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Potential Effects of Agricultural Fungicide (Mancozeb) on Fish *Clarias batrachus*

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Abstract: The subclasses of carbamate pesticides, i.e., dithiocarbamates are being used in pest control programs because of their low toxicity. However, it has announced effects on aquatic organisms, especially on fishes. Therefore in the present study, the effects of exposure of fungicide on *Clarias batrachus* on different tissues have examined. The LC values (LC_{50}) estimated on different life stages of fish which were dose, as well as time dependent. LC_{50} differences has found in both adult and fingerlings, indicated that Mancozeb was more sensitive for fingerlings than adults. The bioassay study has performed after 24 and 72 h. Marked changes have observed in biochemical and enzymatic pathways in a single exposure, 40% of LC_{50} (10.86 mg L^{-1}) due to secondary metabolites of fungicide Mancozeb. The changes in the level of these parameters may because of cellular damage and impairment of enzymes in oxidative metabolisms which effect on the release of enzymes. Therefore, the findings of present investigation show that Mancozeb has potential to damage biochemicals and enzymatic pathways in fish. Therefore, it is suggested that appropriate toxicological risk assessment should be made in those areas where Mancozeb is proposed to be used in pest control activities.

Key words: Dithiocarbamates, fingerlings, Mancozeb, *Clarias batrachus*, India

INTRODUCTION

We are living in a technological era where technologies have made our life easier. Agricultural areas cannot assume without it, today. After green revolution especially in India, the pesticides have emerged as knight armor for crops. These pesticides have immense importance because of their ability to control weeds, pests and other insects. During the last 3 decades, the use of modern organic synthetic pesticides has increased about 40 fold (Srivastava, 2014). Crops receiving the most intensive application of various pesticides were cotton for insecticides, corn for herbicides and fruits and vegetables for fungicides. Fungicides are those chemicals which is used to stop fungal diseases in plants. Fungi and oomycetes are the causal agents of numerous fungal plant diseases, causing losses in agricultural production worldwide. Plant pathogenic fungi and oomycetes have coevolved with their plant hosts and have developed extremely efficient mechanisms to cause an infection to grow, multiply and spread on living plants. Most of the fungi produce various kinds of spores which come in contact with plant tissue, germinate and penetrate into the plant during the infection process. After infection, fungi and oomycetes continue to grow, as mycelium in or on plant organs where they produce their new vegetative or generative, propagation or dormant structures. Nearly all fungicides used in agriculture today show their best effect if applied before the infection occurs. When present on the surface of plant organs, fungicides destroy fungal spores or suppress germination tubes, hyphae and other fungal structures. As a rule, control of fungal diseases with fungicides is aimed to prevent an infection and subsequent disease development and in such way the use of fungicides in plant protection differ from the use of antibiotics or antimycotics in medicine and animal health.

If the credit goes to fungicides that it enhances economic potential with increases production of vegetables and fruits by stopping fungal infections, it has also resulted in serious health implications to man and his environment. Several evidences tell us that these chemicals pose potential risk to humans and other life (Igbedioh, 1991; Forget, 1993; Aktar *et al*., 2009). Exposure of fungicides and its potential in reference to health effects is not completely protected by any population in environment (WHO, 1990). The worldwide deaths and chronic illnesses due to pesticide poisoning has estimated about 2 million per year (US EPA fact sheet Report, 2002). Fungicides residues reach into the aquatic environment and represents, as risk assessment for aquatic flora and fauna. Recent evidence indicates that fish which is the

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most important fauna are quickly becoming scarce. Via food chain, these residues enter in non-targeted animals and make threatening the ecological balance and the biodiversity of the nature (Rand and Petrocelli, 1984). Surface run-off fungicides through rain and by industrial disposal cause adverse effects by endocrine disruptors. Long-term exposure of chemicals induced immune-suppression, hormone disruption, diminished intelligence, reproductive abnormalities and cancer.

Mancozeb [[1, 2-ethanediyl bis-[carbamodithioate]] (2-)] manganese is dithiocarbamates fungicide have a similar action to carbamate insecticides. Carbamates are organic compounds derive from carbamic acid (NH_2COOH). In carbamate group, carbamate ester (e.g., ethyl carbamate) and carbamic acids are functional groups that are inter-related structurally and often are interconvert chemically. It is use to protect many fruit, nut and field crops from a wide spectrum of fungal diseases. The International Programmed on Chemical Saftey (IPCS) in USA, 2002 tell that the dithiocarbamates are mainly use in agriculture and form part of the large group of synthetic organic pesticides that have been developed and produced in large scale in the last 30-40 years (WHO, 2002). It affects the nervous system through its main metabolite, carbon disulfide (Singh and Srivastava, 2013). Dithiocarbamates used as fungicides, being effective against a broad spectrum of fungi and plant diseases caused by fungi.

Mancozeb is marketed by the trade names Dithane, Manzeb, Nemispot and Manzane. Mancozeb, if applied to soil will have a low mobility based on its high adsorption coefficient. If it is released into water, it will tend to adsorb to sediment and suspended solids. It has low soil persistence with a reported half-life of 1-7 days. Again, the primary concern with Mancozeb is its spontaneous degradation into a number of compounds, such as sulfur, 5, 6-dihydro-3 H-imidazol [2, 1-C]-1, 2, 4-dithiazole-3-thione, Ethylenethiourea (ETU) and Ethylenediamine (EDA) in the presence of water and oxygen. ETU has a persistence of 5-10 weeks. While Mancozeb is practically insoluble in water making, it unlikely to contaminate groundwater, its metabolite, ETU has the potential to be mobile in soils. ETU stable has high water solubility and is of particular importance because of its specific toxicity (Singh and Srivastava, 2013). ETU is one of the important residues in plants and in the environment following the agricultural use of Ethylene Bisdithiocarbamates (EBDCs). It is, also a metabolite formed when EBDCs ingested by animals and man. ETU is a stable compound with respect to hydrolytic reactions but easily oxidized to Ethyleneurea (EU) which is considerably more stable, than ETU and can be consider major breakdown products. In animals, ETU also degrade into urea, 2-imidazoline, glycine and oxalic acid. It also degrades into natural substances which affect protein and fat. For this reason, toxicological information of this compound has included in this study on fish *Clarias batrachus*.

MATERIALS AND METHODS

Chemicals: Mancozeb purchased from Syngenta India Ltd., India and all the other chemicals were of analytical grade and obtained from Indian commercial source.

Fish acclimatization: The fresh-water fish *Clarias batrachus* (total size 12-17 cm and weight 35-50 g) for adult and fingerlings (total size 6-8 cm and weight 9-12 g) were brought from local fresh-water pond. They were stored in laboratory tank containing 100 L of de-chlorinated tap water for 3 weeks and then acclimatized to the laboratory conditions for 72 h.

Toxicity testing: Toxicity test were performed by the method of Singh and Agarwal (1988). About 5 fishes were kept in glass aquaria containing 10 L de-chlorinated tap water. Fish exposed for 24-96 h to 4 different concentrations (10, 15, 20 and 25 mg L^{-1}) of pesticides in laboratory. Control fish were kept in similar conditions without any treatment. Each group of fish was replicated 3 times. Mortality was recorded after every 24 h. Dead animals were removed to prevent the decomposition of body in experimental aquarium. The effective doses (LC values, upper and lower confidence limits, slope value and heterogeneity) calculated by POLO PLUS programmed computed calculation probit log method of Russell *et al.* (1977). Product moment co-relation co-efficient was applied in between exposure time and lethal concentration Sokal and Rohlf (1973).

Experimental designing: Fishes exposed to 40% of 24 h LC_{50} doses (10.86 mg L^{-1}). Experiment was conducted from 24-72 h. After completion of treatment the test fishes were removed and washed with water and killed by severe blow on head and operated their liver and muscles were quickly dissected out in ice tray and used for biochemical and enzymatic analyses. Control fishes were kept in similar condition without any treatment. Each experiment was replicated at least 6 times and values expressed as mean±SE of 6 replicates. Following parameters were tested by different methods.

Biochemical estimation: Protein level estimated according to the method of Lowry *et al.* (1951) using

bovine serum albumin as standard. Estimation of total free amino-acids was made according to the method of Spices (1957). Estimation of DNA and RNA was performed by method of Schneider (1957) using diphenylamine and orcinol reagents, respectively. Glycogen was estimated by Anthrone method of Van der Vies (1954).

Enzymatic estimation: Lactic dehydrogenase estimated by the method of Anonymous (1984). Succinic dehydrogenase examine by the method of Arrigoni and Singer (1962). Protease examination has made by the method of Moore and Stein (1954). GOT and GPT by the method of Reitman and Frankel (1957).

Statical analysis: Two ways ANOVA and t-test were performed between control and tested group by the method of Sokal and Rohlf (1973). The significant level was 0.05, i.e., $p<0.05$ both for t-test and for ANOVA.

RESULTS

Toxicity results After treatment all the experimented fishes immediately settled down at the bottom of aquarium. Within 5-10 min, the breathing of fishes was affected and they came to the water air interface for air breathing, the respiratory impairment, probably due to the effect of the pesticides on gills and general metabolisms. After 30-60 min, their swimming activity is also slow down. During exposure with Mancozeb, the loss of equilibrium hypo and hyper activity and vertical position were seen after 48 h. Finally, their activity ceases and fishes died. LC values of Mancozeb for period ranging from 24-96 h on both fingerlings and adult fish *Clarias batrachus* are presented in Table 1. The toxicity in both the cases was time, as well as dose dependent. There was a significant negative correlation between LC values and exposure periods. Thus, with an increase in exposure period the values of LC_{50} of Mancozeb decreased.

Biochemical assay: Biochemical's changes in liver and muscles tissue presented in Table 2. Protein value decreases 87% in muscles and 82% in liver after 24 h while after 72 h the decreasment reached up to 70% in muscles and 62% in liver. Amino acids increase 110% in muscles and 118% in liver after 24 h. It also increases, as 113% in muscles and 118% in liver after 72 h. Level of Glycogen decreases 82 and 70% in muscles and liver, respectively

Table 1: Piscicidal activity of fungicide Mancozeb against different stages of fresh water fish *Clarias batrachus* at different time intervals

	Fingerlings		Adult fish	
Exposure periods (h)	Effective doses (mg L^{-1})	Slope	Effective doses (mg L^{-1})	Slope
24	LC_{50} = 27.174 (24.2-33.6)	7.72±2.22	LC_{50} = 28.594 (21.16-38.3)	3.21±0.94
48	LC_{50} = 21.845 (18.3-2.59)	5.49±1.62	LC_{50} = 19.008 (10.47-24.5)	3.23±0.99
72	LC_{50} = 16.406 (9.69-19.4)	4.90±1.66	LC_{50} = 15.919 (8.54- 20.3)	4.01±1.19
96	LC_{50} = 14.043 (7.34-16.6)	6.36±2.17	LC_{50} = 14.368 (6.89-18.4)	4.38±1.37

Batches of 15 fishes exposed to 4 different concentrations of the fungicides; Concentrations given are the final concentration (v/v) in the aquarium water containing de-chlorinated tap water; Values given in parenthesis are lower and upper confidence of LC values

Table 2: Changes in total protein (μg mg^{-1}), total free amino acids (μg mg^{-1}), glycogen (mg g^{-1}), nucleic acids (μg mg^{-1}) level and activity of protease (tyrosine/mg protein/h), LDH (pyruvate reduced/min/mg protein), SDH (μmoles dye/min/mg protein), GOT (μmoles pyruvate/mg protein/h) and GPT (μmoles pyruvate/mg protein/h) in different tissues of fresh water fish *Clarias batrachus* exposure to 40% of LC_{50} (10.86 mg L^{-1}) of Mancozeb at different time intervals

Parameters	Tissues	Control	24 (h*)	72 (h*)
Protein	Muscle	107.14±0.14 (100)	93.21±0.10 (87)	74.47±0.16 (70)
	Liver	94.20±0.10 (100)	77.24±0.14 (82)	58.40±0.12 (62)
Amino acids	Muscle	60.33±0.16 (100)	66.36±0.18 (110)	71.15±0.20 (118)
	Liver	68.22±0.12 (100)	77.08±0.12 (113)	80.47±0.20 (118)
Glycogen	Muscle	142.34±0.20 (100)	116.71±0.18 (82)	91.09±0.15 (64)
	Liver	182.20±0.20 (100)	127.54±0.16 (70)	109.32±0.14 (60)
DNA	Muscle	139.35±0.09 (100)	111.48±0.10 (80)	91.93±0.12 (66)
	Liver	129.21±0.12 (100)	100.78±0.15 (78)	82.65±0.14 (64)
RNA	Muscle	100.45±0.10 (100)	78.35±0.13 (78)	68.27±0.12 (68)
	Liver	93.20±0.12 (100)	70.83±0.16 (76)	60.59±0.10 (65)
Protease	Muscle	0.97±0.011 (100)	1.06±0.09 (110)	1.14±0.10 (116)
	Liver	1.03±0.015 (100)	1.27±0.17 (124)	1.40±0.15 (134)
LDH	Muscle	4.30±0.16 (100)	4.51±0.10 (105)	4.80±0.15 (111)
	Liver	7.35±0.12 (100)	8.08±0.14 (110)	8.52±0.12 (116)
SDH	Muscle	1.98±0.02 (100)	1.44±0.03 (73)	1.29±0.03 (66)
	Liver	2.01±0.05 (100)	1.30±0.04 (65)	1.23±0.09 (60)
GOT	Muscle	3.9±0.09 (100)	4.29±0.11 (110)	4.48±0.13 (115)
	Liver	2.58±0.07 (100)	3.01±0.20 (117)	3.12±0.15 (121)
GPT	Muscle	1.45±0.01 (100)	1.55±0.05 (107)	1.60±0.07 (112)
	Liver	1.87±0.06 (100)	2.20±0.10 (118)	2.28±0.12 (124)

Values given in parenthesis were percent change in parameters; *$p<0.05$ (significant value)

after 24 h and 64 and 60% in muscles and liver, respectively after 72 h. DNA decreases 80% in muscles and 78% in liver after 24 h and after 72 h, it becomes 66% in muscles and 64% in liver. RNA decreases 78% in muscles and 76% in liver after 24 h while after 72 h the decreasment reached up to 68% in muscles and 65% in liver.

Enzymatic assay: Enzymatic changes in liver and muscles tissue presented in Table 2. Protease value increases 110% in muscles and 124% in liver after 24 h while after 72 h the increments reached up to 116% in muscles and 134% in liver. LDH (Lactic Dehydrogenase) increase 105% in muscles and 110% in liver after 24 h. It also increases, as 111% in muscles and 116% in liver after 72 h. Level of SDH (Succinic Dehydrogenase) decreases 73 and 65% in muscles and liver, respectively after 24 h and 66 and 60% in muscles and liver, respectively after 72 h. GOT (Glutamic Oxalic Transaminase) increases 110% in muscles and 117% in liver after 24 h and after 72 h it becomes 115% in muscles and 121% in liver. GPT (Glutmic Pyruvic Transaminase) increases 107% in muscles and 118% in liver after 24 h while after 72 h the increscent reached up to 112% in muscles and 124% in liver.

DISCUSSION

Because of their constant and direct contact with the aquatic environment fishes are ideal indicators for behavioral assays of various stressors and toxic chemicals exposure (Schlenk and Benson, 2001; Srivastava and Singh, 2013). Behavior provides a unique perspective linking the physiology and ecology of an organism and its environment. Behavioral action is a sequence of quantifiable actions which operated through the central and peripheral nervous systems (Keenleyside, 1979) and the cumulative manifestation of genetic, biochemical and physiologic processes essential to life such as feeding, reproduction and predator avoidance. For the best meet of the challenge of surviving in a changing environment, behavior allows an organism to adjust to external and internal stimuli in order to adapted environmental variables. Selective evolutionary processes have conserved stable behavioral patterns in concert with morphologic and physiologic adaptations (Sancho *et al*., 2003). Since, behavior is not a random process but instead of it is a highly structured and predictable sequence of activities designed to ensure maximal fitness and Survival of the individual. Fish are able to uptake and retain different toxicants dissolved in water via active or passive processes. Sub-lethal concentrations of pesticides in aquatic environments cause structural and functional changes in aquatic organisms and this is more common than mortality. Behavioral modification is one of the most sensitive indicators of environmental stress and many affect survival (Byrne and O'Halloran, 2001). Alterations in fish behavior, particularly in non-migratory species can, also provide important indices for ecosystem assessment. In the present study, Mancozeb shows significant behavioral changes in fish (hyper movement, hypo movement, vertical position and loss of equilibrium). Toxicity data clearly indicate that the fingerlings are more susceptible than adult fish due to the dependence of age and body size. It has been demonstrated that ETU degradation leads to traces of EU and other metabolites in the urine and that 14C-carbon dioxide. It suggested that the metabolites of ETU in the fish produced primarily by fragmentation of the imidazoline ring and decarboxylation of the 4th and 5th carbon atoms. These fragments may incorporate with polypeptide chains and alter its fate. Reduction in level of protein in experimental fish under pesticide influence is indicates hepatic insufficiency and probably malnutrition. Protein reduction might observe, in the present study due to high-energy demand in TCA cycle. The protein is the next alternative source of energy to meet the increased energy demands. Reduction in level of protein in experimental fish under pesticide influence is indicates hepatic insufficiency and probably malnutrition (Srivastava and Singh, 2013). Protein reduction might be due to several pathological processes including renal damage, decrease in liver protein synthesis and even protein elimination in the urine. The fall in protein level during exposure may be due to increased catabolism and decreased anabolism of proteins. Decrease in protein content under toxicity stress has already been reported by Khare and Singh (2002). The decrease in total protein level and increase in free amino acids level in both tissue and liver suggest the high protein hydrolytic activity due to elevation of protease activity. Increase in free amino acids level was the result of breakdown of protein for energy requirements and impaired incorporation of amino acids in protein synthesis and decline in nucleic acids level (Srivastava and Singh, 2013). In the present study, increase level of amino acids and protease has observed. Prasanth *et al.* (2006), observed significant elevation in the levels of free amino acids and protease activity in the Indian major carp (*Cirrhinus mrigala*) in response to cypermethrin. Furthermore, inhibition of DNA synthesis, thus might affect both protein, as well as amino acid levels by decreasing the level of RNA in protein synthesis machinery. The regulatory roles of nucleic acid metabolism, as observed in the different animals when treated with the different pesticides (Das and Mukherjee, 2003). The metabolites of Mancozeb in ETU, EU and

natural products cause depletion of glycogen and fat in fish body. Carbohydrates are the chief source of energy. In stress conditions, its reserves levels depleted to meet energy demand of the fish. Depletion of glycogen in liver and tissue may be due to direct utilization for energy generation during acute hypoxia or physical physical disturbances in the fish (Srivastava and Singh, 2013). During stress conditions, the glycogen reserves are depleted to meet energy demand (Rawat *et al*., 2002; Tiwari and Singh, 2009). The freshwater fish, *Clarias batrachus* has been reported to exhibit significant reduction in the level of glycogen. Saha and Kaviraj (2009) studied effects of cypermethrin on various biochemical parameters. Similar results have been found in this study.

In this study, the level of LDH, GOT and GPT significantly increases under the effect of Mancozeb. Mancozeb has ability to modify the effect of several enzymes. These enzymes are blood soluble enzyme and best indicator of stress conditions (Das and Mukherjee, 2003). LDH may indicate changes and hypofunction of liver under the toxicants effects on the hepatocytes are in the form of tissue damage in which cellular enzymes are released from the cells into the blood serum (Srivastava and Singh, 2013). Increase level of LDH activity has observed in this study. In the present study, the activity of SDH has reduced. It is due to the mitochondrial disruption. SDH activity indicated anoxic hypoxic conditions when the fish was exposed to toxicant and it was possibly, leading to decrease in the activities of oxidative enzymes and an increase in the glycolic enzymes reported by Dubale and Awasthi (1984). Ethylenethiourea of Mancozeb has S-group and this group inhibit the ubiquinone binding site and prevent the transfer of electrons to electron transport system. In general, decrease in succinic dehydrogenase activity during fungicide stress was associated with the inhibition of mitochondrial respiratory mechanisms or derangement in ultra structure and permeability of mitochondria (Srivastava, 2014). In the present study, the level of GOT and GPT enhanced. It might be that GOT and GPT function at the junction between carbohydrate and protein metabolisms (Harper *et al*., 1978). Increase concentration showed probably elevation in gluconeogenesis, through transamination of glucogenic amino acids for energy demand in stress condition. However, some pesticides caused increased transaminase activity (GPT and GOT) levels in liver and muscle tissues. Begun (2004) found the activity levels of GPT and GOT increased in liver and muscle tissues of *Clarias batrachus* during exposed to carbofuran. Rao *et al*. (1989), also found that *Sarotherodon mossambicus* when exposed to sublethal and lethal concentrations of carbaryl showed adaptive elevation in the activity levels of GOT and GPT enzymes, particularly in liver and muscle.

CONCLUSION

The studies show that the fungicide (Mancozeb) which is widely used in crop fields has potential to damage fish physiology and ecology too. It is highly toxic for fingerlings and also adults. It causes severe biochemical and enzymatic alteration in fish. Therefore, researchers should be avoided extensive running off that fungicide in near water bodies.

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