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Toxicity and Reproductive Effect of Combination Pesticide to *Daphnia magna*

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Abstract

Daphnia magna is ubiquitous link in ecological food chain; they are small planktonic crustacean used in ecotoxicological acute tests and chronic reproduction studies. Daphnia species are very sensitive to xenobiotics and very low concentrations will affect their survival. The objective is to investigate the toxicity and reproductive effect of *Daphnia magna* exposed to different concentrations of combination pesticide in M4 medium in accordance to OECD 211 guideline and in exposure water. To maintain the water hardness within the range in the exposure water as per the OECD guideline reverse osmosis water and drinking well water was blended 1:1 ratio. Twenty one days semi-static exposure was conducted with 10 daphnia held individually in each treatment and control. Daphnids were exposed to 0.0000134, 0.000134, 0.00134, 0.0134, 0.134, 1.34 and 13.4 µg/L of Chlorpyrifos 50% + Cypermethrin 5% EC. 10 µL of alga and yeast were fed with for daphnia of water and only alga was provided M4 medium during the semi-static 48 hour medium change. Agilent QQQ GC-MS/MS, Electron Impact Ionization mode was used for active content analysis. Stock solution of 13.4 µg/L was analyzed for its active content chlorpyrifos and cypermethrin. At 0 hour it was 89.7%, 87.5% and at 48 hour it was 85.3%, 72.4%, respectively. 0.0000134 µg/L was below limit of quantification level. Statistical analysis was performed with ECOSTATS program version 2012.06.03 (SAS Version 9.3, SAS Institute Inc., Cary, NC, USA, 2002-2010). EC50 calculated was 0.012 µg/L for M4 medium and 0.003 µg/L for exposure water at the end of 21 days. Parental mortality of daphnia and reproduction rate decline of off-springs collected in M4 medium and exposure water was found to be dose dependent. Significant change ($P < 0.05$) in off-spring reproduction was observed in M4 medium and exposure water by ANOVA Student-Newman-Keuls test. Based on the EC50 it is concluded that the pesticide is toxic to daphnids exposed in water than M4 medium exposure. Pesticide mixture effects and its interactions within mixtures is an area of concern to public and regulatory authorities, hence the combined actions of pesticides need to be addressed in the risk assessment process since mixtures of these chemicals may cause higher toxicity effects than those expected from the single compounds. Endocrine disrupting chemicals are compounds that alter the normal functioning of the endocrine system of both wildlife and humans and the tested pesticide active ingredient is considered as endocrine disruptor; no reliable data is available on the reproduction effect of the combination pesticide and this experiment throws light that exposure water can also be used to assess contamination of pesticide like M4 medium which is used regularly in ecotoxicological studies for pesticide toxicity assessment.

Keywords: Combination Pesticide, *Daphnia Magna*, Reproductive Effect, Toxicity

1. Introduction

Pesticide when applied to crop or used in household activities gets dispersed into the environment and contaminate the aquatic bodies. Adverse effect of the pesticide causes reduction viability and fertility. Organophosphate and synthetic pyrethroid are extensively used in developing countries contaminating

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waterways. Pyrethroids mode of action is based on the toxic pyrethrin which are persistent in the environment; they get attached to soil particles and washed into waterways on sediment. Contamination due to spill of toxic pesticide chlorpyrifos entered. It is a neurotoxic banned insecticide from residential applications in the U.S. after EPA determined serious adverse health outcomes for children[1]. Organophosphate impairs the nerves function consequently affects the normal mobility of organism. Chlorpyrifos is a chlorinated organophosphate insecticide widely in the world on a wide range of crops and also in non-agricultural areas. It is considered to be a priority pollutant in the European Water Framework as per Directive 2000/60/CE. Chlorpyrifos is an endocrine disruptor with antiandrogenic, oestrogenic and also shows effects on thyroid hormones[2]. Active substances under consideration are cypermethrin and chlorpyrifos as endocrine disruptor and insufficient data is available to determine whether they are potential endocrine disruptors[3]. The objective of this study is to assess the impact of the pesticide toxicity and its reproductive effect on the zooplankton *Daphnia magna*. Daphnia are widely grown since it is easy to culture as fish food. Adult female asexually lays parthenogenitcally eggs in its brood chamber and is located beneath the carapace. Embryos hatch after about one day and remain in the brood chamber for further development. Young daphnia off-springs are released through ventral flexion of the post-abdomen. It is the first ecotoxicological study that evaluates the toxicity of a combination pesticide to a *Daphnia magna* parent and off-springs.

2. Materials and Methods

Chlorpyrifos 50% + cypermethrin 5% EC was purchased from commercial market. 13.4 mg of the pesticide was weighed and dissolved in 100 mL of M4 medium and exposure water. From this stock solution, the required concentrations were prepared by serial dilutions. M4 medium was prepared as per OECD guideline[4] and exposure water prepared by mixing reverse osmosis water and well water mixed in a ratio of 1:1. The prepared exposure medium was kept under continuous aeration during the conduct of the experiment.

During culture brood daphnids were reared in 100 mL beaker containing 50 mL of M4 medium and exposure water. Chronic toxicity assay as per OECD guideline[5] was initiated with the third brood of neonates (≤24 h old). Prior to start of the test *Daphnia magna* less than 24 hours old was collected from the cultures maintained in M4 medium and exposure water. 10 Daphnids for each concentration of 0.0000134, 0.000134, 0.00134, 0.0134, 0.134, 1.34 and 13.4 µg/L, control group without pesticide was also maintained. Daphnia off-springs were collected and divided into eight groups. One off-spring in each glass beaker was maintained in semi-static exposure for 21 days. The medium was changed at the end of 48 hour during the experiment. During the medium change daphnids in M4 medium and exposure water were fed with 10 µL of alga (Pseudokirchneriella subcapitata). Exposure water was supplemented with 1 mL yeast solution. Off springs when released from brood daphnids were counted and removed from the exposure beaker. Photoperiod of 16 h light and 8 h darkness was provided using artificial fluorescent light (700-800 LUX) and it is controlled by an automatic timer. pH, temperature, conductivity and dissolved oxygen were analysed using instruments (Eutech pH Testr 30, LaMotte Series-CON 5-WC and Lovibond-Sensodirect Oxi 200) and total hardness by EDTA method[6]. On experiment completion the live daphnids and off-springs were euthanized by transferring it to buffered MS-222 (Tricaine methanesulfonate) solution approximately for 5 minutes before disposal.

Agilent QQQ GC-MS/MS equipped with Electron Impact Ionization mode with Mass Hunter software supplied by Agilent USA was used for system control and data acquisition. Chlorpyrifos and cypermethrin residues are separated using HP-5 MS fused silica capillary column (30 m length, 0.25 mm i.d. and 0.25 µm film thickness). Carrier gas was Helium at 1.8 mL/min, Injector and source temperature was 310°C with a split ratio of 1:5. Column temperature was maintained at 70°C. Sample Injection volume was 3.0 µL. Chlorpyrifos and cypermethrin had a retention time of 11.8 and 24.1. The medium was transferred to 250 mL separatory funnel and residues extracted with 2x25 mL of dichloromethane. Dichloromethane layer collected was transferred into a separate flask and evaporated by turbovap, the residues reconstituted with 2 mL hexane.

3. Result

In M4 medium the temperature range was between 19.5-20°C, pH in the range of 7.2-8.4, conductivity 400-500 µ siemens, hardness between 158-160 mg/L and dissolved oxygen between 7.61-7.85 mg/L. In exposure

water temperature was between 19.35-20°C pH 7.4-8.2, conductivity 600-702 µ siemens, 180-200 and dissolved oxygen ranged between 7.74-7.96 mg/L.

Linearity range was 1 µg/L to 500 µg/L for chlorpyrifos and cypermethrin. The limit of detection and quantification was 1 µg/L with 0.999 correlation co-efficient for chlorpyrifos and cypermethrin. 0.0000134 µg/L was below limit of quantification level for both chlorpyrifos and cypermethrin. For 13.4 µg/L chlorpyrifos active content recovered at 0 hour and 48 hour was 12.07 (89.7%) and 11.7 (87.5%) respectively. Cypermethrin active content recovered at 0 hour and 48 hour was 11.42 (85.3%) and 9.70 (72.4%) respectively.

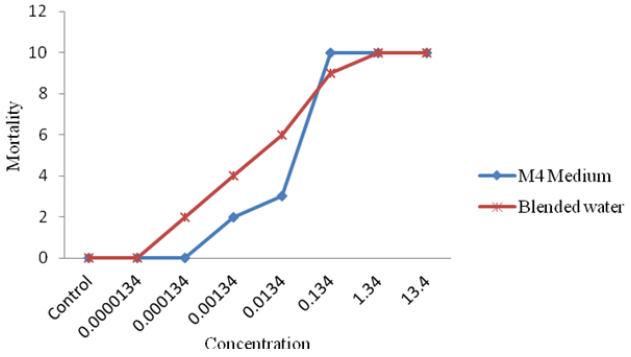

Figure 1. Concentration versus mortality of parent *Daphnia magna*.

No toxicity signs or immobilization, floating, changes in color were observed in control beakers throughout the experimental period of both M4 medium and exposure water. In the treatment beaker immobilized parent daphnids did not exhibit any toxicity signs. Further, immobilization of the parent daphnids were dose dependent in concentrations of 0.0000134, 0.000134, 0.00134, 0.0134, 0.134, 1.34, 13.4 µg/L. In M4 medium no immobilization was observed in 0.0000134, 0.000134 µg/L. 2, 3 daphnids were found immobile in 0.00134 and 0.0134 µg/L and all 10 parent daphnids were found immobile in 0.134, 1.34, 13.4 µg/L. In exposure water no immobilization was observed in 0.0000134 µg/L, 2, 4, 6, 9 daphnids were immobilized in 0.000134, 0.00134, 0.0134, 0.134µg/L. All 10 parent daphnids were found immobile in 1.34 and 13.4 µg/L (Figure 1). Statistical analysis was performed with ECOSTATS program and probit EC_{50} with 95% confidence limits value at the end of 21 days was calculated based on the Parent immobilization. For the M4 medium it was 0.012 µg/L with upper fiducial limit of 0.037 µg/L and lower fiducial limit of 0.005µg/L and for exposure water it was 0.003 µg/L with upper fiducial limit of 0.012 µg/L and lower fiducial limit of 0.0008 µg/L. ANOVA Student-Newman-Keuls test was used to calculate the significant difference $P<0.05$. Reduction in the reproduction rate is represented in Figure 2.

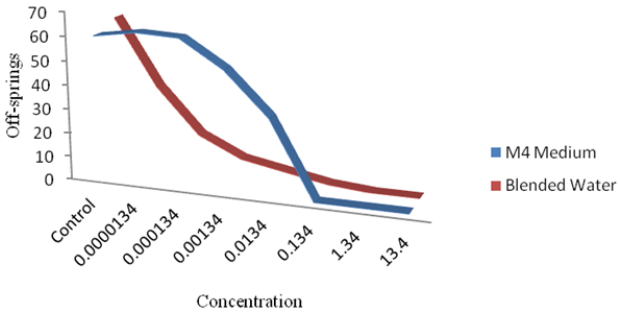

Figure 2. Concentration versus off-spring reproduction.

4. Discussion

Water fleas as primary grazers and primary forage represent ecologically important constituents of food webs[7]. Pulsed nature of agricultural runoff releases precludes drawing any conclusions about the effects of long-term average exposures[8]. It has been reported that neurotoxic, neonicotinoid insecticide has the potential to act specifically on the insect nervous system. Thus, indirectly cause lethality in the aquatic invertebrate population at low, sub-lethal concentrations by impairing movements and feeding[9]. The acute toxicity effects of chlorpyrifos formulation are greater than chlorpyrifos active ingredient and cypermethrin formulation toxicity is greater than cypermethrin active ingredient in *Daphnia magna* and all formulations are considered to be more potent[10].

Organophosphate and pyrethroid are considered not to cause any reproductive effects but it was observed that the pesticide combination had an impact on the number of off-spring production. Metabolism changes could potentially change population's sensitivity to xenobiotics that it has never encountered before, through altered metabolism of toxic compounds[11]. Nevertheless, organophosphorus insecticides synergise pyrethroid for which the carboxylesterases are the major route of metabolism through inhibition of the enzyme[12]. Chlorpyrifos formulation toxicity was greater than chlorpyrifos technical and cypermethrin formulation

was greater than cypermethrin a.i technical[13]. Effect of chlorpyrifos through maternal exposure to embryo has been studied and there was no reduction in the number of offspring per female at concentrations of 0.01and 0.03 µg/L. Significant reduction in the number of off-springs produced per female was observed above a concentration of 0.03µg/L. Moreover, exposure of maternal daphnids to 0.01 mg µg/L of chlorpyrifos over 21 days significantly increased the production of abnormal offspring[14]. *Daphnia magna* exposure to combination pesticide is observed with toxicity and a significant reduction of off-springs in lower concentrations and it is dose dependent. Moreover, no toxicity signs were observed hence feeding was not affected in the surviving daphnids in all the tested concentrations or in the control group.

Arthropods are more sensitive than fish, birds and mammals. This is due to increased sodium channel sensitivity, smaller body size and difference in metabolism, risk increasing with decreasing metabolic rate[15]. Cypermethrin is found to have a synergistic effect when mixed with other compounds. Despite the common occurrence of chemical mixtures only single compounds are considered in a majority of risk assessments[16]. Daphnia 96 hr effective concentrations for cypermethrin was reported as 0.00061 µg/L and No adverse effects on reproduction or survival for 20 days after a 24 hr exposure in concentration up to 1.9 µg/L. Chronic continuous exposure for 21 days of 7-day old Daphnia to cypermethrin significantly reduced the intrinsic population growth rate in a concentration dependent manner. Daphnia < 24 hr old neonates exposed to cypermethrin for 21 days caused significant, sub-lethal reproduction related problems, such as increased time to first brood, reduced brood size and reduced total brood number at 0.0002, 0.002 and 0.2 ng/L cypermethrin, but reported that the intrinsic population growth rate was not significantly affected[17]. Sub-lethal concentrations of 0.1, 1.0 and 10µg/L cypermethrin on the activity of physiological parameters of egg-carrying *Daphnia magna* was studied and even at low concentrations of 0.1 µg/L cypermethrin physiological mechanisms were found impaired[18]. The very low concentration of cypermethrin did not cause detectable and statistically significant toxic effects on reproduction[19]. In aquatic invertebrate cladoceran daphnia embryo lethality investigated was often masked by changes due to adult immobilization or number of offspring[20]. The level of a response of daphnia is related to the degree of contamination and time of exposure to toxics[21]. Sustainability of population should be supported by the success of its reproductive cycle[22]. Chronic bioassays are ideal tools to learn about survival in the changed environmental quality, while, reproduction revealed the response to the pollutants discharged[23]. Further work is required to develop a greater understanding of exposure to pesticide formulations in which more complex interactions occur.

5. Conclusion

It is concluded that the combination pesticide chlorpyrifos 50% + cypermethrin 5% EC caused reduction in the off-spring reproduction in both exposure water and M4 medium. Based on the obtained EC_{50} results the pesticide is observed to be more toxic in exposure water than M4 medium. Based on the data it can be considered as potential endocrine disruptor chemical and to simulate pond water it is suggested that exposure water can also be used for ecotoxicological studies to evaluate the toxicity of pesticides.

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7. Conflict of Interest

Potential conflicts of interest none.

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