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***Research Article** (Open Access)*

EVALUATION ON THE SENSITIVITY OF THE FRESHWATER TROPICAL WORM, *BRANCHIURA SOWERBYI* BEDDARD, 1892TO FLUORIDE

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ABSTRACT
The present study was undertaken to determine the sensitivity of the freshwater tropical worm, *Branchiura sowerbyi* Beddard, 1892 (Oligochaeta: Naididae: Rhyacodrilinae) to fluoride on the basis of 24, 48, 72 and 96h lethal concentrations ($LC_{1, 5, 10, 15, 50, 85, 90, 95, 99}$) and their behavioural responses. The 24, 48, 72 and 96h LC_{50} value of fluoride to the worm were recorded as 249.996, 240.618, 231.757 and 224.227 mg/l respectively. The rate of mortality of the worm was significantly increased (p<0.05) with increasing concentrations and time of exposure (24, 48, 72 and 96h). The study further focussed on estimation of the toxicity factor at different time scale and possible safe level of fluoride to the worm to strengthen the base line data that could be used to set up local water quality criteria (WQC) for the toxicant. The worms showed behavioural changes like clumping tendency, movement and mucous secretion with increasing concentration of the toxicant and the progress of time. The findings can be used in determination of ecological risk assessment for the worm to fluoride toxicity as well as to understand its mode of action in the light of their ethological changes.

Keywords: *Branchiura sowerbyi, Fluoride, Acute Toxicity, Toxicity Factor, Safe Level, Behavioural Responses*

INTRODUCTION
Fluoride is an inherent component of igneous and metamorphic rocks and ranked for the 13th in the order of abundance in earth's crust (Mariappan *et al.*, 2000a). It occurs in the combined form because of its high reactiveness. The concentration of fluoride has been increasing in both ground water and surface waters day to day owing to its expanded industrial discharges from aluminium smelters, thermal power plants, fluoridated municipal waters and plants manufacturing brick, ceramics, glass and fluoride chemicals, and also from other factories like textiles, refrigerants, paper pulps, pharmaceuticals, fertilizers, pesticides, surfactant etc. (Rai *et al.*, 1996; Roy *et al.*, 2000; Camargo, 2003; Kumar *et al.*, 2007; Casellato *et al.*, 2014; Gupta and Poddar, 2014). Today fluoride has been emerged as one of the major pollutants in ecotoxicological studies because of its ubiquity and toxicity (NAS, 1971; Sharma, 2002). It is one of the components, which are toxic at high concentrations (Bagale *et al.*, 2015). Sodium fluoride (NaF) is the most common inorganic toxic form of fluoride to the aquatic organism (Sanders and Cope, 1966). In India, many industrial areas, particularly near the vicinity of aluminium, glass, ceramics, fertilizer factories are identified for containing high quantities of fluoride in ground water (Gupta *et al.*, 1994; Sahu *et al.*, 1998). Excessive fluoride concentrations in ground waters have been reported in 17 states of India, causing serious environmental hazards (Mariappan *et al.*, 2000b; Bhargava, 2010). It has been found that 65 per cent of India's villages are at fluoride risk (Kumar and Shah, 2006).Though fluoride toxicity is endemic to several countries of the world, but concerning effects of elevated fluoride toxicity on various aquatic organisms especially to fill aquatic ecosystem are still under scope in

comparison to humans, live stock and plants (Camargo, 2003). Aquatic organisms are continuously exposed to high concentrations of fluoride in surface waters and face harmful effects due to its entry into food chain and accumulation in the exoskeleton of invertebrates (Chlubek, 2003; Gupta and Poddar, 2014). Aquatic invertebrates living in soft waters are more affected by fluoride pollution than those living in seawater as its toxicity is reduced with increasing water hardness (Camargo 2003). It is now recognized that fluoride ion affects soft tissues cells (NRC, 2006) but it mainly acts as enzymatic poisons that inhibit enzyme activity and ultimately interrupt metabolic processes such as glycolysis and the synthesis of proteins (Chitra *et al.*, 1983; Kessabi 1984; Gupta, 2003; Casellato *et al.*, 2012; Gupta and Poddar, 2014). Several studies have been conducted on the fluoride toxicity in fresh water organisms, especially in fish involving their morphological, behavioural, biochemical, haematological and histopathological changes (Saxena *et al.*, 2001; Tripathi *et al.*, 2004; Kumar *et al.*, 2007; Narwaria and Saksena, 2012; Gupta and Poddar, 2014; Yadav *et al.*, 2014), but the data on aquatic oligochaetes are still scanty, although they have a long history of use as an indicator organism to monitor aquatic pollution (Whitley, 1967; Brkovic-Popovic and Popovic, 1977; Kaviraj and Konar, 1982; Khangarot, 1991; Chapman, 2001; Del Piero *et al.*, 2014; Lobo and Espindola, 2014; Dhara *et al.*, 2019).

In the light of the above, the present investigation was undertaken to determine the sensitivity of the tropical aquatic worm, *Branchiura sowerbyi* Beddard, 1892 to fluoride. This endobenthic worm is broadly distributed in the sediments of freshwater bodies (Tyler, 2009) and can be easily collected and identified due to their large size and prominent posterior gills (Ducrot *et al.*, 2007).They dominates other bottom macroinvertebrates in terms of abundance and biomass (Ducrot *et al.*, 2007). They feed on sediment which involves in the intake of large amounts of substrate (Wang and Matisoff, 1997). Again they are in turn fed on by the higher tropic level organisms like crustaceans, fish etc. and thus they form important links in detritus food chain. In addition, they require little space, are easily adaptable to laboratory conditions and have a low natural mortality rate in the laboratory compared to other tubificids (Bonacina *et al.*, 1994; Lobo and Espindola, 2014). In the study, the acute lethal toxicity during the period of 24, 48, 72 and 96h has been assessed. Besides, their behavioural changes due to toxic stress were also analyzed. The study further focussed on the toxicity factors at different time scale and on the estimation of the possible safe level of fluoride. The findings may help to design environmental monitoring strategies and ecosystem conservation measures.

MATERIALS AND METHODS

Healthy, mature and acclimatized specimens of *Branchiura sowerbyi* (Oligochaeta: Naididae: Rhyacodrilinae) (mean length 2.01 ± 0.56 cm; mean weight 2.13 ± 0.68 mg) collected from single population were undertaken for 96h bioassay.

Analytical grade NaF (assay ≥ 97%, molecular weight 41.99 g/mol; E. Merck India Ltd., Mumbai; Batch no. MJ2K520652) was used as the test chemical.

Static replacement bioassays were conducted in 500 ml Borosil glass beakers each containing 300 ml water under the laboratory condition to determine the sensitivity and behavioural changes of the worms.

Water chemical analysis and the bioassays were done following the methods outlined in American Public Health Association (APHA, 2012). Tap water stored in the glass aquaria (temperature 21 ± 0.45 °C, pH 7.8 ± 0.21, free CO_2 4.0 ± 0.12 mg/l, DO 5.54 ± 0.42 mg/l, alkalinity 220 ± 7.01 mg/l as $CaCO_3$, hardness 175 ± 7.0 mg/l as $CaCO_3$) was used as a diluent medium. During bioassay, the worms were subjected to different concentrations of fluoride. A set of three beakers was exposed to each concentration of the toxicant to make three replicates per concentration. Each set of tests was also accompanied by three replicates of control. Ten test organisms were used in each replicate. Test concentrations were prepared by diluting appropriate aliquot of the stock solution into the test medium. Initially, rough range finding tests were conducted for the toxicant to determine the dose range at which mortality occurs. The selected test concentrations of the toxicant (200, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270 mg/l) were finally used for the determination of 24, 48, 72 and 96h acute lethal concentration to the

worm. The vitality of the worms was frequently checked using soft tweezers, and they were considered dead when there was no response to physical stimulation within 10s (Maestre et al., 2009; Del Piero *et al.,* 2014). The number of dead organisms was counted every 24h and removed immediately from the test medium to avoid any organic decomposition and oxygen depletion (Dhara *et al.,* 2013; Lobo *et al.,* 2016). The test medium was replaced every 24h by freshwater and the desired quantity of respective toxicant was immediately added to the water to maintain a constant concentration of the toxicant in the solution and to avoid the interference of other abiotic factors with the animals' performance (Al-Attar, 2005; Dhara *et al.,* 2013). Finally, cumulative mortality rate of the worms at different concentrations of each toxicant and at different times of exposure (24, 48, 72, 96h) was analyzed using the computer software R version 2.14.0 (US EPA, 1999) and probit analysis by Finney (1971) for determining lethal toxicity ($LC_{1,5,10,15,50,85,\ 90,95,99}$) with 95% confidence limits of fluoride to the test organism. The relation between mortality rate with exposure time and doses was determined by analysis of variance (ANOVA) followed by DMRT (Gomez and Gomez, 1984).

On the basis of acute toxicity values, toxicity factors at different exposure period (24, 48, 72 and 96h) were assessed following the formula coined by Ayoola *et al.,* (2011):

Toxicity factor (TF): (LC_{50} at 24h/ LC_{50} at any other exposure time)

The safe level estimation was calculated by multiplying the 96h LC_{50} with different application factors (AF) based on Sprague (1971), Committee on Water Quality Criteria (CWQC, 1972), National Academy of Sciences/ National Academy of Engineering (NAS/ NAE, 1973), International Joint Commission (IJC, 1977) and Canadian Council of Resources and Environmental Ministry (CCREM, 1991) and also based on the formula developed by Hart *et al.,* (1948).

The behavioural changes of the test organism exposed to various concentrations of fluoride like movement, clumping tendency and mucous secretion were recorded systematically by naked eye observation during the bioassay following the method of Rand (1985).

RESULTS

The acute toxicity of fluoride ($LC_{1,5,10,15,50,85,90,95,99}$ values) with 95% confidence limit to *Branchiura sowerbyi* during the exposure period of 24, 48, 72 and 96h are given in **Table 1, 2, 3** and **4** respectively. No mortality was observed in the control group during the experiment.

Table 1: 24h lethal concentration ($LC_{1,5,10,15,50,85,90,95,99}$) values with 95% confidence limits of fluoride to *Branchiura sowerbyi* (Control group theoretical spontaneous response rate = 0.0000)

Lethal Concentration points	Concentration values with 95% confidence limits (mg/l)	Slope ± SE	Intercept ± SE
LC_1	194.097(153.437-211.076)		
LC_5	209.033(176.988-222.267)		
LC_{10}	217.460(190.842-228.650)		
LC_{15}	223.339(200.672-233.210)	21.165	–45.752
LC_{50}	**249.996(241.566-260.401)**	±	±
LC_{85}	279.834(266.561-317.196)	5.263	12.597
LC_{90}	287.399(271.754-333.685)		
LC_{95}	298.986(279.454-359.939)		
LC_{99}	321.993(294.179-415.314)		

Table 2: 48h lethal concentration ($LC_{1,5,10,15,50,85,90,95,99}$) values with 95% confidence limits of fluoride to *Branchiura sowerbyi* (Control group theoretical spontaneous response rate = 0.0000)

Lethal Concentration points	Concentration values with 95% confidence limits (mg/l)	Slope ± SE	Intercept ± SE
LC_1	182.802(141.890-200.154)		
LC_5	198.125(165.626-211.715)		
LC_{10}	206.813(179.712-218.334)		
LC_{15}	212.891(189.756-223.076)	19.4912	–41.416
LC_{50}	**240.618(231.856-251.594)**	±	±
LC_{85}	271.956(257.932-311.661)	4.836	11.491
LC_{90}	279.949(263.394-329.254)		
LC_{95}	292.225(271.511-357.412)		
LC_{99}	316.720(287.090-417.353)		

Table 3: 72h lethal concentration ($LC_{1,5,10,15,50,85,90,95,99}$) values with 95% confidence limits of fluoride to *Branchiura sowerbyi* (Control group theoretical spontaneous response rate = 0.0000)

Lethal Concentration points	Concentration values with 95% confidence limits (mg/l)	Slope ± SE	Intercept ± SE
LC_1	179.508(148.032-194.735)		
LC_5	193.456(168.159-205.664)		
LC_{10}	201.330(179.851-211.898)		
LC_{15}	206.825(188.092-216.335)	20.967	–44.589
LC_{50}	**231.757(223.216-240.462)**	±	±
LC_{85}	259.694(248.446-284.981)	4.514	10.681
LC_{90}	266.782(253.667-298.016)		
LC_{95}	277.640(261.371-318.718)		
LC_{99}	299.213(276.055-362.031)		

Table 4: 96h lethal concentration ($LC_{1,5,10,15,50,85,90,95,99}$) values with 95% confidence limits of fluoride to *Branchiura sowerbyi* (Control group theoretical spontaneous response rate = 0.0000)

Lethal Concentration points	Concentration values with 95% confidence limits (mg/l)	Slope ± SE	Intercept ± SE
LC_1	170.350(128.327-188.204)		
LC_5	184.629(150.046-198.962)		
LC_{10}	192.725(162.997-205.065)		
LC_{15}	198.389(172.288-209.382)	19.492	–40.819
LC_{50}	**224.227(214.114-232.570)**	±	±
LC_{85}	253.430(242.015-284.029)	4.877	11.496
LC_{90}	260.878(247.275-300.018)		
LC_{95}	272.318(254.999-325.736)		
LC_{99}	295.144(269.698-380.695)		

The effect of different concentrations of fluoride on the mortality of *B. sowerbyi* during different time of exposure (24, 48, 72 and 96h) is presented in **Figure 1**. Significant relationship ($p<0.05$) between mortality rate of the worm and exposure times was recorded at all concentrations of the toxicant.

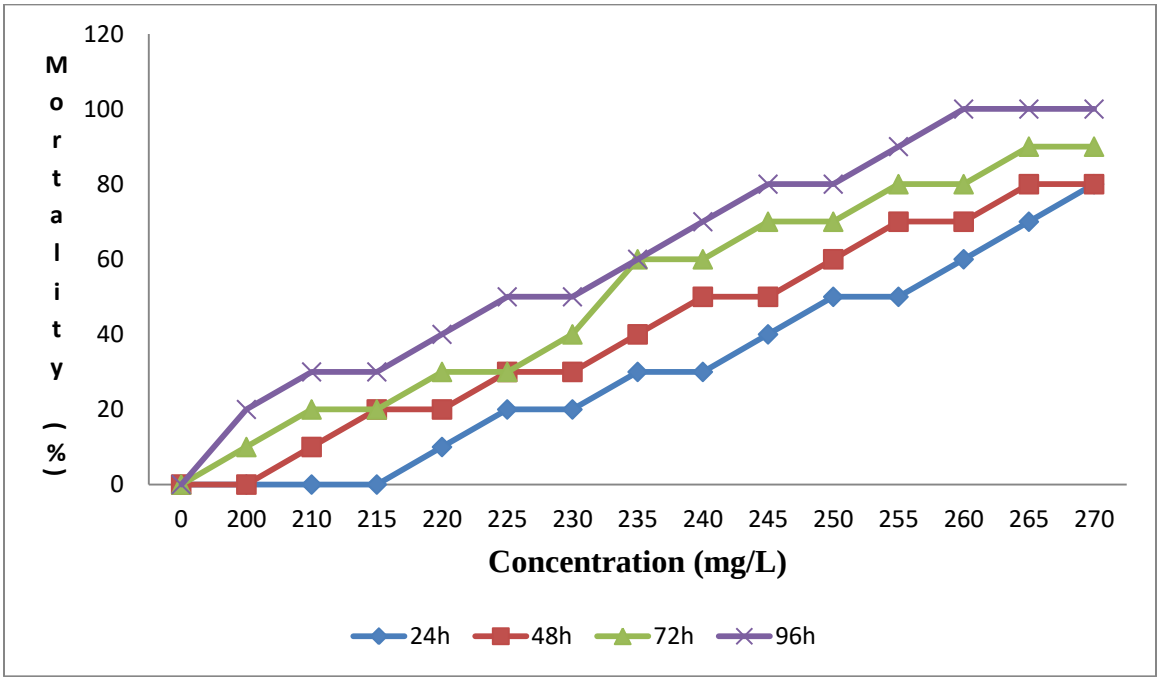

Figure 1: Relationship between the concentrations of fluoride and mortality of *Branchiura sowerbyi* during 24, 48, 72 and 96 hr

The toxicity factors as calculated from the medial lethal toxicity values at different time of exposure are tabulated in **Table 5**.

Table 5: Toxicity factors for *Branchiura sowerbyi* exposed to fluoride at different time scale (24, 48, 72 and 96h).

Exposed time (h)	Toxicity factor value
24	1.000
48	1.039
72	1.079
96	1.115

The estimated possible safe level of zinc for the worm as calculated by multiplying their 96h LC_{50} values with different application factors are recorded in **Table 6**. In the present study, the safe level estimated for the toxicant is varied from 22.423-0.00224 mg/l.

Table 6: Estimate of safe levels of fluoride to *Branchiura sowerbyi* at 96h of exposure time

Name of the test organism	96h LC_{50} value (mg/l)	Method	Application factor (AF)	Safe level (mg/l)
Branchiura sowerbyi	224.227	Hart *et al.*, (1948)*	-	6.687
		Sprague (1971)	0.1	22.423
		CWQC (1972)	0.01	2.242
		NAS/NAE (1973)	0.1-0.00001	22.423-0.00224
		IJC (1977)	5% of 96h LC_{50}	11.211
		CCREM (1991)	0.05	11.211

*(*C = 48h LC_{50} X 0.03/S^2, where C is the presumable harmless concentration and S = 24h LC_{50}/48h LC_{50})*

The behavioural changes observed in the test organisms exposed to various lethal concentrations of fluoride are presented in **Table 7**. The worms in the control group were active throughout the test period and showed clumping tendency with their normal movements. The clumping tendency of *B. sowerbyi* inversely varies with increasing concentration of fluoride and exposure times. It was pronounced at 72 and 96h. The worm showed faster movement initially but decrease in movement was observed with increasing concentration of fluoride and time of exposure than that of control. With the progress of time the treated worms were separated from each other and remained individual after 72h at higher concentrations. The excessive mucous secretion was observed in the treated worms at higher concentrations of fluoride at 72 and 96h of exposure. With the progress of time the body colour of the treated worms was gradually changed from red to white. Finally the worms died.

Table 7: Impact of fluoride on the behavioral responses of *Branchiura sowerbyi* (CT: clumping tendency; M: movement; MS: mucous secretion; –: none; +: mild; ++: moderate; +++: strong) at various concentrations during different hours of exposure.

Dose	24h			48h			72h			96h		
(mg/l)	CT	M	MS	CT	M	MS	CT	M	MS	CT	M	MS
0.000	+++	+++	–	+++	+++	–	+++	+++	–	+++	+++	–
200.0	+++	+++	–	+++	+++	–	+++	+++	–	+++	++	+
210.0	+++	+++	–	+++	+++	–	+++	++	–	++	+	+
215.0	+++	+++	–	+++	+++	–	+++	++	–	++	+	++
220.0	+++	+++	–	+++	++	+	++	++	+	++	+	++
225.0	+++	+++	–	++	++	+	++	++	+	++	+	++
230.0	+++	++	–	++	++	+	++	++	++	++	+	++
235.0	++	++	+	++	++	+	+	++	++	+	+	+++
240.0	++	++	+	++	++	++	+	+	++	+	+	+++
245.0	++	++	+	++	++	++	+	+	++	+	+	+++
250.0	++	++	+	+	+	++	+	+	++	+	–	+++
255.0	++	++	+	+	+	++	+	+	+++	+	–	+++
260.0	+	++	+	+	+	++	+	+	+++	–	–	–
265.0	+	+	+	+	+	++	–	–	–	–	–	–
270.0	+	+	++	+	+	++	–	–	–	–	–	–

DISCUSSION

Endobenthic tubificid worm, *Branchiura sowerbyi* is a useful biological tool in assessing possible ecotoxicological risks of contaminants (Lobo and Espindola, 2014). In the present study, the stress response of the worm to fluoride was probably due to interference of the contaminant with various enzymes present in the worm. Fluoride acts as a metabolic inhibitor by exerting its adverse effects on various nutrient metabolizing enzymes by binding it to the functional amino acid groups that encircles the enzyme's active centre (Kessabi 1984; Barbier *et al.*, 2010; Casellato *et al.*, 2012; Ghosh and Ghosh, 2019). The median lethal concentrations of the worm to fluoride at 24, 48, 72 and 96h recorded in the present study were 249.996, 240.618, 231.757 and 224.227 mg/l respectively **(Table 1,2,3,4).** These values are much higher than the findings of the earlier workers on various freshwater invertebrates, which are also very pervasive (Camargo, 2003). The 24 and 48 h LC_{50} values for the water flea, *Daphnia magna*, which has been the most widely used invertebrate species to study the acute toxicity of inorganic fluoride in laboratory, showed a range between 205–352 and 98–304 mg Fl/l, respectively depending on temperature (15° to 23.2°C) and water hardness (160 to 250 mg $CaCO_3$/l) variation (LeBlanc, 1980; Dave, 1984; Fieser *et al.*, 1986; Kűhn *et al.*, 1989; Camargo, 2003). Gonzalo *et al.*, (2010) reported a much lower LC_{50} value (5.8 mg/L at 17°C) for the gammarid *Dikerogammarus villosus*. The 96h LC_{50}

value for the aquatic oligochaete worm, *Lumbriculus variegates* to fluoride was recorded as 93.5mg/l (The Advant Group, 2000). Del Piero *et al.,* (2014) observed variable 96h LC_{50} values in *B. sowerbyi* at two different temperatures (91.28 and 61.68 mg/l for 17°C and 22°C respectively). On the same species, they also recorded 96h LC_{50} values of 267.63 and 80.07 mg/l at the same temperature variation in present of sediment. Such variation in acute toxicity values for fluoride to the same or different test organisms were probably due to their age and size differences, variation in physico-chemical parameters like pH, temperature, water hardness, alkalinity etc. of the culture medium, presence of sediment, test chemicals used, design of the experiment and also due to species variation (Kaviraj and Konar, 1982;Casellato *et al.,* 1992; Hamelink *et al.,* 1994; Phipps *et al.,* 1995; Camargo, 2003; Rathore and Khangarot, 2003; Meyer *et al.,* 2004; Del Piero *et al.,* 2014; Lobo *et al.,* 2016; Sparling, 2016).

In the present study, it is observed that the fluoride toxicity to the worm increases with increasing concentration and exposure time (**Figure 1).** This is in agreement with the earlier studies on different aquatic organisms exposed to fluoride by various workers (LeBlanc, 1980; Camargo and Tarazona, 1990; Narwaria and Saksena, 2012; Del Piero *et al.,* 2014; Tirumala Rao *et al.,* 2017).

Acute toxicity is the culmination of a series of various physical, chemical and biological processes (Banavathu *et al.,* 2016). LC_{50} value, as an effective tool of toxicity test, is the level of tolerance of population response to contaminant (Reda *et al.,* 2010).In the present study, the degree of tolerance of *B. sowerbyi* to fluoride was determined by the toxicity factor (TF) at different time of exposure (**Table 5**). Tolerance is an important mechanism of the organism by which they react to their surrounding adverse environment. With the progress of time, it increases gradually probably due to decreased uptake, increased excretion or redistribution of the metal to less sensitive target sites in different degree (Enuneku and Ezemonye, 2012).

Camargo (2003) revealed that the safe concentrations of fluoride are significantly different between classes, families, genera, and species. Different responses in biochemical and physiological parameters and differences in sensitivity of individuals within a population were also recorded by Giesy and Graney (989). So, the estimated possible safe level for fluoride recorded in the present study (**Table 6**) may not be conclusive and further a large variation in calculated result depending on various processes also made a controversy over its acceptability (Buikema *et al.,* 1982; Pandey *et al.,* 2005). So it is difficult to extrapolate laboratory data to the field as acceptable concentration as "safe" for the toxicant (Mount and Stephan, 1967; Abou *et al.,* 2001).

Behavioural changes due to environmental stress can be considered as sensitive indicator. Many workers recorded fluoride induced behavioural alterations in the fresh water fish but there is no report on oligochaete worms (Tripathi *et al.,* 2004; Bajpai *et al.,* 2009; Narwaria and Saksena, 2012; Aziz and Jabeen, 2014). The changes in behaviour of the treated *B. sowerbyi* in the present study (**Table 7**) were probably an early indication of their avoidance reaction from the toxicant. The avoidance reaction may be related to narcotic effects or to change in sensitivity of chemo receptors (Suterlin, 1974). The behavioural changes of the worms may also be considered as the neurotoxic effects of the toxicant (Doving, 1992; Bhatnagar and Regar, 2005; Choi *et al.,* 2012). Barbier *et al.,* (2010) reported that the increase of oxidative stress due to fluoride toxicity leads to an increase in the expression of genes responsible for stress response. On exposure to high content of fluoride, it inhibits the cell proliferation and induced apoptosis (Barbier *et al.,* 2010). Jha (2004) recorded DNA and cytogenetic alterations in aquatic organisms impaired enzyme function or general metabolism, immunotoxicity and cytotoxicity. Tripathi *et al.,* (2009) reported that the chromosomal aberrations increased with the increase in fluoride dose. Probably these were reflected in the present study where behavioural changes were gradually increased with the progress of time and increasing concentration of the toxicant (**Table 7**). Excess mucus secretion in the organisms exposed to fluoride may be an adaptive and protective response to avoid the absorption of the toxicant by the overall body surface (Kumar *et al.,* 2007; Sahu *et al.,* 2014). It is probably to prevent the entry of toxicant into the body as the –SH groups present in the mucus acts as protective ion trap (Jayakumar and Paul, 2006). The concentration of specific differences in response to toxicant

observed in the present study may be due to the variation in the formation of mucus-toxicant complex which precipitates over the body wall of worms at different degrees (Whitley, 1967).

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