SODIUM NITRATE INDUCED CHANGES IN THE LIFE HISTORY TRAITS OF INDIAN CRICKET FROG *FEJERVARYA LIMNOCHARIS*

A

Project Report Submitted to

In partial fulfilment of the requirements for
the degree of Master of Science

BY

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Roll No. 202820024013
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UNDER GUIDANCE OF:

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To

My beloved parents

with love

DECLARATION

I, **Queeu Deori,** bearing Roll No. 202820024013, Registration No. 451428220 dated 07-12-2021, hereby declare that the subject matter of the desertation entitled **"Sodium nitrate induced changes in the life history traits of Indian cricket frog *Fejervarya limnocharis*"** is the record of work done by me. The desertation is being submitted to Silapathar Science College for the degree of Master of Science in the Department of Zoology (PG) and not been submitted to any other Institute for obtaining any degree.

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Place: Silapathar Science College **[QUEEN DEORI]**

Date: 23rd July, 2022

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CERTIFICATE

Certified that the desertation entitled **"Sodium nitrate induced changes in the life history traits of Indian cricket frog *Fejervarya limnocharis*"** for the award of Master of Science degree (as final semester practical project) is the outcome of a bonafide research work. This work has not been submitted previously for obtaining any other degree of this or any other institution. I recommend that the project work may be placed before the examiners for consideration of award of the degree.

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INTRODUCTION

"The more clearly we can focus our attention on the wonders and realities of the universe about us, the less taste we shall have for destruction."

--- Rachel Carson

Most of the anurans amphibian have dual mode of life cycle comprising both water and land. Almost all the amphibian species adapted early part of their life cycle in water; therefore, alterations in aquatic environment may directly or indirectly affect the life history traits of anuran amphibians (Egea-Serrano et al., 2012). Environmental pollutions are of multitude in nature and their distribution patterns are even more complex. In recent times, chemical pollution reported to be played a significant role in the ongoing global decline of amphibian biodiversity, especially the anurans (Bankony et al., 2020; Catenazzi, 2015).

According to the first global assessment of the status of amphibian species, more than 40% of the world's 5743 amphibian species have experienced recent declines, a situation far worse than that reported for mammals and birds (Stuart et al., 2004). Amphibian species and population declines are likely the result of a multitude of causes including habitat destruction/alteration, infectious disease outbreaks, altered host-parasite interactions, and introduction of alien species and xenobiotic exposure (Davidson & Knapp, 2007; Relyea & Diecks, 2008; Relyea, 2009). Unprecedented rate of industrialization and population outburst also contributed to this crisis several fold higher than before. Anuran amphibians, specifically the early stages of amphibian development are sensitive to aquatic contaminants due to the presence of permeable eggs, gills and skin; which make them susceptible to contaminants and thus predispose them to be a

good indicator species of environmental health (Welsh Jr. & Ollivier, 1998; Beebeen & Griffiths, 2005; Pollet & Bendell-Young, 2009).

The aquatic systems are contaminated from a variety of sources such as agrochemicals, industrial effluents, and heavy metals from human economic activities as well as geogenic sources. Recent surveys in USA have found that 30-60% of country's shallow groundwater and 60–95% of streams are currently contaminated with at least one pesticide (Gilliom et al., 2007). Because there are thousands of different possible contaminants released to the environment, data on aquatic contamination for any particular contaminant is usually quite limited. Given that large varieties of contaminants find their way into aquatic systems, the relevant question is whether they affect the species diversity in these systems. We need to examine the impacts of this unintended reality. While these contaminants have the potential to affect many aquatic taxa, the impacts on amphibians are of particular concern because they appear to be declining on a global scale (Stuart et al., 2004). In recent times, amphibian declines have emerged as a key example of the global biodiversity crisis. Since they were first brought to the attention of the herpetological and conservation biology communities in early 1990s (Wake, 1991), amphibian population declines have become a focal issue in the scientific community (Corn, 2000). Most researchers now agree that many species and some entire communities (Fisher & Shaffer, 1996) of amphibians are undergoing ecological collapse.

Nitrogen being one of the most essential macronutrients required for normal growth and development of both plants and animals; deficiency of which can lead to severe physiological as well as pathological consequences. On

contrary, excessive use of nitrogenous products and nitrogen based fertilizers in the crop fields may promote eutrophication and consequently decrease in dissolved O_2 of aquatic ecosystem thus affecting native flora and fauna (Rouse, 1999; Ilha & Schiesari, 2014; Van Meter et al., 2019; Wijer et al., 2003; Alton & Franklin, 2017).

Over decades, several biomarkers have been utilized as tools for both the detection of exposure to genotoxic pollution and the effects of such pollution, such biomarkers including the presence of DNA adducts, chromosomal aberrations, DNA strand breaks and micronuclei measurements. In aquatic organisms, blood erythrocytes are mainly used as sentinel markers of genotoxic exposure (Bombail et al., 2001). Among all kind of toxicity genetic toxicity is the most important, because this toxicity tends to transmits one generation to the next and leads to reduction in genetic diversity resulting from strong selection for chemical tolerance or population decline leading to genetic bottleneck and drift. Therefore genotoxicity data is important to identify genetic diversity (Murdech & Hebert, 1994), contamination induced natural selection (Peles et al., 2003) and increased mutation rates (Somers et al., 2002).

REVIEW OF LITERATURE

Increasing diffuse nitrate loading of surface waters and groundwater has emerged as a major problem in many agricultural areas of the world, resulting in contamination of drinking water resources in aquifers as well as eutrophication of freshwaters and coastal marine ecosystems (Spalding & Exner, 1993). Anthropogenic nitrogen inputs have recently been identified as one of the major issues potentially compromising a safe operating space for humanity (Rockström et al., 2009). In many regions, the amount of human-activated reactive nitrogen, primarily via application of synthetic fertilizers and cultivation of leguminous crops, exceeds now the amount of natural nitrogen as a result of population growth and the associated need for food production (Vitousek et al., 1997; Galloway et al., 2003). These anthropogenic nitrogen inputs have significantly impacted the nitrogen cycle in terrestrial and aquatic ecosystems (Galloway et al., 2004; Brut et al., 2008). Empirical correlations relating increased use of synthetic fertilizers, their application rates, land use change, and nitrate leaching suggest that the increased application of synthetic fertilizers is strongly connected with the increase of nitrate concentrations in groundwater and surface waters (Howarth et al., 1996; Donoso et al., 1999), but quantitative data on transfer rates of fertilizer N into the hydrosphere are elusive.

Amphibians are highly susceptible to chemical contaminants during their fresh water life stages (Venturino et al. 2003). A major component of the agricultural fertilisers is nitrogen, which is applied in different forms. Among the various available forms of nitrogen viz., ammonium ion, ammonia, nitrite and nitrate, the nitrate is least toxic and most stable form of nitrogen and occurs in highest concentration in aquatic environments (Camargo and Ward 1992). The

environmental concentration of nitrate in surface water is known to produce chronic and acute effects on several species of amphibians (Rouse et al. 1999). Nitrate toxicity to aquatic animals' increases with increasing concentration and exposure time (Camargo et al. 2005). Nitrate is known to produce chronic effects on physical and behavioural functions of individuals (Hecnar 1995). The chronic effects on behaviour include reduced feeding and mobility resulting in severe weight loss and high mortality of individuals, while the physical abnormalities include bent tail, body swelling and bulging, head and digestive system deformities. The severity of these effects increased with higher concentration of nitrate (Hecnar 1995). Nitrate contamination has contributed directly and indirectly to the demise of some amphibian populations (Hecnar 1995; Rouse et al. 1999; Smith et al. 2005). However, there is a paucity of studies describing the impact of nitrate on amphibian population and its biodiversity crisis in this locality.

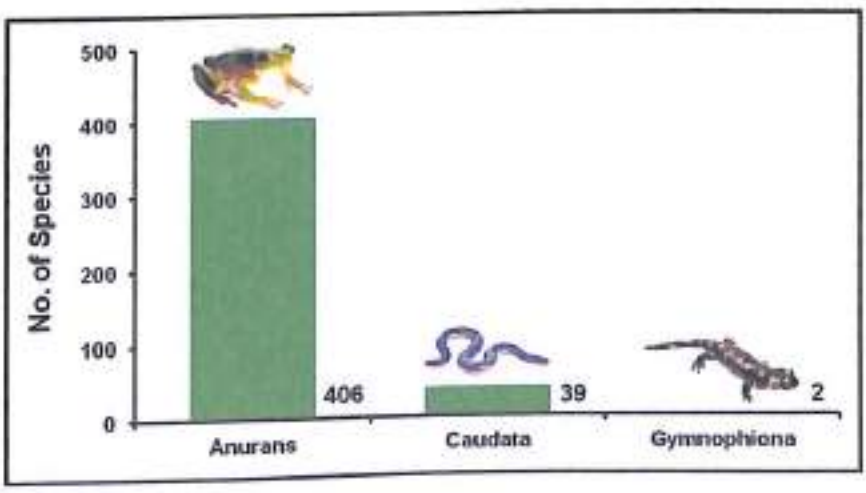

Figure 1: Different orders of amphibian species found in India (Data source: https://www.amphibians.org/news/updated-checklist-of-indian amphibians-2020).

Numerous chemical contaminants like pesticides, insecticides, and other stressors at environmentally relevant concentrations have reported to mediate various toxic and genotxic effects on larval amphibian species (Feng et al., 2004; Lajmanovich et al., 2005). However, no reports available related to the impacts of nitrates on the genetic materials of anuran amphibians.

OBJECTIVES

In the light of the importance of the problem stated earlier and on the basis of the reviewed scientific literature, the major objectives of the present study are:

1. **To determine the effects of Sodium nitrate on the growth, survival and other life history traits of anuran tadpoles.**

2. **To determine the genotoxic effects of Sodium nitrate on anuran tadpoles.**

METERIALS AND METHODS

4.1. Experimental Animal and Rearing:

Larval rearing and toxicity testing has been done as per the standard toxicology protocols, as described elsewhere (Singha et al., 2014). In brief, tadpoles of *Fejervarya limnocharis* (Figure 2) were used for all the experiments. The tadpoles were collected from nearby local ponds and shallow rain fed water bodies. The location of the pond being upstream from the nearby agricultural lands and is unlikely to be contaminated with any pesticides. Following collection, the tadpoles were brought to the laboratory and acclimated to the laboratory conditions in aged well water in polypropylene containers for a period not less than 2-3 days. Developmental stages belonging from Gosner 26-28 (Gosner, 1960) were selected for the experiments (Figure 3 & 4). This period represents to active growth and erythrocyte proliferation. The unused tadpoles were released at the collection site. Whole experiments were performed at 26±1°C and 12h light and dark cycles. Larval tadpoles were fed with crushed fish food pellets or boiled spinach leaf (*Spinacia oleracea*) at every alternate days.

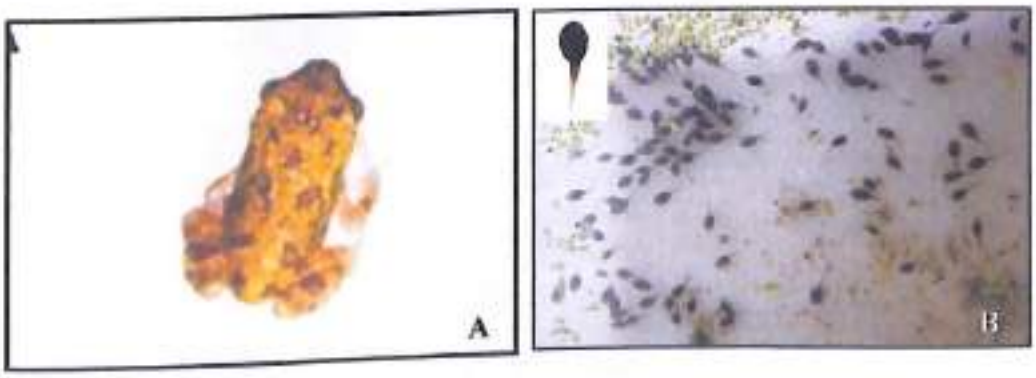

Figure 2: *Fejervarya limnocharis* (A) An adult frog (B) Tadpole larvae.

All the experimental studies of sodium nitrate were carried out using six concentrations of the chemical i.e. 0, 50, 60, 70, 80 and 90 mg/L. Since the LC50 values of sodium nitrate was determined to be very high as compared to the environmental relevant concentrations (Xu and Oldham, 1997). Therefore, treatment doses were selected based on the reported environmental relevant concentrations of 1-100 mg/L of nitrate (Rouse et al., 1999).

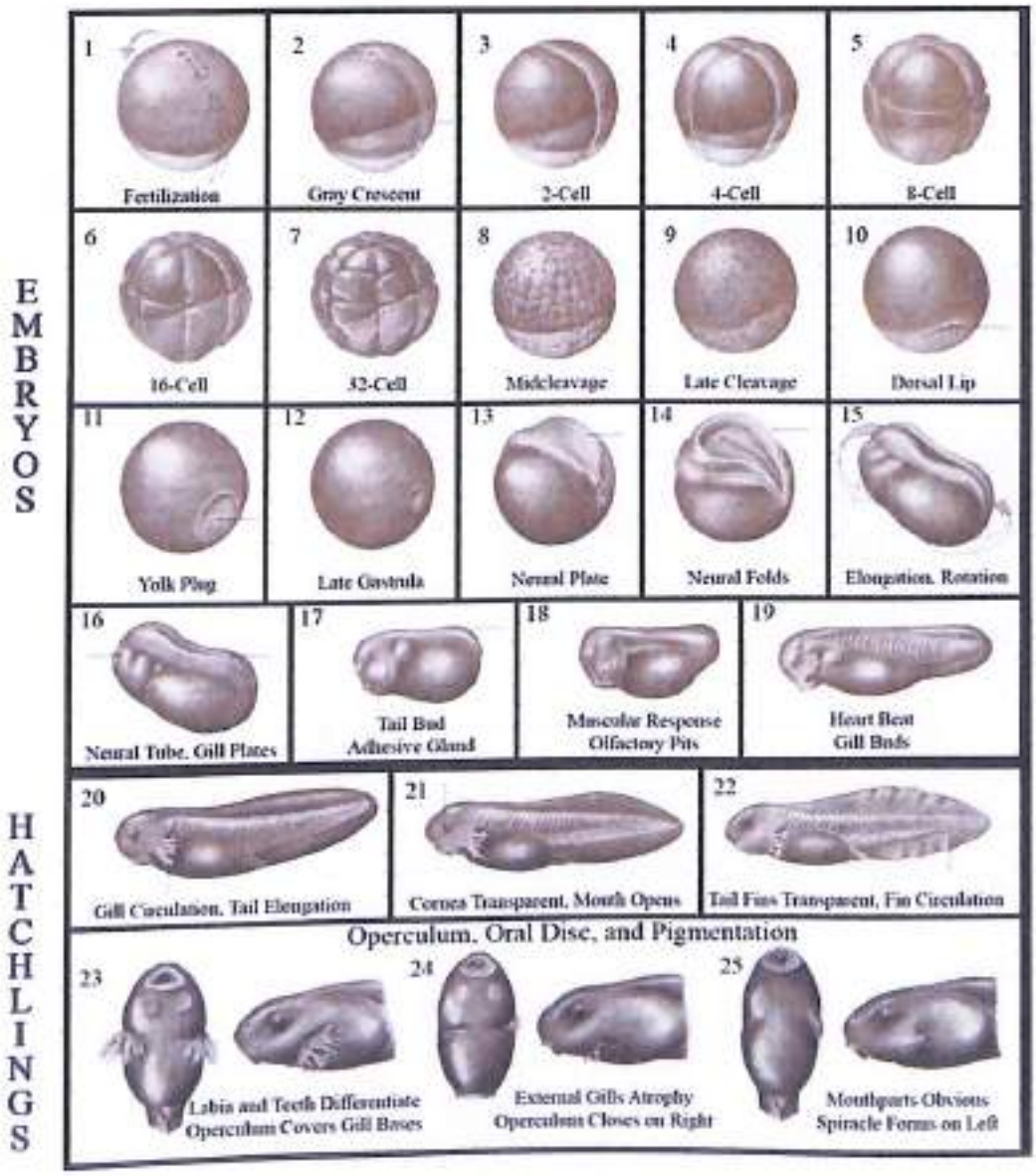

Figure 3: Gosner's reference chart of amphibian development (Part I).

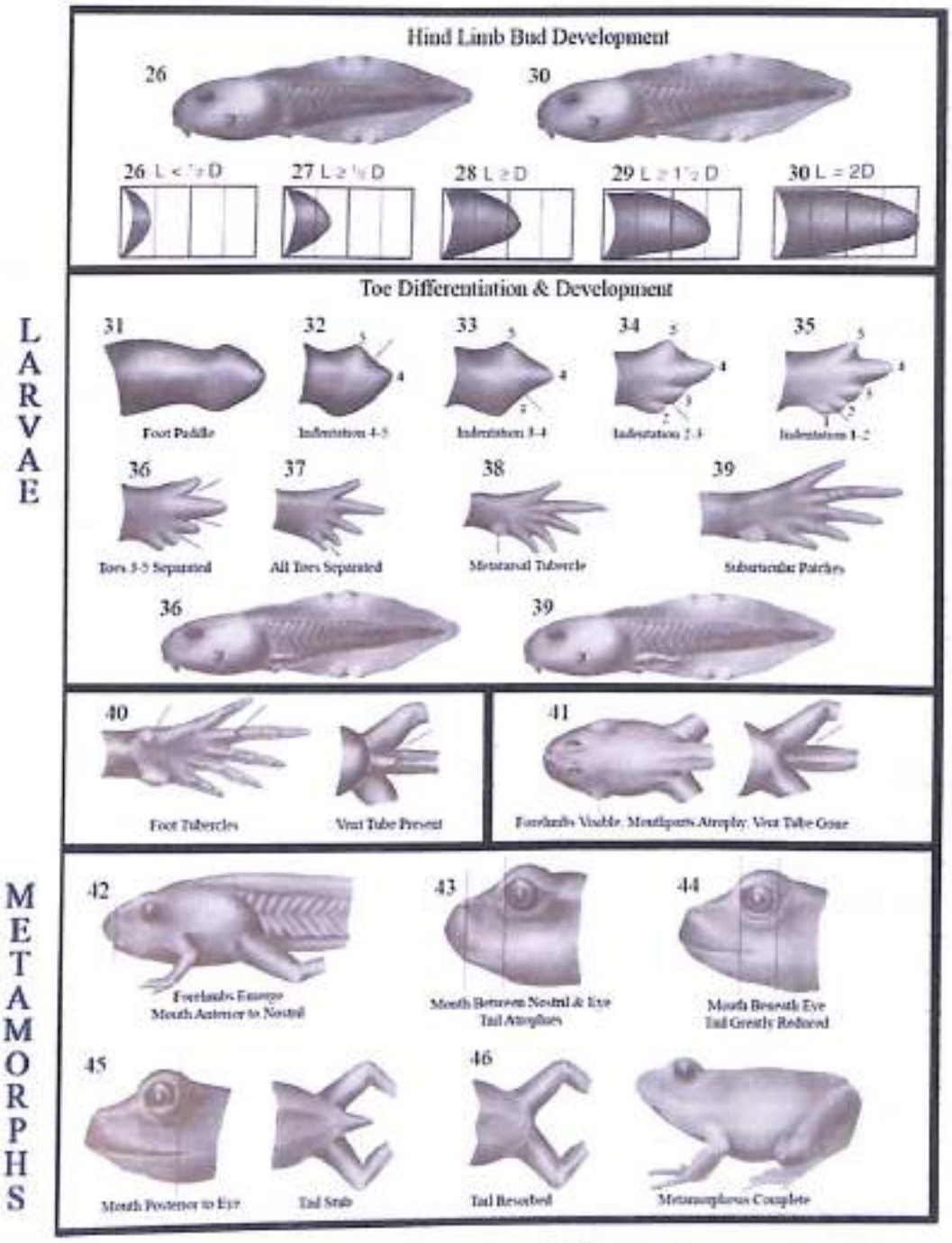

Figure 4: Gosner's reference chart of amphibian development (Part II)

4.2. Tadpole Survival Assay:

The survivals of tadpoles were monitored on daily basis and any dead animal(s) was recorded and carefully removed from the tub with no disturbance

to the remaining tadpoles. Water was being changed in every alternate day (static renewal) and treatments were reapplied. The experiment continued till all the tadpoles either died or completely metamorphosed (Gosner stage 46). The body weights of tadpoles were measured at the beginning of the experiments and at metamorphosis, and the number of days taken for metamorphosis was recorded. The metamorphs were also examined for presence of any gross physical abnormalities if any with special emphasis on snout-vent length development. The dissolved oxygen content was monitored all throughout the experimental period and was always > 8.4 mg/L and pH varied between 7.4–7.8 during assays.

4.3. Genotoxicity Study:

Micronucleus is a small extra-nuclear body formed due to oxidative DNA damage that shears off a chunk of the DNA from the chromosomes (i.e. chromosome breakage) or spindle motor protein dysfunction that might cause a lagging segregation of chromosome prior to karyokinesis, the lagging strand fails to incorporate the nucleic material within the main nucleus, thus forming abnormal number of chromosome in the daughter cells (Figure 5).

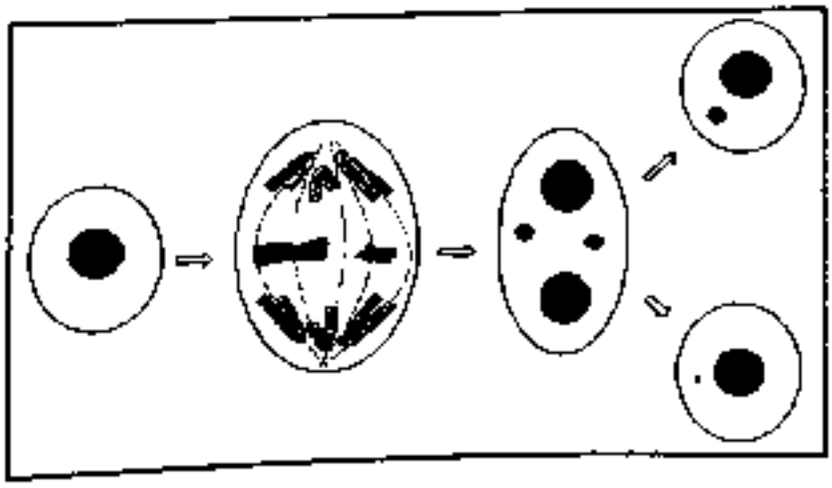

Figure 5: Formation of micronucleus during anaphase of cell division.

Micronucleus assay is considered as a strong genetic tool that provides important information about a chemical's ability to interfere with chromosome structure and function. Evaluation of micronucleus frequency is the primary test in a battery of in vivo genotoxicity tests and recommended by the regulatory agencies around the globe to be conducted as part of product safety assessment (Krishna and Hayashi, 2000).

Polypropylene tubs assigned randomly containing 8 litres of well water to different treatment groups. The treatment groups contained negative control (without any treatment), and sodium nitrate (50, 60, 70, 80 and 90 mg/L). Erythrocytes in amphibians contain nuclei and cell division takes place in the circulation, especially during the larval stages. Since Sodium nitrate found to have very little genotoxic potential in amphibian species hence the treatment groups were being selected after the 72 and 96 h of exposure time. Therefore, 5 tadpoles from each group were anesthetized in 30% ethyl alcohol. Blood samples obtained by cardiac puncture using a magnifying glass to facilitate viewing. Two blood smears for each tadpole were prepared on clean grease free slides, fixed in absolute methanol for 3 minutes and air-dried. We stained the slides in 10% May–Grunwald–Giemsa stain in the following day and determined micronucleus frequency in coded slides. Two thousand erythrocytes from each tadpole (1000 from each slide) were analyzed using 1000× magnification under oil immersion following the criteria described by Lajmanovich et al. (2005).

4.4. Statistical Analysis:

The survival analysis of tadpoles exposed to different concentrations of sodium nitrate done by using Kaplan-Meier product limit estimate. Analysis of variance

(ANOVA) used to analyze the micronucleus data at different concentration levels and time points. Prior to ANOVA, we performed an F- test and data were transformed wherever these did not meet the assumptions of normality. We used ANOVA to compare the change in body weight as well as time to metamorphosis data. The analyses were performed using SPSS® 18.0 statistical software at 95% confidence interval (CI) level. Variances were considered significant at p value less than 0.05.

EXPERIMENTAL FINDINGS

1. Effect of Sodium nitrate on Life History traits:

For determination of acute toxicity of sodium nitrate in the tadpoles of *F.limnocharis*, Gosner 26-30 stages were exposed to 0, 50, 60, 70, 80 and 90 mg/L of sodium nitrate. The survival was monitored up to 15 days. The rate of survival was not affected to any significant extent in the exposed group as compared to the control group except in the highest dose of 90mg/L. (Figure 6).

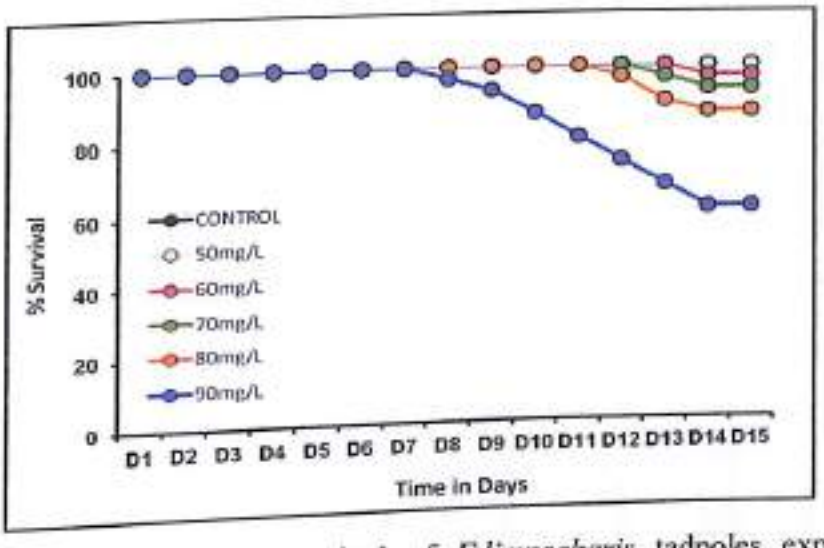

Figure 6: Graph showing survival of *F.limnocharis* tadpoles exposed to different concentrations of sodium nitrate (mg/L); n=30.

Apart from acute toxicity, the life history traits such as time to metamorphosis, body weight at metamorphosis and snout-vent length at metamorphosis was monitored following exposure to different concentrations of sodium nitrate. It was observed that sodium nitrate in the concentration of 90 mg/L accelerated metamorphosis process. However, the decrease in the time to metamorphosis was statistically significant ($p < 0.05$) in the group exposed to 90mg/L of sodium nitrate (Figure 7).

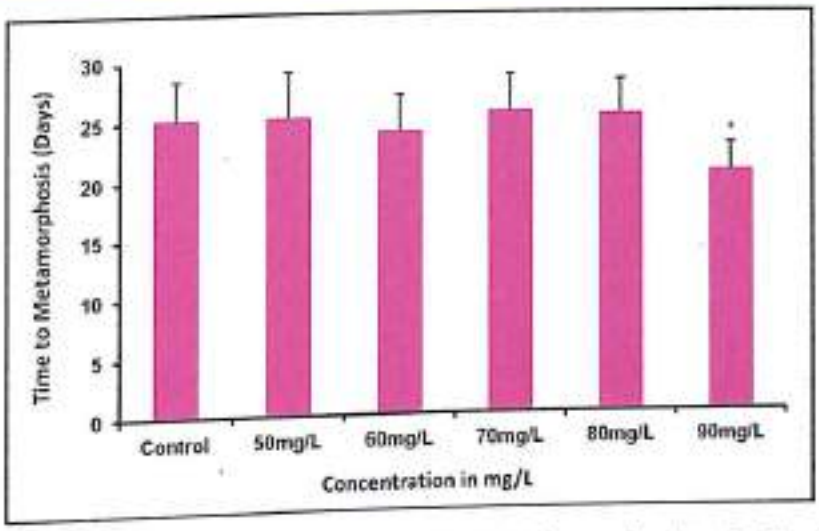

Figure 7: Change in time to metamorphosis in *F.limnocharis* tadpoles following exposure to different concentrations of sodium nitrate; (n = 30). Values significantly different from the control at $p < 0.05$ (*).

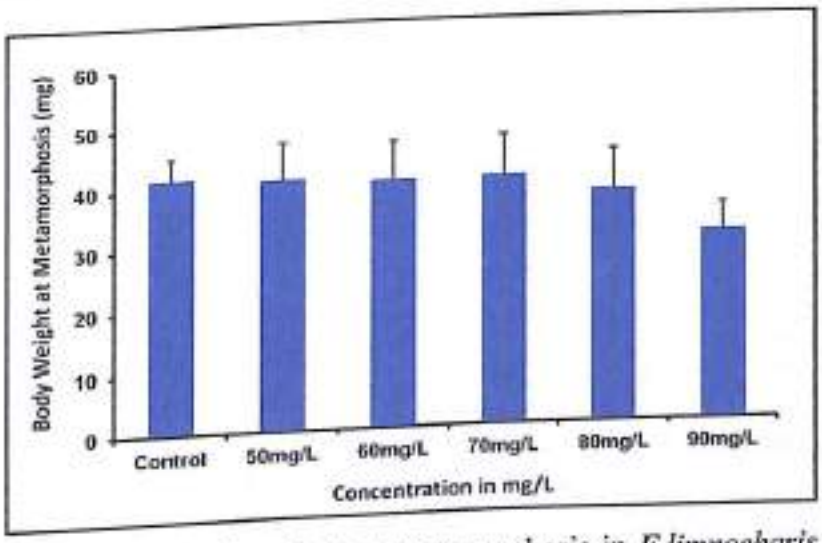

Figure 8: Change in body weight at metamorphosis in *F.limnocharis* tadpoles following exposure to different concentrations of sodium nitrate; (n = 30).

There is an apperent change in the body weight of metamorphs when exposed to highest concentration (90mg/L) of sodium nitrate; though the data is not statistically significant (Figure 8). Similarly, the snout to vent length was found to be small at the highest dose of Sodium nitrate concentration (90mg/L) but the data obtained was not found to be statistically significant (Figure 9).

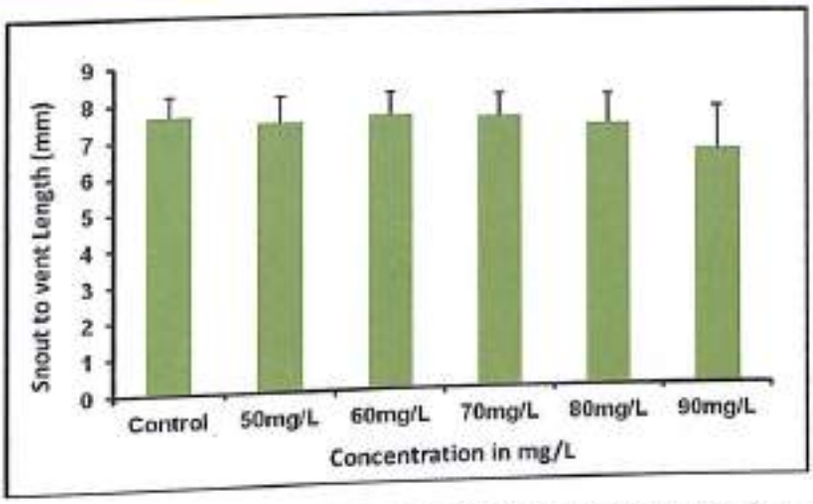

Figure 9: Change in snout-vent length in *F.limnocharis* froglets following exposure to different concentrations of sodium nitrate; (n = 30). Data statistically not significant.

Induction of micronucleus in the peripheral blood erythrocytes was used to test the potential genotoxic effects of sodium nitrate in the tadpoles of *F.limnocharis*. The tadpoles were exposed to different concentrations of sodium nitrate (50, 60, 70, 80 and 90 mg/L) for a period of 72h and 96h of fixation time. The findings are given below (Table 1 and Figure 10). It was observed that exposure of different concentration of sodium nitrate did not cause significant levels of Genotoxicity in various exposed groups of *F.limnocharis* tadpoles.

Table 1: Incidence of micronucleus induced by sodium nitrate in *F.limnocharis* tadpoles. a,b.

	Exposure Time	
Dose	**72H**	**96H**
Control	0.00 ± 0.00	0.00 ± 0.00
N50	0.10 ± 0.08	0.12 ± 0.05
N60	0.13 ± 0.05	0.14 ± 0.06
N70	0.12 ± 0.02	0.13 ± 0.05
N80	0.19 ± 0.07	0.15 ± 0.09
N90	0.20 ± 0.08	0.22 ± 0.11

[a] Control: No treatment was given; Sodium nitrate (N).
[b] Values are frequency of micronucleated erythrocytes (‰) expressed as means ± SD based on 1000 cells per animal (n=5).

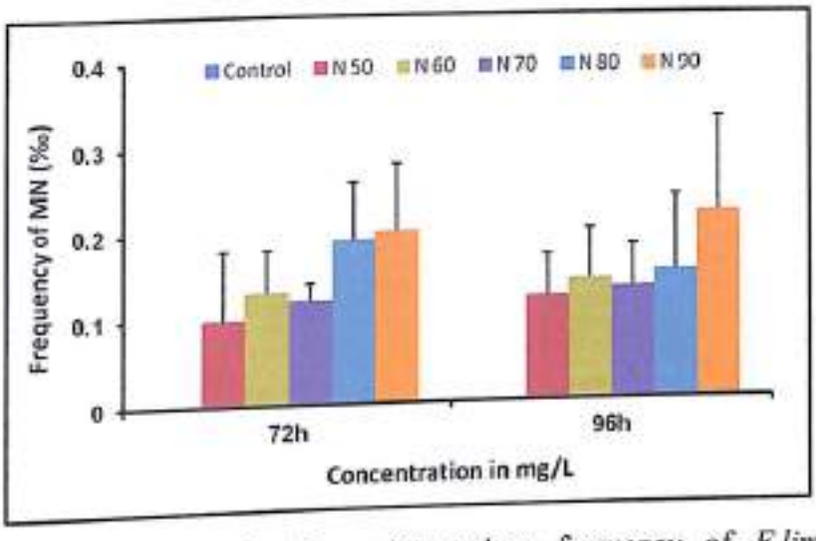

Figure 10: Histogram showing micronucleus frequency of *F.limnocharis* tadpoles exposed to different concentrations of sodium nitrate at 72h and 96h fixation time respectively. (N=Sodium nitrate).

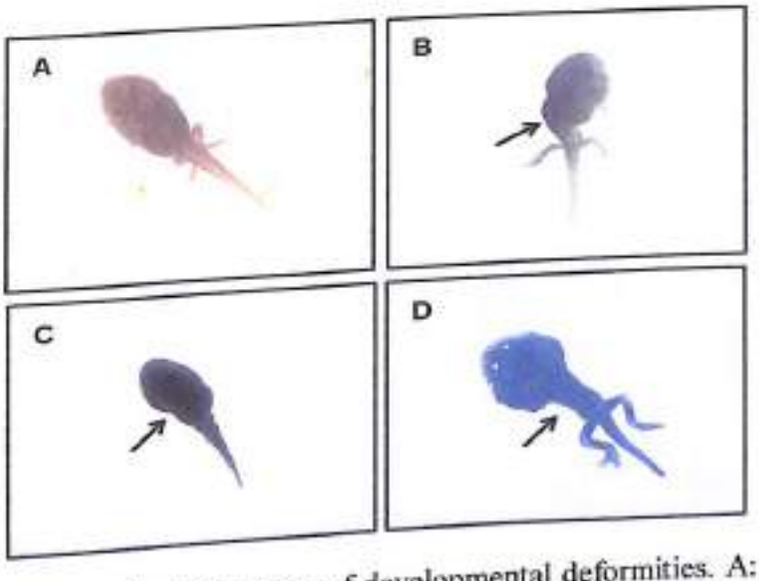

Figure 11: Images showing types of developmental deformities. A: Normal *F. limnocharis* tadpole; B: Bent tail formation; C: Deformed body shape; D: Missing forelimbs.

DISCUSSIONS

Present study enumerates possible deleterious effects of sodium nitrate of anuran amphibian namely *F. limnocharis* at their larval stages. The study parameters included analysis of acute and sub-lethal toxicity, changes in various life history traits and induction of Genotoxicity in the said model species.

Sodium nitrate in the concentration ranges tested (50–90 mg/L) did not cause significant mortality except in the highest dose (90mg/L) in *F. limnocharis* tadpoles during the 15 day exposure period (Figure 5). Similar results were also observed by Garriga et al., (2017) while working with *Alytes obstetricians* tadpole and found significant alterations of growth and survival of that species when exposed to ≥80mg/L of sodium nitate.

Under stressful conditions, amphibians exhibit a great deal of plasticity in life history traits. These could be early (Relyea, 2007; Johansson et al., 2010; Giri et al., 2012; Yadav et al., 2013; Singha et al., 2014) or late (Relyea and Hoverman, 2003; Liu et al., 2011) metamorphosis. The plasticity could depend upon the nature and concentration of the toxicants and/or the species concerned. In this experiment, sodium nitrate at highest concentrations accelerated the process of metamorphois in *F. limnocharis* (Figure 6); which is contrary to those reported for *R. pipiens* (Chen et al., 2009).

Metamorphosis is a perfectly regulated process which can exhibit high degree of plasticity under changed environmental circumstances. There is a dynamic equilibrium exists between growth and development which determine the timing of metamorphosis. Since metamorphosis process has a prospective relevance to the fitness in the post metamorphosis life stages, amphibians strike a balance between growth maximization prior to metamorphosis to maximize

survival and fitness in the later life stages after metamorphosis. Therefore, it is often seen that in order to avoid a higher mortality risk in larval stages compared to adult life prost-metamorphosis, tadpoles adjust the developmental process by an earlier metamorphosis than usual (Reylea 2007).

Phenotypic plasticity may allow an organism to respond to temporally variable opportunities for growth and risks of mortality. However, there are often trade-offs between the benefits afforded by plasticity in one life stage and the fitness related traits which may have long-term costs that accumulate in later stages (Johansson et al., 2010; Morey and Reznick, 2004). These may include smaller body size for earlier metamorphose (Altwegg and Reyer, 2003; Johansson et al., 2010), late reproductive maturity (Smith, 1987), reduced fecundity (Berven, 1981), survival (Altwegg and Reyer 2003), locomotor performance (Chen et al., 2009; Johansson et al., 2010) and other physiological traits like impaired immune system (Gervasi and Foufopoulos, 2008).

Toxicological evaluations of contaminants on genotoxicity in amphibians are scanty (Chang et al., 2009; Giri et al., 2012; Lajmanovich et al., 2005; Yadav et al., 2013). Genotoxicity assessment of pollutants is important because most of the pollutants in aquatic ecosystems are present at sub-lethal toxicity. Therefore, even these pollutants do not produce any obvious effects in the short term; however, chronic exposure over a period of time can cause population level effects. Moreover, alteration in genetic material will have long term hereditary consequences when these occur in germ cells.

Amphibian genotoxicity testing has been used as biomarker for DNA damage related to environmental pollution and pesticide contamination (Maselli

et al., 2010; Giri et al., 2012; Yadav et al., 2013; Singha et al., 2014; Patar et al., 2016). On the other hand, exposure to sodium nitrate apparently induced higher frequency of micronucleus in the peripheral blood erythrocyte. However, these were not statistically significant (Figures 9). This indicates that the environmental contaminants like sodium nitrate can have little genotoxic impacts on experimental species concerned.

CONCLUSION

Environmental pollution is inevitable but mitigating it sustainably is of paramount importance towards the better future. The environment is the place where we all live; we all have mutual interest, and the world which all of us share. As we continue to pollute our environment, it is a general perception that our efforts to take care of the environment are the reparation that we owe to the environment. This is completely a wrong notion. The fact of the matter is that, in an interconnected world, caring for our environment is to care ourselves as our own fate is inseparably linked with the fate of our environment.

Amphibian population crisis is of global concern where declining trend is at alarming rate with almost 40% of the 3 species are facing the danger of extinction. In the present study, toxicity evaluation of one of the most frequent contaminants of the aquatic ecosystems namely Sodium nitrate were evaluated in the tadpoles of *F. limnocharis*. The toxicities evaluated include various lethal and sub-lethal effects of nitrate in the life history traits, Genotoxicity etc. of anuran amphibian tadpoles.

Many toxicity studies do not explore the possible sub-lethal effects of environmental contaminants; instead rely on death as the toxicological endpoint. Understanding the effects of nitrate on aquatic life can act as biological early warning system to assess how anthropogenic sources of nitrate could shape the biodiversity in the affected areas. The present findings are significant in the perspective of amphibian population decline worldwide.

In the present study, it has been found that the accelerated metamorphosis, genotoxicity and teratogenic effects induced by environmentally relevant concentrations of sodium nitrate in *F. limnocharis*

tadpoles could have long-term fitness consequence to the population as a whole. In addition, nitrate may cause reductions in fecundity or viability, or increased mortality and lead to a reduction in the number of breeding adults. More studies on the effects of Sodium nitrate on different species of amphibian as well as other aquatic organisms suggested.

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