups. The heartbeat rate can be an early sign and a reliable indicator in assessing the severity of Cu toxicity for developing zebrafish with minimal laboratory equipment and resources. This protocol may be easily strengthened by specialised image analysis software for automatic zebrafish heartbeat detection and analysis (Pylatiuk et al., 2014; Kang et al., 2018), reducing time and resources for initial evaluation of Cu toxicity on developing zebrafish. It would be interesting to validate this method in other fish species or environmental pollutants for potential reduction of required resources and experimental animals in pilot experiments for toxicological research.

Conclusion

The results of the present study indicate changes of physiological parameters and toxic effects of Copper on the zebrafish embryos which are important for the assessment of water toxicity and the role of heavy metals in aquatic ecosystems and their impact on aquatic organisms. Also, the heartbeat rate of the developing embryos can be a reliable indicator of the



effect of toxicity on the zebrafish. Heartbeat rate may be used as a rapid and convenient criterion for initial screening of toxicological evaluation of Cu on fish embryos or in sighting studies for determining appropriate starting concentration for further toxicological research.

Ethical statement

All fish studies have been approved by the appropriate ethics committee, and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments (Number of ethical permission: 33/07-04-2016).

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Conflict of Interest: None.

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Contribution of Authors

Gouva E: Designed and performed experiments, analysed data and co-wrote the paper.

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