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RESEARCH ARTICLE

MICRONUCLEI INDUCTION IN DIFFERENT TISSUES OF *EUPHLYCTIS CYANOPHLYCTIS* (COMMON SKITTERING FROG) EXPOSED TO SUBLETHAL CONCENTRATION OF MERCURIC CHLORIDE

***Kour Preetpal, Tripathi, N. K. and Poonam**

Department of Zoology, Govt. College for Women, Gandhi Nagar, Jammu, Jammu & Kashmir, India-180006

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ABSTRACT

In the present study acute toxic effect of mercuric chloride was carried out in three different tissues (RBCs, Intestine and kidney) of skittering frog (*Euphlyctis cyanophlyctis*). The study was conducted using the micronucleus test. The frogs were treated with four different sublethal concentrations of mercuric chloride (1.5µg/kg, 3.0µg/kg, 6.0µg/kg, 12.0µg/kg) after calculating the LC_{50} value which was found to be 18µg/kg. Each dose was injected intraperitoneally once in the treatment period for 24, 48, 72 and 96hrs to study its potential toxic effect. It was observed that this heavy metal treatment induced a significant increase in frequency of micronuclei at different concentrations in frog for 24, 48, 72 and 96hrs when compared with the control. The results lead us to the conclusion that there is a dose and time dependent induction of micronuclei frequencies in different somatic tissues of the frog due to genotoxic and cytotoxic properties of this heavy metal.

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INTRODUCTION

Amphibians are considered as excellent "bio indicators" of environmental health (Schuytema and Nebeker, 1999; Tejedo, 2003; Garcia-Munoz *et al.,* 2010. Naturally-occurring metals and metalloids are widely distributed in the environment. Mercury is a naturally occurring metal whose primary store is within the planet. It is regarded to most toxic, non-essential, persistent, immutable and non-biodegradable heavy metal. It is a xenobiotic metal and its environment level is increasing substantially every year as a result of natural weathering process and human activities and it ultimately reaches the aquatic system (Joy and Kirubagaran, 1989). Mercury is used in a variety of consumer, industrial and medical products and processes. Product examples are fluorescent light bulbs and batteries, medical devices (e.g. thermometers, blood pressure instruments), laboratory chemicals, pharmaceutical and dental products, and various temperature and moisture measurement and sensing devices (barometers, hygrometers, flame sensors). Metallic mercury is used to produce chlorine gas and caustic soda and Mercury salts are sometimes used in skin lightening creams and as antiseptic creams and ointments. Mercury emissions come from a range of human activities, primarily coal burning, but also from incineration or disposal of mercury-containing products, cremation, and from natural sources (Joy and Kirubagaran, 1989). High doses can be fatal to humans, but even relatively low doses of mercury containing compounds can have serious adverse neurodevelopment impacts, and have recently been linked with possible harmful effects on the cardiovascular, immune and reproductive systems (Peraza *et al*., 1998; Silva *et al*., 2005). Studies of the sources, accumulation, and toxicological effects of Hg span most taxa and include a growing body of research on wildlife (Wolfe *et al*., 1998; Eisler 2006; Scheuhammer *et al*., 2007). What little data are available; indicate that metal can significantly reduce viability in amphibians through their actions on metabolism, development and gametogenesis (Byrne *et al.,* 1975; Goyer, 1986; Kanamadi and Saidapur, 1991, 1992; Punzo, 1993). Amphibians may be useful indicators of metal contamination (Cooke 1981), especially where fish cannot survive. They bioaccumulate and are particularly sensitive to metals, have obligate aquatic larval stages, and sometimes spend their entire life cycle in a given pond or reach of a stream. The sensitivity of amphibians to

****Corresponding author: Kour Preetpal,***
Department of Zoology, Govt. College for Women, Gandhi Nagar, Jammu, Jammu & Kashmir, India-180006

metallic contaminants may allow environmental monitoring of these compounds, using amphibians as indicators (Niethammer *et al*., 1985). The Indian Skipper Frog or Skittering Frog (*Euphlyctis cyanophlyctis*) is a common frog found in South Asia and is the native frog of Kashmir. They are often seen at the edge of bodies of water with their eyes above the water. *E. cyanophlyctis* was chosen as a test animal in the present study to evaluate the genotoxic potential of mercuric chloride, using the micronucleus test, which is an *in vivo* and *in vitro* short-time screening cytogenetic test, introduced by Heddle (1973) and Schmid (1975) and is a widely used method for assessing genotoxicity of chemicals in organisms (Meier *et al*., 1999). The micronucleus test has been used because it is technically easy to master, reliable, least expensive and extremely rapid screening system.

MATERIALS AND METHODS

Animals and Experimentation

Adult live and apparently healthy specimens of, *E. cyanophlyctis* weighing 30-40gms caught from local unpolluted lentic habitats were acclimatized to laboratory conditions in large well aerated plastic containers for at least 5 days prior to use at ambient temperature and natural photoperiod, according to the time of the year, in which each test was performed, for acclimatization before starting the assay. They were kept in 15L plastic containers. The water was changed daily to avoid accumulation of toxic substances. Frogs were divided into 1 control and 4 treatment groups in separate plastic containers. Four frogs were kept in each group. LC50 value was estimated using standard method of Finney (1980) and was found to be 18µg/kg. Based on LC50 value of mercuric chloride, four sublethal concentrations were arbitrarily chosen and the frogs were treated with these doses (1.5µg/kg, 3.0µg/kg,6.0µg/kg,12.0µg/kg) through an intraperitoneal injection by using 1ml syringe only once in the treatment period. Frogs of all groups were subjected for 24, 48, 72 and 96hrs of exposure periods.

Preparation of erythrocyte micronuclei slides

For each frog, experimental as well control, fresh blood samples were taken after each duration of exposure. The only reliable method of blood collection is by cardiac puncture. In frog the ventricle is located near the xiphisternum. Rinsed syringe with heparin solution (75U/ml) and left approximately 2mm gap at top of syringe column. After feeling the pulsating heart on the ventral side of thoracic region, pricked the needle in the middle of the heart. As blood started flowing into syringe, the dispenser was very gently pulled outwards to collect the blood and smeared onto the clean glass slides. The slides were air dried for 1-2hrs and then fixed in absolute methanol for 10min. After fixing, the same slides were stained in Giemsa (2%) for about 30min. The slides were then rinsed with distilled water; air dried and examined using optical microscopy under 100 x objectives for the presence of micronuclei.

Preparation of micronuclei slides from intestine and kidney

The animals were anaesthetized and dissected from the ventral side so as to remove the intestine and bone marrow (Haertel *et al*., 1974; MacGregor and Varley, 1986). The tissues were chopped into very fine pieces hypotonised with 0.50M NaCl solution for 1hour at room temperature. Fixing of the tissues was done in Conroy's fixative for 45min changing the solution every 15 minutes. The material was then dabbed on clean slides, air-dried and stained with 2% Giemsa stain for 30-35 minutes and differentiated in distilled water and then again air-dried.

Examining and scoring of MN slides

Only micronuclei not exceeding 1/3rd of the main nucleus diameter, clearly separable from the main nucleus and with distinct borders and of the same colour as the nucleus were scored. The frequency of MN in erythrocytes as well in intestinal and kidney tissues was established by calculating the number of MN in at least 1500 interphase cells/ specimen (total of 6000 interphase cells from four specimens used for conc. and duration). The micronucleated interphase cells were photomicrographed at 1000X magnification under a binocular research microscope (Olympus CH20iBIMF) using Sony SSC-DC378P camera. Only cells with intact cellular and nuclear membrane were scored.

Statistical Analysis

Data from the micronucleus test obtained from the experimental as well as controlled group were expressed as mean ±SD. Data were then compared by the non-parametric Kruskal-Wallis test. Statistical analysis of the data was carried out using computer software called 'PRIMERS- 4.0'. $p<0.05$ was considered to be the level of significance. Statistical significance in the frequencies of micronuclei between exposed and control groups after each dose and duration of exposure were evaluated.

RESULTS

Results of MNT in RBCs

Frequencies of MN recorded in the RBCs after different treatment and exposure periods have been shown in Fig.1 and 4 and Table 1. In the frogs treated with 1.5µg/kg of test chemical, frequency of MN was determined as 0.77±0.35, 0.95±0.404, 1.3±0.0 and 1.3±0.571 at 24, 48, 72 and 96hrs respectively. Values of MN frequencies were same at 72 and 96hrs of treatment. MN frequency was found to increase from 1.47±0.670 (at24hrs) to 3.65±0.404 (at96hrs) through 1.82±0.35 (at 48hrs) and 1.97±0.531 (at72hrs) after treatment with 3.0µg/kg of mercury. Minimum (0.95±0.404) and maximum (4.15±0.834) frequency of MN after treatment with 6.0µg/kg of test chemical was recorded at 24 and 96hrs of exposure periods respectively. Similarly, the incidence of MN was found to increase in a time dependent manner from 2.47±0.68 (at24hrs) to 11.62±1.244 (at 96hrs) after treatment with 12.0µg/kg of mercury. Data was found to be statistically significant versus respective controls after exposure periods in all treatment doses (at24 and 48hrs, $p<0.01$ and at 72 and 96hrs, $p<0.001$) as shown in the table. The value of p revealed highly significant result after treatment and exposure periods as compared to respective controls as shown in Table 1 and Fig.4).

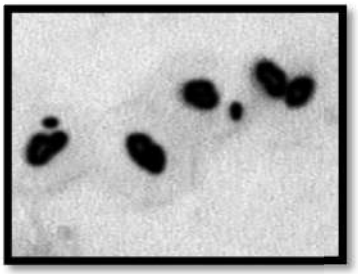

Figure 1. The micronuclei frequency in RBCs

Results of MNT in Intestine

Fig. (2and5) and Table 1 show the values of MN obtained in intestinal tissue after different treatment and exposure periods. MN frequencies observed in frog treated in vivo with 1.5µg/kg was 0.97±0.865, 0.97±0.65, 1.15±0.834 and 1.12±0.35 during 24, 48, 72 and 96hrs respectively. Values of MN were found to be same at 24 and 48hrs of exposure and there was a slight decrease in the frequencies of MN from 1.15±0.834 to 1.12±0.35 during 72 and 96hrs of the same dose treatment. MN frequency was found to increase from 1.3±0.0 (at 24hrs) to 2.3±0.346 (at 96hrs) through 1.65±0.404 (at 48hrs) and 1.97±531 (at 72hrs) after treatment with 3.0µg/kg of test chemical. Similarly, the incidence of MN was found to increase in a time dependent manner from 1.97±0.942 (at24hrs) to 4.15±1.558 (at 97hrs) after treatment with 6.0µg/kg of mercury. Also, the frog treated with 12.0µg/kg of mercury showed an increase in MN frequency from 2.97±1.575 (at24hrs) to 10.47±0.618 (at96hrs) through 6.3±0.852 (at 48hrs) and 7.8±1.840 (at72hrs). Data was found to be statistically significant versus respective controls after different exposure periods in all treatment doses (at24hrs, $p<0.0.5$; at 48hrs and 72hrs, $p<0.01$; at 96hrs, $p<0.001$) as shown in Table 1 and Fig.5.

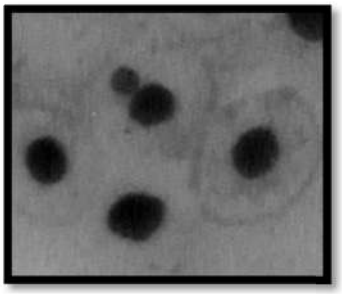

Figure 2. The micronuclei frequency in Intestinal cells

Results of MNT in Kidney

Fig. (3and6) Table 1 show the values of MN obtained in kidney at different sublethal concentrations and durations of exposure. Values of MN were found to be significant from the respective controls (at 24, 48, 72 and 96hrs, $p<0.01$) as shown in the Table 1 and Fig. 6. At sublethal concentrations of 1.5 µg/mg, MN frequencies were 0.45±0.3, 1.3±0.571, 1.12±0.35 and 1.47±0.35 at 24, 48,72 and 96hrs respectively. Frequencies of MN recorded after treatment with 3.0µg/kg were found to increase from 1.65±0.404 (at 24 hrs) to 3.12±0.670 (at 96 hrs) but minimum MN frequency (1.3±0.571) was recorded at 96 hrs. Similarly, values of MN frequency at 6.0µg/kg of treatment of test chemical showed an increase from 2.62±0.531 (at24hrs) to 3.3±0.571 (at 96 hrs) but a minimum value of MN frequency (2.47±0.994) was observed at 96hrs. However, with the increase in dose concentration i.e. at 12.0µgkg treatment of test chemical, the values of MN frequencies were found to increase significantly from 3.47±0.670 (at 24hrs) to 7.65±2.344 (at 96 hrs) through 4.12±0.618(at 48hrs) and 4.97±1.595(at 72hrs).

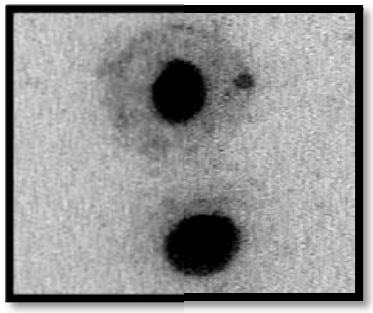

Figure 3. The micronuclei frequency in Kidney cells

DISCUSSION

During the past decades the ecology and ecotoxicology of amphibians started to get attention (Sparling *et al*., 2000) because of global amphibian population declines (Houlahan *et al*., 2000). Frogs and toads are about 90% of all amphibians (McDiarmid and Micthell 2000). They are an important link between human and ecosystem health (Hayes *et al*., 2002) and they are main components of aquatic and terrestrial ecosystems (Unrine *et al*., 2007).

Table 1. Frequencies of micronuclei (%) in different tissues of *Euphlyctis cyanophlyctis* exposed to Mercury

Conc. (µg/kg)	Duration (Hrs)	No. of specimens	Total interphase cells studied	Frequency of micronuclei (Mean±SD)		
				RBC	Intestine	Kidney
Contr.	24	4	6000	0.45±0.3	0.45±0.3	0.3±0.346
	48	4	6000	0.3±0.346	0.3±0.346	0.15±0.3
	72	4	6000	0.45±0.3	0.62±0.531	0.45±0.3
	96	4	6000	0.15±0.3	0.8±0.627	0.3±0.346
1.5	24	4	6000	0.77±0.35^{b}	0.97±0.865^{a}	0.45±0.3^{b}
	48	4	6000	0.95±0.404^{b}	0.97±0.65^{b}	1.3±0.571^{b}
	72	4	6000	1.3±0.0^{c}	1.15±0.834^{b}	1.12±0.35^{b}
	96	4	6000	1.3±0.571^{c}	1.12±0.35^{c}	1.47±0.35^{b}
3.0	24	4	6000	1.47±0.670^{b}	1.3±0.0^{a}	1.65±0.404^{b}
	48	4	6000	1.82±0.35^{b}	1.65±0.404^{b}	1.82±0.35^{b}
	72	4	6000	1.97±0.531^{c}	1.97±0.531^{b}	3.12±0.670^{b}
	96	4	6000	3.65±0.404^{c}	2.3±0.346^{c}	1.3±0.571^{b}
6.0	24	4	6000	0.95±0.404^{b}	1.97±0.942^{a}	2.62±0.531^{b}
	48	4	6000	2.3±0.852^{b}	3.47±0.35^{b}	3.3±0.571^{b}
	72	4	6000	4.12±0.618^{c}	4.12±0.994^{b}	3.3±1.205^{b}
	96	4	6000	4.15±0.834^{c}	4.15±1.558^{c}	2.47±0.994^{b}
12.0	24	4	6000	2.47±0.68^{b}	2.97±1.575^{a}	3.47±0.670^{b}
	48	4	6000	4.65±0.943^{b}	6.3±0.852^{b}	4.12±0.618^{b}
	72	4	6000	6.62±0.531^{c}	7.8±1.840^{b}	4.97±1.575^{b}
	96	4	6000	11.62±1.244^{c}	10.47±0.618^{c}	7.65±2.344^{b}

a=p<0.05,b=p<0.01,c=p<0.001,ns= non-significant(represent values significantly differently from the respective controls; Kruskal-Wallis test; df=4), Contr.= Control

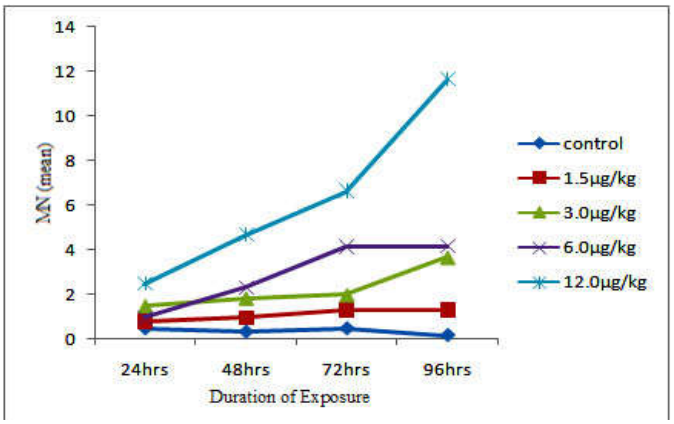

Fig.4. Frequencies of micronuclei in RBC after treatment with Mercury

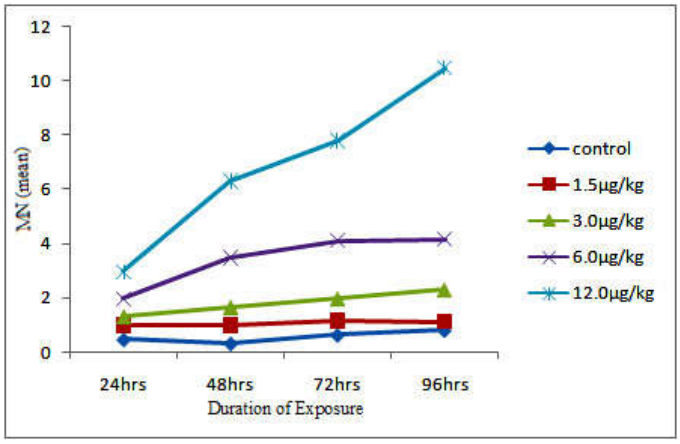

Fig.5. Frequencies of micronuclei in Intestine after treatment with Mercury

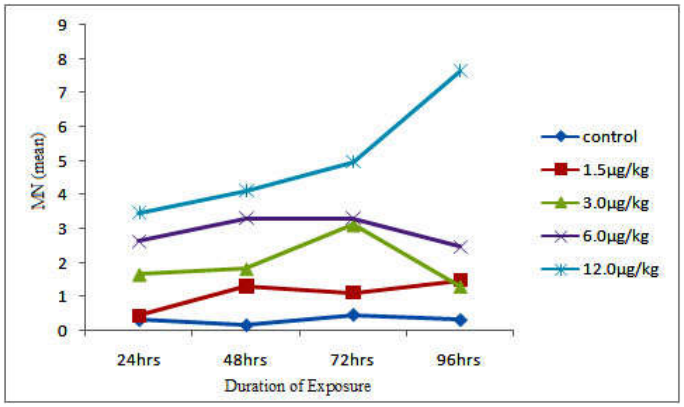

Fig.6. Frequencies of micronuclei in Kidney after treatment with Mercury

Most adult frogs and toads feed on invertebrates, so they are important, energy-efficient trophic link between insects and other vertebrates (Sparling *et al*., 2000). They are sensitive to environmental changes both in terrestrial and aquatic habitats because they have highly semi-permeable skins and different life cycle stages (Alford and Richards 1999). Nevertheless, the information on the effects of environmental contamination on frogs and toads is little known (McDiarmid and Micthell 2000). Heavy metal pollution has likely played an important role in global biodiversity decline, but there remains a paucity of information concerning the effects of metals on amphibian diversity. Toxic chemicals and metals may interact synergistically with other anthropogenic stressors such as habitat degradation, UV-B radiation, and global climate change to affect amphibian populations (Stebbins and Cohen, 1995; Sparling *et al*., 2000). Fish, frog and aquatic wildlife may bioaccumulate mercury (Hg) to levels that adversely affect reproduction, growth, and survival. Hg contamination of amphibians has received little attention despite worldwide population declines (Houlahan *et al*., 2000). Complexity in watershed, trophic, and within stream mercury sorption likely influences the bioavailability, bioaccumulation, and biomagnifications of mercury in lotic ecosystem biota. Watershed and trophic complexities encompass interactions from both within streams and between watercourses and adjacent terrestrial landscapes, including riparian zones (Naiman and De´camps, 1997).

An attempt was made during the present work to assess the time-dose-dependent, tissue specific peculiarities of mercuric chloride genotoxicity in frog, *Euphlyctis cyanophlyctis*, as aquatic model test organism. During the present studies, it is reported that mercury induced the micronuclei formation in the test organism. Sub-lethal concentrations of the heavy metal used clearly increased the micronuclei frequencies in all the tissues which were dose and time dependent, compared to respective controls. A comparison between the micronucleus frequencies in all the tissues revealed highest micronuclei frequencies in RBCs followed by intestine and least in kidney cells (Table 1; Fig.4, 5 and 6). The observation that mercury caused greater increase in micronuclei frequencies in RBCs is most likely due to the fact that the blood of amphibians is very plastic tissue. In fact, variation of several hematological parameters in response to natural changes in the environment have widely described by researchers (Krauter, 1993; Stansley and Roscoe, 1996). Furthermore, the heavy metal is highly reactive; its effects could be attenuated as it travels in the blood to other organs. However, differences in metabolic activation and deactivation properties of these tissues or different sensitivities of the tissues to the mutagens cannot be ruled out as alternative explanations. A dose and a time dependent increase in the micronuclei frequency were observed in the various tissue of the frog studied after treatment with various sublethal concentrations of mercury. In general, the data revealed that with respect to the dose, maximum effect was found to be induced at the highest dose of exposure i.e.12.0mg/kg while prominent effect with respect to the duration of exposure was induced after maximum period of exposure i.e. 96hrs.

Comparison between the micronucleus frequencies induced in the various tissues revealed highest MN frequencies in RBCs (11.62 ± 1.244^{c}), followed intestine (10.47 ± 0.618^{c}) and least in kidney (7.65 ± 2.344^{b}). Individually in all the tissues, the value of MN frequency recorded showed a significant increase with the increase in dose and duration of exposure. However at concentration of 1.5µg/kg, the value of Mn frequencies showed a slight decrease at 48 and 96hrs of duration in intestine but in RBCs and kidney, a significant increase in value of micronuclei was observed except at 72hrs of duration with the same dose of treatment. At concentration of 3.0µg/kg,

a general increase in MN frequency was observed in intestine and RBCs whereas in kidney, maximum MN frequency was observed at 72hrs of duration and a fall in the value of MN was observed at 96hrs of duration. With a treatment dose of 6.0 µg/kg, intestine and RBCs both showed increase in value of MN frequency with the duration of exposure whereas minimum MN frequency was observed in kidney at 96hrs of duration. However the incidence of MN frequencies observed in all the tissues recorded a significant increase in value from 24hrs to 96hrs of duration with 12.0 µg/kg treatment of heavy metal.

In general, while comparing the effect of different concentrations on the RBCs, intestine and kidney, at concentration of 1.5µg/kg, minimum MN frequency was observed in intestine followed by RBCs and maximum was observed in the kidney only at 96hrs of duration. With treatment dose of 3.0µg/kg, minimum MN frequency value was observed in the intestine (at 24hrs of duration) and maximum MN frequency was observed in RBCs at 96hrs of duration. At treatment dose of 6.0 µg/kg, minimum value of MN frequency was observed in RBCs (at 24hrs) whereas maximum value was observed in intestine as well as in the RBCs with a slight difference in value while at treatment dose of 12.0 µg/kg, minimum and maximum value of MN frequency was recorded in RBCs (at 24hrs and 96hrs of duration respectively). Thus, a positive relationship between metal concentrations and micronuclei frequencies were recorded for all the treatment groups with respect to their dose and the exposure periods compared to the control groups. The frogs treated with highest dose of heavy metal treatment i.e. 12.0 µg/kg at 96hrs of duration showed significant results ($p<0.01$) in kidney but the result obtained were highly significant ($p<0.01$) in RBCs and intestine.

Conclusion

The present work investigated the cytotoxic and genotoxic effects of mercuric chloride on erythrocytes and other tissues (intestine and kidney) of the Indian skittering frog, *Euphlyctis cyanophlyctis* using micronucleus test in controlled laboratory conditions with different sub-lethal doses to a maximum of 96hrs. The results and the data evaluated indicates that mercuric chloride, one of the most persistent toxicants in aquatic can be genotoxic at higher concentrations, therefore if inefficiently used in industries, laboratories, medical and monitoring systems might reach levels that pose severe threat to tadpoles and frogs making it a potential threat to water ecosystems and to human health. An investigation of such substances on the genetic material of aquatic organisms may produce meaningful and useful results. Ultimately, the goal of the studies such as this is to provide information that will help mediate the environmental damage resulting from pollutant releases like those of heavy metals.

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