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Full Length Research Paper

Effects of acute toxic pesticide on survival, morphology and behavior of Adult Toad

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The acute toxicity of Endosulfan (organochlorine) and Diazinon (organophosphate) pesticides to adult amphibians, *Bufo regularis* was evaluated to determine uptake and effect of environmentally relevant concentrations on survival, morphology and behaviour. Toxicity characterizations were also assessed using standard indices. Toads were exposed for 96 h to varying concentrations of the pesticides; 0.25, 0.50, 0.75 and 1 mg/l. Mean percentage mortality increased significantly ($p < 0.05$) with concentrations and exposure duration for Endosulfan and Diazinon pesticides and was significantly ($p < 0.05$) different from the control, indicating that pesticide induced lethality. The results showed that Diazinon (LC_{50} = 0.44 mg/l) was more toxic than Endosulfan (LC_{50} = 0.73 mg/l). Derived safe concentrations were 0.07 and 0.04 mg/l for Endosulfan and Diazinon, respectively. Estimated Toxicity index values (TIV) and Hazard Quotients (HQ) for all the concentrations were above one (1) indicating potential risk of the pesticides to the toad. Bioconcentration of the pesticides after 96 h increased with increasing concentrations indicating that uptake was concentration dependent. There was a significant positive correlation between tissue concentration and mortality ($p < 0.01$) for both pesticides. The pesticides also caused dose-dependent deformities and behavioural abnormalities. More pronounced poisoning symptoms were observed in Diazinon and at higher concentrations.

Key words: Acute toxicity, Endosulfan, Diazinon, adult amphibian.

INTRODUCTION

Agricultural pesticides are indispensable in contemporary agriculture. They are beneficial by providing reliable, persistent and relatively complete control against harmful pests with less cost and effort. They have, no doubt, increased crop yields by killing different types of pests, which are known to cause substantial or total crop damage. However, their effects are less than desirable when they leave the target compartment of the agricultural ecosystem. Up to 90% of the pesticides applied never reach the intended targets (Sparling et al., 2001); as a result, many other organisms sharing the same environment as pests are accidentally poisoned. One of the non-target biological groups mostly affected by pesticides is amphibians (Fulton and Chambers, 1985; Berrill et al., 1994; Sparling et al., 2001).

Amphibians are exposed to pesticides by many routes but perhaps the most common route is agricultural runoffs. Agricultural practices affect natural habitat in several ways such as through land conservation, increased fragmentation and agrochemical contamination (Hecnar, 1995; Davidson et al., 2002). Much of the interest on amphibian's declines is currently focused on the role of pesticides on the observed global declines (Houlahan et al., 2001). A diversity of pesticides and their residues are present in a wide variety of aquatic habitats (McConnell et al., 1998; LeNoir et al., 1999; Kolpin et al., 2002). Declining amphibian populations have been correlated with greater amounts of upwind agriculture, where pesticide use is common (Davidson et al., 2001; 2002). While these correlative studies suggest that pesticides may affect amphibian communities, there are

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few rigorous experiments to confirm that pesticides are altering amphibian communities.

This study therefore, concurrently evaluates the effects of Endosulfan, an organochlorine insecticide and Diazinon, an organophosphate insecticide at acute lethal doses on survival, morphology and behaviour of the adult toad *Bufo regularis*. The study was designed to identify the acute toxic effects of these pesticides widely used in Nigeria, and to contribute to the knowledge of the effects of pesticides on amphibians.

MATERIALS AND METHODS

Collection of test organisms

Adult toads, B. regularis of both sexes were collected by hand net from their spawning ponds in unpolluted and non agricultural sites (Harri et al., 1979; Allran and Karasov, 2001; Vogiatzis and Loumbourdis, 1997; Khan et al., 2003).

Toads samples were collected by hand net from their spawning ponds and were transported to the laboratory in a covered basket. Adult toads of the same size and almost the same weight (32.87 ± 0.03 g) were acclimatized in glass tanks (51 × 32 × 33 cm^3) containing 2 L of dechlorinated tap water, for 7 days prior to the experiments (Vogiatzis and Loumbourdis, 1997). Tanks were placed on a slant to provide the option of both aqueous and dry environment (Allran and Karasov, 2001). Water was changed every two (2) days and the tank cleaned thoroughly. Toads were fed with earthworms twice weekly. Uneaten earthworms and faecal wastes were removed and water replenished regularly (Allran and Karasov, 2001).

Test chemicals

Endosulfan and Diazinon pesticides were purchased from Coromandel Fertilizers Limited (North Arcot District, Ranipet 632 401 Tamilnadu, India).

Pesticide toxicity tests (bioassay procedure)

Test water

Water for toxicity tests was dechlorinated tap water. The water was dechlorinated by allowing it to stand and exposed for 36 h (Ezemonye and Enuneku, 2005). This water was used for acclimatization, control tests and for making the various concentrations of the test chemicals. Water qualities assessed during the exposure periods for the bioassay were, temperature, pH, dissolved oxygen, conductivity, turbidity and alkalinity. They were determined using standard methods (APHA, 1998).

Test concentrations

The bioassay procedures started with a range finding test (ASTM, 1996). For the definitive test, stock solutions of the required concentrations were prepared for both pesticides. The stock was then diluted serially into environmental relevant treatment concentrations of 0.25, 0.50, 0.75 and 1 mg/l, for Endosulfan and Diazinon pesticides. Two replicates per test concentration were used to avoid test repetition and to provide a stronger statistical baseline. Each test chamber contained an equal volume of test solution (2 L) and equal numbers of toads (10). Replicate test chambers were physically separated.

Toxicity tests

Acute toxicity tests were conducted according to standard procedures (ASTM, 1996). Adult amphibians were exposed for 96 h to each selected concentration of Endosulfan and Diazinon pesticide solutions. Ten (10) toads each were assigned to individual experimental units containing one of the treatments of Endosulfan and Diazinon pesticides (0.25, 0.50, 0.75 and 1 mg/L). The control contained only dechlorinated tap water. Feeding was discontinued 24 h before the commencement of the experiment and during the test. Observations were made on a daily basis and mortality recorded. Behavioural and morphological changes were also observed.

Mortality rate

Mortality was recorded at an interval of 24 h over a period of four days. Toads were assumed dead when they turned upside down and sank to the bottom of the tank or when they showed no form of movement even when prodded with a glass rod (Harri et al., 1979). The behaviour and morphological changes in exposed toads were assessed at 24 h interval.

Bioconcentration

Concentrations were achieved according to the method described by Harri et al. (1979) and Steinwandter (1990). For the whole-body tissue concentration studies, toads (Dead samples after 96 h) were frozen to -18°C. Deep frozen tissues were grounded while still partially frozen, as this makes the tissue more brittle.

Statistical analysis

The susceptibility of the Adult toads to Endosulfan and Diazinon pesticides were determined using the Probit (Probit Software) method of analysis (Finney, 1971) for median lethal concentration at 96 h. Computation of confidence interval of mortality rate was also obtained from the Probit analysis used to determine the LC_{50}. Student's t-test, Pearson correlation and one-way analysis of variance SPSS (14.0 version), SPSS Inc, Chicago, USA was employed to test the variable at $p < 0.05$ level of significance. Multiple bar graphs and line graphs were also used in this study for the pictorial representation of assessment endpoints.

Risk characterization using standard indices

Safe concentrations at 96 h were obtained by multiplying the lethal concentration by a factor of 0.1 (EIFAC, 1998). Bioconcentration factor was calculated as the concentration of the pesticide in the tissue per concentration of the pesticide in water (Falandysz and Chwir, 1997). Hazard Quotient (HQ) was calculated as the ratio of the concentrations of chemicals in tissues to the Toxicity Reference Value (TRV) [Endosulfan = 0.056 (EPA, 1992), Diazinon = 0.043 (EPA, 1996)] (Moloche, 2008). Toxicity index value was calculated as the concentration of the pesticide in the tissue divide by LC_{50} (Battaglin and Fairchild, 2002).

RESULTS

Behavioural and morphological changes

Observations of the behavioral responses of toads

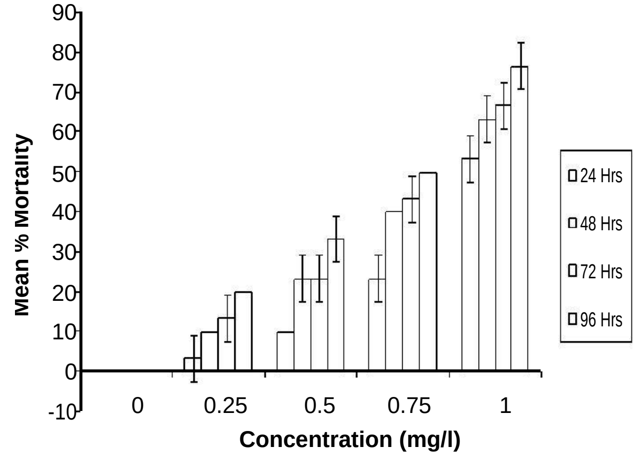

Figure 1. Mean percentage mortality of toads exposed to different concentrations of Endosulfan pesticide.

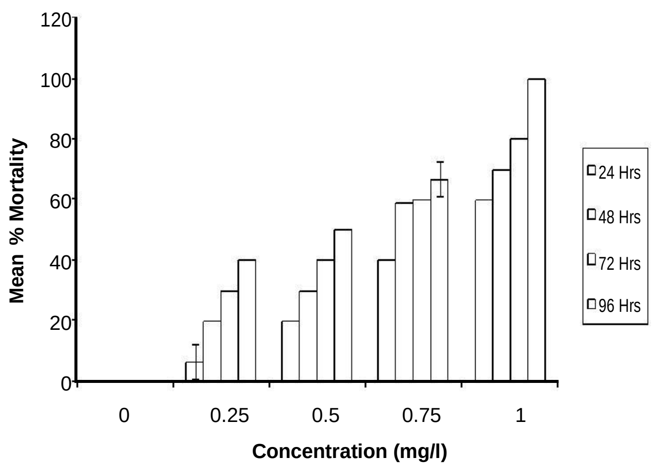

Figure 2. Mean percentage mortality of toads exposed to different concentrations of Diazinon pesticide.

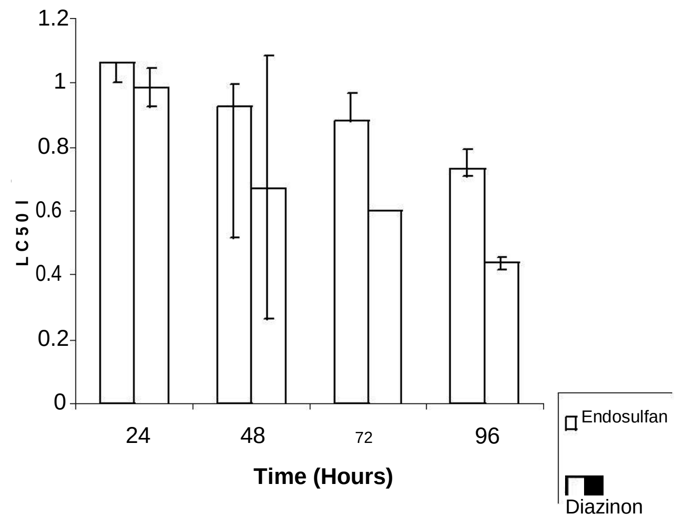

Figure 3. Mean 24, 48, 72 and 96 LC_{50} in adult *B. regularis* exposed to Endosulfan and Diazinon.

exposed to Endosulfan pesticide were conducted during the 96 h acute toxicity test. The control group showed normal behavior during the test period. Toads exposed to the lowest concentration of 0.25 mg/l had close to normal behavior. At 0.50 mg/l and at all concentrations above 0.50 mg/l, less general activity was recorded when compared with the control group. The highest concentration (1.0 mg/l) showed all responses at high levels. Behavioural changes observed at all concentrations above 0.50 mg/l were characterized by initial hyperactivity (thrashing and leaping) followed by loss of coordination in both front and hind limbs, erratic swimming, unusual retention of water, prolonged and motionless laying down on the aquarium bottom. Finally death was defined as lack of response to mechanical stimuli.

Similarly, toads exposed to Diazinon pesticide also showed the aforementioned poisoning symptoms, However it was more severe with skin discoloration, reddening of the snout and the protrusion of the intestine from the anus (heamorrhoid).

Mortality

No mortality or morphological changes were observed in the controls for the 96-h acute toxicity test. Toads in the control experiment appeared active and healthy throughout the test period.

Adult *B. regularis* exposed to varying Endosulfan and Diazinon concentrations recorded mortality in all the concentrations (0.25, 0.50, 0.75, 1.0 mg/l). The mean percentage mortality increased with increase in concentration and exposure duration for both pesticides (Figures 1 and 2). One hundred percent (100%) mortality was observed in Diazinon exposed toads in 1 mg/l at 96 h. This indicated that mortality was concentration dependent. The exposure showed a clear significant positive correlation between dose and mortality ($p < 0.01$, $r = 1$) at all the exposure durations.

Derived 96-h LC_{50} values observed for 24, 48, 72 and 96 h for Endosulan and Diazinon pesticides were 1.06, 0.93, 0.88, and 0.73 mg/l (Endosulfan) and 0.98, 0.67, 0.60, and 0.44 mg/l (Diazinon), respectively (Figure 3). The Probit analysis showed that 96-h LC_{50} values decreased with increase in exposure time. This indicates an increase in toxicity with exposure duration (Figure 1). Safe concentrations at 96 h for Edosulfan and Diazinon pesticides were 0.07 and 0.04 mg/l, respectively (Table 1).

Bioconcentration

The 96 h bioconcentration values obtained increased with

Table 1. Bioconcentration, bioconcentration factors, toxicity index values (TIV) and toxicity quotient (TQ) for *B. regularis* exposed to Endosulfan and Diazinon for 96 h (Mean ± SD).

Test pesticides	Bioconcentration (mg g^{-1})					Bioconcentration Factors (BCF)					Toxicity Index Value (TIV)					Hazard Quotient (HQ)					96 h-LC_{50}	Safe Conc.
	0	0.25	0.50	0.75	1.0	0	0.25	0.50	0.75	1.0	0	0.25	0.50	0.75	1.0	0	0.25	0.50	0.75	1.0		
Endosulfan	0	0.40 ±0.03	1.29 ±0.11	2.44 ±0.01	2.70 ±0	0	1.61 ±0.12	2.57 ±0.21	3.26 ±0.02	27.00 ±0	0	0.56 ±0.04	1.76 ±0.15	3.35 ±0.02	3.70 ±0	0	7.20	22.98	43.63	48.21	0.730 mg/l	0.073 mg/l
Diazinon	0	0.63 ±0	2.19 ±0.08	4.15 ±0.13	4.71 ±0.01	0	2.52 ±0	4.38 ±0.21	5.53 ±0.18	47.07 ±0.12	0	1.47 ±0	5.09 ±0.19	9.65 ±0.30	10.95 ±0.03	0	14.65	50.93	96.51	109.53	0.438 mg/L	0.044 mg/L

increase in concentration of test pesticides (Table 1). There was also a significant positive correlation between accumulated residues and mortality ($p<0.01$) for both pesticides.

Risk characterization

Bioconcentration factors (BCF), toxicity index values (TIV) and hazard quotient (HQ) of Endosulfan and Diazinon pesticides in *Bufo regularis* are shown in Table I. Toxicity index values increased with concentration. The highest TIV of 10.95 was recoded in 1 mg/l of Diazinon. Toxicity index values increased with concentration. The highest TIV of 10.95 was recoded in 1 mg/L of Diazinon.

The bioconcentration factors ranged from 1.61 - 27 for toads exposed to Endosulfan pesticide and 2.52 - 47.07 for toads exposed to Diazinon pesticide. In toads exposed to the highest concentration (1 mg/L) of Endosulfan, BCF was approximately 25 times more than the lowest concentration of 0.25 mg/l, while for Diazinon, BCF was 45 times more.

All the estimated HQs in all the concentrations for both pesticides were above 1 (Table 1). Diazinon pesticide had the highest TQ of 109.53 in 1 mg/l.

DISCUSSION

Behavioural and morphological observations

Toads exposed to different lethal concentrations of Endosulfan and Diazinon pesticides, undoubtedly experienced stress due to their irritating and neurotoxic effects. The increased erratic movements exhibited by the toads may be an attempt to be relieved from such stressful environment. The changes in the animal's behaviour after exposure to toxicants may be related to the consequent alteration in physiological process (Marler et al., 1966). Certain signs of toxicity, such as hyperactive symptoms at the beginning, then loss of balance, followed by motionlessness and finally death were observed in the behaviour of *B. regularis* with higher symptoms occurring for Diazinon exposed toads. Similar behavioural effects in response to laboratory exposure to carbaryl, carbofuran, malathion, dimethoate atrazine and basudin in larvae amphibians have been reported (Bishop, 1992; Saym and Akyurtlaki, 1999; Saym and Kaya, 2006; Ezemonye and Tongo, 2009; Ezemonye and Ilechie, 2007). These behavioural effects are not surprising as most of these pesticides are neurotoxins. It is also known that behavioural changes of these types increase the chances of amphibian predation (Bishop and Pettit, 1992). Morphological changes of skin discoloration observed in Diazinon exposed toads is comparable with the findings of Kaplan and Overpeck (1964) who reported skin discoloration in *Rana pippiens* exposed to near lethal concentrations of aldrin and chloradane pesticides.

Mortality

No mortality was reported in all the control tests in this study, while varying degrees of mortality were reported in the tests concentrations. This is a clear indication that the effects of the pesticides could be regarded as possible cause of death of the test organisms. The results clearly indicate that both pesticides, Endosulfan and Diazinon varied greatly in their effects on survival of *B. regularis.* The highest mortality was found at the highest concentrations, suggesting dose-dependent survival and concentration graded lethality. The 96 h LC_{50} for *B. regularis* exposed to Endosulfan in this study was found to be 0.730mg/l while the 96-h LC_{50} values for Diazinon was 0.438mg/l. The safe concentrations were 0.07 and 0.04 mg/l for Endosulfan and Diazinon, respectively. Using hazard ratings and 96 h LC_{50} estimates (Table 1) from the present study, Endosulfan and Diazinon would be classified as very highly toxic (that is, LC_{50} > 0.1 to 1). The current pesticide water quality criterion of > 0.01 mg/L (FEPA, 1991) however, appears protective of these toad species.

Mortality patterns were pesticide specific. Endosulfan and Diazinon pesticides significantly induced mortality in the toad *B. regularis*, with Diazinon recording complete mortality (100%) at the highest treatment concentration within 96 h. Although, no data on the toxicity of Endosulfan and Diazinon on adult amphibians are available for comparison with the results of this study, mortality rates were however, comparable to those reported for larvae amphibians (Broomhall, 2002; Sparling and Fellers, 2009; Sumanadasa et al., 2008; Harris et al., 1998; Relyea, 2004; Ezemonye and Ilechie, 2007) in which lethality was dose and time dependent.

In comparison with fish for endosulfan pesticide, Nowak and Sunderam (1991) reported LC_{50} values of 2.0 µg/l at 30°C and 4.6 µg/L at 35°C when mosquito fish was exposed to technical grade Endosulfan. Smith (1991), reported LC_{50} for rainbow trout to be 1.4 µg/l. For Diazinon pesticide, Hoque et al. (1993) observed the 96-h LC_{50} values for *Puntius gonionotus* exposed to Diazinon to be 3.67 mg/l, Al-Arabic et al. (1992) reported the 96-h LC_{50} value of *Labio calbasu* fingerlines exposed to Diazinon to be 1.54 mg/l, Svobodova et al. (2001) reported 96-h LC_{50} value of 26.7 mg/l for common carp exposed to Diazinon.

The recorded 96-h LC_{50} values observed for fishes exposed to Diazinon pesticides were higher than the 96-h LC_{50} values of 0.44 mg/l for *B. regularis* observed in this study; this implies that *B. regularis* is more sensitive to diazinon compared to the fish species. Standard toxicity tests show that for some contaminants, amphibians are more sensitive than fish (Birge et al., 2000). However, *B. regularis* was less sensitive to Endosulfan than in the reported fish species.

Higher mortality values recorded for Diazinon may be mainly due to the metabolite Diazoxon that is formed in animals. Diazoxon is a potent enzyme inhibitor capable of killing organisms directly by inhibiting acetylcholinesterase and numerous other important enzymes with molecular structures that are similar to it (Eisler, 1986). Most disorders in animals exposed to organophosphate compound like Diazinon have been linked to their toxic effects in the central nervous system (Berrill et al., 1994; Saglio et al., 1998). The pesticides have been found to concentrate in tissue of frogs with depressed cholinesterase activity (Sparling et al., 2001) and have induced hyperactivity in frogs followed by paralysis (Berrill et al., 1998).

Bioconcentration

The high mortality recorded could also be explained by bioconcentration of these agrochemicals in the tissues. There was a clear significant positive correlation between accumulated residues and mortality ($p < 0.01$). Each of the two focal compounds in this study has been reported to bioconcentrate (Sparling et al., 2001) in the tissues of test organisms and the estimates of tissue concentrations may be more valuable for the assessment of situations in the natural environment (Sparling et al., 2001). Bioconcentration in tissues has serious ecological consequence because these pesticides are retained in the amphibian’s body tissue which when fed on by a predator can lead to the concentration of the chemical from one trophic level to the next (ASTM, 1998; Suter, 1993). The high BCF values observed for Diazinon pesticide may be due to persistence of the chemical in the system (Hall and Swineford, 1980). Results of the measured indices of toxicity (TIV and TQ) indicate that the pesticide Diazinon was highly toxic to the toad *B. regularis* than Endosulfan.

REFERENCES

Al- Arabi SSM, Mazid MA, Alam MGA (1992). Toxicity of Diazinon to three species of Indian major carps. Banglasesh J. Tran. Dev. 5(1): 77-86.

Allran JW, Karasov WH (2001). Effects of atrazine on embryos, larvae and adults of anuran amphibians. Environ. Toxicol. Chem. 20: 761-775.

American Public Health Association (APHA) (1998). Standard methods for the examination of water and waste water 20th Edn American Public Health Association, New York, USA, p.1976.

American Society for Testing and Materials (1996). Standard practices for conducting acute toxicity test with fishes, macro invertebrates, and amphibians In Annual Book of ASTM standards. 11 (5): 1 - 29.

American Society for Testing and Materials (ASTM) (1998). Standard guide for conducting the Frog Embryo Teratogenessis Assay - Xenopus (FFTAX) E1439 volume 1105 E47, Annual book of ASTM standards West Conshohocken PA: Committee on Biological Effects and Environmental fate. E1439-1498

Battaglin W, Fairchild J (2002). Potential toxicity of pesticides measured in midwestern streams to aquatic organisms Water Sci. Technol. 45(9): 95-103.

Berrill M, Coulson D, McGillivray L, Pauli B (1998). Toxicity of endosulfan to aquatic stages of anuran amphibians. Environ. Toxicol. Chem. 17: 1738-1744.

Berrill M, Bertram S, McGillivary L, Kolohan M, Paul B (1994). Effects of low concentrations of forest use pesticides on frogs’ embryo and tadpoles Environ. Toxicol. Chem. 18: 657 -664.

Birge WJ, Westerman AG, Spromberg JA (2000). Comparative toxicology and risk assessment of amphibians, In Sparling DW, Linder G, and Bishop CA (Eds)Ecotoxicology of amphibians and reptiles SETAC Press, Pensacola, FL, USA p. 877.

Bishop CA (1992) The effects of pesticides on amphibians and the implications for determining the causes of decline in amphibian populations In: Bishop CA, Pettit KE, editors Declines in Canadian amphibian populations designing a national monitoring strategy Ottawa ON: Canadian wide life service p. 76.

Bishop CA, Pettit KE (1992). Declines in Canadian amphibian populations: designing a national monitoring strategy, Minister of Supply and Services Canada, Ontario. 61: 243-250.

Broomhall S (2002). The effects of endosulfan and variable water temperature on survivorship and subsequent vulnerability in predation in *Litoria citropa* tadpoles. Aqua. Toxicol., 61: 243-250. Davidson CH, Shafer B, Jennings MR (2002). Spatial tests of the pesticide drift, habitat destruction, UVB and Climate change hypothesis for California amphibian declines. Conservat. Biol. 16: 1588-1601.

Davidson CH, Shafer B, Jennings MR (2001). Decline of the California red-legged frog: climate, UVB, habitat and pesticides hypothesis Ecol. Appl. 11: 464- 479.

Eisler R (1986). Diazinon Hazards to Fish Wildlife and Invertebrates, a synoptic viewRep; 85 (1-9) US Fish and wildlife service, US Department of interior Washington DC p. 37.

Environmental Protection Agency (EPA) (1992). National Recommended Water Quality Criteria Federal Register. 57-60848.

Environmental Protection Agency (EPA) (1996). Ecotox Thresholds Eco update Office of Solid Waste and Emergency Response. EPA 540/D-95/038.

European Inland Fisheries Advisory Commission (EIFAC) (1998). Revise report on fish toxicology testing procedures: EIFAC Tech paper, 24, Rev 1: FAO Rome p. 37.

Ezemonye LIN, Tongo I (2009). Lethal and Sublethal Effects of Atrazine to Amphibian Larvae. Jordan J. Biol. Sci. 2(1): 29-36.

Ezemonye LIN, Enuneku A (2005). Acute toxicity of cadmium to tadpoles of Bufo maculatus and Ptychedena bibroni. Pollut. Health 4(1): 13 - 20.

Ezemonye LIN, Ilechie I (2007). Acute and chronic effects of organophosphate pesticides (Basudin) to amphibian tadpoles (Ptychadena bibroni). Afr. J. Biotech. 6(11): 1554 - 1568.

Falandysz J, Chwir A (1997). The concentrations and bioconcentration factors of mercury in mushrooms from the Mierzeja Wislana sand-bar, northern Poland. Sci. Total Environ. 203(3): 221-228.

FEPA (1991). Guidelines and Standards for Environmental Pollution Control in Nigeria Federal Environmental Protection Agency, Lagos.

Finney DJ (1971). Probit Analysis Cambridge, England, Cambridge University Press pp. 333.

Fulton MH, Chambers JE (1985).The toxic and teratogenic effects of selected organophosphorus compounds on the embryos of three species of amphibians Toxicol. lett. 26: 175-180.

Hall RJ, Swineford D (1980). Toxic effects of edrin and toxaphene on the southern leopard frog Rana sphenocephala. Environ. Pollut. 23(A): 53-56.

Harri MNE, Laitinen J, Valkama EL (1979). Toxicity and retention of DDT in adult frogs, Rana temporaria L. Environ Poll. 20(1): 45-55.

Harris ML, Bishop CA, Struger J, Ripley B, Bogart JB (1998). The functional integrity of northern leopard frog (*Rana pipiens*) and green frog (*Rana clamitans*) populations in Orchard wetlands II Genetics, physiology and biochemistry of breeding adults and young-of-the year. Environ. Toxicol. chem. 17: 1338-1350.

Hecnar SD (1995). Acute and chronic toxicity of ammonium nitrate fertilizer to amphibians from southern Onatara. Environ. Toxicol. chem. 141: 2131-2157.

Hoque MM, Mirja MJA, Miah MS (1993). Toxicity of Diazinon and Sumithion to Puntus gonionotus.. Bangladesh J. Trans. Dev. 6(1): 19-26.

Houlahan JE, Fridlay CS, Schmidt BR, Mayers AH, Kuzmin SL (2001). Quantitative evidence for global amphibian population declines. Nature 404: 752-755.

Kaplan HM, Overpeck JG (1964). Toxicity of halogenated hydrocarbon insecticides for the frog. Rana pipiens Herpetologica 20: 163-169.

Khan MZ, Tabassum R, Shah EZ, Tabassum F, Ahmad I, Fatima F, Khan MF (2003). Effect of Cypermethrin and Permethrin on Cholinesterase Activity and Protein Contents in Rana tigrina (Amphibia). Turk. J. Zool. 27: 43-246.

Kolpin DW, Furlong ET, Meyer MT, Thuranan EM, Zaugg SD, Barber LB, Buxton HT (2002). Pharmaceuticals, hormones, and other organic wastewater contaminant in US Streams 1999–2000, a national reconnaissance. Environ. Sci. Technol. 36: 1202- 1211.

LeNoir JS, McConnell LL, Fellers GM, Cahill TM, Serber JN (1999). Summertime transport of current use pesticides from California's central valley of the Sierra Nevada Mountain range, USA Environ. Toxicol. Chem. 18: 2715 - 2722.

Marler PR, Hamilton WJ (1966). Mechanism of Animal Behaviour New York: John Wiley p. 345.

McConnell LL, LeNoir JS, Datta S, Seibor JN (1998). Wet deposition of current-use pesticides in the Sierra Nevada Mountain range, California, USA Environ. Toxicol. Chem. 17: 1908-1916.

Moloche L (2008). Risk assessment of contaminated soil.http://wwwprsssca/pdf.

Nowak B, Sunderam RIM (1991). Toxicity and bioaccumulation of endosulfan to Mosquito fish *Gambusia affinis* (Baird and Girard). Verh Internat Verein Limnol. 24: 23-29.

Relyea AR (2004). Growth and survival of five amphibian species exposed to combinations of pesticides. Environ. Toxicol. Chem. 23(7): 1737-1742.

Saglio P, Trijasse S (1998). Behavioural responses to Atrazine and diuron in goldfish. Arch Environ contain Toxicol. 35: 484-491.

Sayim F, Akyurtlakli N (1999). Acute toxicity of malathion on the 25th stage larvae of Rana ridibunda. J. Fisheries. Aquatic Sci. 16: 19-29.

Sayim F, Kaya U (2006). Effects of Dimethoate on Tree Frog, Hyla arborea Larvae. Turk. J. Zool. 30: 261-266.

Svoboda M, Luskova V, Drastichova J, Zlabek V (2001). The effect of diazinon on haematological indices of common carp (Cyprinus carpio L). Acta Vet. Brno. 70: 457-465.

Smith AG (1991). Chlorinated hydrocarbon insecticides In: Handbook of Pesticide Toxicology, Volume 2, Classes of Pesticides, Hayes, WJ and Laws, ER (Eds) Publ: Academic Press, Inc pp. 731-915.

Sparling DW, Fellers GM (2009). Toxicity of two insecticides to California, USA, anurans and its relevance to declining amphibian populations. Environ. Toxicol. Chem. 28(8): 1696-1703.

Sparling DW, Fellers GM, McConnell LS (2001). Pesticides and amphibian population declines in California, USA Environ. Toxicol. Chem. 20: 1581-1595.

Steinwandter H (1990). Contributions to the on-line method For the extraction and isolating of pesticide residues and environmental chemicals II Miniaturization of the on-line method. Fresenius J. Anal. Chem. 336: 8-11.

Sumanadasa DM, Mayuri R, Wijesinghe Ratnasooriya WD (2008). Effects of diazinon on survival and growth of two amphibian larvae. J. Natn. Sci. Foundation Sri Lanka 36(2): 165-169.

Suter GW (1993). Ecological risk assessment. Boca Raron FL: Lewis p. 538.

Volgiatzis AK, Loumbourdis N (1997). Uptake, tissue distribution and depuration of cadmium (Cd) in the frog Rana ridibunda. Bull. Environ. Contamination Toxicol. 59: 770-776.