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Evaluation of acute and its sub lethal toxicity effects of Oxygold on certain freshwater ciliates *Oxytricha fallax*and *Blepharisma intermedium*

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ABSTRACT
The present investigation was performed to study the responses of certain selected freshwater ciliates such as Oxytricha fallax and Blepharisma intermedium against Oxygold which is a widely used herbicide in India. The acute toxicity tests were conducted for 3 hours and lethal & sub lethal concentrations were calculated against mortality rate by using probit analysis for further studies. The obtained LC_{50} values of Oxytricha fallax and Blepharisma intermedium against Oxygold was found to be 89.12µg/ml and 85.11µg/ml respectively. Depletion in contractile vacuole and food vacuole formation was observed in concentration dependent manner. The nuclear changes leading to DNA damage were noticed using feulgen fast green technique and the changes were very significant. Number of nuclear aberrations was observed in Oxytricha (61.4±0.84%) at 35µg/ml in comparison with Blepharisma (57±0.67 %) at 32µg/ml. Based on the findings, the contractile vacuole and food vacuoles are highly responsive to the environmental changes; therefore, they should be recommended as one of the parameters in toxicity evaluation. It is further concluded that macronucleus aberration test of Oxytricha fallax and Blepharisma intermedium could be used as a potential biomarker in assessing carcinogenicity and genotoxicity of pesticides.
Keywords: freshwater ciliates, genotoxicity, necrosis, Oxygold, phagocytosis, pulsatory activity, toxicity.

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INTRODUCTION

Zooplanktons are the most important components of the freshwater bodies and act as a link between primary and secondary consumers of food chain. In a freshwater aquatic ecosystem among the different zooplankton, protozoan ciliates are the most sensitive indicators of chemical pollution. These microscopic eukaryotes exist either individuals or colony which feeds on certain bacteria, organic material and debris [1]. The protozoa are also called as "Single celled animals"; perform all the physiological functions in an efficient manner which is also characteristics of all higher-grade animals [2]. Any member of phylum Protozoa are worldwide in distribution [3]. These microscopic animalcules have the ability to move, have a nucleus with cytoplasm and perform reproduction by fission or encystment [4]. The ciliates have also been used as experimental tools to determine toxic effects of natural food dyes, acute effects of pesticides, also for physiological processes, and swimming behaviour from contaminated wastewater. [5, 6 & 7]. In spite of all the above advantages, literature on ciliates in toxicity bioassay studies is limited when compared with those of other organisms [8 & 9].

According to FAO [10], million tonnes of pesticides are being used in agriculture in many countries. The worldwide use of pesticides in 2006-2007 was estimated to be about 2.4 megatons, herbicides constituting about 40%, insecticides 17% and fungicides 10%. Due to the indiscriminate use of various pesticides, there is an increased disruption of environmental balance which effects a series of abiotic and

biotic processes involve in degradation, leaching and runoff leading to contamination of soil, surface and groundwater near to the agricultural fields. Therefore, the studies on environmental risk assessment of these pollutants are essentially recommended [11 & 12].
In view of this, the present study is designed to evaluate the toxicity of Oxyglod using three freshwater ciliates namely *Oxytricha fallax* and *Blepharisma intermedium.*

MATERIAL AND METHODS

Oxygold: It is a Nitro diphenyl ether herbicide applied to control weeds (wine grapes, almonds, and cotton crops) and has great verity of applications. Oxygold disrupt permeability of the cell membrane in the presence of light and cause bio chemical changes leading to death of the plant. This herbicide inhibits haem biosynthesis (plays an important role in haemoglobin, myoglobin and various cytochromes) in animals [13 & 14]. Due to the persistence and toxicity of Oxygold, concerns exist regarding its potential to contaminate the environment and impact on non-target organisms. It is highly baneful to aquatic plants and fish, and moderately to highly toxic to aquatic invertebrates [15].

Selected organisms: Freshwater ciliate *Oxytricha fallax* was collected from different water-logged areas of Osmania University Campus, Hyderabad, Telangana, India. Microbial culture of *Blepharisma intermedium* was purchased from Carolina Biological suppliers, NC, USA. The experimental cells were sub cultured and maintained for further studies.

Preparation of culturing medium: Culturing and growing of experimental cells was done by using Hey infusion media [16]. To 1 liter of boiling distilled water 6 – 8 grams of hay was added and boiled for 30 minutes, cooled at room temperature and filtered using whatman filter paper. Then the filtrate was sterilized in an auto clave for 15 minutes for 15 ponds. Later the auto calved hey filtrate was kept open in the laboratory for 24 hours without any lid. For culturing ciliates, hey infusion media was diluted in the ratio of 1:1andcultures were maintained at room temperature (25 $\pm 2^0$C). In order to induce ciliate multiplication boiled wheat grains were added to culture media and Log phase cultures were used throughout the experimentation.

Test solutions: The test solutions were prepared according to recommendations given by APHA [17]. Stock solution of Oxygold 1000µg/ml was prepared by using distilled water and required sub lethal concentrations were prepared by using stock solution.

Acute toxicity studies: Acute toxicity studies were conducted for 3hours to measure quick cell responses, such as changes swimming pattern, cell motility and cytopathological deformities under pesticide stress as suggested by Apostol [18] and the experiments were repeated thrice. 0.5 ml of known concentration of Oxygold solution was added to 4.5 ml of culture medium containing about 50 organisms. Observations were made and counting was done for every 10min during the first hour and after 20 min interval for next two hours. Total breakdown of the cell was taken as the lethal point (LC_{100}). Quick cytopathological responses were recorded at different concentrations to determine the LC_{50} value by plotting a graph [19]. The data obtained from acute toxicity studies was important in establishing the relative toxicity of pesticides as well as in providing information to carry out further experiments.

Pulsatory vacuole activity: Pulsatory vacuoles are the sub cellular organelles found commonly in all fresh-water ciliates, flagellates, amoebae, and some marine organisms that release excess fluid from cytoplasm [20] hence plays an important role in osmoregulation in ciliates [21 & 22].The test organisms were subjected to different sub-lethal concentrations of Oxygold for 15 minutes, then the single individual organism was taken, and the pulsation rate of contractile vacuole was counted, Equal number of observations was done in control cells. Rate of pulsation is described as the time needed for one complete pulsation i.e., from one contraction to the next. As the ciliates move faster, immobilization of the cells was done by using protamine coated slides, as the test organisms appeared not to be harmed by this procedure [23].

Food vacuole activity: Experimental cells were divided into two groups, 1) Treated cells and 2) Control cells. About 25 organisms were exposed to different sub-lethal concentrations of Oxygold for an hour, to identify the number of food vacuoles formed. Treated organism from each concentration were picked with the help of micropipette, mixed with India ink and kept for 10 minutes. 10 organisms from each concentration were taken, immobilised and number of food vacuoles formed was recorded. Control cells with same molar concentrations of India ink were run simultaneously.

Nuclear aberration study: Nuclear aberration studies were carried out by using the method Feulgen fast green [24] staining technique in the test species. Nuclear studies give an immediate qualitative picture of nuclear changes that have been induced by the pesticide. The organisms were treated with different sub lethal concentrations of Oxygold for one-hour. Carnoy's fixative was used for cell fixation. The treated organisms were first briefly hydrolysed in 1N HCL at 27^0C temperature and washed in distilled water,

slides were transferred into Schiff's reagent and incubated for 1hr.Schiff's reagent was prepared as suggested by De Tomasi [25]. The organisms were immersed in three changes of sodium bisulphate solutions, again rinsed with water, dehydrated in graded alcohol, cleared in xylene and mounted with DPX. The exposed cells have exhibited various macronuclear changes like rod shape, vacuolated, unevenly divided, fragmented macronucleus and karyolitic forms.

RESULTS AND DISCUSSION

Acute toxicity studies

A chemical would be considered acutely toxic when test organisms are killed instantly by its direct action or relatively short-term exposure. In present studies' acute effects of test organisms were evaluated when exposed to different concentrations of Oxyglod for 3hrs. From the experiments it was calculated that lethal concentration of the treated organisms was168µg/ml for *Oxytricha* and 90µg/ml for *Blepharisma* and the LC_{50} value obtained in trials with Oxygold to *Oxytricha fallax* and *Blepharisma intermedium* against mortality curve was foundto be 89.12µg/ml and 85.11µg/ml respectively, which were represented in graph1and 2. Among the tested ciliates *Blepharisma* was found to be sensitive to Oxyglod, while *Oxytricha* was relatively tolerant. Worked out sublethal concentrations for further studies were for *Oxytricha* 20µg/ml, 25µg/ml, 30µg/ml and 35µg/ml and *Blepharisma intermedium* were 17µg/ml, 22µg/ml, 27µg/ml and 32µg/ml.Acute toxicity tests were carried out to observe the immediate cytopathological responses in ciliates the body size, shape and ultra structural deformities were observed in ciliates when exposed to different concentrations of Oxygold. The Oxygold has significantly enhanced the velocities of exposed ciliates many folds in concentration and time dependent manner, may be due to the effect of the pesticide on the cellular metabolism. Behavioural and structural changes such as erratic movement, changes in swimming pattern, loss of coordination and hyperactivity were observed under pesticide stress due to sudden changes in pH & osmotic gradient of the media. Certain cells were swollen and disorganized due to damage to cell membrane and mitochondria, similarly treated organisms showed alteration in their shape, structure, damage to cilia, narrowing of anterior end, oval/round shaped body, blebbing of cell, movement of nucleus to cell periphery, blacking of cytoplasm, oozing of internal contents, complete disintegration of the organisms at different higher concentrations. The acute effect of pesticides on cell membrane may be primarily altering its absorbency allowing entry of fluids into the cell until it leads to lysis. Similarly behavioural changes were observed in *Daphnia magna, Palaemonetes pugio* [15] and in fishes *Oreochromis niloticus, Gambusia affinis* exposed to different concentrations of Oxyfluorfen [26]. Similar results were also reported by various authors [27 & 28] in different experimental ciliates with different

Alterations of pulsatory vacuole activity in *Oxytricha fallax* and *Blepharisma intermedium when* treated with various sublethal concentrations of Oxygold

Pulsatory activity of individuals were performed after exposing the organisms to various sublethal concentrations of Oxygold for 15min and was compared with untreated cells. Pulsatory activity in *Oxytricha* at each concentration, with Mean & SD values was recorded as 3.5±0.35, 2.7±0.27, 2.1±0.65, & 1.9±0.54 against 20µg/ml, 25µg/ml, 30µg/ml and 35µg/ml respectively. The pulsatory vacuole activity of *Blepharisma* to different concentrations was found to be 2.8±0.44at 17µg/ml, 1.7±0.67at 22µg/ml, 1.3±0.44at 27µg/ml and 0.9±0.65at 32µg/ml. A significant variation in contractile vacuole activity under different concentrations was recorded with P value 0.00 which was significant at 5% level and it was concentration dependent. The organisms treated with higher concentrations resulted in slowing down of their pulsatory vacuole activity. The rate of pulsations may reflect the ionic balance in the culturing medium [29]. The pesticide present in the culture medium might have caused damage to the structure and mechanism of the contractile vacuole complex, thereby affecting the frequency of vacuole contractions and perhaps leading to arrest of expulsions. The rate of contraction may reflect the ionic balance in the medium. According to Rouabhi et al [30]. cells consume oxygen levels to make the cell more hydrophilic and this can be explained by detoxification mechanism whereas this mechanism is blocked in the organisms exposed to higher concentration of pesticides. So, there will be reduction in consumption of oxygen thereby decreasing the pulsatory activity. Similarly dose dependent reduction in vacuolar activity was observed in *Paramecim Sp*. & *Tetrahymena.* exposed to different toxicants such as Delfin (25, 50 and 100µg/ml), Carbofuran (100,115 and 135µg/ml), Carboxylate, Dimethoate (0.5 and 1 mg/ml), Cypermethrin (0.05, 0.5,1 and 2µg/l), Cycloxydim (3,6 and 9mg/l) and Novaluron (1,10 and 20µg/ml) by Amanchi & Hussain,[31,32,33 & 34]. An alteration in osmotic pressure of culture medium which is induced by the toxicant causes the osmotic imbalance. Observations from this study indicates that rate of pulsation in freshwater ciliates gets altered by the changes in the external environment of the organism.

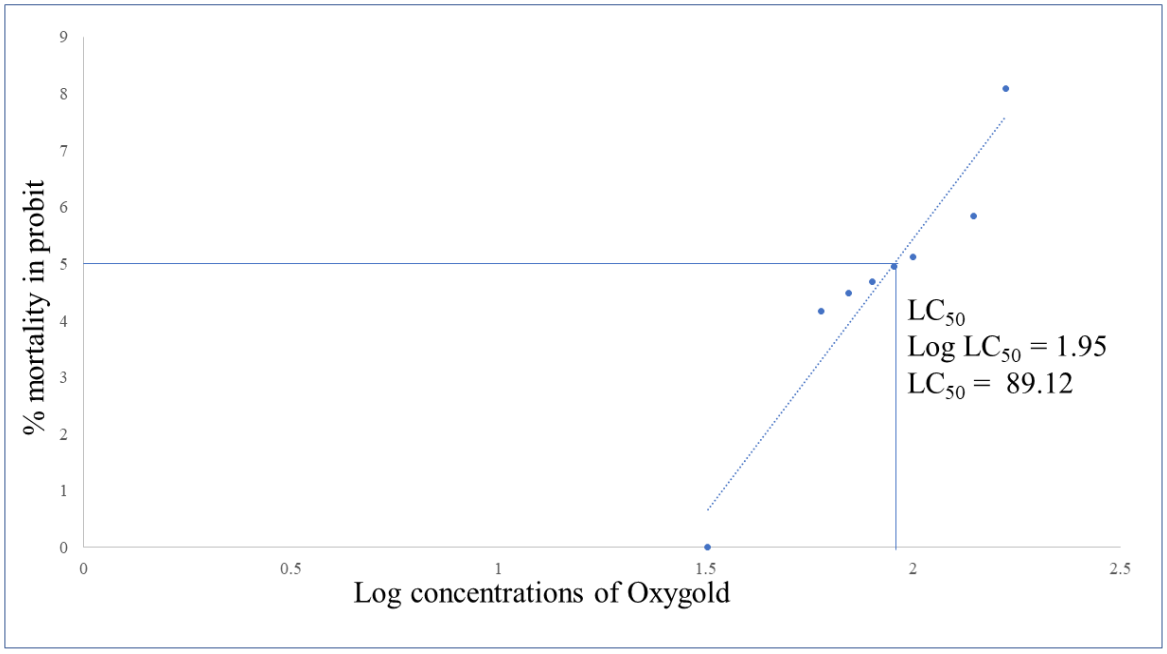


Fig 1: Showing Log LC_{50} Value, *Oxytricha fallax* exposed to different concentrations of Oxygold

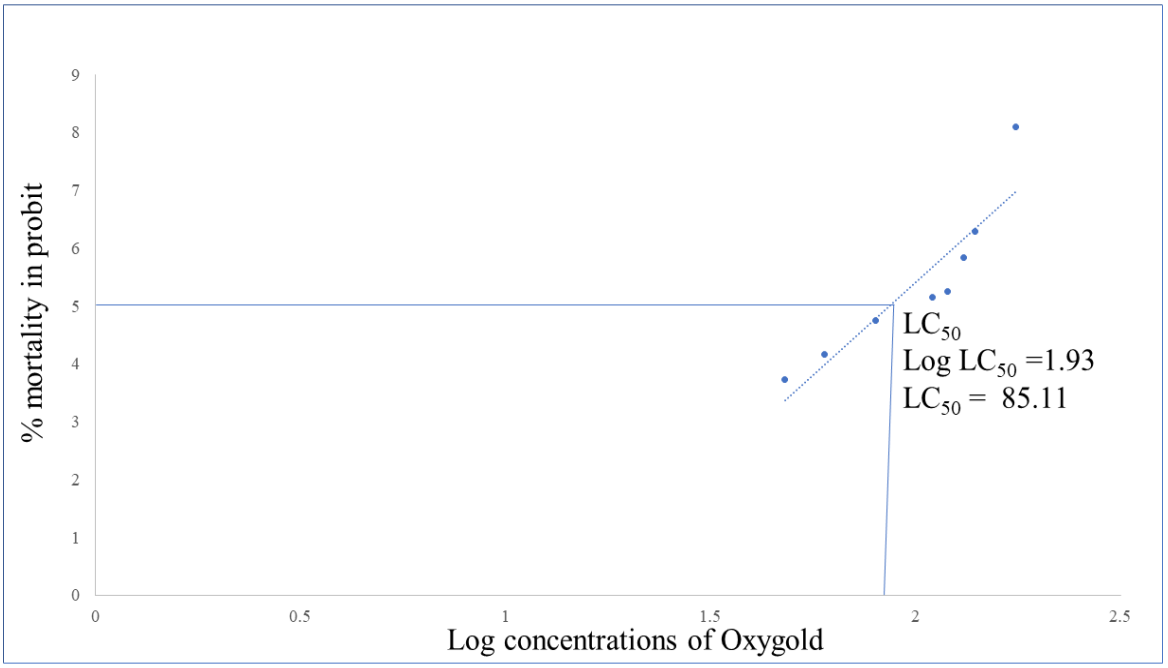


Fig 2: Showing Log LC_{50} Value, *Blepharisma intermedium* exposed to different concentrations of Oxygold

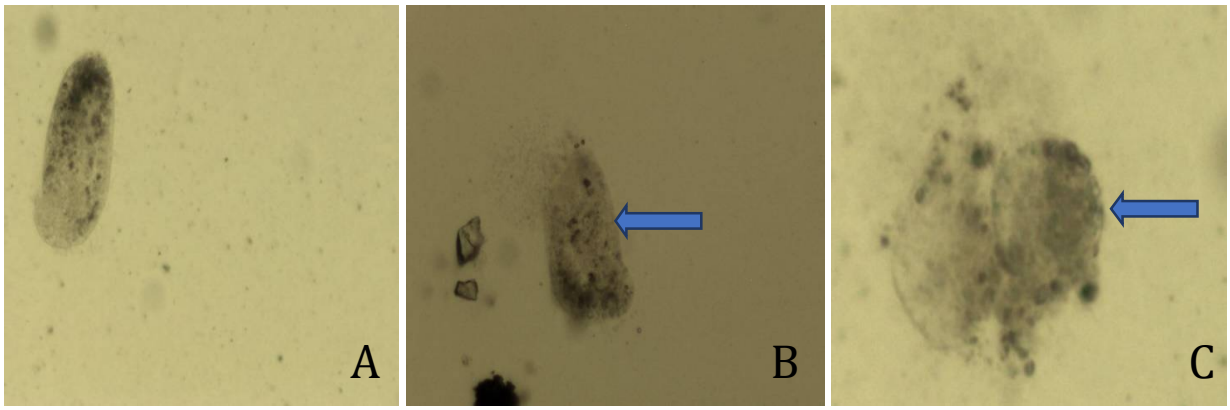
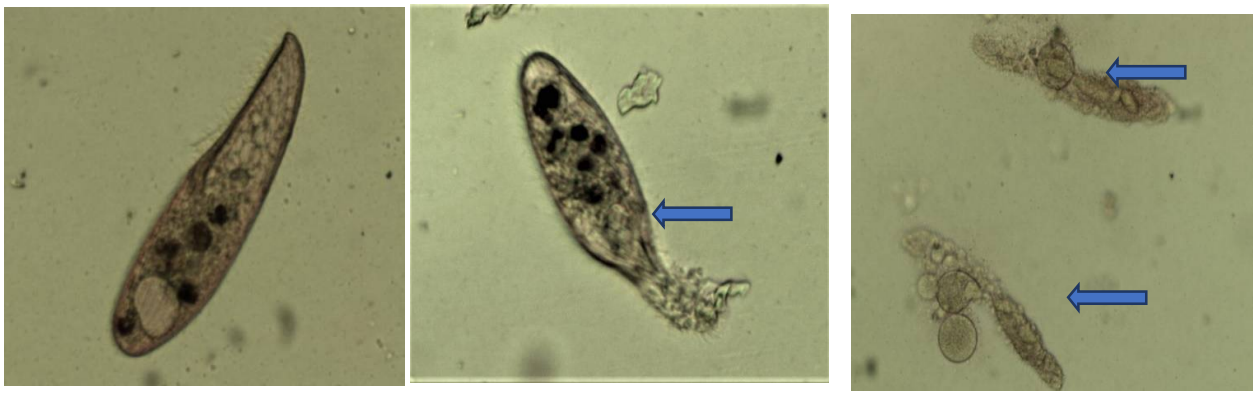


Fig 3 : Cytopathological deformities of *Oxytricha fallax*exposed to different concentrations of Oxygold (100X). A-Control, B-Irregular deformation, C-Total disintegration



A **B** **C**

Fig 4: Cytopathological deformities of *Blepharisma intermedium* exposed to different concentrations of Oxygold (100X). A-Control, B-Damage to cytostome region C-Complete disintegration of the cell.

Table:1. One way ANOVA showing pulsatory vacuole activity in *Oxytricha fallax,* when treated with various sublethal concentrations of Oxygold

Conc.	No. of observations	Mean value	Std. D	Std. Error	95% Confidence Interval for Mean		Mini mum.	Maximum
					Lower limit	Upper limit		
20µg/ml	5	3.5000	.35355	.15811	3.0610	3.9390	3.00	4.00
25µg/ml	5	2.7000	.27386	.12247	2.3600	3.0400	2.50	3.00
30pp	5	2.1000	.65192	.29155	1.2905	2.9095	1.50	3.00
35µg/ml	5	1.9000	.54772	.24495	1.2199	2.5801	1.50	2.50
Control	5	3.8000	.44721	.20000	3.2447	4.3553	3.00	4.00
Total	25	2.8000	.87797	.17559	2.4376	3.1624	1.50	4.00

Table: 2. One way ANOVA Showing pulsatory vacuole activity in *Blepharisma intermedium,* when treated with various sublethal concentrations of Oxygold

Conc.	No.of observations	Mean value	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum.	Maximum.
					Lower limit	Upper limit		
17µg/ml	5	2.8000	.44721	.20000	2.2447	3.3553	2.00	3.00
22µg/ml	5	1.7000	.67082	.30000	.8671	2.5329	1.00	2.50
27µg/ml	5	1.3000	.44721	.20000	.7447	1.8553	1.00	2.00
32µg/ml	5	.9000	.65192	.29155	.0905	1.7095	.00	1.50
Control	5	4.0000	.70711	.31623	3.1220	4.8780	3.00	5.00
Total	25	2.1400	1.27083	.25417	1.6154	2.6646	.00	5.00

Table: 3. One way ANOVA showing assay of phagocytic activity in *Oxytrichafallax* on treatment with different sublethal concentrations of Oxygold for 1hour

Conc.	No. of observations	Mean value	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum.	Maximum
					Lower limit	Upper limit		
Control	10	6.6000	.69921	.22111	6.0998	7.1002	6.00	8.00
20µg/ml	10	4.7000	.67495	.21344	4.2172	5.1828	4.00	6.00
25µg/ml	10	4.1000	.73786	.23333	3.5722	4.6278	3.00	5.00
30µg/ml	10	3.2000	.63246	.20000	2.7476	3.6524	2.00	4.00
35µg/ml	10	2.5000	.52705	.16667	2.1230	2.8770	2.00	3.00
Total	50	4.2200	1.55563	.22000	3.7779	4.6621	2.00	8.00

Table: 4. One way ANOVA showing assay of phagocytic activity in *Blepharisma intermedium*on treatment with different sublethal concentrations of Oxygold for 1hour

Conc.	No. of observations	Mean value	Std. D.	Std. Error	95% Confidence Interval for Mean		Minimum.	Maximum.
					Lower limit	Upper Limit		
Control	10	7.7000	.67495	.21344	7.2172	8.1828	7.00	9.00
17µg/ml	10	5.7000	.48305	.15275	5.3544	6.0456	5.00	6.00
22µg/ml	10	4.2000	.63246	.20000	3.7476	4.6524	3.00	5.00
27µg/ml	10	3.0000	.66667	.21082	2.5231	3.4769	2.00	4.00
32µg/ml	10	2.1000	.56765	.17951	1.6939	2.5061	1.00	3.00
Total	50	4.5400	2.09187	.29584	3.9455	5.1345	1.00	9.00

Table: 5. One way ANOVA showing Oxygold induced nuclear abnormalities in *Oxytricha fallax* treated for one hour

Conc.	No. of observations	Mean value	SD	Std. Error	95% Confidence Interval for Mean		Minimum.	Maximum.
					Lower limit	Upper limit		
20µg/ml	10	29.5000	.52705	.16667	29.1230	29.8770	29.00	30.00
25µg/ml	10	36.7000	.67495	.21344	36.2172	37.1828	36.00	38.00
30µg/ml	10	47.0000	.81650	.25820	46.4159	47.5841	46.00	48.00
35µg/ml	10	62.4000	.84327	.26667	61.7968	63.0032	61.00	64.00
Control	10	3.0000	.47140	.14907	2.6628	3.3372	2.00	4.00
Total	50	35.7200	19.95612	2.82222	30.0485	41.3915	2.00	64.00

Table: 6. One way ANOVA showing Oxygold induced nuclear aberrations (%) in *Blepharisma intermedium* exposed for one hour

Conc.	No. of observations	Mean	SD	SE	95% Confidence Interval for Mean		Min.	Max.
					Lower Bound	Upper Bound		
17µg/ml	10	20.4000	.51640	.16330	20.0306	20.7694	20.00	21.00
22µg/ml	10	37.1000	.87560	.27689	36.4736	37.7264	36.00	38.00
27µg/ml	10	42.0000	.66667	.21082	41.5231	42.4769	41.00	43.00
32µg/ml	10	57.0000	.66667	.21082	56.5231	57.4769	56.00	58.00
Control	10	3.3000	.48305	.15275	2.9544	3.6456	3.00	4.00
Total	50	31.9600	18.68751	2.64281	26.6491	37.2709	3.00	58.00

Food vacuole activity in *Oxytricha fallax* and *Blepharisma intermedium when* treated with various sub lethal concentrations of Oxygold

The results of our study showed that, organisms treated with different sub lethal concentrations of Oxygold showed significant reduction in formation of the food vacuoles, the mean number of vacuoles recordedwere,*Oxytricha*4.7 ± 0.67 at 20µg/ml, 4.1 ± 0.73 at 25µg/ml, 3.2 ± 0.63 at 30µg/ml and 2.5 ± 0.52 at 35µg/ml and *Blepharisma* 5.7 ± 0.48 at 17µg/ml, 4.2 ± 0.63 at 22µg/ml, 3.0 ± 0.66 at 32µg/ml and 2.1± 0.56 at 37µg/ml (Fig. 7, 8 & 9), with respect to control value, indicating that the defecation rate is higher than the uptake rate. The experiment was performed at room temperature with one-hour exposure to

various sub lethal concentrations of Oxygold. Test organisms were fed with India ink, which were clearly visible under Olympus binocular microscope. Since this stain correspond to the size of food vacuole, the numbers of vacuoles containing stained particles were counted to calculate Mean number of food vacuoles and represented against concentration. One-way ANOVA was performed to test the significance of data generated from different exposed and control groups, where p value is < 0.05, which was significant at 5% level. Ossipov et al [35] stated that, system of food recognition seems to be quite complex in protozoa when compared to other organisms. Movement of cilia and mobility of organism attribute and stimulate food vacuole activity under toxicant stress as suggested by Nilsson [36]. The rate of food vacuole activity is directly proportional to activity of cilia, acidity, temperature, etc. The physico-chemical quality of the food affects the uptake rate, this clearly shows that surface alterations influence ingestion while others do not, earlier studies provided evidence that adding proteins, polypeptides and RNA stimulated ingestion rate in protozoa [37].When concentration of pesticide increases the movement of cilia become weaker and irregular, hence the formation of food vacuoles also decreases. Some times to avoid toxic conditions, the organism undergo starvation for few minutes which results in inhibition of phagocytic activity and it is one kind of adaptive strategies in protozoan ciliates. Similar results were reported by Park [38] in different flagellates and ciliates. In the present experiment, a significant decrease in food vacuole formation was observed with increasing concentration of Oxygold. The rate of food vacuole formation is automatically influenced by factors influencing the ciliary action. It has been proved that the ciliary motility assure the movement and development of food vacuoles in the organism.

Oxygold induced nuclear abnormalities (%) in *Oxytricha fallax* and *Blepharisma intermedium* treated for one hour

The treated groups of *Oxytricha*and *Blepharisma* were showed various nuclear changes including vacuolated, fragmented, unevenly divided and karyolysis.The total nuclear abnormalities in treated group of *Oxytricha* were recorded with Mean and SD 29.5±0.53% at 20µg/ml, 36.7±0.67% at 25µg/ml, 47±0.82% at 30µg/ml and 61.4±0.84% at 35µg/ml. The total number of nuclear aberrations (%) recorded in *Blepharisma* at each concentration was 20.4±0.52 at 17µg/ml, 37.1±0.88 at 22µg/ml, 42±0.67 at 27µg/ml and 57±0.67 at 32µg/ml.One-way ANOVA was done for total percent abnormality forms showing significant difference between mean scores of concentrations, the calculated p-value is 0.05 which was significant at 5%level. Nuclear aberrations test is the most commonly used procedures of cytogenotoxicity bioassay studies. The present experiment showed significant changes in the size, shape and general morphology of nucleus in all the treated organisms under pesticide stress leading to chromatin condensation and decrease in size along the longitudinal axis of the macronuclei. Rod shaped, marginalization, unevenly divided, vacuole formation, fragmentation, karyolysis, and total disintegration of nucleus were observed with increasing concentration of Oxygold. Hussain et al., [29] observed similar macronuclear abnormalities such as fragmented, unevenly divided and vacuolated nucleus in *Paramecia* upon exposure to various concentrations of Carbofuran (100, 115 and 135µg/ml). Similar results were reported in other ciliates exposed to different pesticides and heavy metals. **[** 39,40,41 & 42]. The metabolism of Oxygold might have resulted in free radicals production, interacting with the nucleophilic sites of DNA leading to breaks and other damages in ciliates. Extrusion of nucleus and hypertrophy due to pesticide stress on micro tubular material in test species, has suggested that these forms degenerative in nature leading to death.

CONCLUSION

The present findings could be useful to ascertain safer concentrations of Oxygold to various non target organisms including ciliates in freshwater environment and protection of micro zooplankton. It is concluded that *Blepharisma intermedium* was found to be sensitive and *Oxytricha fallax was* relatively tolerant. The contractile vacuoles are highly responsive to the environmental changes, therefore it should be recommended as one of the parameters in toxicity evaluation of water bodies. It is further concluded that macronucleus aberration test of species could be useful as potential biomarker in assessing carcinogenic and genotoxic effect of the certain pesticides.

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