TOXIC AND GENOTOXIC EFFECT OF SODIUM NITRATE ON ANURAN TADPOLES *POLYPEDATES MACULATUS*

A

Project Report Submitted to

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

BY

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To

My beloved parents

with love

DECLARATION

I, **Punamshree Newar**, bearing Roll No. 202820024012, Registration No. 451328220 dated 07-12-2021, hereby declare that the subject matter of the desertation entitled "**Toxic and Genotoxic effect of Sodium nitrate on amphibian tadpoles *Polypedates maculatus***" 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.

Punamshree Newar

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Place: Silapathar Science College

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CERTIFICATE

Certified that the desertation entitled **"Toxic and Genotoxic effect of Sodium nitrate on amphibian tadpoles *Polypedates maculatus*"** 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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Punamshree Newar

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INTRODUCTION

"Progress is impossible without change, and those who cannot change their minds cannot change anything."

--- George Bernard Shaw

Xenobiotic are compounds foreign to an organism's normal biochemistry, such as drugs, poisons and environmental contaminants of both synthetic and natural origin. Organisms have natural mechanisms to metabolise these compounds to avoid any toxic effects. The intermediates in xenobiotic metabolism can themselves be the cause of toxic effects in some cases leading to disease state and even cause lethality. The toxicities can be at biochemical, physiological, endocrine, neuronal or genetic level. Of the various kinds of possible toxicities, genetic toxicity is of vital importance because of the fact that the consequences of genetic defects have the potential to pass on to the next generation and hence affect an entire population. Genotoxicity data is important because environmental contaminants can lead to a reduction in genetic diversity resulting from strong selection for chemical tolerance or population decline leading to genetic bottleneck and drift. In such populations, disease outbreaks can quickly assume the form of epidemic which may threaten the entire population with the possibility of extinction. Therefore, genotoxicity data is important to identify genetic diversity (Murdoch & Hebert, 1994), contamination-induced natural selection (Peles et al., 2003), and increased mutation rates (Somers et al., 2002).

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 or 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).

As humans increasingly alter the environment, we tend to understand more and more of the impacts that these alterations may have on natural populations, communities and ecosystems. Understanding and predicting the impacts of anthropogenic chemicals on non-target organisms is a challenging proposition for ecologists and toxicologists.

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.

Although, a growing number of laboratories across the world recently have started studying the ecological impacts of pesticides on amphibians at species and community level (Relyea, 2005); but very little information is available concerning the genotoxic impact of these contaminants on amphibians. Most studies of chemical contaminants and amphibians have focused on toxicity or developmental effects that lead to poor survivorship or death directly (Rohr et al., 2006; Relyea, 2009). One of the best ways to estimate the risk assessment of environmental contaminants on amphibians is to use biological tests *in vivo*.

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).

In eukaryotes, the micronucleus (MN) assay is a relatively simple and reliable method that can be used to detect genotoxins in a cost and time effective manner. MN are acentric chromosomal fragments or whole chromosomes that are

not integrated within the main nucleus, because they lag behind in mitotic anaphase, and are retained in the cytoplasm where they are isolated by a nuclear membrane. The usefulness of the MN assay has been demonstrated in many non-mammalian vertebrates as well as invertebrate species, including fishes and molluscs (Bolognesi & Hayashi, 2011). Amphibians are considered 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 (Giri et al., 2012; Yadav et al., 2013; Singha et al., 2014; Patar et al., 2016).

REVIEW OF LITERATURE

The earliest amphibians appeared around 395 million years ago (Mya) in the late Devonian and early carboniferous from lobe-finned fish sarcopterygians (class Sarcopterygii, "flesh fins"). The name of Amphibia gets it from the Greek words amphi meaning "two" and bios meaning "mode of life" because many species have a diphasic life history: they spend their entire early larval stages in the fresh-water or aquatic phase followed by an adult phase after metamorphosis, which can be terrestrial. Amphibians are unique group of ectothermic vertebrate animals, comprising three major orders namely- Anura (the tail less amphibians, i.e. frogs and toads), Apoda (the limb less amphibians, i.e. caecilians) also known as Gymnophiona, and Urodela (i.e. salamanders) also known as Caudata. The name of order Anura is derived from Greek words a(n) meaning "without" and oura meaning "tail". Majority of Anurans found almost everywhere around the world except for areas characterized by extreme cold or dry places and in oceanic islands (Pough et al., 2004).

Amphibians are broadly considered as model bio-indicator species (Hopkins, 2007). Amphibian posses several characteristics which make them more susceptible to the environmental disturbances compared to other wildlife species (Rowe et al., 2003). Amphibian possess permeable integument which is responsible for both gaseous exchange and osmoregulation. Therefore, they are highly vulnerable to the changes in contaminants and pathogens and disturbances of any sort may adversely affect the life cycle and finally affecting their populations (Dunson et al., 1992).

The declining of amphibian population over the globe has attracted attention from scientist in coming recent years. According to the recent IUCN Red List data, considerably a higher percentage of amphibians are threatened or

endangered than the birds or mammals, with many of amphibians on brink of extinction (Figures 1).

It is believed the declines of amphibian population are being mostly affected by the multiple stressors. The amphibian decline may vary among the species, life stages within population and are dependent with multiple stressors interacting to drive population declines (Blaustein et. al., 2011). The estimated current total number of well-known amphibian species throughout the world is approximately 8,482. Out of which nearly 7,494 (88.35%) are frogs and toads, 773 (9.11%) are newts and salamanders and only 215 (2.53%) are caecilians. (http://amphibiaweb.org, 6th July, 2022).

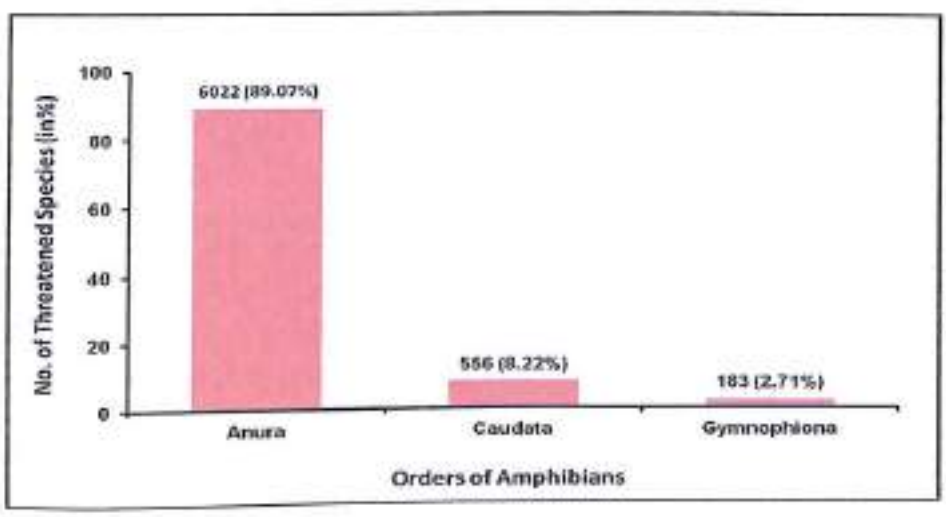

Figure 1: IUCN Red List assessment of Amphibians (Threatened Species) according to taxonomic order (2019)

In addition, recent outburst of population growth and increased levels of anthropogenic activities have dramatically changed the environment, especially aquatic environment. Many terrestrial as well as aquatic species were highly sensitive to these changes leading to their decline or extirpation (Thomas et al., 2004; Hopkins 2007; Acevedo-Whitehouse and Duffus, 2009). Amphibians have

dual mode of life cycle; larval stages spent in water and rest on terrestrial habitats. Therefore, they are supposed to respond towards environmental disturbances more than the rest. Contaminants may have positive or negative effects on various aquatic species depending on the nature of the contaminants and their persistence in the environment (Carey and Bryant, 1995; Strong et al., 2017). Amphibians, especially the larval forms, constantly receive chemical contaminants through their permeable skin from the contaminated environment which may cause physiological and developmental malfunctions (Voccia et al., 1999; Martin et al., 2010). Therefore, extensive research is in progress to evaluate the myriad effects of environmental contaminants on anuran amphibians and how these contaminants can shape their ecotoxicological interations with the surrounding environment.

Rohr et al. (2008) reported that fertilizer pollution may drastically increase the rate of trematode infections in amphibians. Kiesecker (2002) and Budischak et al. (2008) have shown that commercial grade of insecticides namely, malathion and esfenvalerate can induce limb deformaties and immune dysfunction in Ranid tadpoles by elevating the level of trematode parasites infection. Besides, the lethal effects of chemical contaminants, they also have various sub-lethal toxicities in amphibian population and disease dynamics. A synergistic effect of chemical contaminants and parasites is reported to alter the time needed to metamorphosis, smaller body size of the metamorphs in numerous amphibian species (Rohr et al., 2004; Raffel et al., 2010). Smaller body size is imperative from survival point of view, as this may lower fitness of the

individuals by reducing survival and fecundity during terrestrial life (Semlitsch et al., 1988; Berven, 1990; Rohr and Palmer, 2013).

Nitrogen is a ubiquitous element naturally occurring in both ground water as well as in surface water. It is one of the most important nutrient elements required for the normal growth and survival of all living beings. The sources of nitrogen are varied and numerous. Among the various sources of nitrogen pollution, widespread use of nitrogen based fertilizers in the crop field is of major concern for environmental ecologists around the globe (Chen et al., 2005). The increasing demand for the production of large-scale agricultural crops and consequently extensive application of fertilizers in the crop field made the scenario even worse thereby polluting the aquatic ecosystem to many folds greater than before (Vitousek et al., 1997). In aquatic habitats, the most common form of nitrogen is ammonium cation ($NH4^+$), nitrite anion (NO_2^-) and nitrate anion (NO_3^-); found in aquatic bodies naturally or enter via different anthropogenic activities (Gleick, 1993). Biodegradation of organic product leads to the formation of such nitrogen compounds or they may be present due to atmospheric deposition, surface runoffs, ground water leaching and nitrogen fixation by certain cyanobacteria (Wetzel, 2001; Rabalais, 2002). In the past few decades, agricultural input of nitrate has increased manifolds both in developed and developing nations like India (Rouse et al., 1999; Scott & Crunkilton, 2000).

As per the EPA guidelines, the maximum drinking water limit of nitrate in public water supply was 10mg/L for nitrate-nitrogen and 1mg/L for nitrite-nitrogen (USEPA, 2018). The nitrate concentrations in ground water were documented as high as 100mg/L and in surface waters 25mg/L (Steinheimer et

al., 1998). The widespread fluctuations in the concentrations of nitrate and nitrogen-based compounds in aquatic systems were mainly due to seasonal input of these compounds through various anthropogenic activities and occasional inflow due to surface run-off from nearby agricultural practices or domestic and industrial effluents deposition from urban sources (Goolsby et al., 1991). In some extreme cases, nitrogen pollution may lead to the eutrophication which in turn results in the loss of biodiversity and promotes few aquatic organisms to thrive and flourish. Various scientific literatures also documented the adverse effects of nitrogen pollution among the animal kingdom, ranging from fishes (Westin, 1974), molluscs (Soucek, 2012), amphibians (Hecnar, 1995; Marco et al., 1999, 2001) and even daphnia (Scott & Crunkilton, 2000).

Effects of nitrogenous compound on the neural functions and cholinesterase activities of anuran amphibians were less documented; however very few literatures have demonstrated the effects nitrogenous pollution mainly the nitrate contaminations on other organisms. In a study conducted by Fergani and Arab (2017) on the native fish species Barbus setivimensis, demonstrated inhibited cholinesterase activity in the agricultural and urban runoff sites as compared to the non-polluted sites.

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.***
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 *Polypedates maculatus* (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: *Polypedates maculatus* (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).

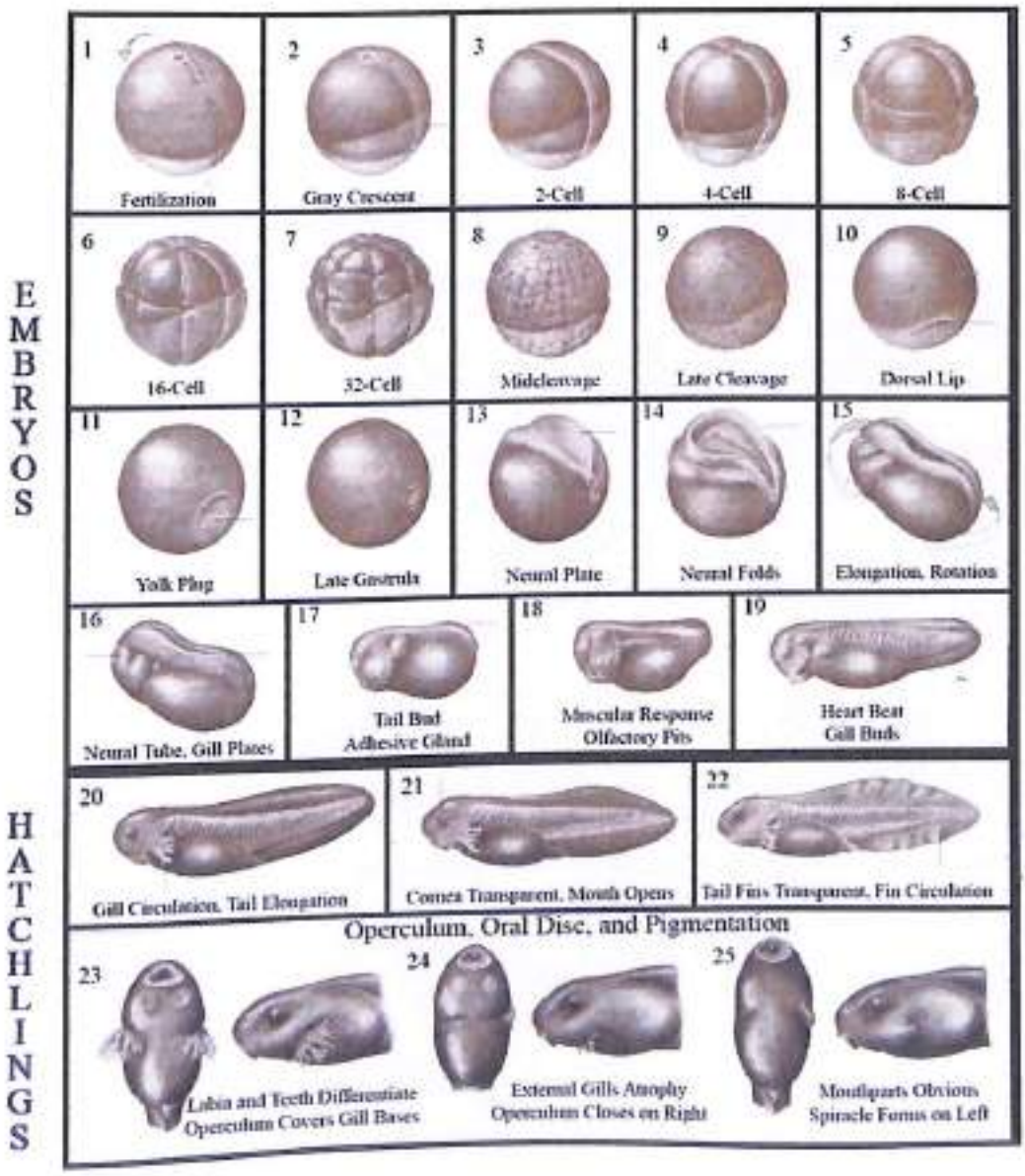

Figure 3: Gosner's reference chart of amphibian development (Part I)

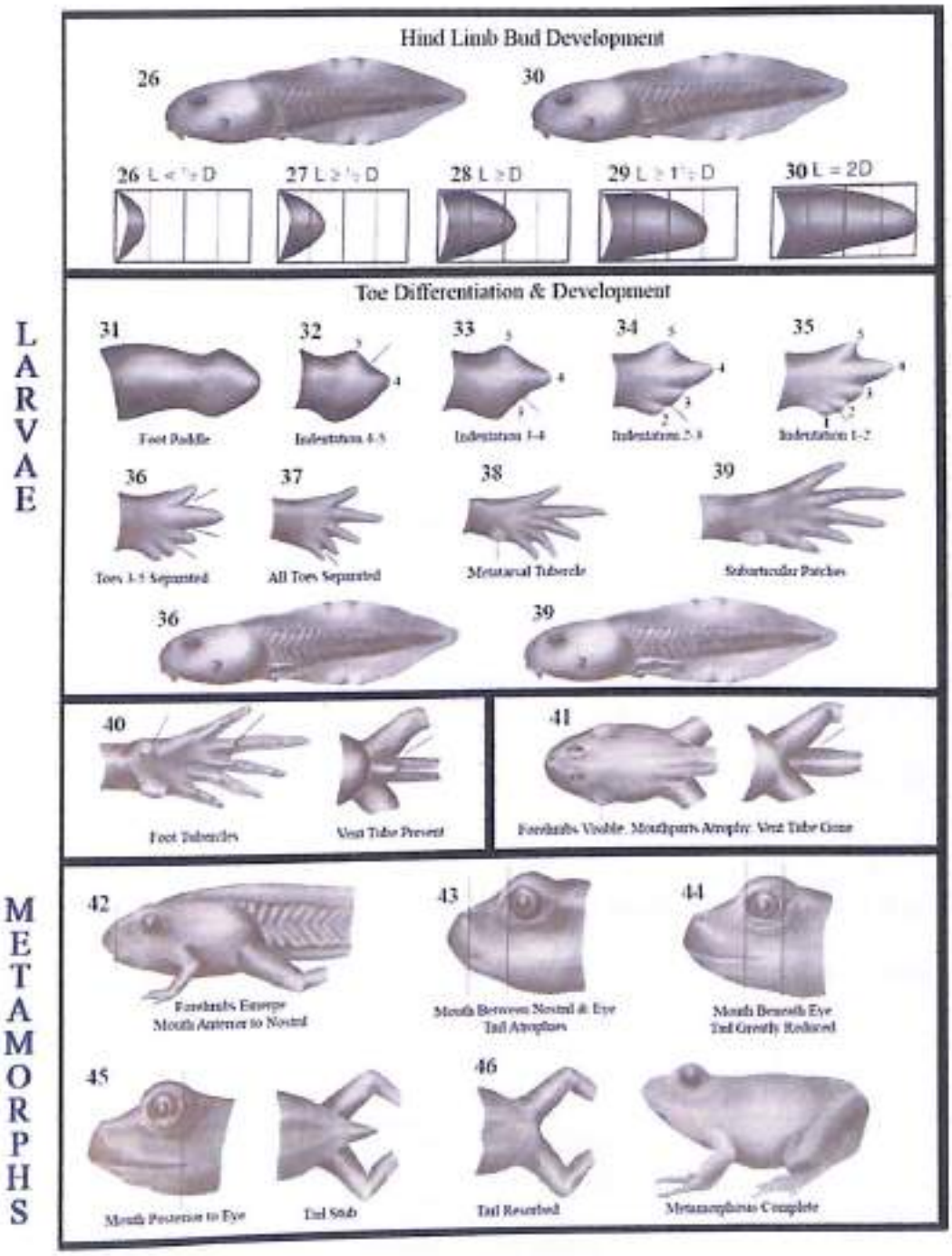

Figure 4: Gosner's reference chart of amphibian development (Part II)

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 minimal disturbance to the

remaining tadpoles. The water in the tub was changed in alternate days (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 5).

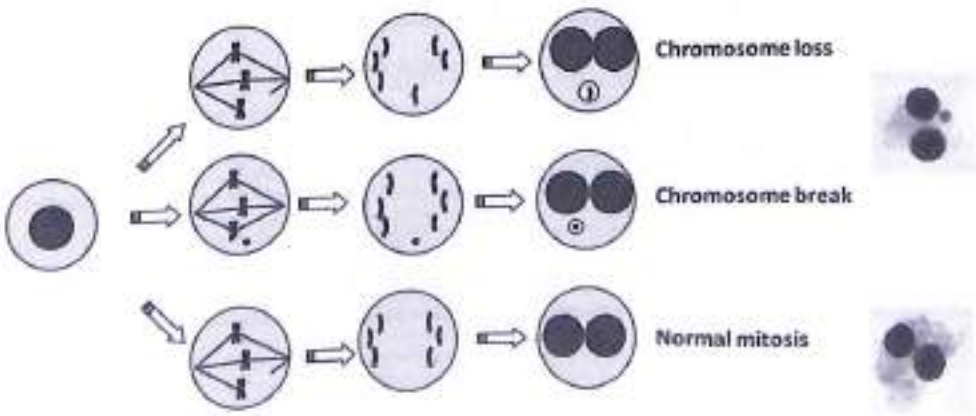

Figure 5: Description of micronucleus formation (Andreassi et al., 2007)

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 *P. maculatus*, 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).

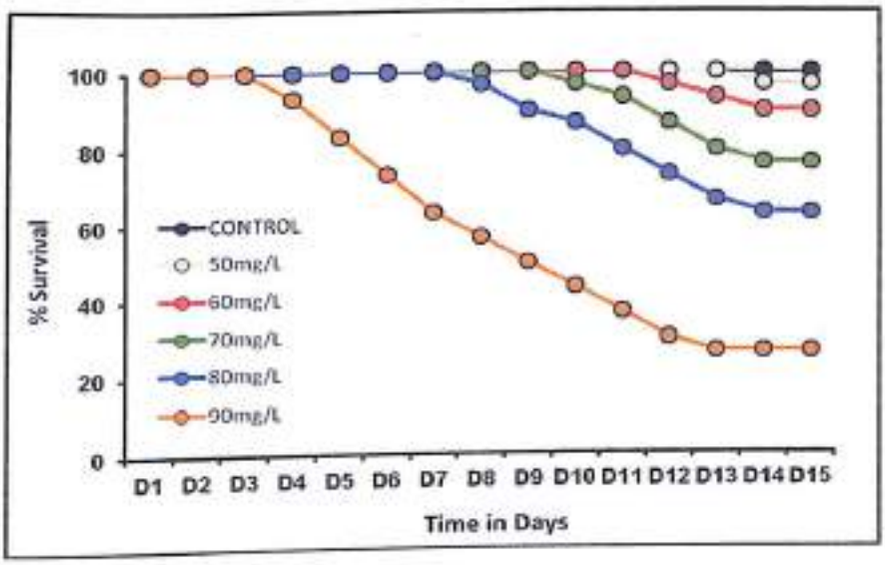

Figure 6: Graph showing survival of *P.maculatus* tadpoles exposed to different concentrations of sodium nitrate (mg/L). n=30

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 ranges between 80 and 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 80 and 90mg/L of sodium nitrate (Figure 7).

Sodium nitrate exposure in the concentration range between 50-80 mg/L did not significantly affect the body weight at metamorphosis as compared to the control group except the highest dose of concentration i.e. 90mg/L (Figure 8).

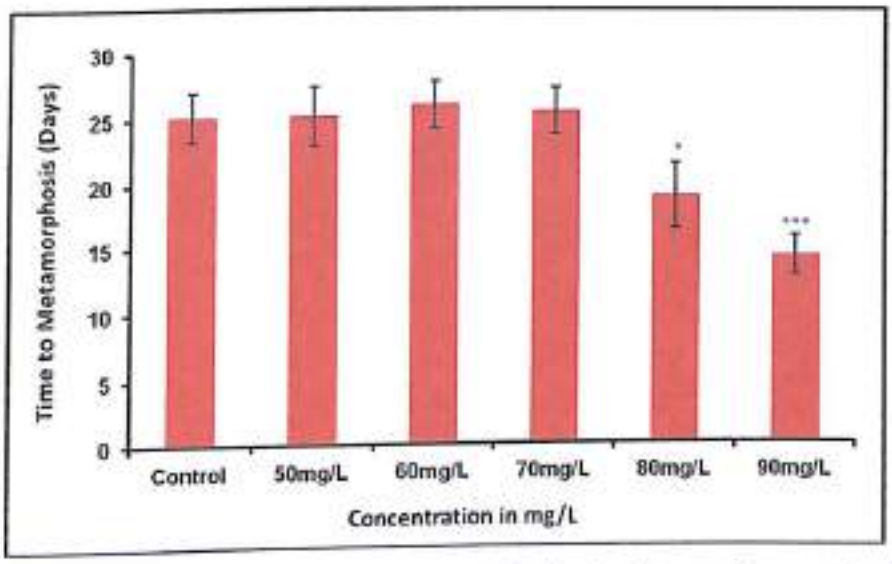

Figure 7: Change in time to metamorphosis in *P. maculatus* tadpoles following exposure to different concentrations of sodium nitrate; (n = 30). Values significantly different from the control at $p < 0.05$ (*) and $p < 0.001$ (***).

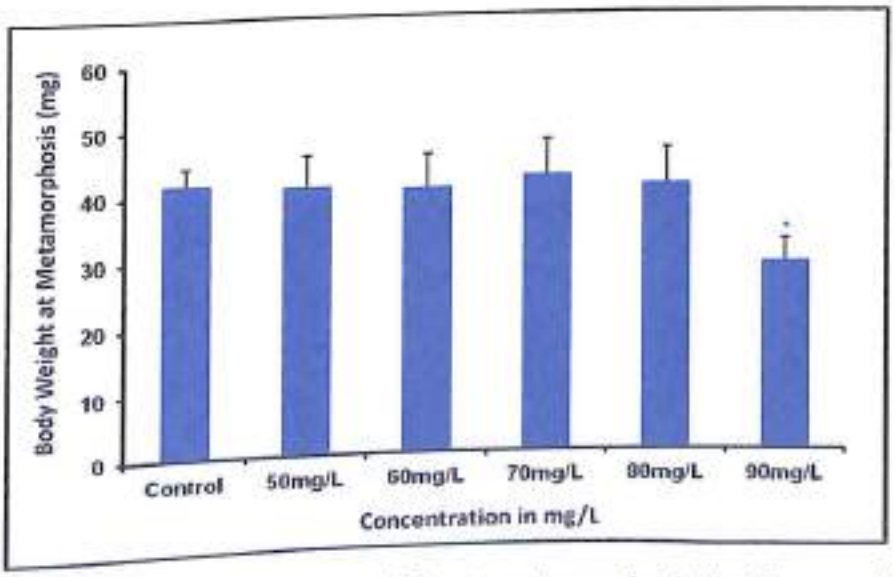

Figure 8: Change in body weight at metamorphosis in *P. maculatus* tadpoles following exposure to different concentrations of sodium nitrate; (n = 30). Values significantly different from the control at $p < 0.05$.

On the other hand, the snout to vent length of the froglets was found to be small at the highest dose of Sodium nitrate concentration which is 90mg/L and determined to be highly statistically significant ($p<0.001$). (Figure 9).

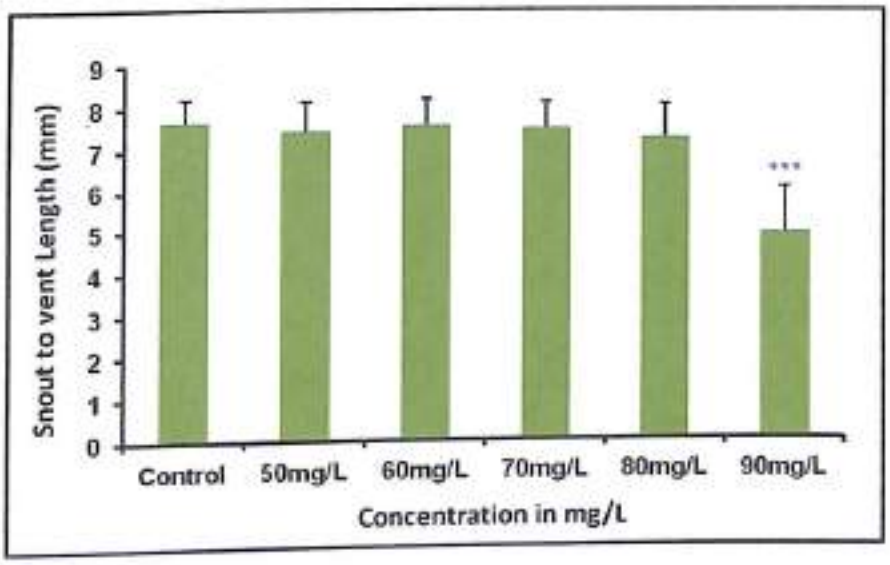

Figure 9: Change in snout-vent length in *P. maculatus* froglets following exposure to different concentrations of sodium nitrate; (n = 30). Values significantly different from the control at $p<0.001$ (***).

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

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

Dose	Exposure Time	
	72H	96H
Control	0.00 ± 0.00	0.00 ± 0.00
N50	0.10 ± 0.08	0.20 ± 0.10
N60	0.13 ± 0.05	0.14 ± 0.06
N70	0.32 ± 0.02	0.34 ± 0.05
N80	0.33 ± 0.11	0.35 ± 0.09
N90	0.29 ± 0.13	0.32 ± 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)

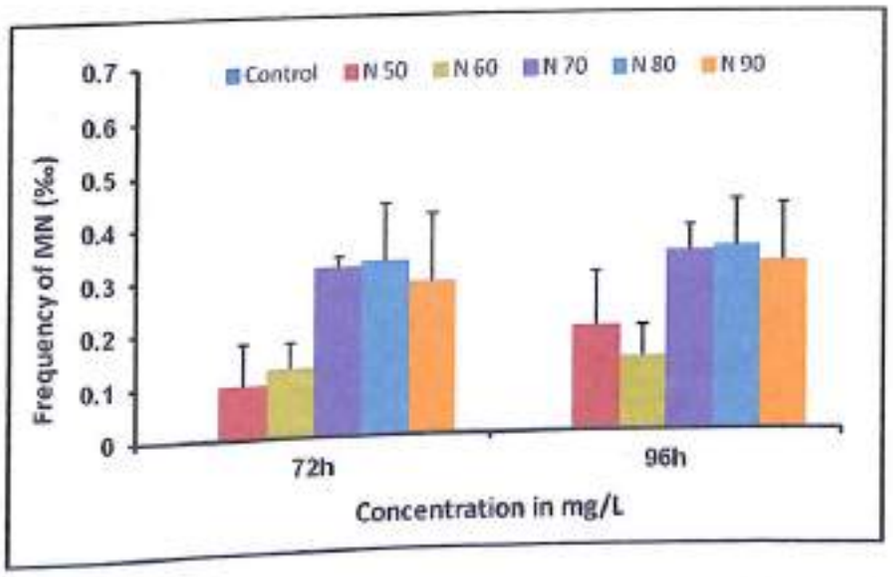

Figure 10: Histogram showing micronucleus frequency of *P. maculatus* tadpoles exposed to different concentrations of sodium nitrate at 72h and 96h fixation time respectively. (N=Sodium nitrate).

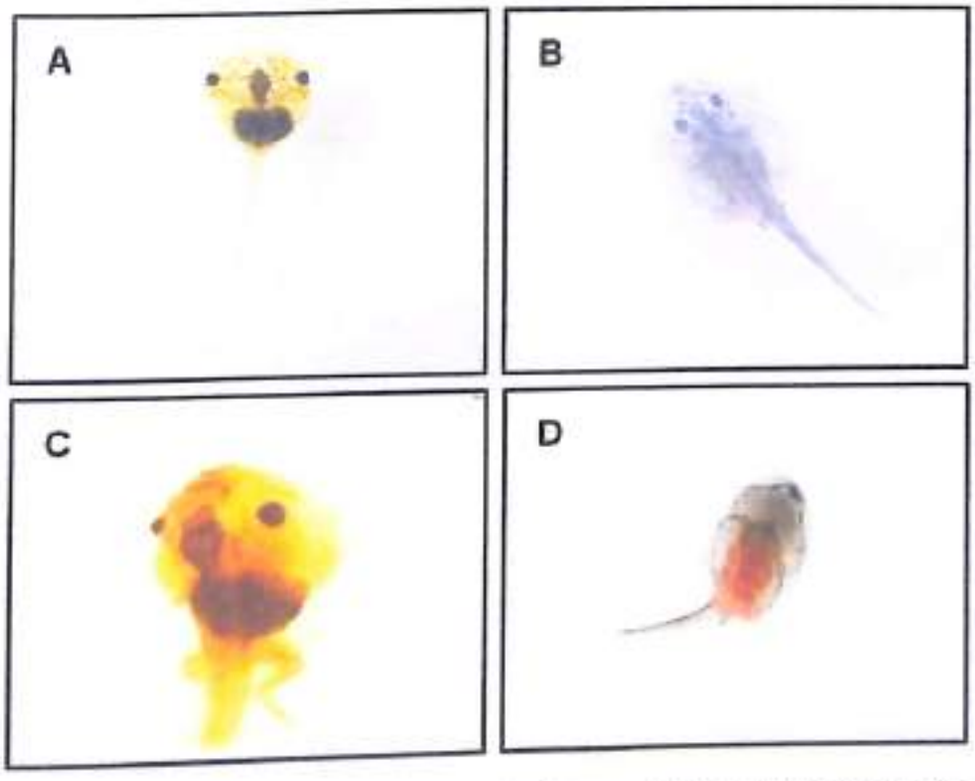

Figure 11: Photographs of types of developmental deformities following exposure of Sodium nitrate to *P. maculatus*. A: Normal; B-C: Edema formation; D: Blood clotting.

DISCUSSIONS

In the present study, possible deleterious effects of the contaminants like sodium nitrate was analyzed in the species of anuran amphibians namely *P. maculatus* 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 *P.maculatus* 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 higher concentrations accelerated the process of metamorphois in *P. maculatus* (Figure 7). The effects however were more prominent in the concentration of 90mg/L of sodium nitrate exposure. The observations are 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).

The pre-term metamorphosis of amphibian tadpoles under conditions of desiccation is the finest example of developmental plasticity. The tadpoles adopt such a strategy to survive against a rapidly drying habitat, which otherwise will lead to the death of all of the tadpoles (Richter-Boix et al., 2011; Morey and Reznick, 2004).

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 scares. In the published literature, reports on genotoxicity of toxicants can be found intermittently (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 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.

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 is having very less or no potential to cause genetic toxicities in that experimental species concerned.

CONCLUSION

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.

In recent years, the amphibian population is declining at an 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 nitrate were evaluated in the tadpoles of *P. maculatus*. The endpoints included lethal and sub-lethal toxicity, changes in life history traits, Genotoxicity etc.

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.

In nature, toxicants do not act in isolation. The toxicant could interact with various inorganic and biotic factors including other toxicants, thus modifying toxicity response.

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 *P. maculatus*

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 nitrate on different species of amphibians and other aquatic organisms suggested.

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