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

HEMALATA DOLEY

Roll No. 202820024008
Reg. No. 450928220, Dated: 07-12-2021

UNDER GUIDANCE OF:

DR. UTSAB SINGHA

Assistant Professor
Department of Zoology (PG)
Silapathar Science College
Silapathar, Dhemaji, Assam
787059

To

My beloved parents

with love

DECLARATION

I, **Hemalata Doley,** bearing Roll No. 202820024008, Registration No. 450928220 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.

Hemalata Doley

Place: Silapathar Science College
Date: 25th July, 2022

[HEMALATA DOLEY]

Dr. Utsab Singha
Assistant Professor
Department of Zoology (PG)

Silapathar Science College
Silapathar, Dhemaji, 787059

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.

Utsab Singha
[DR. UTSAB SINGHA]
Assistant Professor
Department of Zoology (PG)
Silapathar Science College
Dhemaji, Assam, 787059

Place: Silapathar Science College
Date: 23rd July, 2022

External
T. Kanthikal
Associate Professor
CHF, CAU.

singha.utsab@gmail.com
Phone: 9706765389

ACKNOWLEDGEMENT

I express my deep sense of gratitude and sincere thanks to my Project supervisor **Dr. Utsab Singha** of the Department of Zoology (PG), Silapathar Science College, Silapathar, Dhemaji, Assam for his constant guidance and inspiration throughout the course to facilitate this study.

I would like to convey my sincere thanks to all the faculty members of Department of Zoology (PG), Silapathar Science College, for their help, support and considerate opinion on various matters during the course of this study.

I am extremely grateful to my beloved parents, sister and my brother for their blessing and best wishes as well as unparalleled support all throughout.

Last but not the least, the sincere help and support of my colleagues Ms. Bhanushree Chutia, Ms. Punamshree Newar, Ms. Queen Deori and Mr. Dibyajyoti Borah are thankfully acknowledged.

Hemolata Doley

[HEMALATA DOLEY]

CONTENTS

INTRODUCTION

"I only feel angry when I see waste. When I see people throwing away things we could use."

--- Mother Teresa

The unprecedented rate of urbanization and industrialization and the subsequent release of various chemical compounds have dramatically increased the level of pollution of our ecosystems; in which the aquatic ecosystem suffered the most. Excessive use of Pesticides, fertilizers and other agrochemicals thus increased the extent of pollution and can deteriorate the ecosystem even further and negatively impact native inhabitants of aquatic flora and fauna (Nicolopoulou-Stamati et al., 2016). Anurans amphibian, especially toads and frogs have dual mode of life cycle, where they spent the most responsive phase of their life cycle in aquatic environment and are mostly at risk due to the exposure of contaminant related stress (Bókony et al., 2020).

Amphibian populations are declining worldwide (Gardner, 2001; Blaustein & Kiesecker, 2002). Although natural fluctuations may cause demographic variation; chemical pollutants have been implemented as a major contributor to these declines (Semlitsch, 2003). In particular, pollutants associated with agricultural practices have been linked to amphibian decline in multiple regions of the world (Davidson et al., 2002; Hamer et al., 2004). One of the major concern is the dramatic increase of nitrogenous fertilizers and their consequent detrimental effects on wild-life species, especially anuran amphibians (Carpenter et al., 1998) and expected to be ubiquitous in the future (Tilman et al., 2001; Galloway et al., 2003). Thus, understanding the impacts of increasing nitrogenous pollution on wildlife populations is of paramount importance.

Within few decades, increasing numbers of studies have assessed the impact of nitrogenous compounds on amphibians (Marco & Ortiz-Santaliestra, 2009) and have demonstrated that compounds such as ammonium, nitrite and nitrate can significantly affect rate of survival, development, behaviour and even habitat occupancy of amphibians (Egea-Serrano et al., 2012; Denoël et al., 2013). Additionally, amphibian responses to nitrogenous compounds can vary with high levels of inter and intra-specific variation (Johansson et al., 2001; Shinn et al., 2008, 2013).

Exposure to multiple stressors (as expected for natural settings) may lead to unexpected additive or synergistic responses (Berenbaum, 1989; Kiesecker, 2002). For example, the presence of low levels of salinity can reduce the toxicity of nitrite (Shinn et al., 2013). In contrast, the presence of other chemicals (Boone et al., 2005; Egea-Serrano et al., 2009), UV-B radiation (Macias et al., 2007) or low pH (Hatch and Blaustein, 2000) can increase the severity of amphibian larval responses to nitrogenous pollutants. Since amphibians are commonly exposed to multiple pollutants in nature, assessing the consequences of nitrogenous compounds alone and in combination is essential to develope our understanding of amphibian responses to nitrogenous pollution.

Nitrate compounds are readily soluble in water, forming the nitrate ions (NO_3^-) that can be toxic to aquatic organisms including fishes (Westin, 1974) and amphibians (Hecnar, 1995). A major source of nitrates in aquatic habitats stems from the use of nitrogen-based fertilizers and subsequent runoff into surface waters. Nitrate-related compounds can have a wide range of adverse effects on larval amphibians including impairment of growth and developmental (Hecnar,

1995; Marco et al., 1999), feeding (Kiesecker et al., 2004), respiratory physiology (Huey and Beitinger, 1980), and have been associated with carcinogenesis (Westin, 1974).

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) where amphibians are considered as excellent sentinels of ecosystem health (Roy, 2002; Hopkins, 2007). The MN assay has also been carried out in amphibians in many experimental and bio-monitoring studies to detect genotoxic agents and their potential toxicity (Giri et al., 2012; Yadav et al., 2013; Singha et al., 2014; Patar et al., 2016).

REVIEW OF LITERATURE

Amphibian population decline in the recent time is a major subject of concern among the researchers over the globe. Although many declines are due to habitat loss and overutilization other unidentified processes threaten 48% of rapidly declining species and are driving species most quickly to extinction posing a long term ecological damages (Stuart et al., 2004). Among the various factors involved habitat destruction and contaminants are supposed to be at the top of the list. According to International Union for Conservation of Nature (IUCN) criteria, a higher percentage of amphibians are threatened than birds or mammals, with many amphibians on the brink of extinction. The causes for amphibian population declines are complex which may differ among species, populations, and life stages within a population and are also dependent with multiple stressors interacting to drive population declines (Blaustein et. al., 2011). Anthropogenic effects have propelled us into what many have described as the sixth mass extinction, and amphibians are among the most affected groups (Buck et al., 2012). Amphibians continue to fuel our understanding of physiology and cell biology, yet one can argue that their most important role in science today is in helping us understand the ecology of our changing environment. Amphibians are considered to be the excellent bio-indicator model organism throughout the globe for bio as well as environmental monitoring. According to recent data, India is home to about 447 different species of amphibians (Figure 1) out of which 406 species are Anurans, 39 species are Gymnophiona and only 2 species of Caudata. (updated till April, 2020 available at https://www.amphibians.org/news/updated-checklist-of-indian-amphibians-2020).

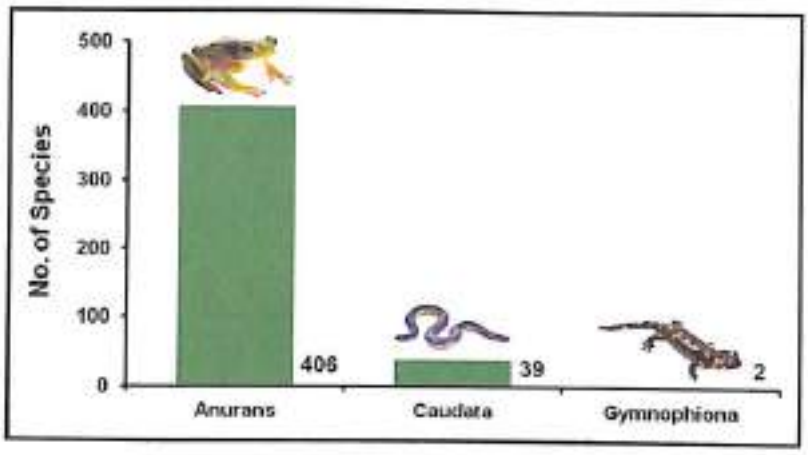

Figure 1: Different orders of amphibian species found in India (Source: https://www.amphibians.org/news/updated-checklist-of-indian amphibians-2020).

After a sound discussion over various factors responsible for amphibian population decline, most scientists now believe that underlying causes rest largely to anthropogenic disturbances (Collins and Storfer, 2003) and aquatic habitats are the first to be affected (Ralph et al., 1996). Amphibians are continuously bombarded with numerous stressors that may affect them directly and indirectly. Natural stress associated with competition, predation, resource availability, reproduction, and disease may be compounded by human induced stresses such as habitat destruction, environmental contamination, invasive species, and climate change. These stressors affect amphibians at the molecular, physiological, individual, population, and community levels (Blaustein et al., 2011). Several works demonstrated that amphibians are sensitive organisms, suitable for detection of genotoxic agents (Machado, 2011). For these reasons, many investigations for evaluating the effects of xenobiotics on organisms use *in vivo* or *in vitro* bioassays. It was, therefore, found that one of the best ways to estimate

the risk assessment of pollutants in the environment is to use biological tests *in vivo*, which give a global response to all chemicals present in the medium (Djomo et al., 2000). Micronucleus test is a useful and widely used tool for the evaluation of short-term and long term environmental mutagenesis and it can estimate the genetic risk followed by xenobiotic exposures.

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.

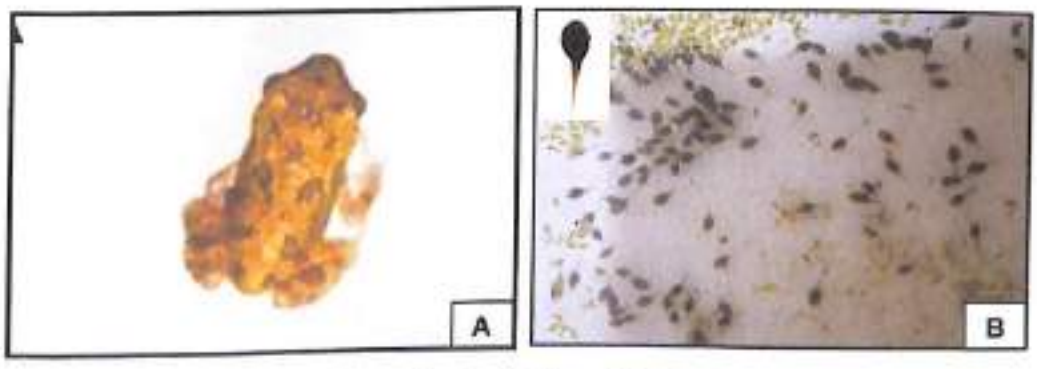

Figure 2: *Fejervarya limnocharis* (A) An adult frog (B) A tadpole larvae.

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).

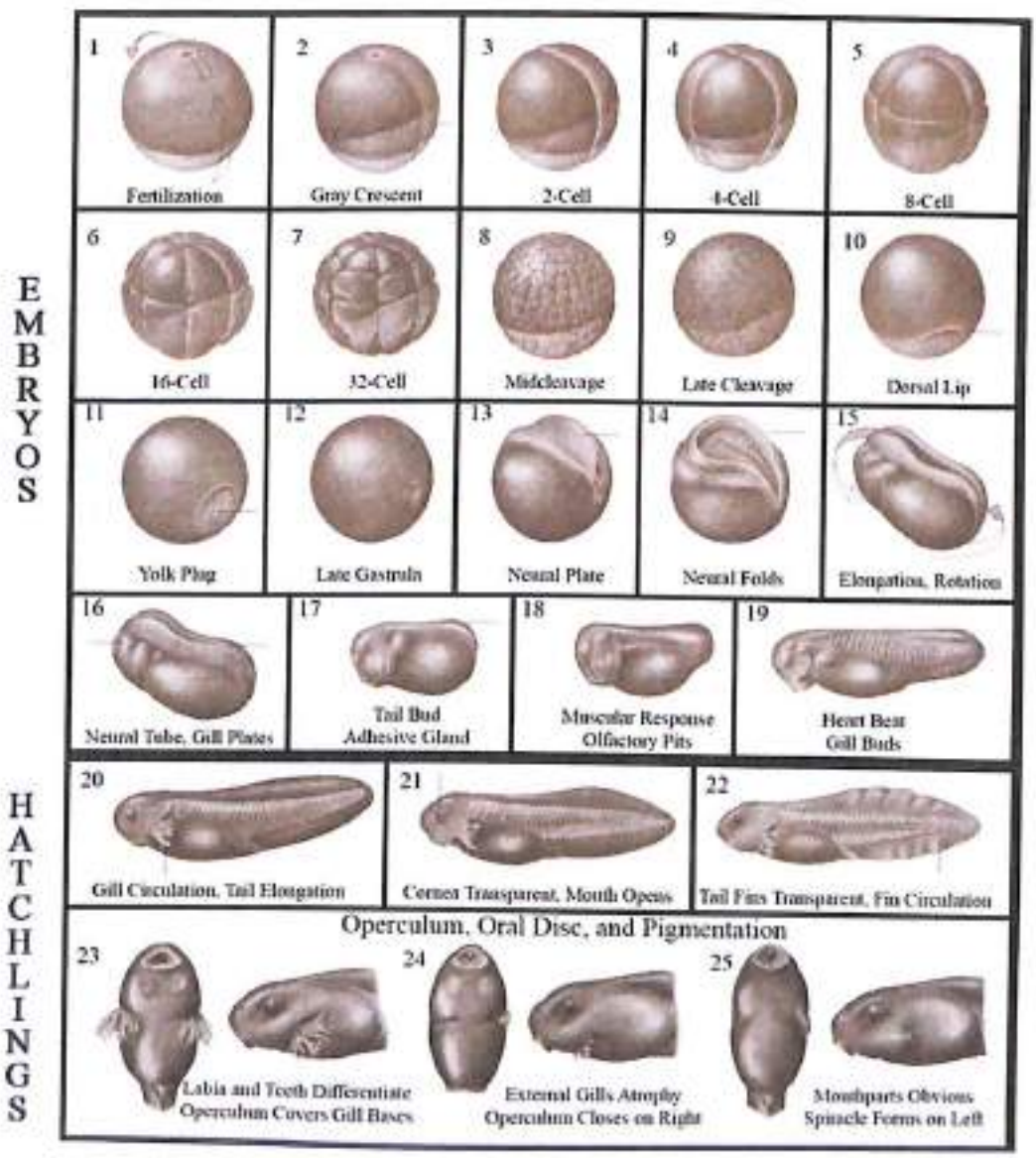

Figure 3: Gosner's reference chart of amphibian development (Part I).

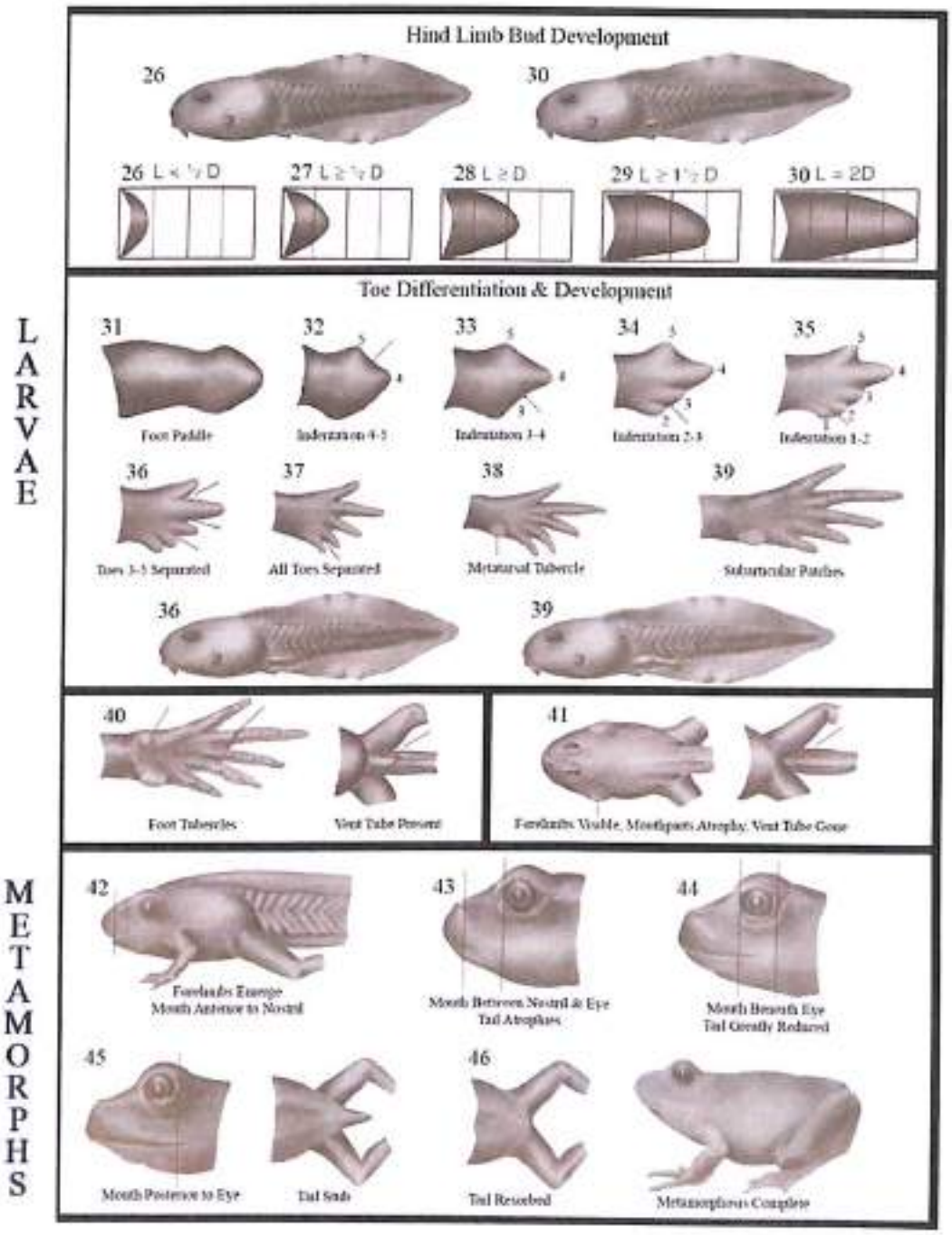

Figure 4: Gosner's reference chart of amphibian development (Part II)

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).

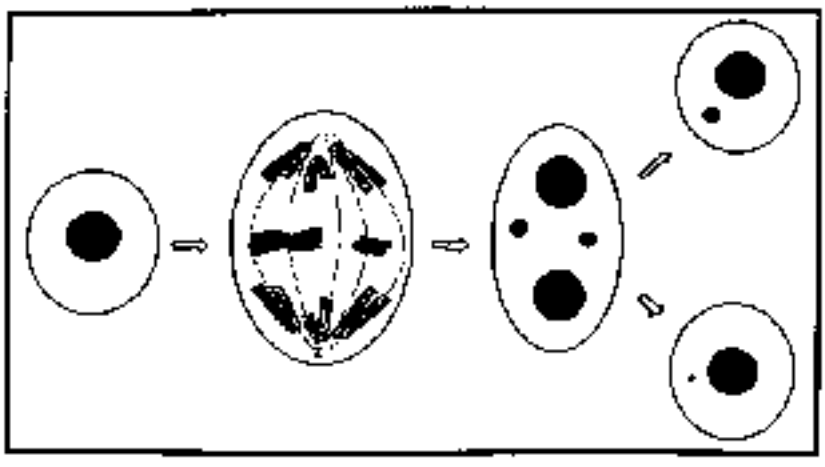

Figure 5: Formation of micronucleus during anaphase of cell division.

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), positive control (cyclophosphamide 2.0 mg/L) 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).

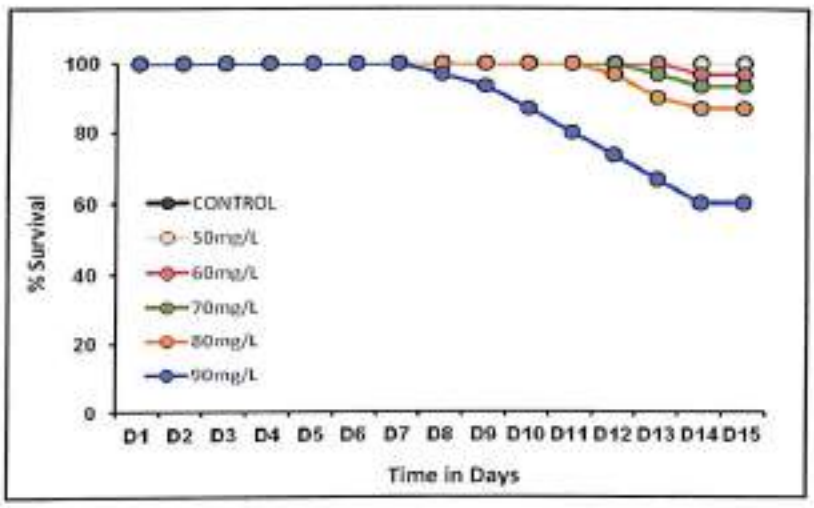

Figure 6: Graph showing survival of *F.limnocharis* tadpoles exposed to different concentrations of sodium nitrate (mg/L); n=30.

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).

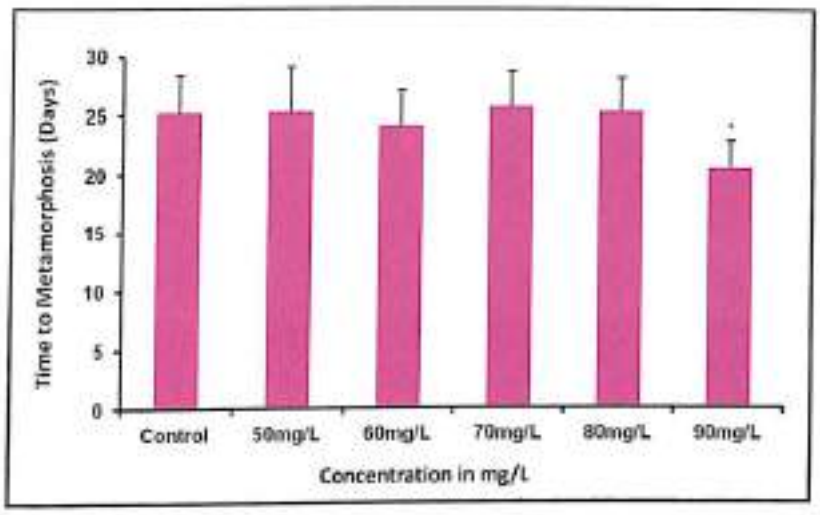

Figure 7: Change in time to metamorphosis in *F.limnocharis* tadpoles following exposure to different concentrations of sodium nitrate; (n = 30). Values significantly different from the control at $p < 0.05$ (*).

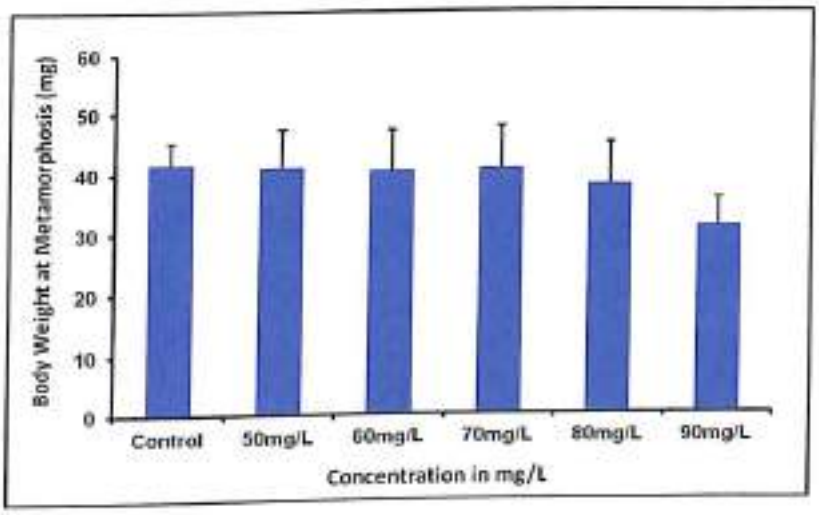

Figure 8: Change in body weight at metamorphosis in *F.limnocharis* tadpoles following exposure to different concentrations of sodium nitrate; (n = 30).

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).

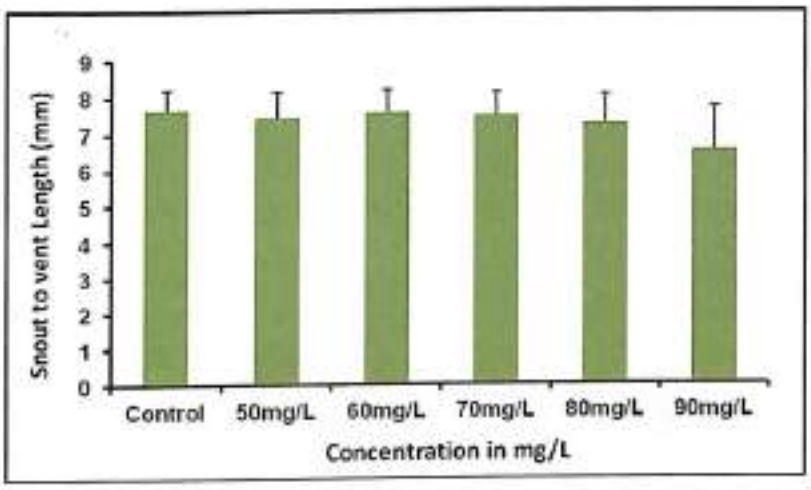

Figure 9: Change in snout-vent length in *F.limnocharis* froglets following exposure to different concentrations of sodium nitrate; (n = 30). Data statistically not significant.

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).

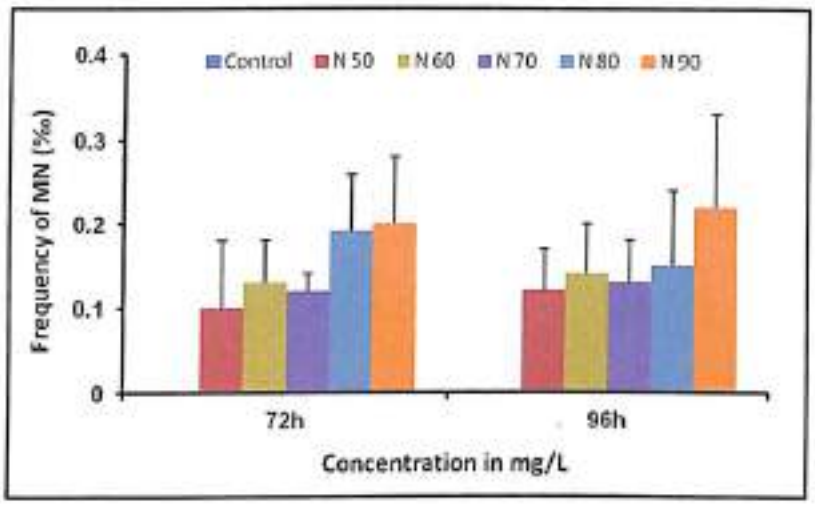

Figure 10: Histogram showing micronucleus frequency of *F.limnocharis* tadpoles exposed to different concentrations of sodium nitrate at 72h and 96h fixation time respectively. (N=Sodium nitrate).

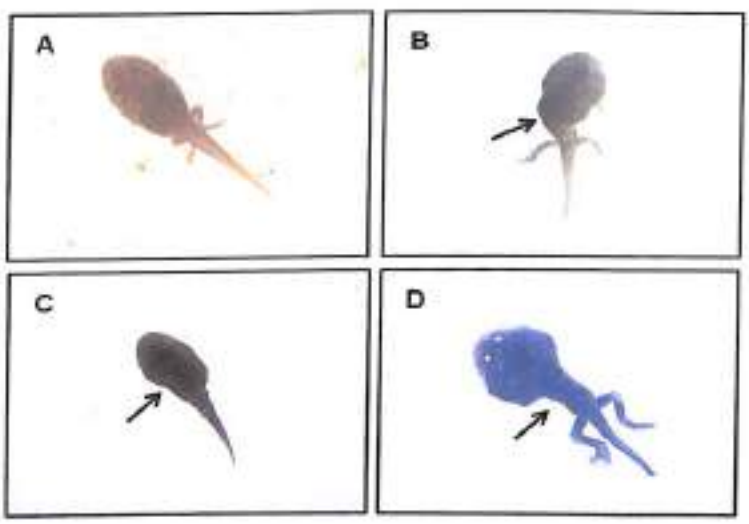

Figure 11: Images showing types of developmental deformities. A: Normal *F. limnocharis* tadpole; B: Bent tail formation; C: Deformed body shape; D: Missing forelimbs.

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 6). 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 7); 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 10). This indicates that the environmental contaminants like sodium nitrate can have little genotoxic impacts on experimental species concerned.

CONCLUSION

Environmental pollution is widespread but mitigating it in a sustainable way 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 environmental

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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