ISSN: 0976-3031

*Available Online at http://www.recentscientific.com*

**CODEN: IJRSFP (USA)**

*International Journal of Recent Scientific Research*
*Vol. 9, Issue, 11(A), pp. 29451-29456, November, 2018*

**International Journal of Recent Scientific Research**

**DOI: 10.24327/IJRSR**

**Research Article**

# BIOCHEMICAL CHANGES IN THE TISSUES OF FROG *HOPLOBATRACHUS TIGERINUS* EXPOSED TO SUB-LETHAL CONCENTRATION OF IMIDACLOPRID

**Padmaja B*[1]., Ch Vivek[2]., K Usha Rani[1] and K Veeraiah[2]**

[1]Department of Zoology, D.N.R College, Bhimavaram, West Godavari, A.P, India
[2]Department of Zoology, Acharya Nagarjuna University, Guntur, A.P, India

**DOI: http://dx.doi.org/10.24327/ijrsr.2018.0911.2865**

**ARTICLE INFO**

***Article History:***
Received 15th August, 2018
Received in revised form 7th September, 2018
Accepted 13th October, 2018
Published online 28th November, 2018

***Key Words:***

Insecticide, Acute and chronic toxicity, Amphibians, Risk assessment.

**ABSTRACT**

Pesticides penetrate into aquatic ecosystem by several ways viz., drift, wash off and drain from agro-ecosystem or by conscious application in the water. *Hoplobatrachus tigerinus* live in freshwater and thus continuously get exposed to the contamination by contact, respiration and by contaminated food intake. We therefore hypothesized that these sub-lethal yet environmentally significant concentrations of imidacloprid has toxicological implications on *Hoplobatrachus tigerinus*. The validation of the study was to assess glycogen and proteins analysis in vital organs of *Hoplobatrachus tigerinus* exposed to Imidacloprid, after 24, 48, 96 h, 8, 15 and 30 days of exposure. The results obviously exemplify that exposure to sub-lethal concentration of imidacloprid ($1/10^{th}$ of 96 h $LD_{50}$) was reflected in tissue concentrations of pesticide with significant alterations in the biochemical parameters glycogen and proteins. The total glycogen levels decreased on exposure to sub-lethal concentration of Imidacloprid. Among the various test tissues, higher glycogen content was observed in liver. This is due to the involvement of liver in glycogen synthesis and utilization. Maximum decrease of proteins was noticed in liver and muscle. The minimum decrease was almost equal in brain and kidney tissues. The depletion in the protein may be due to metabolic utilization of the ketoacids in gluconeogenesis pathway for the synthesis of glucose or may be due to directing the synthesis of proteins from free aminoacids. These results are in agreement with several studies reported in the literature. Risk assessment of pesticides in aquatic ecosystem achievable by the estimation of acute and chronic toxicity.



## INTRODUCTION

The increasing use of pesticides for the control of pests in agriculture including commercial and household production of vegetables causes potential health hazards to live stock, especially to fish, frogs, birds and mammals. As a consequence, the structure and functions of communities, in an eco-system and populations are altered. The reproductive potential, growth and other physiological activities, like behavior, hormonal regulation and protuctions, thermo-regulation etc., will be changed. The present study intended to help fill the gap on the scarcity of information concerning the Imidacloprid toxicity and health hazards to frogs. Imidacloprid is a relatively new, systemic chloro-nicotinyl insecticide (Caroline Cox, 2001). It is used as a crop and structural pest insecticide, a seed treatment and a flea control treatment chemical. Primarily used for the control of sucking insects including rice hoppers, aphids, thrips, white flies, termites, soil insects and some beetles. The selected pesticide most habitually used on rice, cereal, maize, potatoes, vegetables, sugarbeet, fruit, cotton etc. On February 18, 1985, Bayer chemists synthesized this new compound for the first time. It was first introduced for the agricultural use in Europe and Asia in 1992 (Spain, Japan) and in USA in 1994. Depletion in the food reserves was observed in the amphibian species exposed to these toxicants. Among the various test tissues, higher glycogen content was observed in liver. This is due to the involvement of liver in glycogen synthesis and utilization. Glycogen and proteins are major storage form of energy in animals and stored mainly in liver and muscle. Hence, the concentrations of NH3, NO2 have some and NO3 in water need to be monitored in water quality in aquaculture practices. Interestingly, the amphibians are the largest and smallest known genomes of all tetrapods. This frog serves as a suitable bioindicator of environmental pollution since it meets all the criteria. The main aim was to examine the effects of $1/10^{th}$ of 96 h $LD_{50}$ sub-lethal concentration of Imidacloprid on the different tissues of *Hoplobatrachus tigerinus*, thus enriching

**Corresponding author:* **Padmaja B**
Department of Zoology, D.N.R College, Bhimavaram, West Godavari, A.P, India

our knowledge about the impact of pesticide on the energy metabolism of the Indian bull frog.

## MATERIALS AND METHODS

### *Collection of test organism*

The wild fresh water Indus Valley bull frog or Indian bull frog *Hoplobatrachus tigerinus* of both sex were collected by hand net from their spawning ponds in unpolluted and non-agricultural sites of Bhimavaram, West Godavari district Andhra Pradesh, India. The frogs were transported to the laboratory in covered baskets and acclimatized to the laboratory conditions for a period of 7 days. Adult frogs of the same size and almost same weight (35.87± 0.04 g) were acclimatized in glass tanks ($51\times32\times33cm^3$**)** containing two liters of dechlorinated tap water for seven days prior to the experiment (Vogiatzis and Loumbourdis, 1997). Tanks were placed on a slant to provide the option of both aqueous and dry environment. Water was changed for every two days and the tank was cleaned thoroughly. Frogs were fed with earth worms twice in a week. Uneaten earth worms and faecal wastes were removed and water replenished regularly**.** In any batch during acclimatization, if 5% mortality observed, the total batch was discarded.

### *Preparation of imidacloprid (Tatamida 17.8% SL):*

Imidacloprid, a soluble pesticide was dissolved in acetone without any agitation immediately prior to use. Doses of Imidacloprid were prepared and incubated into the experimental animals according to the design of the experiment.

### *Selection of sub-lethal concentrations*

The lethal concentrations ensure death even before noticing the behavioral abnormalities. Anderson and Peterson (1969) reported that sub-lethal exposures to longer periods may be more dangerous than lethal concentrations to the organisms. Even when the animal is exposed to low doses continuously, many behavioral abnormalities and physiological alterations will occur.

In the present study, $1/10^{th}$ of 96 h $LD_{50}$ value was taken as sub-lethal concentration to study the behavioral alterations and physiological alterations (As per the recommendations of committee on toxicity studies - Anon, 1975). The data on the mortality rate of frogs were recorded. The dead frogs were removed immediately. The toxicity tests were conducted to choose the mortality range from 10% to 90% for 24, 48, 72 and 96 h. Finney's probit analysis (Finney, 1971) as recorded by Roberts and Boyce (1972) was followed to calculate the $LD_{50}$ values. The respective probit values were taken from Table IX of Fisher and Yates. For the determination of the 95% confidence limits, $LD_{50}$ values and a normal variant of 1.96 were taken into consideration.

### *Route of Administration*

Imidacloprid was given orally to all the experimental animals. At sub-lethal doses after every test period of 24 h, the pesticide was administered orally with the help of a syringe fitted with a 16 gauze oral blunt feeding needle. The oral feeding needle was placed into the mouth and passed back into the stomach; this is called oral intubation. Control animals of were treated with distilled water without giving pesticide.

### *Biochemical Changes*

The present study, the levels of glycogen and proteins in the five per cent homogenates of brain, muscle, and 2% homogenates of liver and kidney tissues of the frog *Hoplobatrachus tigerinus* were measured.

### *Estimation of glycogen*

The glycogen was estimated by the method of Kemp *et al.,* (1954). Five per cent homogenates of brain, muscle, and 2% homogenates of liver and kidney tissues were prepared in 80% methanol and centrifused at 3000 rpm for 10 minutes. The tissue residue was suspended in 5 ml of trichloroacetic acid (TCA) and boiled for 15 minutes at $100^0$C and then cooled in running water. The solution was made up to 5 ml with TCA to compensate for evaporation and then centrifused. From this, 2 ml of supernatant was taken into the test tube and 6 ml conc. $H_2SO_4$ was added and the mixture was boiled for 10 minutes. The mixture was cooled and the optical density was measured at 520 nm. The standard graph was plotted with D-glucose (Analar supplied by B.D.H. Bombay) by the foresaid method. The glucose obtained was converted to glycogen by the multiplication factor 0.98 (Hawks, 1951) and is expressed as mg of glycogen/gm wet weight of the tissue.

### *Estimation of Total Protein content*

Total protein content was estimated by the modified method of Lowry *et al.,* (1951). Five per cent homogenates of muscle and brain and 2% homogenates of liver and kidney were prepared in 5% trichloroacetic acid and centrifused at 3000 rpm for 10 minutes. The supernatant was discarded. The suspended protein residue was dissolved in 1 ml of 1N NaOH, 0.2 ml of the extract was taken into the test tube and 5 ml of alkaline copper solution (50 ml of 2% $NaCO_3$ and 1ml of 0.5% $CuSO_4$. $5H_2O$ in 1% sodium potassium tartrate) was added. The contents were mixed well and allowed to stand for 10 minutes. To this, 0.5 ml of 50% diluted folin phenol reagent (diluted with distilled water 1:1 ratio) was added. After 30 minutes, the optical density was measured at 540 nm in a spectrophotometer against a blank. The standard graph was plotted by the method of Lowry *et al.,* (1951) with bovine serum albumin supplied by Sigma chemical Company, U.S.A. The values were expressed as mg/g wet weight of the tissue.

Analysis of variance (ANOVA) with repeated measures and Scheffe and Dunnetts comparison test were used to compare the means. Differences were deemed statistically significant at $p<0.05$. Statistical analysis were carried out with SPSS 16.0 for windows.

## RESULTS AND DISCUSSION

### *Biochemical Changes*

### *Total Glycogen*

The results of the present study along with calculated values for glycogen in control and exposed frog along with per cent change over control, Error bars with standard error and standard deviations were graphically represented in figure. 1 and 2. respectively, for 24, 48, 96 h, 8, 15 and 30 days. The total glycogen levels of brain, liver, kidney and muscle were

more or less stable in control frog during the 30 days cycle of the experiment. The total glycogen levels decreased on exposure to sub-lethal concentration of Imidacloprid. They also showed a tendency of decrease with the increase in exposure of time. The maximum level of total glycogen was found in liver and minimum in Brain.

In liver of control frog the glycogen content was more and then was followed by muscle, kidney and brain. Among the various test tissues, higher glycogen content was observed in liver. This is due to the involvement of liver in glycogen synthesis and utilization. Glycogen is the major storage form of energy in animals and stored mainly in liver and muscle. These results are in concurrence with several studies reported in the literature. The muscle glycogen cannot contribute directly to the blood glucose as the enzyme glucose-6-phosphatase is missing from muscle, but can do so indirectly when lactate is formed during anaerobic contractions.

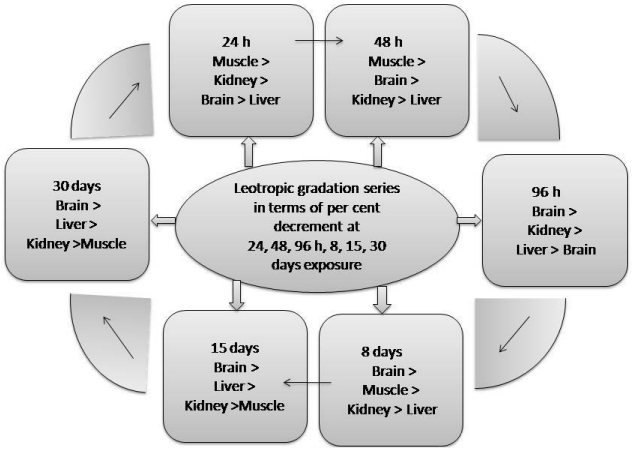


**Fig.1.Changes in Glycogen content (mg/g wet weight) in test tissue of frog, *Hoplobatrachus tigerinus***

In the present study it was observed that sub-lethal exposures were showing more effect on muscle tissue glycogen and it was followed by Kidney, brain and liver for 24 h. On sub-lethal exposure to Imidacloprid the total glycogen level was found to decrease in all the tissues of *Hoplobatrachus tigerinus*. The glycogen content, after exposure to sub-lethal dose in frog *Hoplobatrachus tigerinus* at 96 h, 8 days was found to decrease highest in brain, liver and lowest in muscle. The depletion of glycogen may be due to utilization of stored carbohydrates in liver for energy production as a result of pesticide-induced hypoxia. The results indicated that the liver, a vital organ of carbohydrate metabolism, was drastically affected by Imidacloprid. The glycogen content in liver of the exposed frog for sub-lethal dose was reduced. Frog liver is a primary organ for detoxification (Kabeer *et al*., 1979). Hence, it might be due to the presence of the toxicant in the liver, through hepatic portal system in abundance for detoxification and disposal. The impairment in the glycogen content of liver has also influenced the glycogen content in the brain. In almost all the tissues of the organs i.e. brain, liver, muscle and kidney tested at sub-lethal doses of Imidacloprid a decrease in glycogen values was noticed during the exposure periods 24, 48, 96 h, 8, 15 and 30 days.

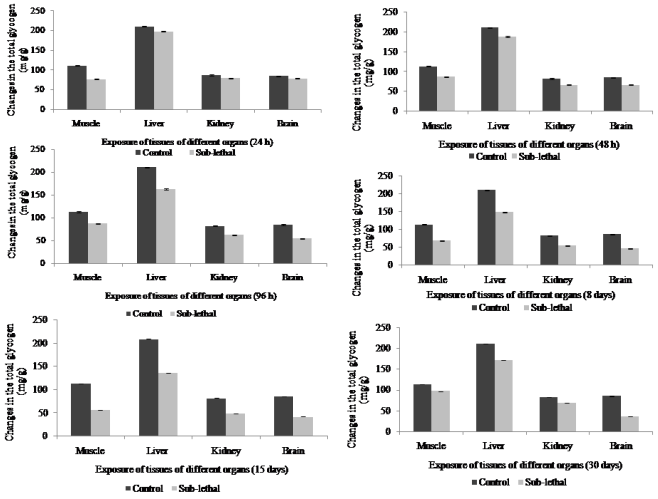


**Fig 2** Changes in Glycogen content (mg/g wet weight) in test tissue of Frog, *Hoplobatrachus tigerinus* exposed to sub-lethal dose of Imidacloprid for 24h, 48 h, 96 h, 8 days, 15 days and 30 days.

There was a gradual decline in the content of liver glycogen observed in the experimental fish, *Labeo calbasu* exposed to aflatoxin (Amjad fatmi and Ruby, 2011). Lakshmanan *et al.* (2013) reported that the total glycogen level of brain, liver, muscle, gill and kidney of *Labeo rohita* were decreased on exposure to sub-lethal concentration of cypermethrin. Gijare *et al*. (2011) reported a notable alteration in liver and intestine glycogen of *Ophiocephalus punctatus* exposed to sub-lethal concentration of cypermethrin. Israel Stalin and Sam Manohar Das (2012) reported glycogen utilization was maximum in the liver tissue under exposure to lebaycid (fenthion). Binukumari and Vasanthi (2014) reported that the carbohydrate level was decreased. Liver glycogen content decreased progressively during exposure period, this might be due to toxic stress (Ganeshwade, 2011).

David *et al.* (2005) stated that there was a decrease in the level of glycogen content in all tissues and the depletion in glycogenolysis either through hormonal imbalance or the other influencing factor, results primarily in the depletion of carbohydrate energy reserves of *Labeo rohita* under exposure to acute concentration of fenvalerate. Rawat *et al.* (2002) observed that the glycogen level in the fish was decreased continuously with the increase in concentration of endosulfan. Sobha Rani *et al.* (2000) observed a significant depletion in glucose and glycogen levels in various tissues of freshwater teleost *Tilapia mossambica* under sub-lethal concentration of sodium arsenate and stated that these changes were tissue specific and time dependent. Hashem *et al*. (1993) reported that the inhibition of amylase activity would in turn reduce glucose level through decreasing the hydrolytic rate of glycogen.

The reduction of carbohydrates suggests the possibility of active glycogenolysis and glycolytic pathway to provide excess energy in stress condition (Remia *et al*., 2008). Saravanan *et al.* (2010) reported that the neem leaf extract of biopesticide, *Azadiracta indica* caused significant alteration in glycogen and protein content of liver and muscle of freshwater fish, *Labeo rohita.* Anita Susan (2010) observed that the liver, a vital organ of carbohydrate metabolism was drastically affected by fenvalerate in *C.mrigala* and *Labeo rohita*. She also stated that a highly significant decrease in glycogen content was noticed

in sub-lethal concentrations of technical grade fenvalerate in most of the tissues in both the experimental fish. The decrease in glycogen content was higher in sub-lethal concentrations than in lethal concentrations. Anthony Reddy, *et al.* (2015) observed that the liver a vital organ of carbohydrate metabolism was drastically affected by confidor. Frog liver is the primary organ for detoxification. Hence it might be due to this stress, the glycogen levels were decreased more in liver of frog, *Hoplobatrachus tigerinus*. The reduction in glycogen of the liver cells after prolonged exposure in higher concentrations of pesticides became more pronounced and the glycogen content displayed faint stainability and became hard to detect (Sridhar and Joice, 2012).

Megahed *et al.* (2013) observed a significant decrease in glucose content in *Spodoptera littoralis* (Boisd.) exposed to aemamectin, benzoate and abamectin throughout all the tested periods. Muthukumaravel *et al.* (2013) observed that there was a decrease of carbohydrate content in all tissues of *Labeo rohita* exposed to monocrotophos. He also stated that in fish, generally the carbohydrate reserves may be rapidly utilized under unfavourable conditions and the great variations found in the tissues indicate that the level of mobilizable carbohydrate reserves may fluctuate widely and rapidly in response to fluctuations in the nutritional state of the animal. In the present study, it was observed that exposure to sub-lethal dose of Imidacloprid, in the frog, *Hoplobatrachus tigerinus* caused moderately changes in the total glycogen level which is due to toxic stress, resulting in the disruption of enzymes associated with carbohydrate metabolism.

***Total Proteins***

The calculated values for total proteins and per cent change over control along with standard deviation and error bars with standard error were graphically represented in figure I.2 for 24, 48, 96 h, 8, 15 and 30 days. In control frog, the total proteins levels of brain, liver, kidney and muscle of control frog were more or less stable during the 30 days cycle of the experiment. The maximum level of total proteins was found in liver and minimum in brain. The variation in distribution suggests the gradual difference in metabolic calibers of various tissues. The present trend in the tissue is justifiable in the wake of mechanical tissue of muscle intended for mobility and it does not participate in metabolism. Liver is the seat for the synthesis of various proteins, and also the regulating center of metabolism.

Under exposure to sub-lethal dose of Imidacloprid the total protein was found to decrease in all the tissues at 24, 48, 96 h, 8, 15 and 30 days. Maximum decrease was noticed in liver and muscle. The minimum decrease was almost equal in brain and kidney tissues. The per cent changes over controls at six test periods were in the order of

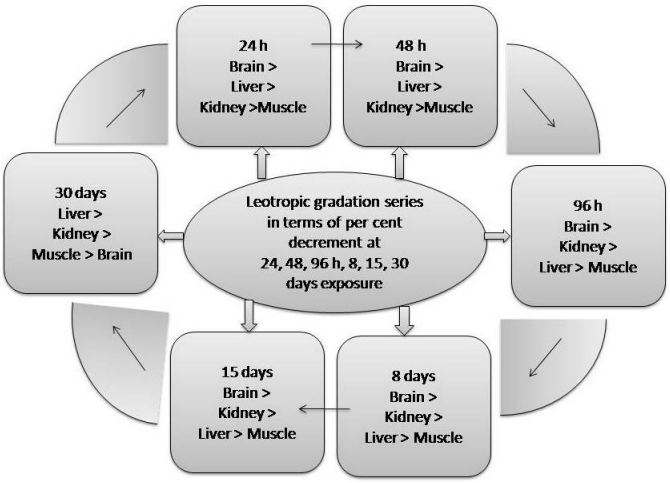


**Fig.3.Changes in Protein content (mg/gram wet weight of the tissue) in different tissue of the frog, *Hoplobatrachus tigerinus*.**

In the present study liver and muscle tissue of the frog *Hoplobatrachus tigerinus* evidenced a significant per cent change in the protein content under sub-lethal dose of Imidacloprid. Changes in brain, liver, kidney, muscle and brain in the frog, *Hoplobatrachus tigerinus* suggests that they were moderately affected under sub-lethal dose of Imidacloprid toxicity. The decreased trend of the protein content as observed in the present study in most of the tissues is due to metabolic utilization of the ketoacids in gluconeogenesis pathway for the synthesis of glucose or due to directing free amino acid for the synthesis of necessary proteins or for the maintenance of osmotic and ionic regulation. Rafat, (1986) stated that the fish is an important source of food for human nutrition. Frog proteins are well balanced with essential amino acids and are incomparable to other proteins of animal origin. The liver is the prime location for detoxifying pesticides in frog. The protein, one of the main sources of energy for the frog, helps in body tissue building, Muscle glycogen and protein response appear particularly suitable for measuring stressful level of pollutants and have been used as indicators of stress in frog.

Significant differences were observed in protein metabolism of *Labeo rohita* exposed to Dimethoate 30% EC concentration of 0.398 ppm for 24, 48 and 72 h respectively Binukumari and Vasanthi (2014). Sudhasaravanan and Binkumari, (2015) observed maximum decrease of glycogen in freshwater fish, *Lepidocephalichthyes thermalis,* kidney during 30 days exposure and minimum decrease in muscles during 10 days exposure to sub-lethal concentration of (4mg/L) detergent rin. Aruna and Sudha Singh (2002) observed the decrease in protein content which suggested an increase in proteolytic activity and possible utilization of its products for metabolic purpose. The fall in protein level during toxicant exposure might be due to increased catabolism and decreased anabolism of proteins. Satyanarayan (2005) described that the physiological status of animal was usually indicated by the metabolic status of protein. The depletion of protein fraction in liver, brain and kidney might have been due to their degradation and possible utilization for metabolic purposes under pesticidal stress.

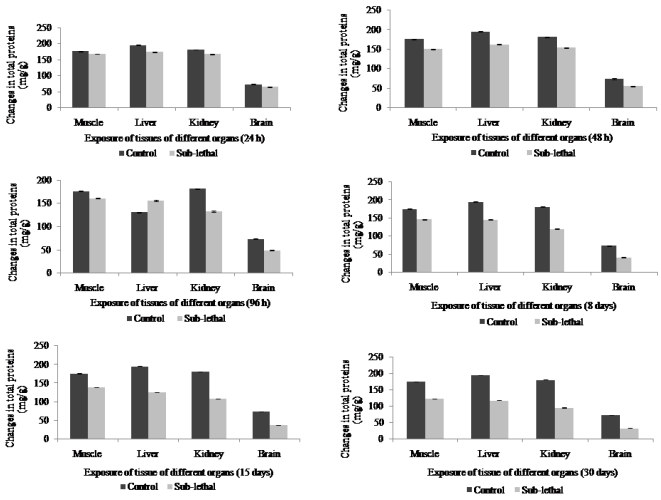


**Fig 4** Changes in Protein content (mg/gram wet weight of the tissue) in different tissue of the frog, *Hoplobatrachus tigerinus* on exposure to sub-lethal dose of Imidacloprid for 24 h.

The protein content was decreased after *Labeo rohita* exposed to cypermethrin (Veeraiah and Durgaprasad, 1996). Rao (2006) reported that the levels of protein decreased significantly in liver, kidney and muscle *Catla catla* treated with endosulfan. Aruna Khare *et al.* (2000) observed a significant increase in total protein content in kidney of exposed fish *Clarias batrachus* during the first week and thereafter a gradual decrease in protein content was observed in the later periods of exposure to sub-lethal concentrations of Malathion. The sub-lethal concentrations of fenvalerate depleted the protein content of *L. thermalis* (Jeba Kumar *et al*., 1990). The level of protein decreased significantly in liver, kidney and muscle of *Catla catla* treated with Endosulfan (Rao, 2005).

Riaz Hussain *et al.* (2009) observed the decreased the total protein contents in the adults *(Tribolium castaneum)* of both the strains exposed to the lower and higher sub-lethal doses of biopesticide. Saravanan *et al*. (2010) observed that leaf extract of *Azadirachta indica* caused significant alterations in glycogen and protein content of liver and muscles of fish *Labeo rohita.* Anita Susan *et al.* (2010) reported that there was a significant decrease of total proteins in all tissues of *L. rohita* and *C. mrigala*. Changes in brain, gill and kidney in both the fish were relatively less affected than hepatic tissue under fenvalerate toxicity. Total proteins are major biochemical components necessary for an organism to develop, grow and perform its vital activities. The reduction of protein content might be due to inhibition of DNA and RNA synthesis (Elbarky *et al*., 2008). Muthukumaravel *et al.* (2013) reported that the decrease in protein levels was noted in all the tissues of fish, *Labeo rohita* exposed to the monoctrotophos.

Rathod (2013) observed that the biopesticide, *Azadirachta indica* exposure to freshwater cat fish, *Heteropneustes fossilis* showed decreased trend in total protein in liver and muscle tissue. A significant decrease was observed in muscle and liver tissues due to the disturbance of biochemical and physiological activity of those organs and proteolysis in freshwater cat fish. The maximum decrease (per cent change) of proteins was observed in brain, kidney and liver during the all time periods of 24, 48, 96 h, 8, 15 and 30 days. All these investigations support the present study, of decreasing trend of proteins in the frog, *Hoplobatrachus tigerinus,* exposed to sub-lethal doses of Imidacloprid.

## SUMMARY AND CONCLUSIONS

In the present investigation, the frog, *Hoplobatrachus tigerinus* were exposed to Sub-lethal concentration ($1/10^{th}$ of 96 h static $LD_{50}$) of Imidacloprid for 24, 48, 72, 96 h and 8, 15 and 30 days. The total glycogen levels of brain, liver, kidney and muscle were more or less stable in control fish during the 30 days cycle of the experiment. The total glycogen levels decreased on exposure to sub-lethal concentration of Imidacloprid. In liver of control frog the glycogen content was more and then was followed by muscle, kidney and brain. Among the various test tissues, higher glycogen content was observed in liver. This is due to the involvement of liver in glycogen synthesis and utilization. Under exposure to sub-lethal dose of Imidacloprid the total protein was found to decrease in all the tissues at 24, 48, 96 h, 8, 15 and 30 days. Maximum decrease was noticed in liver and muscle. The minimum decrease was almost equal in brain and kidney tissues. The depletion in the protein may be due to metabolic utilization of the ketoacids in gluconeogenesis pathway for the synthesis of glucose or may be due to directing the synthesis of proteins from free aminoacids. The changes and decrease in protein level might also be due to inhibition of metabolizing enzymes by administration of toxicants.

**Conflict of interest statement**

The authors affirm that there are no conflicts of interest.

**Acknowledgements**

We would like to thank manual labor that helped during the development of the research. Our special appreciation goes to Department of statistics, ANU for their valuable contribution to the statistical analysis. We thank the Department of Zoology and Aquaculture, Acharya Nagarjuna University for their support.

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