colloid is the large glycoprotein thyroglobulin which contains thyroid hormones within its molecule. Once the secretion has entered the follicles, it must be absorbed back through the follicular epithelium into the blood before it can function in the body. The thyroid gland has a blood flow of about five times the weight of the gland per minute, and the supply of blood is fairly compared to the other area of body, with an exception to adrenal cortex.

The thyroid cells are typical protein secreting glandular cells, as illustrated. The endoplasmic reticulum and golgi apparatus synthesize and secrete into the follicle, a large glycoprotein molecule called thyroglobulin with a molecular weight of 6,70,000. Each molecule of thyroglobulin contains 140 tyrosine amino acids, and these are the major substrates that combine with iodine to form the thyroid hormones. These two hormones, they formed within the thyroglobulin molecule i.e., the thyroxin (T4) and tri-iodothyronine (T3). In addition to secreting the thyroglobulin, the glandular cells also process the iodine and provide the enzymes and other substances necessary for thyroid hormone synthesis.

**Pathology of thyroid gland under imidacloprid toxicity:**

The pathological changes observed in the *Hoplobatracus tigerinus* after administration of sublethal dose of imidacloprid for 30 days include, hyperplasia of epithelial cells, marked hypertrophy, degeneration of some of the cells, formation of blood streaks, appearance of vacuoles, shrunken epithelial cells rupture in blood vessels and necrosis.

Kamrin, *et al.* (1997) stated that the crucial steps in thyroid follicular cell carcinogenesis include, mutagenicity, perturbations in thyroid and pituitary hormones or a combination of two. Pamela *et al.* (1998) identified 24 pesticides out of 240 pesticides to
produce thyroid follicular cell tumors in rodents. The potential antithyroid sites of action are, inhibition of thyroid uptake into the thyroid, thyroid peroxidase, inhibition, damage to thyroid follicular cells, and inhibition of thyroid hormone release from the thyroid. Kackar et al. (1997) reported thyroid cell tumors and damage to follicular cells in male rats exposed to 3 pesticides namely amitrole, ethelene thiourea and mancozeb. The Australian pesticides and veterinary medicines authority in its chronic and carcinogenicity studies on Tetraconazole, noticed a fungicide in rats noticed cystic follicular hyperplasia and follicular epithelial hypertrophy in the thyroid of *Rana hexadactyla*.

All these studies are in agreement with the present observed changes in the thyroid gland to the test organism, *Hoplobatrachus tigerinus*. Thus, when the organisms are exposed to pesticides they cause irreparable architectural changes in various vital organs making them less fit for better survival. These histopathological changes can alter the various physiological activities of the organisms, such as release of various enzymes and the metabolic processes.

**IV.5.2. General Histology of Pituitary gland:**

Hypophysis is the chief center of the endocrine system. On one hand it is in direct connection with the central nervous system through the hypothalamus, while on the other almost all endocrine glands are under the regulatory influence of its trophic hormones. Thus, pituitary forms an important link between the nervous and the endocrine system and acts as a transductor maintaining their functional integration. Considering its important position in the neuroendocrine system it was also referred to as master gland;
however, this concept is completely discarded owing to the fact that it is subservient to the nervous system and even to some of the endocrine glands.

The pituitary gland in man is a small, nut-like structure situated at the base of the brain. It is in communication with the floor of third ventricle by a stalk, the hypothalamus. The body of pituitary develops from two embryonic sources: first is an ectoderm glandular diverticulum- Rathke’s pouch which is an inward invagination of the primitive buccal cavity. It meets with a similar neural down growth from the floor of the third ventricle. The connection of buccal cavity disappears during later stages of the development; however, neural connection persists throughout the life as the hypothalamus.

From the Rathke’s pouch, anterior lobe of pituitary gland develops which in turn is composed of two parts- anterior pars tubularis and posterior pars distalis. The combined structure is also referred to as adenohypophysis which is also called anterior lobe and neurohypophysis possess Pars intermedia, Pars nervosa and Infundibulum which is also called Posterior lobe.

The neural region develops into the posterior lobe including parts like median eminence continuing into infundibulum and pars nervosa. The posterior lobe is also called as neurohypophysis. Pars intermedia are one more structure associated with the neurohypophysis; however, it is derived from the Rathke’s pouch. In poikilothemrs, this part is prominently developed whereas in birds and mammals although anatomically distinguishable, physiologically it is insignificant.

Studies have revealed that pars distalis contains two kinds of acidophils and four types of basophils. Two groups of acidophils are distinct cytologically and secrete STH
and prolactin respectively. The basophils secrete FSH, LH, TSH and ACTH. Studies based on detailed cytochemical analysis and electron microscopy have revealed that each cell type is specific for the secretion of a particular hormone.

**Table IV.5.1.** Adenohypophysis secretes six different hormones with distinct physiological effects.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Symbol</th>
<th>Cell type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyrotropic or Thyroid-stimulating</td>
<td>TSH</td>
<td>Basophil cells</td>
</tr>
<tr>
<td>Adrenotropic or Adrenocorticotropic</td>
<td>ACTH</td>
<td>Basophil cells</td>
</tr>
<tr>
<td>Growth hormone or Somatotropic</td>
<td>STH</td>
<td>Basophil cells</td>
</tr>
<tr>
<td>Lactogenic, Luteotropic or Mammothropic, Prolactin</td>
<td>LTH</td>
<td>Acidophil cells</td>
</tr>
<tr>
<td>Follicle stimulating</td>
<td>FSH</td>
<td>Basophil cells</td>
</tr>
<tr>
<td>Luteinizing</td>
<td>LH</td>
<td>Basophil cells</td>
</tr>
</tbody>
</table>

**Histopathological Alterations of Pituitary gland under imidacloprid toxicity**

Histopathological investigations showed no obvious histological change in the control pituitary gland. The histological sections of control pituitary gland showed many intensely stained dark basophils and acidophil cells present in pars distalis. However, frogs exposed to imidacloprid showed different degrees of poisoning symptoms (Plate IV.1A & B). Histopathological changes were characterized by severe degeneration of dark-stained basophils and acidophils. Poisoning symptoms were concentration dependent.
Histopathological investigations have proved to be a sensitive tool to detect direct effects of chemical compounds within target organs of organism in laboratory experiments. The exposure of aquatic organisms to very low levels or sublethal concentration of pesticides in their environment may result in various histological alterations in vital tissues (Saravana, 2010). As with biochemical studies, histopathological studies on amphibians are scarce, most of the available works are on fish. Very little information exists for amphibians.

Srivastav et al. (2014) evaluated the effect of cadmium exposure on the histopathology of prolactin cells in the freshwater catfish, *Heteropneustes (H.) fossilis* and noticed degranulation, vacuolization and cytolysis on day 28 following cadmium treatment. Narayanaswamy (2003) investigated on the Hypophysis of fish *Glossogobius giuris* during spawning phase, on the treatment with sublethal concentrations of Malathion (0.05, 0.25 and 0.5 ppm) for 24, 48, 72 and 96 hrs intervals and recorded degranulation and hypertrophy of cells and nuclei, appearance of extensive intercellular spaces in gonadotrophs (GTH) and prolatin secreting (PRL) cells of hypophysis.

The results of this study indicated that exposure of *Hoplobatrachus tigerinus* to sublethal concentrations of imidacloprid may have a direct effect on the histology of the pituitary gland, thereby affecting its metabolism. The severe reduction of basophils and acidophils in the pars distalis of pituitary gland which were evident in this study may be proof of the endocrine disruption in the nature of the pesticide.
Fig. IV.1. A. Pituitary gland

Fig. IV.1. B. Pars distalis

Fig. IV.1. C. Pars intermedia
IV.5. Residue Analysis

The results of the thin layer chromatography Rf values of imidacloprid in four different solvent systems were given in Table.IV.6.1. The results were analyzed with high pressure liquid chromatographic (HPLC) analysis in the tissue of lungs, brain, liver, kidney, and muscle tissues of the frog, *Hoplobatrachus tigerinus*. Under exposure to sub-lethal dose, the order of accumulation of residues of imidacloprid was

Kidney > Liver > Lungs > Muscle > Brain

Under sub-lethal exposure to commercial grade, imidacloprid (17.8% SL, Tatamida) for 24, 48, 96 h, 8, 15 and 30 days it was observed that the kidney tissue followed by liver accumulated more residue than the other tissues and least amount of residue was found in brain. The other peaks which appeared in the HPLC chromatogram may be the metabolites of the parent compound imidacloprid or other pesticide residues with similar structures. In TLC confirmation tests also, the residues of imidacloprid appeared as brown spots, prominently in kidney sample and less prominently in other samples.

The variations in the residue analysis are attributed to the factors like difference in uptake rate and lipid content of respective animal tissue. The chemical structure, solubility, frog interaction and metabolic pattern are responsible for pesticide uptake. The results of the present study revealed that prolonged exposure to sub-lethal concentrations led to increase in the accumulation of residue. This is in agreement with the earlier reports by Bradbury *et al.* (1987), Tripathi (1992); Tilak *et al.* (2003 and 2004); Rose *et al.* (2013) and Enbaia *et al.* (2014). The accumulation is a factor responsible for changes in biochemical actions or pathological changes and also disturbance of overall biochemical
reactions which were cumulative causing lethal actions even when the concentrations are at sub-lethal.

Priya and Maruthi (2012) reported similar results of significant increase in the level of imidacloprid residues in exposed fresh water teleost, *Channa punctatus* liver samples and stated that the increase of residue levels was proportional to the concentration of imidacloprid and duration of exposure. The residues accumulated in kidneys may cause toxicity to frog which ultimately results in the disturbance of homeostasis of the organism. It is also clear that the imidacloprid insecticide may concentrate in the kidneys and may cause toxicity to the organisms. Shalaby *et al.*, (2010) also recorded low amount of residues on day 5 and no residues were detected in the kidneys at the end of 10 days of recovery period in rats exposed to thiamethoxam insecticide (Neonicotinoid compound). The presence of residues levels in the kidney confirms that of Yassa *et al.*, (2011) who reported highest levels of diazinon residues in kidney than in liver and muscle tissue of rats administered orally @ 10 mg/kg bw/day. The presence of below the limit of quantification on day 4 of experimental period in the kidney of all the groups of test mice.

Muhammed *et al.*, (1990) also reported pesticide residues in liver, lung and kidneys of cattle reared in pesticide spraying areas of Faisalabad, Pakistan. The residue levels of pesticides found in paddy fish caused ill-effects in the health of farmers in Malaysia. In the present investigations, the relative residue level of imidacloprid on day 1 and below the level of quantification on day 4 of experimental period indicates that the kidneys play an essential role in the excretion of imidacloprid. Moffat *et al.*, (2004) reported the elimination of imidacloprid in urine (70-80%) and feaces (20-30%) in
mammals. According to Tilak et al. (2001), in the freshwater fish *Labeo rohita, Catla catla, Cirrhinus mrigala, Aplocheilus punchex* and *Ctenopharyngodon idellus*, exposed to fenvalerate to both lethal and sub-lethal concentrations, the pesticide residues bioaccumulated in the lipid tissues of the fish. Among the above-mentioned species, *Labeo rohita* showed more accumulation followed by *Catla catla* and *Cirrhinus mrigala*. Tilak et al. (2003) exposed the freshwater major carps, *Labeo rohita, Cirrhinus mrigala* and *Catla catla*, to sub-lethal concentrations of chlorpyrifos for eight days, and noticed that *Labeo rohita* tissues bioaccumulated more amount of pesticide, compared to *Catla catla* and *Cirrhinus mrigala*. They also observed that in all the three fish, the brain tissue accumulated more residue than the liver. According to Bagheri (2007) residues of organophosphate (OP) insecticides in the fish species and the water depend on the physiochemical characteristics of water, time of application, pH of water and the ambient temperature.

Tilak et al. (2004) also observed that the residues of chlorpyrifos accumulated more in brain than in liver of *Catla catla, Labeo rohita* and *Cirrhinus mrigala*. Mohammed Sweilum (2007) studied the effect of sub-lethal toxicity and bioaccumulation of dimethoate and malathion in Nile tilapia *Oreochromis niloticus* and stated that pesticide residues in the liver, gills and muscles of fish increased with increased pesticide concentrations in fish ponds. Their bioaccumulation in the liver was higher than in gill or muscle, which had the lowest residues for these pesticides.

Essumang et al. (2009) observed pesticide residues in the water and fish (*Lagoon tilapia*) samples from lagoons in Ghana. Afful et al. (2010) identified and quantified an organochlorine pesticide residues namely gamma-HCH, delta-HCH, heptachlor, aldrin,
gammachlordane, p,p’-DDE, alpha-endo-sulfan, dieldrin, endrin, endrin-aldehyde, endosulfan-sulfate, p,p’-DDT, endrketone and methoxychlor in six fish species namely *Heterotis niloticus, Channa obscura, Hepsetus odoe, Tilapia zilli, Clarias gariepinus and Chrysichthys nigrodigitatus* collected from Densu basin, Ghana.

Suneetha (2012) reported organochlorine and nitrogen containing pesticide residues in *Labeo rohita*, and traced the residues of DDT, endosulfan, p-methyl, cypermethrin, deltamethrin, atrazine and isoproturan in fish hatchery of Pakistan. Similar findings were also reported by Mahboob *et al.* (2011) in *Cirrhinus mrigala*. Suneetha (2012) observed residues in *Labeo rohita* exposed to sub-lethal concentration of endosulfan after 15 days of exposure. It was observed that liver tissue accumulated more residue than the other and minimum was noticed in muscle.

Liver is the main detoxifying tissue containing relatively high levels of detoxifying enzyme. It is also the first organ to face the effect of pesticides being carried through the portal circulation may be the reason of the greater accumulation. Mono oxygenase enzymes are found in high concentration in the liver and many tissues such as gonad, kidney intestine, gill and heart (Lindstrom Seppa *et al.*, 1981). These enzymes decrease the lipid solubility of organic contaminants thereby facilitating excretion of the pollutants (Verma and Gupta, 1976). The rapid loss of dimethoate from liver was reported by Ghousia Begum *et al.* (1994). Rose *et al.* (2013) assessed the levels of OCs in water, sediment, invertebrates (crayfish shrimps and crabs) and twelve species of fish, and reported that the most bioaccumulated OCs in the fish and invertebrates were beta-HCH and p, p’DDE. Choudhury *et al.* (2013) studied the presence of pesticide residues in
Table. IV. 6.1.

**Pesticide Standard Confirmation and the protocols of analysis**

<table>
<thead>
<tr>
<th>Layer</th>
<th>: Silica gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent</td>
<td>: Hexane + Acetone and water (90+5+5 V/V)</td>
</tr>
<tr>
<td>Front</td>
<td>: 10 Cm</td>
</tr>
<tr>
<td>Impregnated reagent</td>
<td>: 0.3 Per cent Silver nitrate</td>
</tr>
<tr>
<td>Time</td>
<td>: 30 minutes</td>
</tr>
<tr>
<td>UV light exposure</td>
<td>: 10 minutes</td>
</tr>
<tr>
<td>Hours and Exposure</td>
<td>: 24, 48, 96 h and 8, 15 and 30 days</td>
</tr>
</tbody>
</table>

**Rf Values of standard in different solvent systems**

<table>
<thead>
<tr>
<th>Solvent System</th>
<th>Ratio $\frac{U}{V}$</th>
<th>100x Rf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane + Acetone</td>
<td>9:1</td>
<td>58.2</td>
</tr>
<tr>
<td>Acetone + Water</td>
<td>1:1</td>
<td>76.4</td>
</tr>
<tr>
<td>Hexane + Acetone</td>
<td>1:1</td>
<td>78.3</td>
</tr>
<tr>
<td>Acetonitrile + Water</td>
<td>5:5</td>
<td>86.6</td>
</tr>
</tbody>
</table>

**Tissue 100xRf**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>100xRf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lungs</td>
<td>73.2</td>
</tr>
<tr>
<td>Brain</td>
<td>58.4</td>
</tr>
<tr>
<td>Liver</td>
<td>82.6</td>
</tr>
<tr>
<td>Kidney</td>
<td>84.4</td>
</tr>
<tr>
<td>Muscle</td>
<td>61.8</td>
</tr>
</tbody>
</table>
Fig.IV.6.1. Calibration curve of imidacloprid.

![Calibration curve of imidacloprid](image)

\[ y = 1798.2x + 1649.5 \]
\[ R^2 = 0.9983 \]

Fig.IV.6.2. RP-HPLC standard chromatogram of imidacloprid.

![RP-HPLC standard chromatogram of imidacloprid](image)

Table.IV.6.2. Retention time and Sensitivity of Imidacloprid.

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>( t_R ) (min)</th>
<th>Equation</th>
<th>( R^2 )</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imidacloprid</td>
<td>4.5 to 5.2</td>
<td>( y = 1798.2x + 1649.5 )</td>
<td>0.9983</td>
<td>LOD (µg/L)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LOQ (µg/L)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5.01</td>
</tr>
</tbody>
</table>
fish samples *Puntius sophore, Amblyparyngodon mola, Cirrhinus mrigala, Catla catla, Labeo rohita, Labeo goniufl, Cyprinus carpio* and *Labeo calbasu*.

Akan *et al.* (2013a and b) detected eleven organochlorine pesticide residues in all the fish *Clarias gariepinus, Heterotis niloticus, Oreochromis niloticus* and *Tilapia zilli*. Endosulfan was the most abundant pesticide residue found in tissues of all the fish species. He also reported some organophosphorus pesticides, dichlorvos, diazinon, chlorpyrifs and fenitrothion in the flesh, liver, stomach and gills of four commercial valuable species, *Clarias gariepinus, Heterotis niloticus, Oreochromis niloticus* and *Tilapia zilli* from Alau Dam, Borno State.

The frog tissue aliquot concentrations of Imidacloprid (17.8% SL) spiked increased concentrations and measured at various dilutions. The frog tissue aliquot concentrations were 0.364, 3.82, 20.78, 50.20 and 72.56 μg/ml. The coefficient of variation is 0.54. The eluted time minimized compared to that TNA-C method, that Rt minimized from 9.75 min to 4.9-5.2 min. The linear correlation coefficient was 0.9227. Limit of detection (LOD) of imidacloprid was calculated at a single-to-single ratio of 3, while the limit of quantification (LOQ) was obtained at a single-to-single ratio of 10. The LOD and LOQ for imidacloprid were 0.04 mg/Kg and 5.01 μg/Kg respectively.

The present modified method is helps for estimation of residual concentrations of commercial grade pesticide Imidacloprid 17.8% SL in freshwater frog *Hoplobatrachus tigerinus*. All the above standard parameters were followed, the commercial grade imidacloprid (17.8% SL) was eluted at 4.9 and 5.2 min. Compared to PDA detector, in UV detector minimized the eluted time and mobile phase utilization.
Enbaia et al. (2014) studied the estimation of organochlorine pesticide residues in Libyan fish Round Sardinella (Sardinella aurita), European Pilchard (Thynnus thynnus), Yellow Fin Tuna (Thunnus albacares) and Bogue (Boops boops). Organochlorine pesticides, endosulfan, heptachlor, methoxychlor, dieldrin, residues were found in those fish. Akan et al. (2014) reported the presence of organochlorine and organophosphorous pesticide residues, dichlorodiphenyl dichloroethylene, (o,p’-DDE), 4,4'-DDD, 4,4'-DDT), dichlorvos, diazinon, chlorpyrifos, fenitrothion, α-BHC, γ-BHC, metoxichlor, lindane, endosulfan sulphate, dieldrin and aldrin in organs of liver, gills, stomach and flesh of Tilapia zilli, Clarias gariepinus, Hetroits niloticus and Oreochromis niloticus from Lake Chad.

The results of the present study indicate that prolonged exposure to sub-lethal concentrations of imidacloprid in freshwater frog, Hoplobatrachus tigerinus, has leads to increased accumulation of residues. This is in corroboration with the earlier reports of insecticide residues. Thus the uptake and persistence of imidacloprid not only depends on a number of physical and chemical conditions, but also varies according to the biological conditions. Lipid and water contents are different among fish (FDA, 1999). Even for the same fish species, the lipid content could vary due to seasonal or physiological changes (Mendez and Gonzalez, 1997). Besides, pesticides may differ in their hydrophilic or hydrophobic characteristics, residue levels in fish vary with species due to differences in lipid content, biology (trophic level habitat and reproductive season), time of exposure, detoxification, capability, and ecology (Nowak and Julli, 1991). The differences in residue levels may also be a reflection of different exposure period and innate individual differences in metabolism.
Over the years, pesticides have been determined by many conventional as well as modern methods like spectrophotometry (Randhir Kumar and Banerjee, 2012), Polarography, Gas chromatographic technique, using various detectors or in combination with MS (Wang et al., 2012). Extensive research has been carried out on the analysis of pesticide residues in foodstuffs and environmental matrices (Wang et al., 2012 and Curl et al., 2003).

Pesticides, organochlorines, organophosphasates and carbamates have emerged as human-made potential threat to the biota and its environment and their use has been clearly identified as a principal driving force behind the drastic reduction of biodiversity in different parts of the world (Wahid Abdul, 2004). The frog, *Hoplobatrachus tigerinus*, is one of the non-target organisms to be affected by the pesticides that are used in the fields. The aquatic organisms that inhabit different aquatic bodies are facing the problem with the invasion of pesticides used in high quantities for agricultural practices. The frog affected by the pesticide could pose a health problem to the macroconsumers who consume the frog from the contaminated environment.

The results of the present study revealed that prolonged exposure to sub-lethal concentrations of imidacloprid to *Hoplobatrachus tigerinus* has lead to increased accumulation of residues. This is in corroboration with the earlier reports of OP residues. A thorough literature search revealed that repeated or continuous exposure to low concentrations of pesticides can lead to high residue concentrations without mortalities. Thus the uptake and persistence of imidacloprid depends not only on a number of physical and chemical conditions, but also varies according to the biological conditions.
All systemic compounds have the effects with time of exposure. However, only the persistent chemicals (fipronil, neonicotinoids and some OPs) have cumulative effects over time, since the non-persistent compounds are quickly degraded in soil and water. For risk assessment of these compounds it is important to understand their chronic impacts. Unlike traditional protocols based on acute toxicity, the persistent activity of the parent and toxic metabolites requires that exposure time must be taken into consideration (Halm et al., 2006). Concerns about the impacts of dietary feeding on honey bees and other non-target organisms are thus justified (Alix and Vergnet, 2007; Cresswell, 2011; Rortais et al., 2005), because the accumulation of small residue levels ingested repeatedly over time will eventually produce a delayed toxic effect (Tennekes and Sanchez-Bayo, 2012).

It was also relevant to the impact of small residues of those insecticides that have cumulative effects on aquatic ecosystems. Because of the short life-cycle of many zooplankton species, the negative population parameters that result from sub-lethal and chronic effects on such organisms can lead their local populations to extinction (Stoughton et al., 2008). Immediate reductions in populations and species may not always be apparent due to the small residue concentrations and the delayed effects they cause. For example, in recent surveys of pesticide residues in freshwaters of six metropolitan areas of USA, fipronil appears regularly in certain states (Sprague et al., 2008). Imidacloprid was reported in 89% of water samples in agricultural areas of California, with 19% exceeding the US Environmental Protection Agency’s chronic invertebrate Aquatic Life Benchmark of 1.05 μg/L (Starner and Goh, 2012). There is already a widespread contamination of waterways and estuaries with persistent systemic insecticides. The first consequence of such contamination is the progressive reduction, and possible elimination, of entire
populations of aquatic arthropods from the affected areas. As time is a critical variable in this type of assessment, it is envisaged that, this contamination continues at the current pace over the years to come, the biodiversity and functionality of many aquatic ecosystems will be seriously effected (Miranda, et al, 2011). Secondly, as these organisms are a primary food source of a large number of vertebrates (e.g. fish, frogs and birds), the depletion of their main food resource will inevitably have indirect impacts on the animal populations that depend on them for their own survival. The case of the partridge in England was an example of how a combination of herbicides and insecticides can bring the demise of a non-target species by indirectly suppressing its food requirements (Potts, 1986). Therefore, warnings signs of the possible role of environmental contamination with systemic and neonicotinoids in steeply declining populations of birds, frogs, hedgehogs, bats and other insectivorous ani mals are not far fetched and should be taken seriously (Tennekes, 2010b).

Chen and Chang (2013) developed the granular formulations of butachlor, phorate, chlorpyrifos, carbofuran, terbufos, terbufos, disulfoton and diazinon which are frequently used in Taiwan. The concentrations of the active ingredient are from 7% to 26% which are higher than those used before. The carrier was provided by Oil-Dri corporation of America. The stabilizer was also applied on the formulation using glycols. The results showed that the granules of butachlor, phorate, carbofuran, terbufos and disulfoton are chemically stable in the aging test. The granules of chlorpyrifos and diazinon, however, are not stable.

The present study has brought some light on the direct, sub-lethal and indirect effects that systemic insecticides have on species populations and ecosystems. Some long-
term impacts have been known for some time, but it is the rapid increase in the usage of systemic products that poses a new challenge to the ecological risk assessment of agrochemicals. Indeed, current risk protocols, based on acute, short-term toxic effects are inadequate to cope with the chronic exposure and cumulative, delayed impacts of the new compounds. Awareness of the increasing contamination of the environment with active residues of these chemicals should help regulators and managers to implement new approaches for risk assessment of these substances.

Repeated exposure to imidacloprid can result in reduced egg production in test frog and hatching, nest and brood abandonment, lower resistance to disease, decreased body weight, hormonal changes, and reduced avoidance of predators. The overall consequences of sub-lethal doses of pesticides can be reduced adult survival and lowered population abundance.
In the present study, the different tissues of frog, *Hoplobatrachus tigerinus* under Imidacloprid intoxication showed marked alterations in metabolites and enzyme systems of both protein and carbohydrate metabolism including AChE ase activity, T3, T4, TSH during initial time periods of exposure. The levels of these compounds restored to normaly during subsequent periods of exposure suggesting the operation of physiological homeostatic mechanisms in he frogs to counter the effects of Imidacloprid.

In the present study, an attempt has been made to evaluate the toxicity of the Insecticide, Imidacloprid to the test species, *Hoplobatrachus tigerinus*. From the studies toxicity evaluation, it is clear that Imidacloprid is moderately toxic to the test fish, *Hoplobatrachus tigerinus*. In the present study it was observed that the LD$_{50}$ values decreased with the increase in exposure period. Higher values in static system are due to bioaccumulation by frog, pesticide absorption to toxicant chamber walls and degradation of the compound. On the basis of acute toxicity, amphibians are less sensitive than mammals, fish, and sensitive aquatic invertebrates.

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. The
leotropic gradation series in terms of per cent decrement at 24, 48, 96 h, 8, 15, 30 days exposure was

24 h   Muscle > Kidney > Brain > Liver
48 h   Muscle > Brain > Kidney > Liver
96 h   Brain > Kidney > Liver > Brain
8 days Brain > Muscle > Kidney > Liver
15 days Brain > Liver > Kidney > Muscle
30 days Brain > Liver > Kidney > Muscle

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

24 h   Brain > liver > Kidney > Muscle
48 h   Brain > Liver > Kidney > Muscle
96 h   Brain > Kidney > Liver > Muscle
8 days Brain > Kidney > Liver > Muscle
15 days Brain > Kidney > Liver > Muscle
30 days Liver > Kidney > Muscle > Brain

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.
In the present study, the DNA content was found to be decreased in all the tissues in response to Imidacloprid sub-lethal exposure at time periods of 24, 48, 96 h, 8, 15 and 30 days. Decrement in the DNA level might be due to activation of some dormant regulating factors or increase in activity of the essential factors controlling DNA synthesis or may be degeneration of hepatic cells due to the effect of Imidacloprid. Decrease in nucleic acid suggests the decrease in protein synthesis and damage to the liver, is the major metabolic organ of drug detoxification.

Under exposure to sub-lethal dose of Imidacloprid for 24 h the amount of DNA content was found to decrease in all the tissues of the frog, *Hoplobatrachus tigerinus* and the decrement was in the order of

Muscle > Liver > Kidney > Brain

Under exposure to sub-lethal dose of Imidacloprid, for 48 h the DNA content was decreased in all tissues of the frog, *Hoplobatrachus tigerinus*. The decrement was in the order of

Muscle > Kidney > Brain > Liver

Under exposure to sub-lethal dose of Imidacloprid, for 96 h, the decrease of DNA in the test tissues of the frog, *Hoplobatrachus tigerinus* was in the order of

Liver > Kidney > Muscle > Brain

Under exposure to sub-lethal dose of Imidacloprid, for 8 days, the decrease of DNA in the test tissues of the frog, *Hoplobatrachus tigerinus* was in the order of

Muscle > Liver > Brain > Kidney

Under exposure to sub-lethal dose of Imidacloprid, for 15 days, the decrease of DNA in the test tissues of the frog, *Hoplobatrachus tigerinus* was in the order of
Muscle > Liver > Brain > Kidney

Under exposure to sub-lethal dose of Imidacloprid, for 30 days, the decrease of DNA in the test tissues of the frog, *Hoplobatrachus tigerinus* was in the order of

Liver > Muscle > Kidney > Brain

Under exposure to sub-lethal doses of Imidacloprid, the total RNA content was decreased in most of the tissue of the test frog, *Hoplobatrachus tigerinus*. Maximum decrease was noticed in muscle and minimum in brain at 24, 48, 96 h and 8, 15 and 30 days. The percent changes over controls at four test periods were in the order of,

- 24 h: Liver > Brain > Muscle > Kidney
- 48 h: Kidney > Muscle > Liver > Brain
- 96 h: Liver > Kidney > Brain > Muscle
- 8 days: Liver > Muscle > Kidney > Brain
- 15 days: Muscle > Kidney > Brain > Liver
- 30 days: Muscle > Liver > Brain > Kidney

The alterations in DNA levels in the present study could be due to the disturbances in the normal synthesis and turnover rate of DNA besides degenerative changes. The RNA levels reflect the intensity of protein synthesis and metabolic activity of the tissue. The depletion of RNA level suggests increased proteolysis and possible utilization of the products of their degradation for metabolic purposes. The changes in the biochemical markers like DNA and RNA which may be due to the increased activity of the enzyme DNAase and the inhibition of RNA polymerase function.

Under exposure to sub-lethal dose of Imidacloprid, the total LDH levels were increased in most of the tissue of the test frog, *Hoplobatrachus tigerinus*. Maximum
increase was noticed in liver and minimum in muscle at 24 and 48 h. The per cent changes over controls at six test periods were in the order of,

24 h  Liver > Kidney > Muscle > Brain
48 h  Liver > Kidney > Brain > Muscle
96 h  Liver > Kidney > Brain > Muscle
8 days  Liver > Kidney > Brain > Muscle
15 days  Liver > Kidney > Brain > Muscle
30 days  Liver > Kidney > Brain > Muscle

In the present study also, it was observed that the activity of LDH in the frog, Hoplobatrachus tigerinus under exposure to sub-lethal doses of Imidacloprid was increased in 24, 48, 96 h, 8, 15 and 30 days exposure indicating that the anaerobic respirations arrived and aerobic respiration inhibited there by to meet the increased metabolic stress, to overcome the toxic stress and the energy demands as the aerobic oxidation are lowered. Under anaerobic condition this enzyme converts pyruvic acid to lactic acid. SDH is one of the important enzymes involved in Kreb’s cycle.

Under exposure to sub-lethal doses of Imidacloprid, the AAT activity showed a marked elevation from the control frogs. The increase in AAT activity was noticed in all the tissues tested and at all the test periods. The maximum increase of AAT activity was noticed in liver (24 h and 8, 15 days), and minimum in kidney and brain. The per cent increase of AAT activity in different tissues at six test periods were in the order of,

24 h  Liver > Muscle > Brain > Kidney
48 h  Muscle > Liver > Kidney > Brain
96 h  Muscle > Kidney > Liver > Brain
The ALAT activity in control frog was maximum in brain and minimum liver. Under exposure to sub-lethal doses of Imidacloprid the ALAT activity was elevated. The ALAT activity increased progressively throughout the test period with maximum increase in liver and minimum in muscle. The per cent increase of ALAT activity over controls during the four test periods was in the order of,

- **24 h** Muscle > Brain > Liver > Kidney
- **48 h** Muscle > Kidney > Liver > Brain
- **96 h** Muscle > Kidney > Brain > Liver
- **8 days** Muscle > Liver > Kidney > Brain
- **15 days** Liver > Muscle > Kidney > Brain
- **30 days** Liver > Muscle > Kidney > Brain

Increased activities of AAT and ALAT in different tissues of frog, *Hoplobatrachus tigerinus* suggest either increased operation of transamination or increased synthesis of amino acids from other sources like glucose or fatty acids during Imidacloprid intoxication. The alterations in activities of aminotransferases as observed in the present study is in an agreement with earlier reports, demonstrating a consistent increase in the activities of these enzymes under conditions of enhanced gluconeogenesis. The significant change induced in protein metabolism due to pesticide stress is known to affect the aminotransferases. AAT and ALAT are located in mitochondrial and cytosol fractions of the cell.