Research Article [Anandhan *et al.*, 4(4): April, 2013]
CODEN (USA): IJPLCP **ISSN: 0976-7126**

INTERNATIONAL JOURNAL OF PHARMACY & LIFE SCIENCES

Effect of aluminium on development of Zebrafish, *Brachydanio rerio* (Ham.)

R. Anandhan*, S. Hemalatha, V. Kavitha and Gyaneswar Bhuyan
Department of Zoology, Annamalai University, Annamalainagar, (TN) - India

Abstract

The Zebrafish (*Brachydanio rerio*) has been extensively studied and well described for environmental toxicity studies. Molecular biology and genetics have recently been used to elucidate the underlying mechanisms of toxicity in zebrafish and to predict effects in mammals. The application of *Brachydanio rerio* embryo development technique to research on environmental science was summarized in this paper. This testing technique was approved as one of standard methods for chemical toxicity test, by international organizations (OECD,). There are many advantages of this testing, e.g., low cost, easy operation, high sensitivity and multiple sensible endpoints, comparing with other toxicity testing. Also, it was used to judge toxic mechanism of chemicals. The embryo development of zebrafish can be affected obviously by pollution, heavy metal, pesticide, organic reagent and complex chemicals. Among them, aluminium appeared stronger toxicity. The results are consistent with behavioral mixed pollutant monitoring and anatomical analyses of zebrafish *(Brachydanio rerio)* development.

Key-Words: Aluminium, Animal model, Toxicity, Heavy metal and *Brachydanio rerio*.

Introduction

In most fish, fertilization and embryogenesis occur outside the body of the female. Their development is readily affected by environmental factors such as heavy metal Aluminium present the water may severely impair fertilization, normal development and survival of embryos and also larvae. In a study concerning egg size, incubation period, and growth in the zebrafish, *Brachydanio rerio* (Hamilton), we encountered the problem of having to strip eggs from the females to synchronize the fertilization of the eggs artificially *Brachydanio rerio*, a freshwater fish, has become the most widely used standard model to study developmental genetics (Eisen,1991), indicating the presence of one or more dominant fish species usually present in a particular zone and these fish species monitor the unpolluted, less polluted, polluted and intensely polluted zones of the streams. Several other parameters such as fish population size, growth rate, condition factor and diversity are also indicative of the overall health of water and prevailing environmental conditions.

*** Corresponding Author**
Email:raman_anandhan@yahoo.co.in, gyana.iit@gmail.com

Its embryonic development and larval culture have been extensively studied. Yet reports detailing the various effects of embryogenesis are lacking Westerfield (1995). The present study addresses the effects of different aluminium on morphological and cytological aspects of development of the zebrafish. Over the past twenty years, the zebrafish (*Brachydanio rerio*) has emerged as a pre-eminent vertebrate model for studying genetics and development (Anandhan., 2009) and more recently, for human disease and the screening of therapeutic drugs (Penberthy *et al*., 2002; Sumanasa and Lin, 2004). A number of favorable attributes, including its small size, rapid development and generation time, optical transparency during early development, tractability in forward genetic screens, and genetic similarity to humans (Lamason *et al*., 2005) have fueled its rise in popularity for biomedical studies. The toxicity of aluminium to fish is primarily due to effects on osmoregulation by action on the gill surface (McDonald *et al*., 1989). Aluminum is readily accumulated on and in the gill, but little is found in blood or internal organs.

Thus the embryo is the life stage least sensitive to aluminum, whereas the fry stage (small larval fish) is the most sensitive; then sensitivity decreases with age (Cleveland *et al*., 1986). Aluminum toxicity is interactive with that of the hydrogen ion and usually occurs at pH values ranging from 0.3 to 0.6 pH units

above that at which the hydrogen ion causes some lethality. The toxicity of aqueous Al is reduced by Ca and dissolved oxygen (Spry and Wiener, 1991). Given the considerable importance of zebrafish as an experimental model, along with the significant economic costs associated with their large-scale use and the establishment and maintenance of culturing facilities, it is to some extent surprising that their husbandry is poorly developed. When compared with other commonly cultured fish species, such as tilapia (Lim and Webster, 2006) and channel catfish (Tucker and Robinson, 1990), published husbandry standards are wholly inadequate. The exchange of information about husbandry techniques between the numerous research facilities housing Zebrafish has been largely non-existent and any advances that have been made are often employed in isolation and without the benefit of peer review. An unfortunate outcome of this situation is that many zebrafish facilities likely operate at sub-optimal levels, a situation that is only further exacerbated when investigators new to the model are unable to consult scientifically rigorous resources when devising standard operating procedures for newly established culturing facilities.

The natural history of zebrafish a more holistic understanding of zebrafish in their native environment, including habitat preferences, reproductive behavior, and diet is necessary for both the refinement of husbandry standards and the optimization of their use in a wide array of biomedical and behavioral genetics studies. However, published information on the natural history of zebrafish is limited, and lags behind the significant amount of genetic and developmental data available for this species. In practice, little of the available behavioral and ecological information on zebrafish has been used to develop husbandry protocols, but this trend must change as the model continues to grow in popularity and is applied to different areas of research, such as behavioral genetics (Miklosi and Andrew, 2006). The importance of such information to husbandry is exemplified by the fact that data from the few field studies that have been conducted are referenced several times or more in various sections of the work.

The following is a brief summary of the available data, along with suggestions for further work that could be used to improve the care of zebrafish in research facilities. A fuller treatment of the behavior and ecology of the zebrafish is given by Lawrence.,C (2007) and Spence *et al*., (2006) Lifespan to date, there have been no reported studies detailing the lifespan of zebrafish in the wild. Spence *et al*. (2006) speculated that zebrafish are primarily an annual species based upon size distribution of specimens collected in monthly samples over the course of a year, and assessment of their reproductive strategy (spawning continuously upon maturity). However, these data are limited, and refer to only a single population. Documented life spans for laboratory zebrafish can exceed five years (Gerhard *et al*., 2002). Further collection of age-class data for wild populations will facilitate comparisons of the physiological ages between wild caught and laboratory housed animals, which will be useful for the design of studies particularly those that involve aging.

Material and Methods

The zebrafish, *Brachydanio rerio* (Hamilton) were procured from a local ornamental fish dealer kept at 26.0 ± 1.0°C in 50-liter aerated aquaria subdivided in two compartments by perforated plastic dividers, one female per compartment to enable identification and male another zebrafish, *Brachydanio rerio* were isolated. No visual contact was permitted between sexes. All individuals were subjected to 12 h of light per day and were generously fed "TetraMin AZ 100" and with ornamental fish pellet, twice a day. Water exchange and tank-cleaning were made as required in the morning. To ensure fertility, each fish was initially permitted to spawn naturally.

A total of 10 fertile females and 10 fertile males were used. Matured females and males were placed in the late evening in 'breeding traps' in 10 l tanks, to prevent them from eating the eggs laid. The fish usually laid eggs in the early morning of the following day. The naturally-spawned and fertilized eggs were transferred into Petri dishes containing egg water, 0.03% instant ocean salt mix. The eggs were checked under a stereomicroscope and the cleaving eggs alone were used. Adult zebrafish and zebrafish embryo are used for determination of acute toxicity test of aluminium. The acute toxicity testing includes determination of the LC_{50} value (the concentration that is lethal to 50% of the test fishes) Hamilton *et al*., 1977. A similar approach may be used for screening of acute toxicity of drugs. There is standard toxicity tests described and recommended by the International Standardization Organization (ISO), 1996.

The study consisted of four experiments. In the first series, fifty 2- to 4-cell embryos were transferred to Petri dishes containing 50 ml test solution with 0.02, 0.04, 0.06, 0.08 and 0.10ppm aluminium and incubated. In the second series, 50 same stage embryos were transferred to petridishes containing 50 ml test solution with 0.02, 0.04, 0.06, 0.08 and 0.10ppm aluminium incubated for 1–2 h and then returned to egg water. In the third and fourth series, 50 blastulae

and 50 gastrulae were incubated in 50 ml test solution with 0.02, 0.04, 0.06, 0.08 and 0.10ppm aluminium for 1–2 h and then returned to egg water. Control embryos were incubated in egg water the results represent the mean of three replicates per aluminium test. The standard error of mean (SEM) is also presented.

Results and Discussion

When 2 to 4-cell embryos were incubated till hatching at different aluminium level, the embryos cleaved and developed normally, as did the control at, although the survival was slightly lower (Table 1). In contrast, none of the embryos developed normally at higher than 0.02 ppm. The embryos incubated at 0.02, 0.04, 0.06,0.08 and 0.10ppm aluminium cleaved synchronously only during the initial 4 to 5 cell cycles, i.e. up to 16 to 32-cell stage, and then the cleavage became asynchronous; some blastomeres started disintegrating from the blastoderm at around 128-cell stage. Subsequently, the peri-vitelline space expanded, the ooplasm gradually collapsed and turned opaque, and all the embryos were dead before gastrulation, i.e. around 4 h after exposure. Apparently, the embryos incubated at aluminium higher than 0.02ppm were not able to undergo gastrulation. When the 2 to 4-cell stage embryos were exposed to 0.04ppm for 1 h, their cleavage rate slightly slowed down. When the control embryos reached 32 to 64-cell stage, they were at 16 to 32-cell stage. Around 72% of the treated embryos underwent gastrulation, and 65% hatched (Table 2). When 2 to 4-cell stage embryos were exposed to 0.04ppm for 2 h, 50% of them turned opaque and succumbed within 2 h, and 22% continued cleaving. Both the cleavage rate and pattern were affected, for the cleavage was slower than that of the control and the blastomeres arrayed irregularly. Although all the 14% of the cleaving embryos gastrulated, only 7% hatched (Table 2).

In addition, the hatched larvae failed to form conspicuous somites in the trunk (data not shown). When 2 to 4-cell embryos were exposed to 0.08 ppm for 1 h, only nil of them cleaved asynchronously and irregularly, but underwent gastrulation. Among them, not developed to hatching stage. In contrast, none of the same staged embryos exposed to 0.10ppm for 2 h survived. Similarly, all the 2 to 4-cell stage embryos exposed to 0.10ppm above for 1–2 h were dead. When the blastulae were incubated at 0.08 for 1 h, they developed synchronously, but the hatching was 6%, which was slightly lower than that of the control. When the embryos were incubated at 0.08ppm for 2 h, 2% of them developed to gastrulae and hatched. But in the embryos at 0.08 and 0.10ppm aluminium for 1 h, the development slowed down sharply and nil of the hatched at 62 h post-fertilization; this was around 6 h later than that of the control. At aluminium higher than 0.10 ppm aluminium for 2 h, all the embryos died prior to gastrulation (Table 3). When the gastrulae were exposed to 0.02, 0.04, 0.06, 0.08 and 0.10ppm aluminium for 1 h, they developed and hatched synchronously, as did the control, and the hatching success was 100, 100, 94, 76 and 40%, respectively. When the gastrulae were exposed to 0.02, 0.04 and 0.06ppm aluminium level for 2 h, their development was normal but slower, and hatching time was around 6 h later than the control. The hatching for the embryos exposed at gastrula at 0.02, 0.04 and 0.06ppm for 2 h was 100, 82 and 37% respectively (Table 4). For the gastrulae exposed to 0.08and 0.10ppm for 2 h, the embryos neither gastrulated nor survived. Zebrafish gastrulae were more toxic change than blastulae and the blastulae more than the cleaving embryos. Obviously, the aluminium of the zebrafish embryos improved with advancing development. Successful stripping was recorded at all times over the morning, afternoon, and early evening. After the stripping, without exception, a pair would commence natural spawning immediately upon introduction regardless of the time of day.

As discussed earlier, many of the freshwater fishes in the tropics spawn only in the rainy season. During this period, there is an addition of flooded lowland suitable for the deposition of eggs, an increase in the dissolved oxygen favorable for embryological development and an increase of organic and inorganic nutrients which promote growth of food plankton. Rain would also dilute any metabolic wastes accumulated during the dry season. Zebrafish are asynchronous, batch spawners that, under favorable conditions, spawn continuously upon attainment of sexual maturation (Breder and Rosen, 1966). Females are capable of spawning on a nearly daily basis. Eaton and Farley found that females would spawn once every 1.9 days if continuously housed with a male Eaton and Farley, (1974) and Spence and Smith (2006) showed that females in their experiments were capable of producing viable clutches every day over a period of at least 12 days, though variance in egg production was substantial. This interval is likely to be greater when the environment (water quality, diet, social situation, etc.) is sub-optimal or if the fish are used for production frequently.

In this context, metabolites may serve as a controlling factor, repressing ovulation until the rainy season when environmental conditions are more favorable for both embryo development and larval growth. There are many reports of Al lethality to fish especially from Scandinavia (Henriksen *et al*., 1984; Skogheim *et al*., 1984). The relative contribution of low pH and

elevated Al is difficult to determine and varies among geographic regions (Schindler, 1988). The results of the present study clearly indicate the stimulatory effect of aluminium heavy metal inhibitory effect of metabolites on development in the *Brachydanio rerio*. Further studies are needed to clarify the route of action of the development. In the present study, a sudden level aluminium increase does not seem important in stimulating development in the zebrafish. In the zebrafish, visual or auditory and lateral line stimuli between sexes do not seem important in enhancing ovulation, although some of these factors may be pertinent in eliciting the proper behavior during the actual spawning act.

Many fish, including zebrafish, use olfactory cues to differentiate between kin and non-kin, and this mechanism may be utilized during breeding to avoid mating with close relatives. For example, female rainbow fish Melanotaenia eachamensis and guppies Poecillia reticulate prefer unrelated over related males based on visual and olfactory cues (Hughes *et al*., 1999; Arnold, 2000). Zebrafish also appear to use olfactory cuts in social and mating contexts. Using odor plume tests, Gerlach and Lysiak., (2006) showed that adult female zebrafish chose the odors of non-related, unfamiliar (reared and maintained separately) males over those of unfamiliar brothers for mating. The underlying genetic basis of this preference is unknown, but may be the major histocompatibility complex (MHC) genes that are important in skin recognition in other fish species (Apanius *et al*., 1997).

It has been proposed that such direct behavioral interactions are highest at intermediate densities (Pickering, 1992) as territories are easiest to defend at low densities and impossible to defend at higher densities (McCarthy *et al*., 1992). Zebrafish have been shown to exhibit antagonistic behavior and to establish dominant– subordinate relationships (Larson *et al*., 2006), and so the possibility that these relationships between density and aggression may contribute to stress in captivity deserves further investigation. Reduced performance and impaired health of fish at elevated densities may also be caused by increased competition for resources and a decrease in water quality (Ellis *et al*., 2002). Because zebrafish in research settings are typically fed ad libitum, competition for food is less likely to be a factor than is water quality. In the absence of formal data for zebrafish, one may at least initially distinguish between density problems related to water quality and those caused by social environment and behavior by examining growth rates and size variation in clutches (Jobling, 1995). In this model, initially proposed by Jobling for rainbow trout, clutches with high mean growth rates and high variance in size indicate good water quality and social environment. Clutches with low mean growth rates and low variance are indicative of poor water quality, whereas clutches with low mean growth rates and high size variance are more suggestive of a poor social environment. A further issue involving rearing densities is the effect that they may have on sex ratio. Environmental conditions, including temperature, pH, behavioral interactions, and rearing densities have been shown to influence primary sexual differentiation in some fishes Anandhan., (2009). It has been suggested that zebrafish are also sensitive to rearing densities, based upon anecdotal observations that clutches reared at low densities often show a female bias (Brand *et al*., 2002). Although recent work suggests that it is actually growth rates caused by variations in food consumption that influence the expression of the sexual phenotype in zebrafish, densities do have an effect on the amount of food that each fish consumes, and thereby may influence growth rate and, in turn, sexual development. The early stages of fish exhibit high sensitivity to many inorganic toxicants (Jezierska *et al*.,2006). It is important to note that classifications of densities in zebrafish research tend to vary considerably depending on the experimental setting. Therefore, the raw data must be taken into account when interpreting the results of density related studies, especially if such data are to be applied to developing standards for husbandry. Given the potential effects of density on the health and productivity of zebrafish at different developmental stages, standard rearing and holding densities should be established to help minimize stress in laboratory populations.

Heavy metal contamination of the environment, which has been recognized as a serious pollution problem, is capable of exerting considerable biological effects even at low levels because of their pervasiveness and persistence nature. In the present study the data revealed that the lowest concentrations of aluminium capable of delivering an unrecoverable effect during a longer period of time to the test organisms. These concentrations prevailing in the creeks will alter the growth and development of the bottom dwellers. The results of present study show that heavy metals in the environment cause: reduction of the development and growth rates, developmental anomalies (scoliosis and skeletal ossification inhibited by aluminium), reduction of fish survival, especially at the beginning of exogenous feeding.

Acknowledgement

The authors thankful the Professor and Head, Department of Zoology Annamalai University, Annamalainagar.608 002,Tamilnadu, India for providing the necessary laboratory facilities.

References

1. Anandhan R., (2009). Studies on the effect of aluminium on embryonic development, histological and biochemical constituents in selected tissues of the freshwater Zebrafish *Branchydanio rerio* (Ham.). Ph.D thesis, Department of Zoology, Annamalai University, Annamalainagar-608 002. Tamilnadu. India.
2. Apanius V, D. Penn, P. R. Slev, L. R. Ruff and W. K. Potts, (1997). The nature of selection on the major histocompatibility complex. Crit. Rev. *Immunol*, 17: 179–224
3. Arnold K. F., (2000): Kin recognition in rainbowfish (*Melanotaenia eachamensis*): sex, sibs and shoaling. *Behav. Ecol. Sociobiol*, 48: 385–391.
4. Brand M., M. Granato and C. Nüsslein-Volhard (2002). Keeping and raising zebrafish. In: Nüsslein-Volhard, C., Dahm, R. (Eds.), Zebrafish: A Practical Approach. Oxford University Press, Oxford, pp. 7–37.
5. Breder C. M. and D. E. Rosen (1966). Modes of Reproduction in Fishes. The Natural History Press, New York. pp 941.
6. Cleveland L., E. E. Little, S. J. Hamilton, D. R. Buckler and J. B. Hunt (1986). Interactive toxicity of aluminum and acidity to early life stages in brook trout. *Trans. Am. Fish. Soc*, 115: 610–620.
7. Eaton R. and R. D. Farley. (1974). Spawning cycle and egg production in zebrafish, *Brachydanio rerio*, reared in the laboratory. *Copeia*, 1: 195–204.
8. Eisen J. S., (1991). Developmental neurobiology of the zebrafish. *J. Neurosci*, 11: 311–317.
9. Ellis T., B. North, A. P. Scott, N. R. Bromage, M. Porter and D. Gadd (2002): The relationships between stocking density and welfare in farmed rainbow trout. *J. Fish Biol*, 61: 493–531.
10. Gerhard G. S., E. J. Kauffman, X. Wang, R. Stewart, J. L. Moore, C. J. Kasales, E. Demidenko and K. C. Cheng (2002). Life spans and senescent phenotypes in two strains of Zebrafish (*Danio rerio*). *Exp. Gerontol*, 37: 1055–1068.
11. Gerlach G., N. Lysiak (2006). Kin recognition in zebrafish based on phenotype matching. *Anim. Behav*, 71: 1371–1377.
12. Hamilton M. A., R. C. Russo and R. V. Thurston. (1977). Trimmed Spearman-Karber method for estimating medium lethal concentrations in toxicity bioassays. *Environ. Sci. Technol*. 11:714-719
13. Henriksen A., O. K. Skogheim and B. O. Rosseland. (1984). Episodic changes in pH and aluminum-speciation kill fish in a Norwegian salmon river. *Vatten*, 40: 255–260.
14. Hughes K. A, L. Du, F. H. Rodd and D. N. Reznick .(1999). Familiarity leads to female mate preference for novel males in the guppy, *Poecilia reticulata*. *Anim. Behav*, 58: 907–916.
15. ISO, 1996. Water quality Determination of the acute lethal toxicity of substances to a freshwater fish [Brachydanio rerio Hamilton-Buchanan (Teleostei, Cyprinidae)] -- Part 1: Static method. 7346
16. Jezierska B., K. Lugowska, P. Sarnowski and M. Witeska .(2006). The effect of short-term water contamination with heavy metals on ford uptake rate of common carp larvae. Electron. *J. Icthiol*, 1:1-11.
17. Jobling M., (1995). Simple indices for the assessment of the influences of social environment on growth performance, exemplified by studies on Arctic charr. *Aquac. Int*, 3: 60–65.
18. Lamason R. L., M. A. Mohideen, J. R. Mest, A. C. Wong, H. L. Norton, M. C. Aros, M. J. Jurynec, X. Mao, V. R. Humphreville , J. E. Humbert, S. Sinha, J. L. Moore, P. Jagadeeswaran, W. Zhao, G. Ning, I. Makalowska, P. M. McKeigue, D. O'donnell, R. Kittles, E. J. Parra, N. J. Mangini, D. J. Grunwald, M. D. Shriver, V A. Canfield and K C. Cheng. (2005). SLC24A5, a putative cation exchanger, affects pigmentation in zebrafish and humans. *Science*, 310: 1782–1786.
19. Larson E. T., D. M. O'Malley and R. H. Melloni. (2006). Aggression and vasotocin are associated with dominant-subordinate relationships in zebrafish. *Behav. Brain Res*, 167: 94–102.
20. Lawrence C., (2007). The husbandry of zebrafish (*Danio rerio*): A review. *Aquaculture*, 269:1-20.
21. Lim C. E. and C. D. Webster (Eds.). (2006). Tilapia: Biology, Culture, and Nutrition.

Complete Book. *Haworth Press, Binghamton*, NY. pp 703.

22. McCarthy I. D., C. G. Carter and D. F. Houlihan. (1992). The effect of feeding hierarchy on individual variability in daily feeding of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J. Fish Biol*, 41: 257–263.
23. McDonald D. G., J. P. Reader and T. R. K. Dalziel. (1989). The combined effects of pH and trace metals on fish ionoregulation. In: Morris, R., Taylor, E., Brown, D., Brown, J. (Eds.), Acid Toxicity and Aquatic Animals. *Cambridge University Press, Boston*, pp. 221–242.
24. Miklosi A. and R. J. Andrew. (2006). The zebrafish as a model for behavioral studies. *Zebrafish*, 3: 227–234.
25. Penberthy W. T., E. Shafizadeh and S. Lin. (2002). The zebrafish as a model for human disease. *Front. Biosci*, 7: 9–14.
26. Pickering A. D., (1992). Rainbow trout husbandry: management of the stress response. *Aquaculture* 100: 125–139.
27. Schindler D. W., (1988). Effects of acid rain on freshwater ecosystems. *Science*, 239: 149–157.
28. Skogheim O. K. B. O. Rossseland and I. H. Sevaldrud. (1984). Death of spawners of Atlantic salmon in River Ogna, SW Norway, caused by acidified aluminum-rich water. *Rep. Inst. Freshwater Res. Drottningholm*, 61: 197–202.
29. Spence R. K. F. Runa, M. Reichard, K. A. Huq, M. A. Wahab, Z. F. Ahmed and C. Smith. (2006). The distribution and habitat preferences of the zebrafish in Bangladesh. *J. Fish Biol*, 69: 1435–1448.
30. Spence R. and C. Smith. (2006). Genetic analysis of male reproductive success in relation to density in the zebrafish, Danio rerio. *Front. Zool*, 3: 5.
31. Spry D. J. and J. G. Wiener. (1991). Metal bioavailability and toxicity to fish in low-alkaline lakes: a critical review. *Environ. Pollut*, 71: 243–304.
32. Sumanasa S. and S. Lin (2004). Zebrafish as a model system for drug target screening and validation. *Drug Discov. Today Targets*, 3: 89–96.
33. Tucker C. S. and E. H. Robinson. (1990). Channel Catfish Farming Handbook. Van Nostrand Reinhold, *New York*. pp 454.
34. Westerfield M., (1995). The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (*Danio rerio*), 3rd edition. *University of Oregon Press, Eugene*, OR. pp 385.

Table 1: Development of 2 to 4-cell zebrafish embryos incubated until hatching at different level of Aluminium

Aluminium (ppm)	Exposed embryos (no)	Developmental stage (%)		
		Cleavage	Gastrulation	Hatching
Control	100	100	100	93
0.02	100	70	67	62
0.04	100	60	55	46
0.06	100	57	50	45
0.08	100	55	34	31
0.10	100	50	12	09

Table 2: Development of the 2 to 4-cell stage Zebrafish embryos incubated until hatching at different Consentration of Aluminium (0.02,0.04,0.06,0.08 and 0.10ppm) for 1–2 h

Aluminium (ppm)	Exposed embryos (no)	Treatment duration (h)	Developmental stage (%)		
			Cleavage	Gastrulation	Hatching
Control	100	---	100	100	100
0.02	100	1	81 ± 1.34	72± 1. 76	65± 1.11
0.02	100	2	50 ± 2.05	36 ± 1.20	21± 2.14
0.04	100	1	46 ± 2.00	31 ± 1.86	18 ± 2.97
0.04	100	2	22 ± 1.56	14 ± 2.44	07 ± 1.07
0.06	100	1	09 ± 1.32	06 ± 2.64	03 ± 1.91
0.06	100	2	04 ± 1.02	03 ± 0.07	0

0.08	100	1	0	0	0
0.08	100	2	0	0	0
0.10	100	1	0	0	0
0.10	100	2	0	0	0

Table 3: Development of blastulae stage exposed to (0.02,0.04,0.06,0.08 and 0.10ppm) for 1–2 h

Aluminium (ppm)	Exposed embryos (no)	Treatment duration (h)	Developmental stage (%)		
			Cleavage	Gastrulation	Hatching
Control	100	---	100	100	100
0.02	100	1	90 ± 0.07	85± 1.18	72± 1.24
0.02	100	2	75 ± 1.05	66 ± 0.20	56± 1.98
0.04	100	1	53 ± 1.00	44 ± 1.34	40 ± 2.22
0.04	100	2	38 ± 2.06	35 ± 1.20	26 ± 1.00
0.06	100	1	22 ± 1.91	19 ± 2.89	15 ± 1.35
0.06	100	2	16 ± 3.00	13 ± 0.02	09 ± 2.56
0.08	100	1	08 ± 0.05	06 ± 1.97	03 ± 1.00
0.08	100	2	05 ± 0.08	02 ± 0.56	0
0.10	100	1	0	0	0
0.10	100	2	0	0	0

Table 4: Development of gastrulae exposed to (0.02,0.04,0.06,0.08 and 0.10ppm) for 1–2 h

Aluminium (ppm)	Exposed embryos (no)	Treatment duration (h)	Developmental stage (%)		
			Cleavage	Gastrulation	Hatching
Control	100	---	100	100	100
0.02	100	1	100	100	100
0.02	100	2	100	100	100
0.04	100	1	100	98 ± 2.89	94± 3.76
0.04	100	2	100	82 ± 1.00	76 ± 1.52
0.06	100	1	100	53 ± 2.33	42 ± 2.05
0.06	100	2	100	37 ± 3.02	20 ± 2.62
0.08	100	1	100	19 ± 1.00	11 ± 2.07
0.08	100	2	100	0	0
0.10	100	1	100	0	0
0.10	100	2	100	0	0