TOXIC AND GENOTOXIC EFFECT OF SODIUM NITRATE ON ANURAN TADPOLES *DUTTAPHRYNUS MELANOSTICTUS*

A

Project Report Submitted to

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

BY

DIBYAJYOTI BORAH

Roll No. 202820024005
Reg. No. 450628220, 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, **Dibyajyoti Borah,** bearing Roll No. 202820024005, Registration No. 450628220 dated 07-12-2021, hereby declare that the subject matter of the desertation entitled **"Toxic and Genotoxic effect of Sodium nitrate on Anuran tadpoles *Duttaphrynus melanostictus*"** 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.

Dibyajyoti Borah

[DIBYAJYOTI BORAH]

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

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

Silapathar Science College
Silapathar, Dhemaji, 787059

CERTIFICATE

Certified that the desertation entitled **"Toxic and Genotoxic effect of Sodium nitrate on Anuran tadpoles *Duttaphrynus melanostictus*"** 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

V. Kantibala
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, sisters and 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. Queen Deori, Ms. Hemalata Doley and Ms. Punamshree Newar are thankfully acknowledged.

Dibyajyoti Borah

[DIBYAJYOTI BORAH]

CONTENTS

INTRODUCTION

"The bad news is time flies. The good news is you're the pilot".

—Michael Altshuler

The loss of populations and extinctions of species are occurring at unprecedented rates (May 2010). Some scientists believe that we are observing a major extinction episode mirroring the five mass extinction events that have occurred during the Earth's history despite widespread interest in understanding extinctions and the recognition that population decline may be complex phenomena, research efforts directed at understanding species losses typically focus on the direct effects of single factors. This is illustrated by attempts to understand the global decline of amphibian population (Alford & Richards 1999; Wake & Vredenburg 2008). One estimate suggests that the extinction rates of amphibians may be 211 times the background rate of extinction (McCallum 2007).

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 (Stuart et al., 2004). Amphibian species decline is a global concern and contain multitude of factors. These may include habitat destruction, pollution, introduced exotic species, disease oubreak, climate change, associated atmospheric processes, and overexploitation, including collecting for the pet and food industry (Stuart et al., 2004). All these causes may have interacting cofactors. Moreover, causes for population declines may differ from region to region and even in different populations of the same species. There may be

inter-specific differences and even differences between life stages in how amphibians react to stressors. Amphibians are continuously bombarded with numerous stressors that may affect them directly and indirectly. Some species have disappeared; others are no longer found (Houlahan et al., 2000).

Nitrogen is the most abundant chemical element of the Earth's atmosphere, and also one of the essential components of many key biomolecules (e.g., amino acids, nucleotides). It ranks fourth behind carbon, oxygen and hydrogen as the commonest chemical element in living tissues (Campbell 1990). Ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) are the most common ionic (reactive) forms of dissolved inorganic nitrogen in aquatic ecosystems (Wetzel, 2001). Nitrogen is a major component of agricultural manure and occurs in the form of nitrate, nitrite, ammonium ion, and ammonia (Rouse et al., 1999). Nitrate (NO_3^-), the most stable and abundant of the nitrogen ions present in cropland and manure run-off, may pose a threat to larval stages of amphibians when it is converted to NO_2^- in the gut. The ion NO_2 changes haemoglobin in the blood to methaemoglobin and reduces the ability of the blood to carry oxygen.

Nitrate at concentrations detected in surface waters has both acute and chronic toxic effects on several species of amphibians. Hecnar (1995) showed that physical and behavioural abnormalities developed at concentrations as low as 3mg/L of nitrate exposure in 96-hr LC50 tests. These effects included reduced feeding and mobility resulting in severe weight loss and high mortality of the individuals. In addition to reduced swimming and feeding behaviour, developmental deformities including bent

tails, body swelling and bulging, head deformities, and digestive-system deformities occurred. The severity of the effects was positively correlated with increasing concentrations of nitrate (Rouse et al., 1999; Edwards et al., 2006; Krishnamurthy et al., 2008).

REVIEW OF LITERATURE

Amphibians play an important role in ecosystem functioning via nutrient cycling; energy flow and pest control (Valencia-Aguilar et al., 2013). Increasing concerns have therefore been raised with worldwide declining amphibian populations, with about one third of species currently under threat of extinction (IUCN 2017). Several underlying reasons for these declines have been identified, including emerging infectious diseases, climate change, invasive species, habitat loss and pollution and many more (Araújo et al., 2014; Whitfield et al., 2016). In rural landscapes, agrochemicals such as pesticides and fertilisers have been suggested to pose a high risk to amphibian communities (Camargo and Alonso 2006; Ortiz-Santaliestra et al. 2018; Hedge et al., 2019).

Despite of the above, few studies have been conducted so far into the toxicity of agrochemicals to amphibians when compared to other taxonomic groups (Ilha and Schiesari 2014; Ortiz-Santaliestra et al., 2018). This lack in research appears to hold true especially for tropical amphibian species, even though amphibian biodiversity and population declines are higher in the tropics than anywhere else (Sańchez-Domene et al., 2018; Schiesari et al., 2007; Whitfield et al., 2016). In addition, life history traits differ between temperate and tropical amphibians, which dictate that their responses to agrochemical stressors are likely to differ (Schiesari et al., 2007).

Nitrogen is one of the most abundant chemical elements of Earth's atmosphere. Increasing nitrogen enrichment has become one of the largest and most pervasive threats to our environment (Galloway et al., 2002; Holland et al., 2005). Mineral nitrogen in aquatic environments can cause declines in species diversity and changes in community composition, through both direct and indirect effects on individuals and populations via increased productivity, acidification, and potential

toxicity (Camargo and Alonso 2006).Amphibians are an important group of aquatic organisms that may be susceptible to nitrogen enrichment. Amphibians are undergoing widespread declines; some have been caused by habitat loss and over-exploitation, but many declines are as yet unexplained (Stuart et al., 2004). Uncovering interactions between pollutants such as nitrogen and amphibian survival may be critical to the long-term management and recovery of amphibian populations. Laboratory evidence is growing on the toxic effects of nitrate on amphibians (Johansson et al., 2001; Griffis- Kyle 2005; Smith et al., 2006); however, these laboratory studies do not include the interacting factors that might either mitigate or exacerbate relationships in the Wild.

Amphibians have the highest proportion of species on the verge of extinction among the world's vertebrates (Stuart et al., 2004), currently estimated as one in three species under the 2004 IUCN Red List (Baillie et al., 2004). Globally, amphibians have suffered massive, widespread, often unexplained, and probably irreversible, declines over the last several decades (Collins and Storfer 2003; Beebee and Griffiths 2005). In total, 21% of amphibian species are critically endangered or endangered, whereas the proportions for mammals and birds are only 10% and 5%, respectively, and this high level of threat might be an underestimate, as 23% of amphibians could not be assessed because of insufficient data (Baillie et al., 2004). Habitat loss, fragmentation and degradation, which often result from urbanisation, currently impact 88% of threatened amphibians (Baillie et al., 2004), and are therefore among the greatest threats to amphibian populations (Stuart et al., 2004; Beebee and Griffiths 2005; Cushman 2006).

During the past two centuries, and especially over the last five decades, humans have substantially altered the global nitrogen cycle (as well as the global cycles of other chemical elements), increasing both the availability and the mobility of nitrogen over large regions of Earth (Howarth et al., 2000; Galloway and Cowling 2002). Consequently, in addition to natural sources, inorganic nitrogen can enter aquatic ecosystems via point and nonpoint sources derived from human activities. Nonpoint sources generally are of greater relevance than point sources since they are larger and more difficult to control (Howarth et al., 2000; National Research Council 2000). Moreover, anthropogenic inputs of particulate nitrogen and organic nitrogen to the environment can also result in inorganic nitrogen pollution (National Research Council 2000; Smil 2001). Concentrations of inorganic nitrogenous compounds (NH_4^+, NO_2^-, NO_3^-) in ground and surface waters are hence increasing around the world, causing significant effects on many aquatic organisms and, ultimately, contributing to the degradation of freshwater, estuarine, and coastal marine ecosystems (Howarth et al., 2000; National Research Council 2000; Smil 2001; Anderson et al., 2002; Philips et al., 2002; Constable et al., 2003; Jensen 2003; Smith 2003).

Amphibians have the highest proportion of species on the verge of extinction among the world's vertebrates (Stuart et al., 2004), currently estimated as one in three species under the 2004 IUCN Red List (Baillie et al., 2004). Globally, amphibians have suffered massive, widespread, often unexplained, and probably irreversible, declines over the last several decades (Collins and Storfer 2003; Beebee and Griffiths 2005). In total, 21% of amphibian species are critically endangered or endangered, whereas the proportions for mammals and birds are only 10% and 5%,

respectively, and this high level of threat might be an underestimate, as 23% of amphibians could not be assessed because of insufficient data (Baillie et al., 2004). Habitat loss, fragmentation and degradation, which often result from urbanisation, currently impact 88% of threatened amphibians (Baillie et al., 2004), and are therefore among the greatest threats to amphibian populations (Stuart et al., 2004; Beebee and Griffiths 2005; Cushman 2006).

Nitrates could disrupt the gonadal functions and steroid synthesis in many vertebrate species (Guillette & Edwards, 2005). It has been reported that nitrate inhibits gonadal testosterone synthesis in rodents and bulls (Zraly et al., 1997; Panesar & Chan, 2000). Larval amphibians exposed to environmentally relevant concentrations of nitrates show altered sperm cell and ovarian follicle maturations (Orton et al., 2006).

Very few reports were available related to nitrogen toxicity and their effect on the immune functions on anuran amphibians. Studies revealed the effects of pulse nitrate exposure on larval amphibians showed the depressed immune response and RBCs in amphibians. Decreased levels of circulating white blood cells and decreased haemoglobin content also observed in bullfrogs and leopard frogs when exposed to 9-26 g/L nitrates for 3 weeks (Dappen, 1983).

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 *Duttaphrynus melanostictus*.**

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

MATERIALS 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 *Duttaphrynus melanostictus* (Figure 1) 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 2 & 3). 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 1: *Duttaphrynus melanostictus* (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).

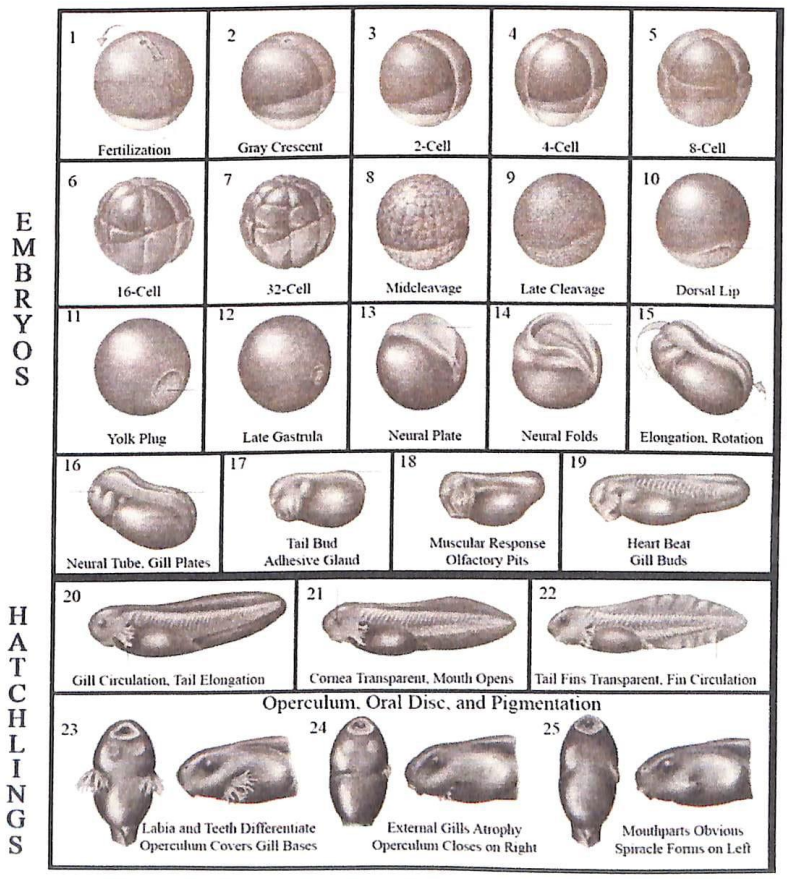

Figure 2: Gosner's reference chart of amphibian development (Part I)

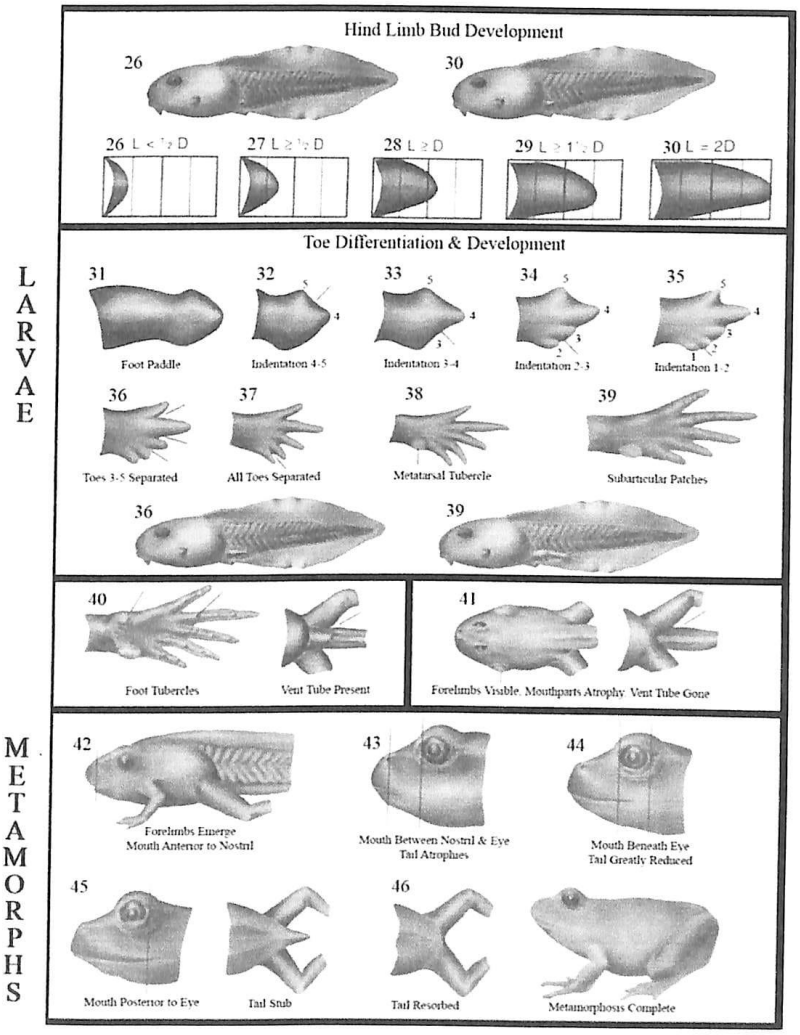

Figure 3: Gosner's reference chart of amphibian development (Part II)

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 minimal disturbance

to the remaining tadpoles. The water in the tub changed in every alternate day (static renewal) and the 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 4).

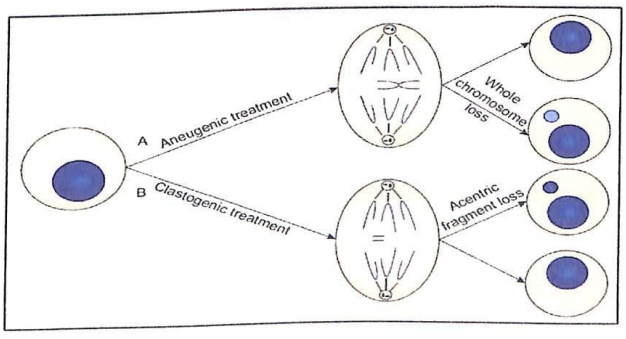

Figure 4: Schematic presentation of micronucleus formation (D'Costa et al., 2019)

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

Effect of Sodium nitrate on Life History traits:

For determination of acute toxicity of sodium nitrate in the tadpoles of *D. melanostictus*, 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 (Figure 6).

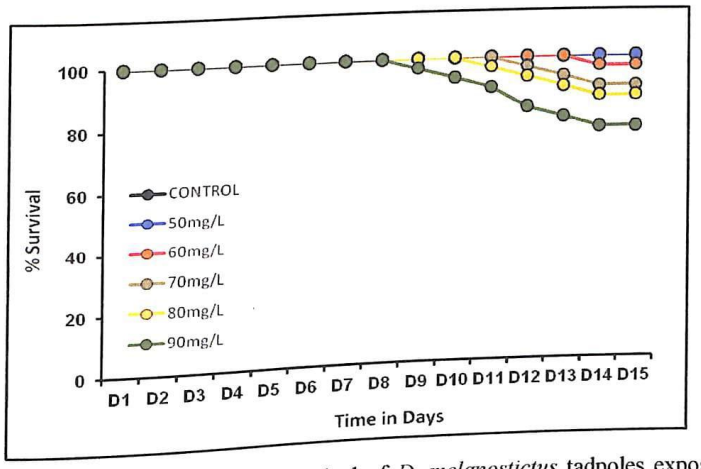

Figure 6: Graph showing percent survival of *D. melanostictus* tadpoles exposed to different concentrations of sodium nitrate (mg/L); n=30.

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 did not accelerate metamorphosis process among the various treatment groups except 90mg/L. The decrease in the time to metamorphosis was statistically significant ($p < 0.001$) in the group exposed to 90mg/L of sodium nitrate (Figure 7).

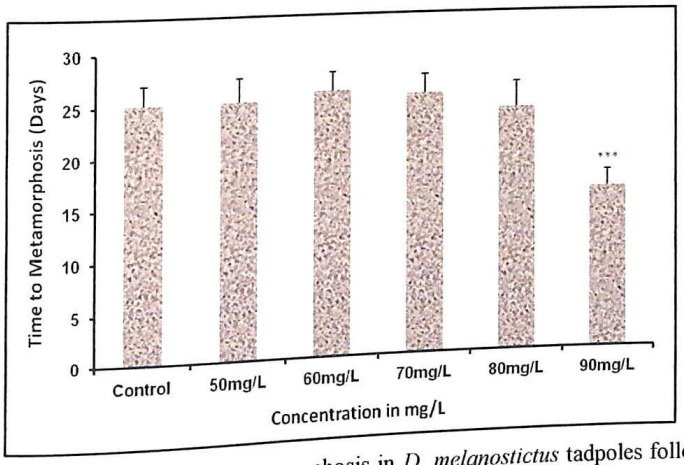

Figure 7: Change in time to metamorphosis in *D. melanostictus* tadpoles following exposure to different concentrations of sodium nitrate; (n = 30). Values significantly different from the control at $p < 0.001$ (***).

Sodium nitrate exposure in concentration range between 50-90 mg/L did not significantly alter the body weight at metamorphosis as compared to the control in all the groups exposed (Figure 8).

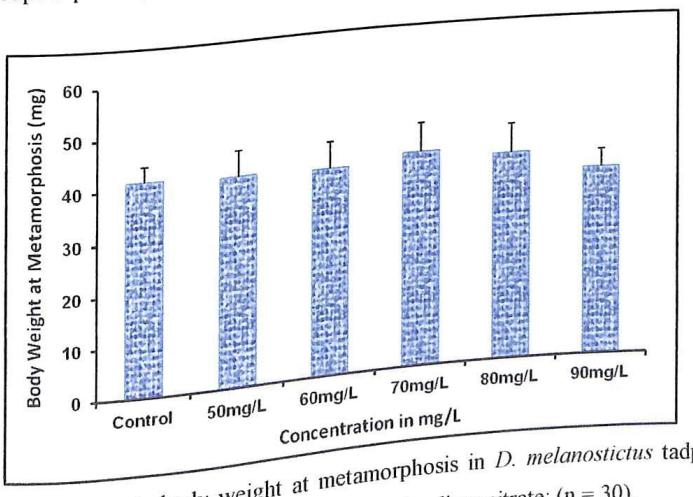

Figure 8: Change in body weight at metamorphosis in *D. melanostictus* tadpoles following exposure to different concentrations of sodium nitrate; (n = 30).

Similarly, there are no alterations in the snout to vent length of the froglets following exposure of different concentrations of Sodium nitrate and the data obtained also not statistically significant (Figure 9).

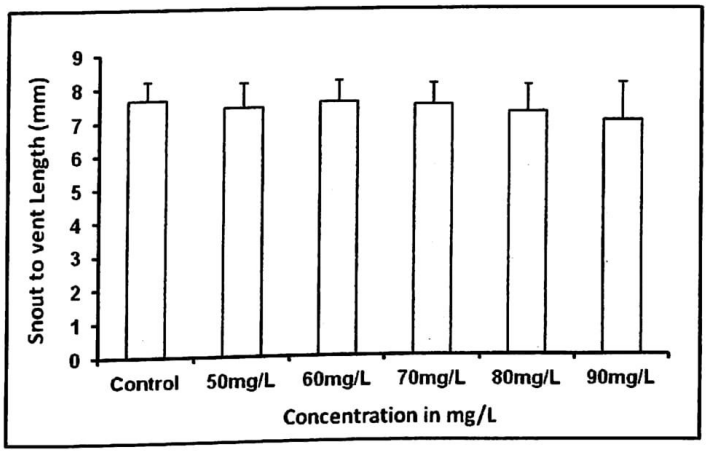

Figure 9: Change in snout-vent length in *D. melanostictus* froglets following exposure to different concentrations of sodium nitrate; (n = 30).

Induction of micronucleus in the peripheral blood erythrocytes was used to test the potential genotoxic effects of sodium nitrate in the tadpoles of *D. melanostictus*. 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 tadpoles.

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

	Exposure Time	
Dose	**72H**	**96H**
Control	0.00 ± 0.00	0.00 ± 0.00
N50	0.10 ± 0.03	0.12 ± 0.03
N60	0.13 ± 0.05	0.13 ± 0.06
N70	0.14 ± 0.02	0.15 ± 0.05
N80	0.12 ± 0.03	0.17 ± 0.04
N90	0.14 ± 0.02	0.18 ± 0.05

[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)

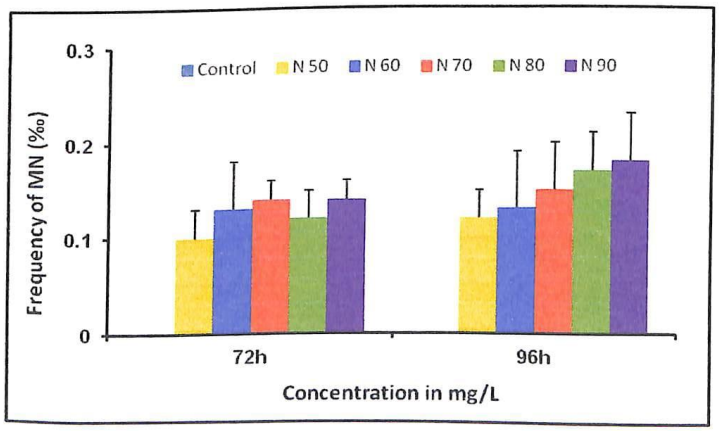

Figure 10: Histogram showing micronucleus frequency of *D. melanostictus* tadpoles exposed to different concentrations of sodium nitrate at 72h and 96h fixation time respectively. (N=Sodium nitrate).

DISCUSSIONS

The global decline in amphibian populations, and more recently the disturbing number of deformed amphibians, has caused many researchers to believe that they may be early indicators of serious environmental problems. Species have been reported to have disappeared from Europe, America, Australia, Asia, Africa and elsewhere (Alford et al., 2001). Various factors have been implicated in global amphibian declines. The animals are sensitive to pollution, because they live at the interface of two environments – land and water – and can easily absorb pollutants through the skin. The skin absorbs chemical contaminants from agricultural run-off that results from an indiscriminate use of pesticides, herbicides and fertilizers.

The deleterious effect of pollutants and chemicals have mostly been associated with finding frogs with missing legs, extra legs, misshapen legs, paralyzed legs that stuck out from the body at odd places, legs that were webbed together with extra skin, legs that were fused to the body, and legs that split into two half-way; frogs have also been found with missing eyes and with one external eye, the second eye growing inside the throat (Cohen 2001). Researchers have discovered that the level of nitrogen-based compounds which the United States Environment Protection Agency (EPA) found in agricultural areas as a result of using crop fertilizers is enough to kill some species of amphibians, especially at their more vulnerable larval stages (Blaustein et al., 2011). When exposed to moderate amounts of nitrates and nitrites, some tadpoles and young frogs reduce their feeding activity, swim less vigorously, experience disequilibrium, develop physical abnormalities, suffer paralysis and eventually die.

Now a report on one of the top-selling weed killers, atrazine, shows that it affects sexual development in frogs at concentrations 30 times lower than that allowed by the EPA, thereby raising concerns about the heavy use of this herbicide (Hayes et al., 2002). Hayes and co-workers have showed that atrazine demasculinizes tadpoles and turns them into hermaphrodites. The herbicide also lowers levels of the male hormone testosterone in sexually mature male frogs by a factor of 10, to levels lower than those in normal female frogs. It is unclear whether these abnormalities lead to reduced fertility.

In the present study, possible deleterious effects of the contaminants like sodium nitrate was analyzed in the species of anuran amphibians namely *D. melanostictus* 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 in *D. melanostictus* 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 nitrate.

Under stressful conditions, amphibians exhibit a great deal of plasticity in life history traits. These could be early (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 (90mg/L)

concentrations accelerated the process of metamorphosis in *D. melanostictus* (Figure 7). The observations are contrary to those reported for *R. pipiens* (Chen et al., 2009).

Moreover, in this study sodium nitrate did not cause statistically significant alterations in body weight as well as snout-vent length parameters following the concentration of doses from 50-90mg/L (Figure 8 & 9).

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 overt 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.

Additionally, exposure to sodium nitrate did not alter in the level of frequency of micronucleus in the peripheral blood erythrocyte (Figures 10). This indicates that the environmental contaminants like sodium nitrate is having very less or no potential to cause genetic toxicities in that experimental species concerned.

CONCLUSION

In this interconnected world pollution of any kind is obviously going to impact the living flora and fauna; directly or indirectly and amphibians are no exception. Amphibians have survived five mass extinctions and the only species that invaded land first in history. They are very sensitive to environmental contaminants and considered as a sentinels biomarker of environmental pollution. Therefore, analyzing the effects of contaminants on anuran amphibians may act as an early warning signal for bio monitoring and environmental crisis.

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 *D. melanostictus*. 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 *D. melanostictus* 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.

BIBLIOGRAPHY

Alford RA and Richards SJ (1999). Global amphibian declines: a problem in applied ecology. Annu. Rev. Ecol. Syst. 30:133–165.

Alford RA, Dixon PM and Pechmann JH (2001). Ecology.Global amphibian population declines; Nature (London) 412: 499–500.

Anderson DM, Glibert PM and Burkholder JM (2002). Harmful algal blooms and eutrophication: nutrient sources, composition, and consequences. Estuaries. 25:704–26.

Araújo CVM, Shinn C, Moreira-Santos M, Lopes I, Espindola ELG and Ribeiro R (2014). Copper-driven avoidance and mortality in temperate and tropical tadpoles. Aquat Toxicol. 146:70–75.

Baillie JEM, Hilton-Taylor C and Stuart SN *(Eds.)* (2004). 2004 IUCN Red List of Threatened Species. A Global Species Assessment. IUCN, Gland, Switzerland and Cambridge, UK.

Beebee TJC and Griffiths RA (2005). The amphibian decline crisis: a watershed for conservation biology? Biological Conservation. 125:271–285.

Blaustein AR, Han BA, Relyea RA, Johnson PTJ, Buck JC, Gervasi SS and Kats LB (2011). The complexity of amphibian population declines: Understanding the role of cofactors in driving amphibian losses. Annals of the New York Academy of Sciences. 1223: 108 -119.

Camargo JA and Alonso A (2006). Ecological and toxicological effects of inorganic nitrogen pollution in aquatic ecosystems: a global assessment. Environ Int. 32:831–849.

Campbell NA (1990). Biology. *2nd edition*. Redwood City (CA): The Benjamin/ Cummings Publishing Company.

Chen TH, Gross JA and Karasov WH (2009). Chronic exposure to pentavalent arsenic of larval leopard frogs (Rana pipiens): bioaccumulation and reduced swimming performance. Ecotoxicology. 18: 587-593.

Cohen Jr MM (2001). Frog decline, malformations, and a comparison of frog and human health. Am. J. Med. Gen.104: 101–109.

Collins JP and Storfer A (2003). Global amphibian declines: sorting the hypotheses. Diversity and Distributions 9:89–98.

Constable M, Charlton M, Jensen F, McDonald K, Craig G and Taylor KW (2003). An ecological risk assessment of ammonia in the aquatic environment. Hum Ecol Risk Assess. 9:527–48.

Cushman SA (2006). Effects of habitat loss and fragmentation on amphibians: a review and prospectus. Biological Conservation. 128:231–240.

Dappen GM, Sundberg MW, Wu TW, Babb BE and Schaeffer JR (1983). A diazo-based dry film for determination of total bilirubin in serum. Clinical Chemistry. 29: 37-41.

D'Costa A, Kumar MKP and Shyama SK (2019). Genotoxicity assays: The micronucleus test and the single-cell gel electrophoresis assay. Advances in Biological Science Research. https://doi.org/10.1016/B978-0-12-817497-5.00018-5.

Edwards TM, McCoy KA, Barbeau T, McCoy MW, Thro MJ and Guillette Jr. LJ (2006). Environmental context determines nitrate toxicity in Southern toad *(Bufo terrestris)* tadpoles. Aquat Toxicol. 78: 50–58.

Feng Y, Ueda A and Wu CF (2004). A modified minimal hemolymph-like solution, HL3.1, for physiological recordings at the neuromuscular junctions of

normal and mutant Drosophila larvae. Journal of Neurogenetics. 18: 377-402.

Galloway JN and Cowling EB (2002). Reactive nitrogen and the world: 200 years of change. Ambio. 31:64–71.

Garriga N, Montori A and Llorente GA (2017). Impact of ammonium nitrate and sodium nitrate on tadpoles of Alytes obstetricians. Ecotoxicology. 26: 667-674.

Giri A, Yadav SS, Giri S and Sharma GD (2012). Effect of predator stress and malathion on tadpoles of Indian skittering frog. Aquatic Toxicology. 106-107: 157-163.

Gosner KL (1960). A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica. 16: 183–190.

Griffis-Kyle KL (2005). Ontogenetic delays in effects of nitrite exposure on tiger salamanders *(Ambystoma tigrinum tigrinum)* and wood frogs *(Rana sylvatica)*. Environ Toxicol Chem. 24:1523–1527.

Guillette Jr LJ and Edwards TM (2005). Is nitrate an ecologically relevant endocrine disruptor in vertebrates? Integrative and Comparative Biology. 45: 19-27.

Hayes TB, Collins A, Lee M, Mendoza M, Noriega N, Stuart AA and Vonk A (2002). Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proc. Natl. Acad. Sci. USA. 99: 5476–5480.

Hecnar SJ (1995). Acute and chronic toxicity of ammonium nitrate fertilizer to amphibians from southern Ontario. Environmental Toxicology and Chemistry. 14: 2131-2137.

Hegde G, Krishnamurthy SV and Berger G (2019). Common frogs response to agrochemicals contamination in coffee plantations, Western Ghats, India. Chem Ecol. 35:397–407.

Holland EA, Braswell BH, Sulzman J and Lamarque J (2005). Nitrogen deposition onto the United States and western Europe: synthesis of observations and models. Ecol Appl. 15:38–57.

Houlahan JE, Findlay CS, Schmidt BR, Meyer AH and Kuzmin SL (2000). Quantitative evidence for global amphibian population declines; Nature. 404:752–755

Howarth RW, Anderson BD, Cloern JE, Elfring C, Hopkinson CS, Lapointe B, Malone T, Marcus N, McGlathery K, Sharpley AN and Walker D (2000). Nutrient pollution of coastal rivers, bays, and seas. Iss Ecol. 7:1-15.

Ilha P and Schiesari L (2014). Lethal and sublethal effects of inorganic nitrogen on gladiator frog tadpoles *(Hypsiboas faber*, Hylidae). Copeia. 2014:221–230.

IUCN (2017). IUCN Red list of threatened species, v 2017. 3. International Union for Conservation of Nature, Gland, Switzerland, http://www.iucnredlist.org.

Jensen FB (2003). Nitrite disrupts multiple physiological functions in aquatic animals. Comp Biochem Physiol. 135:9-24.

Johansson M, Rasanen K and Merila J (2001). Comparison of nitrate tolerance between diVerent populations of the common frog, *Rana temporaria*. Aquat Toxicol. 54:1–14.

Krishna G and Hayashi M (2000). In vivo rodent micronucleus assay: protocol, conduct and data interpretation. Mutatation Research. 455: 155-166.

Krishnamurthy SV, Meenakumari D, Gurushankara HP and Vasudev V (2008). Nitrate-Induced Morphological Anomalies in the Tadpoles of Nyctibatrachus major and *Fejervarya limnocharis* (Anura: Ranidae). Turk J Zool. 32: 239-244.

Lajmanovich RC, Cabagna M, Peltzer PM, Stringhini GA and Attademo AM (2005). Micronucleus induction in erythrocytes of the Hyla pulchella tadpoles (Amphibia: Hylidae) exposed to insecticide endosulfan. Mutation Research. 587: 67-72.

Lajmanovich RC, Cabagna M, Peltzer PM, Stringhini GA and Attademo AM (2005). Micronucleus induction in erythrocytes of the Hyla pulchella tadpoles (Amphibia: Hylidae) exposed to insecticide endosulfan. Mutation Research. 587: 67-72.

Liu WY, Wang CY, Wang TS, Fellers GM, Lai BC and Kam YC (2011). Impacts of the herbicide butachlor on the larvae of a paddy field breeding frog (Fejervarya limnocharis) in subtropical Taiwan. Ecotoxicology. 20: 377–84.

May RM (2010). Ecological science and tomorrow's world. Philos. Trans. Royal. Soc. B. 365: 41–47.

McCallum ML (2007). Amphibian decline or extinction? Current declines dwarf background extinction rate. J. Herpetol. 41:483–491.

National Research Council (2000). Clean coastal waters: understanding and reducing the effects of nutrient pollution.Washington, DC: National Academic Press.

Ortiz-Santaliestra ME, Maia JP, Egea-Serrano A and Lopes I (2018). Validity of fish, birds and mammals as surrogates for amphibians and reptiles in pesticide toxicity assessment. Ecotoxicology. 27:819–833.

Orton F, Carr JA and Handy RD (2006). Effects of nitrate and atrazine on larval development and sexual differentiation in the Northern Leopard Frog Rana pipiens. Environmental Toxicology and Chemistry. 25:211-217.

Panesar NS and Chan KW (2000). Decreased steroid hormone synthesis from inorganic nitrite and nitrate: studies in vitro and in vivo. Toxicology and Applied Pharmacology. 169: 222-230.

Philips S, Laanbroek HJ and Verstraete W (2002). Origin, causes and effects of increased nitrite concentrations in aquatic environments. Rev Environ Sci Biotechnol. 1:115–41.

Relyea RA and Hoverman JT (2003). The impact of larval predators and competitors on the morphology and fitness of juvenile treefrogs. Oecologia. 134: 596-604.

Rouse JD, Bishop CA and Struger J (1999). Nitrogen pollution: an assessment of its threat to amphibian survival. Environmental health perspectives. 107: 799-803.

Rouse JD, Bishop CA and Struger J (1999). Nitrogen pollution: an assessment of its threat to amphibian survival. Environmental health perspectives. 107: 799-803.

Sańchez-Domene D, Navarro-Lozano A, Acayaba R, Picheli K, Montagner C, Rossa-Feres DC, da Silva FR and de Almeida EA (2018). Eye malformation baseline in *Scinax fuscovarius* larvae populations that inhabit agroecosystem ponds in southern Brazil. Amphib–Reptilia. 39:325–334.

Schiesari L, Grillitsch B and Grillitsch H (2007). Biogeographic biases in research and their consequences for linking amphibian declines to pollution. Conserv Biol. 21:465–471.

Singha U, Pandey N, Boro F, Giri S, Giri A and Biswas S (2014). Sodium arsenite induced changes in survival, growth, metamorphosis and Genotoxicity in the Indian cricket frog (Rana limnocharis). Chemosphere. 112: 333-339.

Singha U, Pandey N, Boro F, Giri S, Giri A and Biswas S (2014). Sodium arsenite induced changes in survival, growth, metamorphosis and Genotoxicity in the Indian cricket frog (Rana limnocharis). Chemosphere. 112: 333-339.

Smil V (2001). Enriching the Earth. Cambridge: The MIT Press.

Smith GR, Temple KG, Dingfelder HA and Vaala DA (2006). Effects of nitrate in the interactions of the tadpoles of two ranids *(Rana clamitans* and *R. catesbeiana)*. Aquat Ecol. 40:125–130.

Smith VH (2003). Eutrophication of freshwater and coastal marine ecosystems: a global problem. Environ Sci Pollut R. 10:126–39.

Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues ASL, Fischman DL and Waller RW (2004). Status and trends of amphibian declines and extinctions worldwide. Science. 306:1783–1786.

Valencia-Aguilar A, Corteś-Gómez AM and Ruiz-Agudelo CA (2013). Ecosystem services provided by amphibians and reptiles in Neotropical ecosystems. Int J Biodivers Sci Ecosyst Serv Manag. 9:257–272.

Wake DB and Vredenburg VT (2008). Are we in the midst of the sixth mass extinction? A review from the world of amphibians. Proc. Natl. Acad. Sci. USA. 105: 11466–11473.

Wetzel RG (2001). Limnology. *3rd edition*. New York: Academic Press.

Whitfield SM, Lips KR and Donnelly MA (2016). Amphibian decline and conservation in Central America. Copeia. 104:351–379.

Xu Q and Oldham RS (1997). Lethal and Sublethal Effects of Nitrogen Fertilizer Ammonium Nitrate on Common Toad (Bufo bufo) Tadpoles. Archives of Environmental Contamination and Toxicology. 32: 298-303.

Yadav SS, Giri S, Singha U, Boro F and Giri A (2013). Toxic and genotoxic effects of Roundup on tadpoles of the Indian skittering frog (Euflictis cyanophlyctis) in the presence and absence of predator stress. Aquatic Toxicology. 132-133: 1-8.

Zralý Z, Bendová J, Svecová D, Faldíková L, Vězník Z and Zajícová A (1997). Effects of oral intake of nitrates on reproductive functions of bulls. Veterinární medicína. 42: 345-354.