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ORIGINAL ARTICLE

ACUTE TOXICITY BIOASSAY AND BEHAVIOURAL CHANGES ON ZEBRA FISH, *Danio rerio* (Hamilton) UNDER ARSENIC TRIOXIDE

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ABSTRACT

The acute toxicity tests (24, 48, 72, and 96 hr) were conducted using static and renewal bioassay method to determine the lethal toxicity (LC_{50}) of metalloid compound Arsenic (III)oxide to the zebrafish, *Daneo rerio* (Hamilton) and to study its behavioural response during toxicity test. The safe level concentration was also determined. The dose and dose-time dependent increases in mortality rate was observed in the zebrafish in response to Arsenic (III)oxide. Behavioural parameters under investigations showed prominent changes as erratic swimming activity, increase in opercular movement, exudation of mucous over the bodies, loss of equilibrium and body dispigmentation in fish exposed to various lethal concentration of Arsenic(III)oxide. The prominent features of death were respiratory distress, paralysis and loss of equilibrium. Further, the observed behavioural alterations were dose-dependent effects in zebrafish, exposed to As(III)oxide. It is necessary, however, to select behavioural indices of monitoring that relate to the organism behavior in the field in order to derive a more accurate assessment of the arsenic pollution that contaminated may pose in natural system.

Key words: As(III)oxide, Acute toxicity, *Daneo rerio*, Behavioural indices

1.INTRODUCTION

Arsenic, an important environmental contaminant, is present in the aquatic environment as a result of geogenic and anthropogenic processes (Gonzaliz *et al.*, 2006; Singh and Banerjee, 2008) and it has been reported as one of the most alarming chemical (ATSDR, 2002). In the environment, arsenic is present in different forms and the toxicity depends upon its chemical form and oxidation states (Agusa *et al.*, 2008). The toxicity of arsenical in animals depends on species, sex, age, exposure dose, duration of exposure, organic or inorganic form, valence state, etc., (Luh *et al*., 1973 and Allen *et al.*, 2004). In natural water, arsenic is mostly exist in inorganic and organic form (Luh *et al.*, 1973) and the inorganic form has been found to be more toxic (Liao *et al.*, 2004). In the aquatic environment, the semimetal arsenic exists either in arsenite (the trivalent form, As^{3+}) or arsenate (the pentavalent form, As^{5+}), which are inter-converted through redox and methylation reaction (Reuther, 1992; Bears *et al.*, 2006). Trivalent arsenic (As^{3+}) is more toxic than pentavalent arsenic (As^{5+}) (Bertelero *et al.*, 1981; Liao *et al.*, 2004). Among the various arsenical compounds, arsenic trioxide ($As_2 O_3$) is mostly used in synthesis of various inorganic and organic compounds and in agricultural chemicals. It is also used as a chemotherapeutic agent for the treatment of hematological malignancies (List, 2002). Arsenic trioxide (As_2O_3) was a dominant species in most of the arsenic contaminated area in India and which is almost 50% of the total area arsenic level (Chatterjee *et al.*, 1993). The contamination of aquatic ecosystem by arsenicals and their impact on the aquatic organisms has now emerged as a serious environmental problem (Allen *et al.*, 2004).

A higher concentration of arsenic in the aquatic environment is lethal to many organisms (Pedlar *et al.*, 2002; Bhattacharya and Bhattacharya, 2007). Bears *et al*. (2006) indicate that fish can serve as vital indicators of arsenic toxicity as they are continuously exposed to arsenic through gill respiration and ingestion of arsenic contaminated food. In aquatic toxicology the traditional LC_{50} test is often used to measure the potential risk of a chemical. Although the toxicity studies and the determination of the lethal concentration for 50% (LC_{50}) of fishes have been worked out in different fish species (Roy *et al.*, 2006; Ghosh *et al.*, 2006; Sivakumar et al., 2006; Karuppasamy,2001). The effects of arsenic pollutant on definite fish function systems are yet to be clarified (Datta *et al.*, 2007).

The information about acute toxicity in fish abundant (Liao *et al.*, 2004; Roy *et al.*, 2006 and Ghosh *et al.*, 2006). The use of biological test system for monitoring pollution is gaining important worldwide (ISO,1982) by employing toxicity test model with use of a key species of fish (Verma et al., 1996).The different stages of fish were used in literature in evaluating $LC_{50,}$ the species

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comparison for acute toxicity is difficult. Fish tolerance to chemical exposure is increasing with developmental stage.

Parameters	Value
Temterature (ºC)	25±1ºC
pH	7.1-7.3
Dissolved oxygen (mg/L)	6.7-7.6
Salinity (mg/L)	0.3-0.35
Alkalinity as $CaCo_3$ (mg/L)	165-170
Total hardness as $CaCo_3$ (mg/L)	185-200
Photoperiod (hrs)	14 hr L:10 hr D

Sensitivity of fish to arsenic is variable in terms of 96 hr $LC_{50,}$ with

Table 2. Specification of the test chemical As(III)oxid

Chemical	Physical properties
Name :Arsenic (III)oxide	Colour :Colorless to white
Empirical formula : As_2O_3	Appearance :Crystalline (or) powder
Valence state :3,5	Melting Point :312.3ºC
Grade : Analytical,95.0%	Boiling point :465ºC
Supplier : Himedia Che ltd., Chennai, India	Density : 3.74(g/cm^3)
	Solubility : 37g/L at 20ºC,115 g/L at 100ºC

Sensitivity of fish to arsenic is variable in terms of 96 hr of LC_{50} with range of 10.8 to 105 mg/L. Colorado squawfish *Ptychocheilus lucius* was the most tolerant fish to arsenic exposure, with 96 hr LC_{50} of 150 mg/L arsenate at their larval stage (Hamilton and Buhl, 1997b). Rainbow trout *Salmo gairdneri* is the most sensitive species to arsenic exposure, and the LC_{50} is 10.8 mg/L (USEPA, 1985). In contrast to fish species, invertebrate species are very sensitive to arsenical compounds. Therefore, the arsenic toxicity to animals depends on species and the test condition. The test fish *Daneo rerio* is popular among the aquarium hobbyist and steady gain in recognition as a choice model of vertebrate. It is more comparable to humans than invertebrate model species (Barbazuk et al., 2000). Zebrafish has attracted little attention form their wide tolerance associated with genetic basis of behaviour under polluted environment. Generally, the zebrafish behavior is largely known from studies in natural environment, although few studies are available on zebrafish in toxicant contaminated environment. Thus, it is not clear in what respect and what extend of zefrafish may differ from other wild fish species to the response of toxicant environment. From the above information, the present study aims to assess the survival response in terms of acute toxicity test (LC_{50}) and behavioural response of zebrafish *Danio rerio* in view of arsenic(III)oxide exposure in laboratory condition..

2.MATERIALS AND METHODS

Experimental fish

Healthy adult wild-type zebrafish *Danio rerio* 4±1 cm length and 0.78 ±0.05g weight were purchased from Red hills fish farm, Chennai, Tamilnadu,India. Fishes were separately maintained at 25±1ºC in 150 capacity glass tank filled with dechlorinated tap water having the specific physic-chemical parameters listed in Table 1 and continuously aerated at least one month perior to the experiments. The test water parameters were measured according to the experimental procedure described by APHA (1998). The laboratory photoperiod was 10 hr D; 14 hr L. Fishes were fed with goldfish flake food (or) frozen brine shrimp twice per day *ad libitum.* During routine husbandry, $^1/_3$ water was renewed every day with filtered tap water. Feeding was suspended 24 hr before to start the experiment on mortality test for the fish.

Exposure chemical

The test chemical used for the experiments was analar grade Arsenic (III)oxide, which has specification listed in Table 2. It was chosen in the present study because today large quantities of arsenic waste are disposed into aquatic system through industrial activities (ATSDR, 2002). A stock solution of AS(III)oxide was prepared by dissolving 1 g in deionized water and then diluted with tap water to obtain the desired concentration.

Acute toxicity test

In the present study, 24, 48, 72 and 96 hr LC_{50} value and their 95% confidence limits of As(III)oxide for adult zebrafish, *Danio rerio* was determined following Probit analysis method of Finney, (1971). Five to ten concentration of As(III)oxide solutions were prepared for initial range tests. Each test with appropriate control was performed in duplicate using ten fish in each test tank. Definitive test s were subsequently performed using 9 to 10 various concentration of AS(III)oxide solution. Parallely, the controls were also carried out in duplicate with 10 test fish per vessel. The safe level of As(III)oxide at 96 hr of exposure was calculated by the methods of Sprague,(1971),CWQC, (1997), NAS/NAE, (1973) and IJC, (1997).

3.RESULTS

Acute toxicity test

The values of LC_{50}, upper and lower confidence limits, slope function and regression co-efficient results of As(III)oxide on zebrafish *Danio rerio* at different time intervals are presented in Table 3. The LC_{50} values of As_2O_3 on the adult fish were found to be 21.88, 16.21, 10.96 and 8.91 mg/L As(III) oxide respectively for, 48, 72 and 96hr of exposure. The 24 hr LC_{50} value of 21.88 mg/L As(III) oxide is approximately thrice that of 96 hr LC_{50} value of 8.91 mg/L. Toxicity of As_2O_3 revealed that the rate of mortality is increased with the increasing in the concentration of As(III) oxide. The mortality data were subjected to probit analysis and plotted against the log of dose concentration resulting in a straight line (Fig. 1). The safe level concentration of As_2O_3 was obtained by different methods for adult zebrafish (Table 4) at 96 hr exposure. The 100 per cent survival (LC_{100}) of fish for more than 96 hr was observed only belov at a concentration of 5.50 mg/L As_2O_3.

The 100 per cent mortality (LC_{100}) was observed over 96 hr of exposure at a concentration of 13.60 mg/L As_2O_3. Calculated chi-square values for the fitted regression were found to be in good fit

of the fitted regression which is in correlation with the observed arsenic concentration to the response of fish.

Danio rerio. Acute lethal toxicity test and the determination of LC_{50} value is the most reliable test for assessing the potential

Table 3 : LC_{50} value (with 95% confidence limit) and regression equation for *Danio rerio* treated with As(III)oxide

Period (hrs)	LC_{50} (mg/L)	95% confidence limit (mg/L)		Regression co-efficient (y)	Slope function (SF)	Calculated chi-square (x^2)
		Lower	Upper			
24	21.88	19.62	23.98	-13.38 + (3.49)x	3.94	0.97
48	16.21	14.12	18.62	-8.783 + (2.72)x	2.72	0.98
72	10.96	8.91	12.38	-3.673 + (1.22)x	1.22	0.97
96	8.91	7.11	10.22	-2.653 + (0.92)x	0.92	0.98

Table 4 : Estimates of safe levels of As(III)oxide at 96 hr of exposure

96 hr LC_{50}	Method	AF	Safe level (mg/L)
	Sprague (1971)	0.1	0.891
8.91mg/L	CWQC (1972)	0.01	0.0891
	NAS/NAE (1973)	0.1 to 0.0001	0.891 to 0.000891
	IJC (1997)	5% of 96 hr LC_{50}	0.4455

Behavioural response

The test fish *Daneo rerio* exposed to various lethal concentration (sublethal, median lethal and above median lethal concentration) of As(III)oxide during 24 hrs exposure, exhibited altered behavioural responses. During the exposure time, fish initially at all tested concentrations showed rapid movement, faster opercular activities, hyper excitability and tendency of escaping from the As(III)oxide toxicant water. However, the effects of above activities of *Danio rerio* related to concentration of As(III)oxide in the present observation (Table 5). It was observed that in median and above median lethal concentration, hyper behavioural activities were relatively increased initially (upto 6 hrs of exposure) and subsequently reduced expressing the sign of distress. Besides an interesting observations was that the fish had visible increase in body dispigmentation along with profuse mucus secretion all over the body with an increased exposure time and concentration (median lethal and above median lethal) at the end of exposure (24 hr) period, the fish struggled hard for breathing, with their reduction of swimming performance and this followed by a loss of equilibrium. Therefore, fish became progressively lethargic and lost their sense of equilibrium completely in both median and above median lethal concentrations. These behavioural effects are higher in higher concentration of As(III)oxide than the use of median lethal concentrations. Ultimately the fish laid down on the bottom of aquaria under the exposure of lethal concentrations

4.DISCUSSION

Acute toxicity on lethality

Result of this study provided several important contributions to the field of environmental toxicity. Acute toxicity test (LC_{50}) was conducted to determine LC_{50} values of As_2O_3 to the adult zebrafish, adverse effect on aquatic life (Brungs and Mount, 1978). Further, it is an important test to determine the concentration of a toxicant which may be allowed in receiving waters without adverse effects on living resources (Reish and Oshida, 1987). There is relatively little information about the acute toxicity of arsenic trioxide to fish, with only a few species having been tested. Fathead minnow *Pimphales promelas* (LC_{50} =25.6 mg/L) were found to be intermediate between mosquito fish *Gambusia affinis* (LC_{50} = 49.0 mg/L) and rainbow trout *Salmo gairdneri* (LC_{50} = 10.8 mg/L) in terms of acute arsenic toxicity (USEPA, 1985).

In the present study the median lethal concentration (LC_{50}) value of As_2O_3 to the *Danio rerio* was found to be 21.88, 16.21, 10.96 and 8.91 mg/L at 24, 48, 72 and 96 hr respectively (Fig.1). The 95% confidence limits for the LC_{50} values have been shown in table 3. The safe levels, estimated by different methods at 96 hr exposure are listed in table 4. Analysis of degree of scatter of the observed median lethal concentration and the regression coefficient in the fitted regression are in correlation with the tested As_2O_3 concentration to the response of fish. The inclination of the regression lines indicated that increase in As_2O_3 concentration enhanced the mortality of the fish. Calculated chi-square values for the fitted regression were found to be in good fit of the fitted regression lines.

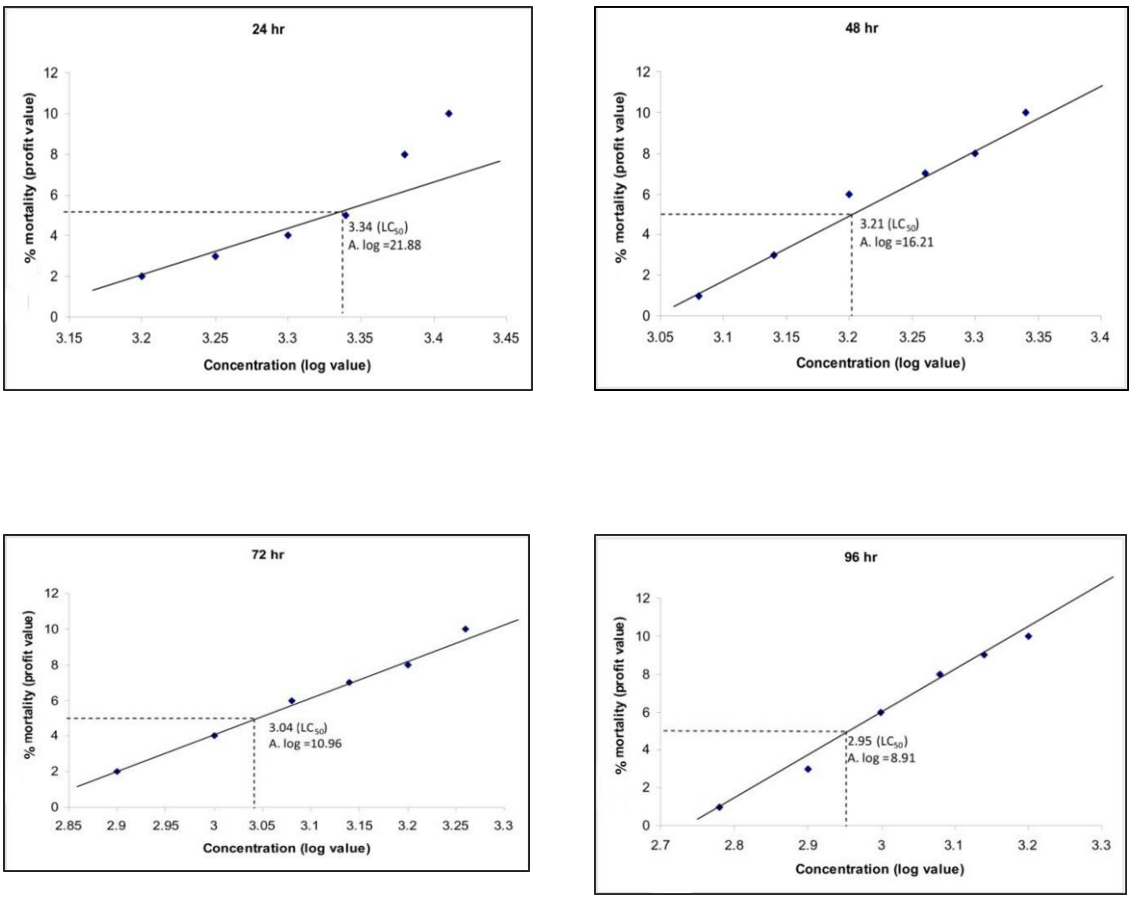
In the current study the 96 hr LC_{50} of arsenic trioxide to the fish *Danio rerio* was found to be 8.91 mg/L. This result of the present work strongly concurrent with the finding of the earlier report on different fish species tested with arsenic compounds. According to the earlier findings, a 96 hr LC_{50} of arsenic trioxide was found to be 14.9 mg/L for stickleback *Apelts quadricus* and 1.60 mg/L for Juvenile Atlantic silverside *Menidia menidia* (EPA, 1980), 2.73 mg/L for *Labeo rohita* (Vutukuru *et al.*, 2007), 10 mg/L for *Carassius auratus* (Maeda *et al.*, 1990), 30.03 mg/L for *Oryzias*

latipes (Suhendrayatna *et al.*, 2002), 21.0 mg/L for *Oncorhynchus mykiss* (Wang *et al.*, 2004), 8.4 mg/L for *C. batrachus* (Ghosh *et al.*, 2006), and 18.21 ppm for *Anabas testudineus* (Akter *et al.*, 2008). In addition, the LC_{50} of arsenate in rainbow trout *Oncorhychus mykiss* and northern *Ptychocheilus oregonensis* ranged from 58-114.1 and 54.8-73.4 mg/L, respectively, depending on temperature and hardness (McGeachy *et al.*, 1989). In the current experiment, the 96 hr LC_{50} of arsenic(III)oxide was 8.91 mg/L.Consequently, zebrafish, *Danio rerio* are much more sensitive to arsenic trioxide under the conditions tested in terms of acute toxicity compared with other fish species studied to date.

The previous literature clearly indicates that the acute toxicity of arsenicals is varying from one species to another species and even in strains of the same species. In general, toxicity of arsenic compounds depends on species, sex, age, dose, exposure period, their valence, nature, concentration and organic and inorganic form (Luh *et al.*, 1973). Gupta and Sastry (1981) have reported that difference in acute toxicity may be due to changes in water quality and test species. Among the different forms, inorganic arsenicals are more toxic than the organic compounds (Allen *et al.*, 2004).

In the present study, the safe levels estimated by different methods at 96 hr exposure showed a large variation for As_2O_3 to the zebrafish, *Danio rerio*. The estimated safe level of AS(III)oxide determined by different methods can not be guaranteed. The extrapolation of laboratory data to field is not always meaningful and hence it is difficult to decide on an acceptable concentration based on the laboratory experiments that may be considered "safe" in the field.

Fig.1 Relation between concentrations of arsenic trioxide and probit mortality of *Danio rerio*



Behavioural changes

The alternations in the behaviroral pattern are the most sensitive indicator of potential toxic effects. The behavioural changes observed in fish increased with the dose and duration of exposure (Table 5). Behavioural responses are important to survival because they are necessary to perform essential life functions (Little *et al.*, 1985). Since appropriate behavioural responses is obviously crucial to the survival of different life stage of fishes.

On initial exposure at higher concentration of As_2O_3 (24 hr exposure) the fish exhibited characteristic avoidance behaviour by rapid and erratic swimming with jerky movements and hyper-excitability. Enzymatic as well as ionic disturbances in blood and tissues may be associated with such abnormal behaviour and altered movements (Larsson *et al.*, 1981), while heavy exudation of mucus over the body and dispigmentation after 3 hr of exposure is attributed to dysfunction of the endocrine (pituitary) gland under toxic stress (Pandey *et al.*, 1990). Further, the swimming in

imbalanced manner and lethargic of arsenic(III) oxide exposed fish which might be due to inactivation of acetylcholinesterase leads to accumulation of acetylcholine at synaptic junctions. The heavy metal As(III) oxide is known to stimulate the peripheral nervous system and as a result the increases of metabolic activities and more oxygen utilization. Likewise, the faster opercular movement in fish has been reported to increase considerably following the exposure of toxins (Pandey *et al.,* 2005). Karuppasamy (2001) has observed the characteristics of restlessness, increased activity and opercular movements of fish put into hypoxic conditions.

Table 5: Effect of Arsenic (III)oxide on the behavioural activity of *Danio rerio* during 24 hr exposure to the various lethal concentration of As(III)oxide

Nature of behaviour	Exposure concentration			
	Control	24 hr HSC (7.29 mg/L)	24 hr MLC (21.88 mg/L)	24 hr <MLC (27.99 mg/L)
Hyper excitability	-	+	+ +	+ + +
Opercular movement	-	+ +	+ +	+ + +
Fin movement	-	+ +	+ +	+ + +
Dispigmentation	-	+	+ +	+ + +
Mucus secretion	-	+	+ +	+ + +
Imbalanced swimming	-	+	+ +	+ + +
Mortality	-	-	++	+++

HSC - High sublethal concentration; MLC- Median lethal concentration (LC_{50} of 24 hr);
<MLC -above Median lethal concentration
- = None (or) Normal; + = Mild effect; ++ = Moderate effect; +++ = High effect

The movement of the fish to the bottom of the tank under As_2O_3 stress in the present experiment clearly indicates the avoidance behaviour of the fish, which was reported by Pickering (1980) in fathead minnow. Abnormal behaviours were also observed in different fishes treated with various heavy metals (Santha *et al.,* 2000; Karuppasamy, 2001; Subathra and Karuppasamy, 2003; Sivakumar *et al.,* 2006). The abnormal behaviors were probably caused by the neurotoxic effects and also by the irritation to receptor system of the body. Jumping out and to and fro movement signified the avoidance reaction of the fishes to the toxicants. Secretion of excessive mucus over the body was probably due to irritation of the skin because of with the stress of As(III) oxide. Further, the accumulation and increased secretion of mucus in the fish exposed to As_2O_3 may be an adaptive response perhaps providing additional protection against corrosive nature of the metal and to avoid the absorption of the toxicant by the general body surface. This agrees to the earlier findings of Karuppasamy (2001); Subathra and Karuppasamy (2003); Sivakumar *et al.,* (2006). Lateral swimming and loss of equilibrium were probably due to the impairment of nervous system (Sinha and Kumar, 1992), which is controlling all the vital activities. Finally, the fish were found dead scattered at the bottom of the tank with their mouth wide open. The control fishes i.e., untreated fish remained alive and active throughout the experimental period.

In general, the determination of any persistent toxicant concentration in aquatic environment may not provide information on the severity of contamination. Biological monitoring using a series of assays having different end points in a "key species" could allow a sensitive approach to predict the potential risk of persistent chemicals like heavy metals. Acute toxicity studies are very first step in determining the water quality requirement of fish. Behavioural characteristics are obviously sensitive indicators of toxicant effect. It is necessary, however, to select behaviuoral indices of monitoring that relate to the organism behaviour in the field in order to derive a more accurate assessment of the hazards that contaminant may pose in natural system. Thus, it can be concluded from the present study that the fish *D. rerio* are highly sensitive to As(III)oxide. Therefore it is suggested that sufficient care must be taken to avoid the release of arsenical compounds into the aquatic system.

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