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Haematological Assessment on Toxicity of Fungicide Hexaconazole on Post larvae of *Macrobrachium rosenbergii* (de Man)

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Abstract

The objective of the study was to determine the LC_{50} (96 h) of the fungicide Taqat (Captan, Hexaconazole) on post larvae of *Macrobrachium rosenbergii* (de Man). Prawns of average length 1.98 cm were exposed to taqat at concentrations of 0, 0.02, 0.04, 0.06 and 0.08 mg L^{-1}. The LC_{50} of Hexaconazole at 96 h was found to be 0.064 mg L^{-1}. Total haemocyte count (THC) of prawns in response to exposure to sublethal concentrations of taqat increased in test groups (7.87±5.96 to 1877.5±1622.3) were noticed. Significant changes in physiological and behavioural patterns were noticed. Differential haemocyte count (DHC) showed elevated agranulocytes (63.66±6.60 to 81.76±1.60) when compared to granulocytes (5±1.7 to 34.72±3.6) in experimental groups. Changes in blood clotting time (30.00±2.32 to 166.66±2.88) were also noticed between control and test groups indicating possible signs of stress in exposed animals. Present results demonstrate the risks involved in farming of *M. rosenbergii* near rice farms where these fungicides are routinely used.

Keywords: Fungicide; taqat; *Macrobrachium rosenbergii*; lethal toxicity

Introduction

Kuttanad, a low lying wetland area extending over 53, 639 ha, spreads in the districts of Alappuzha, Kottayam and Pathanamthitta of Kerala, South India. The region is considered as one of the most fertile lands of the world where rice is cultivated. Kuttanad region and its community were facing severe agrarian distress for the last five decades owing to a variety of factors (Swaminathan, 2007). There is no systematic pest control in the region and therefore, farmers indiscriminately apply pesticides in their farms. This unscientific and non-judicious application of pesticides was reported to be ineffective as well as unwanted as they caused severe damage to the Kuttanad ecosystem as highlighted Kuttanad Water Balance Study Project (KWBSP, 1990).

Study conducted in selected areas of Kuttanadu is recorded 130 species of plants, 67 species of phytoplanktons and 7 species of zooplanktons (Sreejith, 2013). The extensive food chains and rich biodiversity provide unique habitats for a wide range of flora and fauna (Narayanan et al., 2011). The water bodies in the area serve as nursery grounds for larvae and juveniles of major fishes and shellfish. The post larvae and juveniles of *M. rosenbergii* have been reported to be utilizing the polders adjacent to Vembanad lake as nursery grounds, where they feed upon rice grains and small worms (John, 1957; Raman, 1967). However, a heavy decline in its stock size to mere 39 t was reported during eighties (Kurup et al., 1992). The considerable stock depletion was attributed mainly to impact of man-made changes in its ecosystem such as habitat alteration, reduction in natural grow-outs, reclamation, pollution from agricultural effluents, recruitment over-fishing and also operation of a salinity barrier at Thanneermukkam (Kurup et al., 1992). Agrochemical effluents from extensive polders were also attributed to the dwindling stock of this species (Swaminathan, 2007).

Acute toxicity of organophosphate pesticides on *Channa marulius* was observed by Kurup et al. (1990) from Kuttanad wet land areas. Toxicity of pesticides like monocrotophos and 2,4-D in *Etroplus suratensis*

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were reported by Nair et al. (2000) and *Anabas testudineus* collected from Kuttanad waters by Mercy et al. (2001). Taqat or hexaconazole is a fungicide used widely in Kuttanad with chemical composition, captan (70%), hexaconazole (5%) and sodium salt of naphthalene (5%) as major constituents (Varghese, 2012).

Taqat is a systemic fungicide used for the control of many fungi particularly ascomycetes and basidiomycetes. It is a broad spectrum fungicide highly useful to prevent diseases on fruits and vegetables of various crops. Fungicide taqat is well suited for soil and seed borne diseases too (Kengar et al., 2014). It has been reported that taqat is highly toxic to fish and aquatic species which killed 50% of rainbow trout within 24 and 96 h at concentrations of 0.57 and 0.38 mg L^{-1} respectively (Boran et al., 2012). The aim of the study was to assess the effects such as alterations in behaviour, physiology and immune system in response to chronic exposure to sub-lethal levels of taqat by assessing the total haemocyte count (THC), differential haemocyte count (DHC) and blood clotting time in the surviving prawn.

Materials and Methods

The post larvae of freshwater prawn, *Macrobrachium rosenbergii* were procured from commercial hatchery at Azheekode, Kerala, India. The prawns were acclimatized in laboratory for 2 weeks before experiments. Five groups of prawns (6 nos. each) of mean size 1.98 cm were kept in glass aquaria (100 L capacity) containing 20 L of test solution comprising taqat at concentrations ranging from 0 to 0.08 mg L^{-1}. Temperature was maintained at 26-28°C and the prawns were fed with commercial diet (32% crude protein) twice daily at the rate 3% of body weight. The animals were examined every 12 h, and were considered dead if they did not respond to mechanical stimulation. The dead animals were removed and the cumulative percentage mortality for every 12 h was recorded. The 96 h LC_{50} value was calculated statistically by probit analysis (Finney, 1971) using SPSS 16 software. Sub lethal concentrations of taqat 0, 0.002, 0.004, 0.006 and 0.008 mg L^{-1} were prepared by adding calculated doses of stock solution prepared fresh every day. 50% of the water was changed every day and the study lasted for 4 days. During the experiment, water temperature was maintained at 26-28°C, pH at 6.52 ± 0.41, dissolved oxygen at 7.5 ± 0.25 mg L^{-1}, total hardness at 4 ± 2 mg L^{-1} and alkalinity at 6 ± 3 mg L^{-1} following standard methods (APHA, 1998).

Haemolymph were drawn at 24, 48, 72 and 96 h post-exposure to taqat using capillary tube of diameter 1.1 to 1.2 mm from the rostral sinus and the base of the second walking leg of the prawns. Total haemocyte count (THC) was determined by following the procedure of Huang et al. (2010), using the Neubaucer type's Haemocytometer (Pacific Path Surgi Co. New Delhi) by using an anticoagulant solution (10 ppt. trisodium citrate). The cells were counted under a light microscope of magnification 10 x and 100 x. Differential haemocyte count (DHC) was determined using a fixative and Wright stain. Twenty µl of haemolymph was drawn from each animal by a capillary tube pre-filled with 1 ml fixative and it was drawn into a microfuge tube on ice. The contents were stored at 4°C for 1 h and then centrifuged at 8000 rpm for 5 min. Thick smears were prepared from the concentrate on glass slides and stained with dilute Wright's stain of pH 7.2. From each slide haemocyte cells were observed under microscope (100 x) and the percentage was calculated (Mix & Spark, 1980). Haemolymph clotting time was assessed by following the procedure of Smith et al. (1995) using 25 ml of haemolymph inside a pre-cooled glass capillary tube of diameter 1.1-1.2 mm and length 75 mm.

Results and Discussion

Relationship between prawn mortality probability and taqat concentration (mg L^{-1}) after 96 h of exposure was indicated in Fig. 1. The LC_{50} value (96 h) estimated for taqat was 0.064 mg L^{-1}. High THC was recorded in prawns exposed to sub lethal concentrations of taqat in contrast to control. The mean values of THC in control and test organisms are given in Table 1. THC registered an increasing trend with 103.23 and 1877.5 x 10^{-6} cells per unit at taqat concentrations of 0.002 to 0.008 mg L^{-1} respectively as given in Table 1. THC in control was 7.87 ± 5.96. High mean DHC in agranulocytes (81.76% ±1.6) was observed in control group but not in prawns exposed (63.66% ± 6.60) to sub lethal concentrations of taqat. Low DHC in terms of granulocytes was observed in control group (5% ± 1.7) than in prawns exposed (34.72% ± 3.6) to sub lethal concentrations of taqat. The mean ± SD values (%) observed in control and test organisms are given in Table 1. Blood clotting time was prolonged in prawns exposed to sub lethal concentrations of taqat

than control group (Table 1). Blood clotting time recorded an increasing trend showing values of 101.66 ± 2.32 and 166.66 ± 2.88 sec for taqat concentrations of 0.002 and 0.008 mg L^{-1} respectively; whereas the control group showed low clotting time of 30.00 ± 2.32 sec. During the toxicity tests, prawns showed stress symptoms like escaping by jumping, crawling to walls and clinging on to net covering.

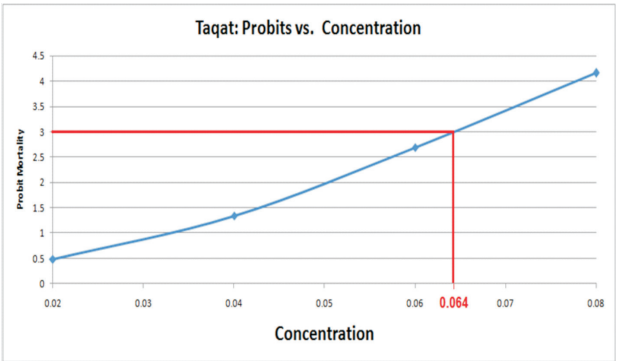

Fig. 1. Mortality of *M. rosenbergii* post larvae after 96 h exposure to taqat concentrations (mg L^{-1}) using probit analysis

In the present study, the prawns exposed to taqat showed LC_{50} value of 0.064 ppm. Ballantyne (2004) reported 96 h LC_{50} values for Hexaconazole as 5.94 mg L^{-1} in minor carp, 7.67 mg L^{-1} in rainbow trout and 5.4 mg L^{-1} in sheepshead minnow. LC_{50} values in *M. rosenbergii* in respect of endosulfan and malathion were 0.006 and 0.013 ppm respectively (Natarajan et al., 1992). Comparable values of LC_{50} have also been reported by Venugopal et al. (2003) in *M. malcolmsonii* for pesticides dichlorovos and monocrotophos as 0.02 ppm and 0.55 ppm respectively (KWBSP, 1990).

M. rosenbergii is a true denizen of Kuttanad Rivers and is known to support a very lucrative fishery in Vembanad Lake adjacent to Kuttanad wet land areas. The exploited stock of *M.rosenbergii* from Vembanad Lake and 5 km. stretch of the confluent rivers *viz.,* Pampa, Manimala, Meenachil and Muvattupuzha was quantified at annual average landings of 121.14 t (Harikrishnan & Kurup, 2003). In the present study, *M. rosenbergii* exposed to higher concentrations of taqat showed symptoms of severe distress and intolerance. Boran et al. (2012) found similar reaction in rainbow trout exposed to captan at 0.35 mg L^{-1} or higher concentrations where the fish exhibited signs of restlessness, erratic swimming, dark pigmentation, flashing, convulsions, increased respiratory rates and loss of body balance. It has been reported that pesticides cause neuromuscular disturbances due to the effect on acetylcholine esterase (AChE) an enzyme involved in the deactivation of acetylcholine at nerve endings, preventing continuous nerve firing, which is vital for normal functioning of sensory neuromuscular systems (Comoglio et al., 2005).

In the present study, THC values of prawn exposed to sub lethal concentrations of taqat were significantly higher than control. Elevated THC may be a strategy to mitigate the cause of stress (Bharghavan, 2008). Cheng (1988) reported that eastern oyster *Crassostrea virginica*, after 3 to 14 days of exposure to 1 mg L^{-1} cadmium showed increased THC and indicated that the increase was due to an increase in number of hyaline cells. Bharghavan (2008) observed similar change in green mussel *Perna viridis* exposed to the highest sub lethal concentration of copper (30 µg L^{-1}) showed increased THC. Auffret et al. (2006) reported an increase in THC in mussels collected from polluted stations. Mussels

Table 1. Mean ± SD of Total haemocyte count (THC), Differential haemocyte count (DHC) and Blood clotting time (Sec.) of *M. rosenbergii* post larvae exposed to sub lethal concentrations of taqat

Conc. (mg L^{-1})	Mean THC (10^6cells mL^{-1})	Mean DHC (%)		Clotting Time (sec)
		Small (Agranulocytes)	Large (Granulocytes)	
0	7.87 ± 5.96	81.76 ±1.60	5 ± 1.7	30.00 ± 2.32
0.002	103. 23 ± 18.1	73.00 ± 3.36	10 ± 2	101.66 ± 5.77
0.004	251. 12 ± 4.794	73.02 ± 7.60	12.9 ± 2	128.33 ± 17.55
0.006	775. 95 ± 298.4	66.66 ± 7.76	22.53 ± 2.8	146.60 ± 14.43
0.008	1877. 5 ± 1622.3	63.66 ± 6.60	34.72 ± 3.6	166.66 ± 2.88

exposed to heavy metals had elevated circulating haemocytes (Coles et al., 1994; Pipe et al., 1999). *Mytilus edulis* on exposure to fluoranthene, cadmium and temperature stress resulted in increased total cell counts (Cheng, 1988). Auffret & Oubella (1997) reported that increase in THC might be due to migration of haemocytes from interstitial tissues into circulation as a consequence of cell disturbance due to loss in adhesion properties. Elevated THC can also be due to haemopoiesis and proliferation of the cells as well (Pipe & Coles, 1995).

Mean DHC values recorded in the present study was comparable to values reported in other similar species. However, the majority of haemocytes observed were agranulocytes (78.35% ± 3.94%) compared to granulocytes. Similar observation was also made by Bharghavan (2008) who reported a drastic decrease of granulocytes in contrast to predominant increase of agranulocytes in green mussel *Perna viridis* exposed to copper and mercury. On the contrary, DHC in *Mytilus edulis* revealed a dominance of granular haemocyte, varying between 70 to 93% (Wootton et al., 2003). In the scallop *Chlamys ferreri,* the granulocytes of 14.43 μm size constituted about 66% of cells in the haemolymph (Xing et al., 2002).

As reported in other studies, blood clotting time in *M. rosenbergii* was significantly higher in experimental groups exposed to sub lethal concentrations of taqat in which the prawn survived (mean time 135 ± 26.95 sec) than in control group (30 ± 2.32 sec). Changes in number of circulating haemocytes and clotting time are considered as indicators of stress in crustacean (Smith et al., 1995; Jussila et al., 1999). It has also been reported that food intake, disease outbreak, pollutants and environmental stress also affect the quantity and quality of circulating haemocytes of crustaceans (Le Moullac & Haffner, 2000; Cheng & Chen, 2001).

The present study showed that low concentration of the fungicide causes severe stress, intolerance and mortality of the species. Taking into account the quantity of fungicide released into the Kuttanad waters, the problem of fungicide toxicity needs to be addressed immediately.

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References

APHA (1998) Standard method for the examination of water and wastewater, 20th edn., American Public Health Association, Washington DC, USA

Auffret, M. and Oubella, R. (1997) Haemocyte aggregation in the Oyster *Crassostrea gigas*: in-vitro measurement and experimental modulation by xenobiotics. Comp. Biochem. Physiol. 118A (31): 705-712

Auffret, M., Rousseau, S., Boutet I., Tanguy, A., Baron, J., Moraga D. and Duchemin, M. (2006) A multiparametric approach for monitoring immunotoxic responses in mussels from contaminated sites in Western Mediterranea. Ecotoxicol. Environ. Saf. 63(3): 393-405

Ballantyne, B. (2004) Toxicology of fungicides. Pesticide Toxicology and International Regulation. Chicester, West Sussex, England: Wiley. 249-261

Bharghavan, P.V. (2008) Haematological responses of green mussel *Perna viridis* to heavy metals copper and mercury. pp 22-23, Ph.D thesis submitted to Cochin University of Science and Technology, Kochi, India

Boran, H., Capkin, E., Altinok, I. and Ertugrul, T. (2012) Assessment of acute toxicity and histopathology of fungicide captan in rainbow trout's. J. Exp. Toxicol. Pathol. 64: 175-179

Cheng, T.C. (1988) In vivo effects of heavy metals on cellular defence mechanisms of *Crassostrea virginica:* Total and differential cell counts. J. Invertebr. Pathol. 51: 207-214

Cheng, W. and Chen, J. C. (2001) Effect of intrinsic and extrinsic factors on the haemocyte profile of the prawn *Macrobrachium rosenbergii.* Fish Shell fish Immunol. 11: 53-63

Coles, J. A., Farley, S.R. and Pipe, R.K. (1994) Effects of fluoranthene on the immunocmpetance of the common marine mussel *Mytilus edulis*. Aquatic Toxicol. 30: 367-379

Comoglio, L., Amim, O., Roque, A., Betancourt-Lozano, M., Anguas, D. and Haro, B. M. (2005) Evaluation of sub lethal biomarkers in *Litopenaeus vannamei* on food borne exposure to methyl parathion. Ecotoxicol. Environ. Saf. 62: 66-74

Finney, D.J. (1971) Probit Analysis. Third Edition, Cambridge University Press. London. 13-28

Harikrishnan, M. and Kurup. B.M. (2003) Temporal and spatial distribution of male and female morphotype *of Macrobrachium rosenbergii* (de Man) in Vembanad Lake. Indian J. Fish. 50(1): 11-19

Huang, J., Yang, Y. and Wang, A. (2010) Reconsideration of phenoloxidase activity determination in white shrimp *Litopenaeus vannamei*. Fish Shellfish Immunol. 28(1): 240-244

John, M.C. (1957) Bionomics and life history of *Macrobrachium rosenbergii* (de man). Bull. Cent. Res. Inst. Univ. Travancore, Trivandrum. Ser. 5(1): 93-102

Jussila, J., Paganini, M., Mansfield, S. and Evans, L.H. (1999) On physiological responses, haemolymph glucose, total haemocyte count and dehydration of *Cherax tenuimanus* to handle and transportation under simulated conditions. In: Freshwater Crayfish. 12: 154-167

Kengar, Y.D., Kamble, A.B. and Sabale, A.B. (2014) Effect of hexaconazole and triazophos on seed germination and growth parameters of spinach and gaur. Ann. Biol. Res. 5(5): 89-92

Kurup, B.M., Sebastin. T.M., Sankaran, N and Rabindranath, P. (1992) Fishery and biology of *Macrobrachium spp.* of the Vembanad Lake. In: Fresh Water Prawn (Silas E.G., Ed) pp 78-79, Kerala Agricultural University, Kochi, India

Kurup, B.M., Sebastin. T.M., Sankaran, N and Rabindranath, P. (1990) Exploited fishery resources of Vembanad lake. Final report presented to Indo-Dutch Co-operation programme. pp 144-145

Kuttanad Water Balance Study Project (1990) Draft final report, 62 p, The Netherlands Ministry of Foreign Affairs, Netherlands

Le Molluac, G. and Haffner, P. (2000) Environmental factors affecting immune response in Crustaceans. Aquaculture. 191: 121-131

Mercy, T.V.A., Kurup, B.M., Korath, A. and Nair, J. R. (2001) Acute toxicity of organophosphate pesticides on the juveniles of *Channa marulius*. Deraniyagala. Indian J. Fish. 48(2): 227-230

Mix, M. C. and Spark, A. K. (1980) Haemocytes classification and differential counts in the Dungeness Crab *Cancer magister*. J. Invert. Pathol. 35: 134-143

Nair, J.R., Mercy, T.V.A. and George, R.M. (2000) Individual and combined lethal toxicity of monocrotophos and 2, 4-D on the juveniles of *Etroplus suratensis* (Bloch). Fish. Technol. 37(2): 116-120

Narayanan, S.P., Thomas, A.P. and Sreekumar, B. (2011) Ornithofauna and its conservation in the Kuttanad wetlands, southern portion of Vembanad-Kole Ramsar site, India. J. Threat. Taxa. 3(4): 1663-1676

Natarajan, E., Biradar, R.S. and George, J.P. (1992) Acute toxicity of pesticides to giant freshwater prawn *Macrobrachium rosenbergii* (de Man). J. Aquacult. Trop. 7 (2): 183-188

Pipe, R. K., Coles, J.A., Carissan, F.M.M. and Ramanathan, K. (1999) Copper induced immunomodulation in the marine mussel *Mytilus edulis,* Aquat. Toxicol. 46: 43-54

Pipe, R.K. and Coles, J. A. (1995) Environmental contaminants influencing immune function in marine bivalve molluscs. Fish Shellfish Immunol. 5: 581-595

Raman, K. (1967) Observations on the fishery of giant fresh water prawn *Macrobrachium rosenbergii* (de Man). In: Proc. Symp. Crustacea. Mar. Biol. Assoc.India. pp 253-279

Smith. V.J., Swindlehurst, R.J., Johnston, P.A. and Vethaak, A.D. (1995) Distribution of host defence capability in the common shrimp *Crangon crangon* by exposure to harbour dredge spoils. Aquatic. Toxicol. 32: 43-58

Sreejith, K.A. (2013) Human impact on Kuttanad wetland ecosystem – An overview. Int. J. Sci. Environ. Technol. 2(4): 679-690

Swaminathan, M.S. (2007) Measures to mitigate agrarian distress in Alappuzha and Kuttanad wetland ecosystem. In: A study report by Swaminathan Research Foundation. 26-38

Varghese, S.A. (2012) Studies on toxicity of pesticides in Kuttanad on *Macrobrachium rosenbergii.* M.Phil. dissertation submitted to Cochin University of Science and Technology. Kerala, India

Venugopal, G., Narasimhacharyulu, V. and Venkateswaran, K. (2003) Acute toxicity of dichlorovos and monocrotophos and their effect on respiratory metabolism of *Macrobrachium malcolmsonii*. Indian J. Fish. 50(4): 461-464

Wootton, E.C., Dyrynda, E.A. and Ratcliffe, N.A. (2003) Bivalve immunity: comparison between the marine mussel *Mytilus edulis*, the edible cockle *Cerastoderma edule* and the razor-shell *Ensis siliqua.* Fish Shellfish Immunol. 15: 195-210

Xing, J., Zhan, W. B. and Zhou, L. (2002) Endoenzymes associated with haemocyte types in the scallop *Chlamys farreri.* Fish Shellfish Immunol. 13: 271-278