Growth Inhibition and Recovery of Alga *Pseudokirchineriella Subcapitata* Exposed to a Combination Pesticide

Rajini .A[1], Revathy .K[2] and Chitrikha .T[3]

[1]Sathyabama University Chennai 600119
[2]Department of Advanced Zoology, Ethiraj College Chennai 600008
[3]International Institute of Biotechnology and Toxicology, Padappai 601301
E-mail: [1]rajinisathyabama@yahoo.com, [1]rajitox@gmail.com,
[2]reva63@rediffmail.com, [3]Chitrikha@gmail.com

Abstract—*The objective of this static algal bioassay is to assess the Chlorpyrifos 50% + Cypermethrin 5% EC pesticide effect on Pseudokirchneriella subcapitata. 1 × 10^4 cells per mL algae were inoculated into conical flask containing 100 mL of 0.0000134, 0.000134, 0.00134, 0.0134, 0.134, 1.34 and 13.4 µg/L concentrations followed by a 7 days recovery period. Growth rate inhibition and yield in the exposed concentrations was found to be dose dependent. Based on Yield 0.004 µg/L E_yC_{50} and based on growth rate 0.180 µg/L E_rC_{50} were statistically derived. At 0 hour Chlorpyrifos and cypermethrin recovery was 89.9%, 76.3% and at 72 hour it was 78.8%, 49.8%, respectively. No published data related to the toxicity is available. Based on the results it is observed that pesticide mixtures can affect freshwater alga of an ecosystem.*

Keywords*: Alga, Inhibition, Recovery, Growth rate, Combination Pesticide*

1. INTRODUCTION

Increased usage of combination pesticide has elicited extensive research into pesticide effects on non-target organisms. Algae are important in aquatic ecosystems and are primary producers. Unicellular algae in aquatic food chain are sensitive to a wide range of pollutants and are important for hazard assessment and environmental protection of aquatic ecosystem. Toxicity data from laboratory tests with single chemicals provide an essential input to scientific assessments of chemical risks to aquatic life. However, they are rarely exposed to only one single contaminant, but typically to mixtures of numerous manmade chemicals with varying constituents, ratios and concentrations [4]. Inhibition and recovery of aquatic phytoplankton alga exposed to the pesticide was studied based on OECD and OCSPP guidelines [14,15]. Exposure to mixtures of dissimilarly acting pesticides at concentrations considered environmentally acceptable can have significant effects on the biota. Chemical monitoring alone cannot assess the quality of water impacted by anthropogenic mixtures. Changes in the structure and productivity of the algal community may induce changes in the ecosystem and also affect water quality [8]. The combinations of an organophosphorus ester or a carbamate with either another organophosphorus ester or a synthetic pyrethroid have been identified for deviations from concentration addition [5]. Chlorpyrifos is one of the widely used pesticides for insect control in the world and is registered in about 100 countries worldwide to control insects that threaten agricultural food supply and houses. Cypermethrin is a synthetic pyrethroid which is also worldwide used extensively for pest control. Due to potential effects on aquatic ecosystems and usage synthetic pyrethroid have generated public concerns [6]. Number and types of esters present in organophosphate and pyrethroid and their stereochemistry regulates pesticide potency. Annihilation of natural populations of some organisms and noxious groups increase can cause an imbalance in the population dynamics thereby leading to serious problems in the ecosystem.

Microalgae non target organisms are affected by pesticides [12]. As primary producers, they are the key component of food chain in aquatic ecosystems [23]. Pesticide application for the plants and soil protection can cause adverse effects on aquatic ecosystems in areas nearby agricultural fields [22]. Direct effects of pesticide exposure are sometimes difficult to detect, nevertheless indirect effects are even more complicated to describe [9]. Humans and animals are exposed to complex and variable combinations of chemicals and exposures to each chemical may be below toxicity level. To envisage the toxicological properties of chemical mixtures, complete information on the composition of the mixture, the mechanism of action, potency of each compound, as well as proper exposure data is required[1]. No robust evidence is available that exposures to chemicals present at or below their zero effect levels is of environmental concern. Impact of chemical mixtures on human and ecosystem health has been highlighted

by the scientific community and brought to the attention of the European Commission concern [20]. Algae provide oxygen and organic substances to other life forms. It is widely used in ecological risk assessment in recent years to evaluate the impacts of metal, herbicide, xenobiotic contamination and bioavailability in aquatic systems [16]. Species sensitivity distributions are applicable to combination pesticides. The species sensitivity describes the variation to which group of different organisms is sensitive to the effects of the pesticide. On the basis of these species sensitivity distributions, concentrations safe for the environment is determined. However, a lot of data are required to determine the adverse effects of pesticides to aquatic organisms, which are usually not available. Cultures of algal species in exponential growth phase were exposed to the pesticide and the effect on growth rate for freshwater algae was assessed.

2. MATERIALS AND METHODS

Chlorpyrifos 50% + Cypermethrin 5% EC was purchased from commercial market and *Alga* obtained from Department of Ecotoxicology, IIBAT. Preculture was inoculated in OECD TG 201 medium and maintained for four days in growth cabinet with continuous illumination. Static exposures with three replicates per concentration along with six replicates for control were maintained.Test concentrations were prepared by serial dilutions. 100 mL of prepared test solution in OECD TG 201 medium was transferred into sterile 250 mL conical flask. Alga cells were observed and counted using Light microscope (Lawrence & Mayo Model: LYNX/LM-52-801). 1×10^4algal cells were inoculated under aseptic conditions in control and treatment conical flask Algae cells were inoculated to 0.00134, 0.0134, 0.134, 1.34 and 13.4 µg/L concentrations. At the end of the experiment algistatic effect was determined in all the concentrations exhibiting inhibition. 0.5 mL aliquots of test solution with growth inhibited algae were inoculated into a new test conical flask containing 100 mL fresh medium. Also the control alga was transferred to fresh medium simultaneously and these subcultures were incubated in the same test conditions. Once growth occurs the test will be discontinued. pH, temperature and Light intensity (LUX) were analysed using instruments (Eutech pH Testr 30 and Lutron LUX meter LX-101).

The average specific growth rate for exponentially growing culture was calculated using µi-j = (ln X j - ln X i)/ tj - ti x(day-1) where, µi – j is average specific growth rate from time i to j, Xi biomass at time i (0 hour), Xj is biomass at time j (72 hours), ti is time period (0 hour), tj is time period (72 hours). The percentage inhibition of average specific growth rate (% I_r) was calculated as, % $I_r = [(\mu_C - \mu_T)/ \mu_C] \times 100$. Where, % I_r = Percentage inhibition in average specific growth rate, µC is the mean value for average specific growth rate (µ) in the control group and µT is the average specific growth rate (µ) for the treatment replicate. The yield was calculated as $Y_{i-j} = X_j - X_i$. Where, Y_{i-j} biomass from the start of the test to the end of the test. Xi biomass (cells/mL) at time i (0 hour), Xj biomass (cells/mL) at time j (72 hours). The percent inhibition of the yield (% I_y) was calculated as, % Iy =[(YC - YT)/ Y_C] x 100. Where, % I_y = Percent inhibition of yield, Yc = Mean value for yield in the control group, Y_T = Value for yield for the pesticide treatment replicate. Statistical analysis was performed on the inhibitions of yield and growth rate, E_yC_{50} (0 - 72 h), E_rC_{50} (0 - 72 h) and their 95% confidence limits were calculated using software ECOSTATS Program Version 2012.06.03 (SAS VERSION 9.3, SAS Institute Inc., Cary, NC, USA, 2002-2010). The No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) for yield and growth rate were calculated by Dunnett test and Dunn's test, respectively using analysis of variance (ANOVA).

Stock solution 13.4 µg/L prepared in OECD TG 201 medium was analysed for stability of the pesticide in Agilent QQQ GC-MS/MS, Election Impact Ionization mode with Mass Hunter software, USA. Chlorpyrifos and cypermethrin residues were separated using HP-5 MS fused silica capillary column (30m length, 0.25mm i.d. and 0.25µm 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 residues extracted with 2x25 mL of dichloromethane and the dichloromethane layer was evaporated by turbovap and residues reconstituted with 2 mL hexane was analysed for active content.

3. RESULT

Initial pH of OECD TG 201 medium and test item concentrations was in the range of 8.01- 8.16 and the final pH measurement range in control was 7.58 and treatments was 7.15 - 7.57. The shaker incubator temperature was of 22.4 to 22.8°C and light intensity was in the range of 6824 to 6890 Lux. The alga cell observed for appearance was normal in control and all test item concentrations at 24, 48, 72 hours. Initial cell count in control flasks was 1×10^4 (10000) cells/mL. The average final cell count in the control flasks at 72 hours was 163×10^4 cells/mL. Maximum inhibition of yield in 13.4 µg/L was 95.9% and in 0.0000134 µg/L a minimum of 2.4% was recorded. Similarly, maximum inhibition of growth rate observed in 13.4 µg/L was 60.1% and minimum of 0.5% was observed at 0.0000134 µg/L and 13.4 µg/L. The inhibition of yield and growth rate based on cell count is represented in **Table 1 & 2**. Statistical analysis of inhibition based on yield, growth rate, NOEC and LOEC at 72 hours with different concentrations Chlorpyrifos 50% + Cypermethrin 5% EC are presented in **Table 3.** The percent coefficient of variation (% CV) for section by section specific growth rate in the controls (0 - 24 hours, 24 - 48 hours and 48 - 72 hours) was 29.47% (less than 35% as per the OECD guideline). The percent co-efficient of variation of average specific growth rate during the whole test period (0 - 72 hours) in replicate control flasks was 0.4% (less than 7% as per the

OECD guideline) which validates the results. Chlorpyrifos and cypermethrin active content at 0 hour in 13.4 µg/L stock was 89.9%, 76.3% at 72 hour it was 78.8%, 49.8%, respectively.

Table 1: Percent inhibition of Yield

Concentrations (µg/L)	Day 0	Day 3	Day 3 - Day 0	Iy (%)
Control	10000	1638333	1628333	-
0.0000134	10000	1600000	1590000	2.4
0.000134	10000	1380000	1370000	15.9
0.00134	10000	1176667	1166667	28.4
0.0134	10000	613333	603333	62.9
0.134	10000	216667	206667	87.3
1.34	10000	83333	73333	95.5
13.4	10000	76667	66667	95.9

Table 2: Percent inhibition of Growth Rate

Treatments (µg/L)	0h	72h	Ln 0 h	Ln 72 h	(Ln (72h) - Ln (0h))/3	Ir (%)
Control	10000	1638333.33	9.21	14.31	1.6996	-
0.0000134	10000	1600000.00	9.21	14.29	1.6917	0.5
0.000134	10000	1380000.00	9.21	14.14	1.6424	3.4
0.00134	10000	1176666.67	9.21	13.98	1.5893	6.5
0.0134	10000	613333.33	9.21	13.33	1.3721	19.3
0.134	10000	216666.67	9.21	12.29	1.0253	39.7
1.34	10000	83333.33	9.21	11.33	0.7068	58.4
13.4	10000	76666.67	9.21	11.25	0.6790	60.1

Table 3: Growth inhibition

Parameter	Toxicity end points	Toxicity end points results (µg/L)	95 % confidence limits(µg/L)		NOEC (µg/L)	LOEC (µg/L)
			Lower	Upper		
Based on Yield*	E_yC_{50}	0.004	0.002	0.008	0.0000134	0.000134
Based on Growth rate **	E_rC_{50}	0.180	0.139	0.221	0.0134	0.134

*Bruce &Versteeg Weighted model Fit by MARQUARDT for EC50/Dunnett for NOEC

** OECD Model 2 Fit by MARQUARDT for EC50/Dunn's for NOEC

4. DISCUSSION

According to REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) a European Union regulation by the year 2018, from 30 000 to 100 000 substances in total must have basic ecotoxicological information, including data concerning growth inhibition of aquatic plants and algae. Industrial chemicals, synthetic nano particles already used in various consumer products needs evaluation for health and environmental effects. Bioassays need to be included, based on the outcome of a risk assessment of the specific water body their selection should be made taking into account of the sources of pollutants such as agriculture, industry, household or hospital. Expected concentrations, methods cost, technical time, and concentration range applicability needs to be considered in bioassays [2]. Acute tests on microalgae, daphnids and fish are the common feature of all regulatory systems. In risk assessment of pesticides, assessment of individual substances is focused more than the joint toxic action of mixtures and recently much attention has been drawn to this issue. Organophosphate pesticides are potent inhibitors of photosynthesis and it has been demonstrated in laboratory experiments of Cyanobacteria [10]. Expired insecticides EC_{50} values were high indicating less toxicity when compared with unexpired; this might be due to less potent degraded products which may be formed from the active ingredients or impurities [19]. Comet assay was used to study the genotoxic effects of insecticides chlorpyrifos to *Pseudokirchneriella subcapitata* and it was reported that higher concentration of chlorpyrifos affected the measured cell viability and exerted genotoxic effect when compared to fungicide Tebuconazole [17]. The quality standard according to Water Framework Directive to protect the aquatic Ecosystem against any adverse effects from short-term exposure for groundwater standards that are currently applied for pesticides are 0.1 µg/L for any individual compound and 0.5 µg/L for the sum of all individual pesticides detected [3]. The concept of concentration addition for describing the joint effect of pesticides on aquatic organisms, although from a theoretical point of view may be invalid when dealing with mixtures of compounds with dissimilar modes of action. Many studies are available with herbicides toxicity to algae very few studies are available for organophosphate and pyrethroid toxicity. Blue green algae *Spirulina platensis* was exposed to Chlorpyrifos and cypermethrin pesticide. Chlorpyrifos exposure resulted in significant reduction of growth rate above 5 ppm and for cypermethrin significant reduction was observed at 10 ppm. Maximum acceptable toxicity concentration was calculated with LOEC and NOEC values and it was estimated at 0.707, 7.07 ppm for chlorpyrifos and cypermethrin [11].

Chlorpyrifos and cypermethrin formulations effects on pesticides on the local aquatic ecosystems should be considered. There is little information available about the effects of formulations on microorganisms in spite of their ecologic importance. It is very important to have information about the ecotoxicity of insecticides towards algae in order to explore combined effects with others agrochemicals products such as herbicides and fungicides. Degradation products of pyrethroids have been documented as more toxic to bacteria, fungi and algae. Cypermethrin commercial formulation caused

algicidal effects. Recovery from toxic exposure could be seriously affected and algal population in aquatic ecosystem would not be able to recover after a short exposition to the commercial formulation [7]. Dose response of cyanobacteria *Microcystis wesenbergii* was investigated to the exposure of organophosphate pesticide chlorpyrifos and dichlorvos generally considered non-toxic to plants and algae and the exposure caused growth inhibition [21]. Multispecies algal culture was exposed to chlorpyrifos and its detrimental effect to its population growth rate has been observed and was found to decrease [13]. Similarly, in this experiment under laboratory condition inhibitory and recovery effect of combination pesticide to the tested algal species reveals the toxic effect of the pesticide.

5. CONCLUSION

Algae play important role in the protection of aquatic ecosystem and Algal bioassays are extensively used in environmental risk assessment. It is concluded that for holistic characterization of water quality and ecological assessment freshwater algae can be adopted. The combination pesticide tested at various concentrations had inhibitory effects on the yield and specific growth rate of *Pseudokirchneriella subcapitata*.

6. ACKNOWLEDGEMENT

The author thanks IIBAT management for facility.

7. CONFLICT OF INTEREST

Potential conflicts of interest none

8. REFERENCES

[1] Combined toxic effects of multiple chemical exposures, Norwegian Scientific Committee for Food Safety 06/404-6 final, 2008. www.vkm.no/dav/15ec300082.pdf.

[2] Carvalho, R. N., Arukwe, A., Ait-Aissa, S., Bado-Nilles, A., Balzamo, S., Baun, A., Lettieri, T. (2014). Mixtures of chemical pollutants at European legislation safety concentrations: how safe are they?. Toxicological Sciences, 141(1), 218-233. 10.1093/toxsci/kfu118.

[3] European Union, 2006. Directive 2006/118 of the European Parliament and of the Council of 12 December 2006 on the protection of groundwater against pollution and deterioration. Off. J. Eur. Comm. L372, 1–19.

[4] Faust M., Altenburger R., Backhaus T., Blanck H., Boedeker W., Gramatica P., Hamer V., Scholze M., Vighi M., Grimme L.H.2003. Joint algal toxicity of 16 dissimilarly acting chemicals is predictable by the concept of independent action. Aquatic Toxicology 63 (2003) 43- 63

[5] John W Deneer (2000) Toxicity of mixtures of pesticides in aquatic systems Pest management Science Volume 56, Issue 6, pages 516–520

[6] Luo, Y., and Zhang, M. (2011). Environmental modeling and exposure assessment of sediment-associated pyrethroids in an agricultural watershed. PLoS ONE 6(1):e15794. Doi:10.1371/journal.pone.0015794.

[7] María Elena Sáenz, Walter Darío Di Marzio, Jose Luis Alberdi 2012 Effects of a Commercial Formulation of Cypermethrin used in Biotech Soybean Crops on Growth and Antioxidant Enzymes of Freshwater Algae IJEP Vol.2 No.1 PP.15-22

[8] Nyholm N., Petersen H. G., 1997. Laboratory bioassays with microalgae. Chapter 9. Wang W., Gorsuch J. W. and Hughes J. S. (eds), Plants for Environmental Studies. Lewis Publishers, Boca Raton, FL.

[9] Nina Akerblom 2004 Agricultural pesticide toxicity to aquatic organisms - a literature review http://info1.ma.slu.se/IMA/Publikationer/internserie/2004-16.pdf

[10] Nafisa Shoaib, Pirzad Jamal Ahmed Siddiqui, Halima Khalid 2012: Toxicity of chlorpyrifos on some marine cyanobacteria species pak. J. Bot., 44(3): 1131-1133

[11] Nidhi Raj Yadav, Sunita Sharma (2013): Toxic Effect of Organophosphate, Pyrethroids and Organochlorine Pesticides on *Spirulina Platensis* Growth Rate. Volume 2, Issue 6, pages 286–287

[12] Neha Chaurasia 2014. Effect of Locally Used Fungicide Mancozeb And Insecticide Profenofos In Rice Fields Of Meghalaya On The Soil Microflora Int J Pharm Bio Sci 2014 July; 5(3): (B) 1049 – 1060

[13] Naik Ayaz A., Wanganeo A. (2014) Occurrence of some pesticides in Bhoj wetland Bhopla and their effect on phytoplankton community: An ecological perspective. Journal of Toxicology and Environmental Health Sciences, Vol. 6(9), pp 170-180

[14] Organization for Economic Co-operation and Development, (2006) OECD Guidelines for the Testing of Chemicals. “Freshwater Alga and Cyanobacteria, Growth Inhibition Test”, OECD Guidelines for the Testing of Chemicals. No.201, Adopted 23rd March, 2006.

[15] OCSPP 850.4500 (2012) Algal Toxicity. http://www.epa.gov/test-guidelines-pesticides-and-toxic-substances/series-850-ecological-effects-test-guidelines

[16] Qian Haifeng, Jingjing Li, Liwei Sun, Wei Chen, G. Daniel Sheng, Weiping Liu, Zhengwei Fu 2009: Combined effect of copper and cadmium on Chlorella vulgaris growth and photosynthesis-related gene transcription. Aquatic Toxicology 94 (2009) 56–61

[17] Ricardo Santiago Martinez, Walter Dario Di Marzio, Maria Elena Saenz Genotoxic effects of commercial formulations of Chlorpyrifos and Tebuconazole on green algae. Ecotoxicology. January 2015, Volume 24, Issue 1, pp 45-54

[18] Stauber J.L. 1995. Toxicity Testing Using Marine and Freshwater Unicellular Algae, Australasian Journal of Ecotoxicology Vol. 1, pp 15-24, 1995.

[19] Satyavani G., Chandrasehar G., Krishna Varma K., Goparaju A., Ayyappan S., Neelakanta Reddy P., BalakrishnaMurthy P. 2012 Toxicity Assessment of Expired Pesticides to Green Algae *Pseudokirchneriella subcapitata* International Scholarly Research Network Volume 2012, Article ID 247072, 10 pages doi:10.5402/2012/247072

[20] SCHER, SCCS, SCENIHR, Opinion on the Toxicity and Assessment of Chemical Mixtures, 2012. http://ec.europa.eu/health/scientific

committees/environmental_risks/docs/scher_o_155.pdf [Accessed on 25.02.2015].

[21] Sun KF, Xu XR, Duan SS, Wang YS, Cheng H, Zhang ZW, Zhou GJ, Hong YG 2015: Ecotoxicity of two organophosphate pesticides chlorpyrifos and dichlorvos on non-targeting cyanobacteria Microcystis wesenbergii. Ecotoxicology. 2015 Oct; 24(7-8):1498-507.

[22] Vagi M.C., Kostopoulou M.N., Petsas A.S., Lalousi M.E., Rasouli Ch., Lekkas T.D (2005) Toxicity of Organophoshorous pesticides to the marine alga Tetraselmissuecica. Global NEST Journal, Vol 7, No 2, pp 222-227

[23] Villem Aruoja 2011: Algae *Pseudokirchneriella Subcapitata* in Environmental Hazard Evaluation of Chemicals and Synthetic Nanoparticles. Ph.D thesis Estonian university of life sciences. www.geoeco.ut.ee/1090551