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Combination effects of dimethoate and atrazine on pigment fluorescence of *Anabaena doliolum* Bhar: Prediction of toxicity

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ABSTRACT

Effects of an organophosphorus insecticide dimethoate (O,O-dimethyl-S-(N-methyl carbamoylmethyl) phosphorodithioate) and herbicide triazine (2-chloro-4-ethylamino-6-isopropylamino -S-triazine) were measured separately and also in combination on growth and pigment fluorescence of the cyanobacterium *Anabaena doliolum* with 4 days exposure. Both the pesticides reduced the growth and chl-*a* content of the cultures but enhanced the fluorescence from phycobilisomes (PBS), PS II and PS I. PS II fluorescence at 580 nm excitation was increased more than that of PBS and PS I and atrazine more effectively accelerated PS II fluorescence than that of dimethoate. The EC_{50}s were 33.17±1.85 µM and 11.94±0.83 µM for dimethoate and atrazine, respectively, determined from a significant first order polynomial relation between PS II fluorescence and pesticide concentrations. The predicted EC_{50}s, calculated from concentration addition equation, ranged between 12.76 and 28.16 µM and the observed EC_{50} in combination of the pesticides ranged between 12.62±0.93 µM and 28.74±1.39 µM. It was found to have additive effects in the combination.

1. Introduction

Pesticides and herbicides have become the integral parts of modern agricultural systems. The registration of pesticides for agricultural use worldwide is based on the ecological risk assessment of chemicals applied alone, even though the protection of crops against pests is known to be achieved by the administration of many pesticides together or in succession. On the other hand, it is impossible to test every possible combination of pesticides at all possible concentration levels for prediction of their environmental toxicity. Information on the possible interactive effects of pesticide mixtures have been gained from several single-species work in the laboratory (Mohapatra and Mohanty, 1992; Mohapatra and Schiewer, 1996; 2000; Panda *et al.*, 1998; Backhaus *et al.*, 2004; Dong *et al.,* 2009; Jena *et al*., 2012).

Atrazine is a triazine herbicide widely used for the control of weeds and grasses in crops (Mehlera *et al.*, 2008; Dong *et al.*, 2009). It also causes damage to the gill epithelium and kidney, and increases the renal excretion of sodium, chloride and proteins in the rainbow trout (Fisher-Scherl *et al.*, 1991) and carp (Neskovic *et al.*, 1993). The herbicide is known to decrease the photosynthetic efficiency and accelerate pigment fluorescence of *Anabaena doliolum* and has many other interactive phytotoxicity (Van den Brink, *et al.,* 2009; Nayak and Mohapatra, 2011; Moore and Locke, 2012). Dimethoate is a conventional organophosphorus insecticide widely used to control a variety of pests on agricultural and animal farms. Researchers have demonstrated that organophosphorus insecticides significantly affect the health and safety of animals (Farag *et al.*, 2003; Tian *et al.*, 2005; Ali *et al.*, 2009) and plants (Mohapatra and Mohanty, 1992; Mohapatra *et al.*, 1997;

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2010; Pandey and Gopal, 2012). The residues of the pesticides have also been reported in surface and ground water (Miller *et al.*, 1999; Du Preez *et al.*, 2005; Banks *et al.*, 2005; Murphy *et al.*, 2006; Van den Brink *et al.,* 2009).

Effects of combined application of atrazine and other chemicals have shown additivity, synergism as well as antagonism (Anderson and Zhu, 2004; Fu *et al.,* 2013; Xing *et al.,* 2013). Atrazine and lindane combination increased abundance of the phytoplankton taxa *Cyclotella* sp. at the highest treatment level, which was due to synergistic effects on the microinvertebrate population. Dimethoate, malathion, parathion and chlorpyrifos has imposed synergistic effects on the toxicity of atrazine to many micro-invertebrates and animal species via the inhibition of acetylcholinesterase (AChE) (Cometa *et al.*, 2007; Choung *et al.,* 2011; Fu *et al.,* 2013). However, there is no report of the combination effect of atrazine and dimethoate to cyanobacteria. The present paper attempts to reveal if at all there is synergism of atrazine and dimethoate as reported in case of microinvertebrates and animals, in the cyanobacterium *Anabaena doliolum* Bhar.

2. Materials and methods

The filamentous and heterocystous cyanobacterium *Anabaena doliolum* Bhar. was maintained in non-absorbent cotton stoppered 500 ml borosilicate conical flasks containing 250 ml BG-11 medium (Rippka *et al.*, 1979) spiked with micronutrients. Actively growing cells were harvested and diluted with fresh medium 2 hours before inoculation to an initial inoculum density of $0.5x10^6$ cells/ ml. All stock and experiment cultures were grown in a culture room at 25 ± 2^0 C under continuous illumination with white light fluorescent tubes providing an irradiance of 40 $\mu E/m^2$ s and were hand shaken twice daily to keep the cultures under active phase of growth.

The commercial formulation of atrazine (2-chloro-4-ethylamino-6-isopropylamino-S-triazine) and dimethoate (O, O-dimethyl-S- (N-methyl carbamoylmethyl) phosphorodithioate) were used for treatment in liquid medium. Stock solution (10 mM) of each pesticide was always freshly prepared in aqueous medium by using sterile BG11 medium and was used to achieve the desired treatment concentrations (0-100 µM for dimethoate and 0-50 µM for atrazine) for toxicity assessment of individual toxicant. The treatments were made in 50 ml cotton stoppered borosilicate culture tubes, each containing 20 ml of the culture and were incubated for 4 days. The combination effect was also measured after 4 days treatment by taking the pesticides in ratios determined by the concentration addition equation of Berenbaum (1985). The ratio of the components in the binary mixture as well as the predicted EC50 were determined by the equation

$$EC_{50z} = (EC_{50x} \times EC_{50y} \times 100)/(P_1EC_{50y} + P_2EC_{50x})$$

Where

x, y and z are dimethoate, atrazine and the combination, respectively. P_1 and P_2 are the percentage of dimethoate and atrazine in the mixture, respectively.

The EC_{50} values of dimethoate and atrazine were determined from the PS II fluorescence response of the cyanobacterium to graded concentrations of the pesticides

The absorbance of the homogeneous suspension was measured at 678 nm by a uv-vis spectrophotometer (systronics, India) against freshly prepared sterile BG11 medium as blank. The measurement of chlorophhyll *a* was made following the standard extraction protocol for cyanobacteria and the absorbance values of the extracts were converted to biomass following the equations of Hirschberg and Chamovitz (1994). Photosynthetic pigment fluorescence was measured with the help of a varian spectrofluorimeter (Varian, Australia) after standardizing the cultures into equal cell density (10^7 cells/ ml). Two millilitre of the homogenious suspension was taken in a spectrofluorimeter cuvette and was dark adapted for five minutes. Fluorescence emission from PS II and phycobilisomes (PBS) was measured at 685 nm and 660 nm, respectively on excitation of culture with a 580 nm monochromatic beam. Similarly chlorophyll *a* specific fluorescence emission from PS II and PS I was also measured at 685 nm and 725 nm, respectively on excitation of culture with a 440 nm monochromatic beam (Mohapatra *et al.*,1997). The excitation and emission band passes were 5 nm in each case.

Three replicates were taken for each treatment. The mean values of the replicates are presented in tables. The EC_{50} of individual pesticide was calculated from the PS II fluorescence response of the cyanobacterium. The combination treatments were made by taking the pesticides in different ratios and the predicted and observed EC_{50}s were compared by independent t test.

3. Results and discussion

Dimethoate, at all concentrations, caused reduction in the growth of the cyanobacterium, which was found significant at ≥ 10 µM. Consequently significant reduction in chl-*a* content was observed in this concentration range (Table 1). On the other hand, there was a concentration dependent increase in fluorescence yield from PS I, PS II and PBS, measured with excitation of phycobiliproteins and chl *a*. Comparison of PBS and PS II fluorescence at 580 nm

excitation showed that PS II fluorescence yield was enhanced more than of PBS. As a result there was a continuous reduction of the PBS/PS II fluorescence ratio which was, however, not significantly different from each other. At concentrations ≥ 50 µM, severe reduction in pigment content resulted in lower fluorescence yield. With 440 nm excitation there was enhancement of PS I and PS II fluorescence yield, which was found concentration dependent. PS II fluorescence was enhanced more than of PS I resulting in significant decrease of PS I/PS II fluorescence ratio.

In higher plants and cyanobacteria, dimethoate is known to increase fluorescence due to limited PQ function (Mohapatra *et al.,* 1997, 2010; Mohapatra and Schiewer, 2000). The insecticide enhances fluorescence emission from both the photosystems as well as from PBS primarily by membrane perturbations (Mohapatra *et al.*, 1996, 1997; 2010; Pandey and Gopal, 2012). Such fluorescence enhancement is attributed to the acceptor limitation of photosystems caused by reduced PQ cycle and delinked PBS-PS II electron flow. In *Solanum melongena* and *Chlorella vulgaris* it has been observed that dimethoate severely impaired photosynthesis more like a herbicide affecting PS II-PS I electron flow (Mohapatra *et al.*, 2010; Jena *et al.*, 2012).

Atrazine was found more effective than dimethoate to reduce the growth and chl *a* content of *A. doliolum* and at all selected concentrations the reduction was found significant (Table 1). The herbicide also caused enhancement of PS II and PBS fluorescence emission with 580 nm excitation, which was significantly higher than the enhancement effected by the corresponding concentration of dimethoate. PS II fluorescence was strongly induced compared to PBS resulting in continuous concentration dependent decrease of PBS/PS II fluorescence ratio. With chl-*a* excitation also there was significant induction of PS II and PS I fluorescence. As expected the PS II fluorescence enhancement was significantly higher than that of PS I resulting in decrease of the PS I/PS II fluorescence ratio with increase of atrazine concentration.

Atrazine is known to enhance PS II fluorescence yield like other herbicides (DCMU, simazine) by blocking the PS II-PS I electron flow at the level of Q_A (Roberts *et al*., 1990; Lazar, 2003; Nayak and Mohapatra, 2011). Interaction of the herbicide with D1 protein of PS II blocks Q_A-Q_B electron flow resulting higher single turn over events at the level of Q_A. The increase in PBS fluorescence in the present case with atrazine treatment is an indication of impaired PBS-PS II excitation transfer, presumably due to membrane perturbations as observed with other hydrophobic chemicals (Mohapatra *et al*., 1997; Lazar, 2003; Jena *et al*., 2012; Pandey and Gopal, 2012).

PS II fluorescence yield per mg chl-*a* was taken as the parameter for determination of the effective concentrations (EC) of pesticides. The parameter could be well correlated with pesticide concentrations through first order polynomial function (Fig. 1). In case of both the pesticides there was significant correlation between the fluorescence rise and the pesticide concentrations. The EC_{50} (at 95 % CI) of dimethoate and atrazine were found to be 33.17 ± 1.85 µM and 11.94 ± 0.83 µM, respectively.

Assessment of fluorescence of individual photosynthetic components with specific pigment excitation is the most efficient way to evaluate adverse impact of insecticides on photosynthetic activity. It represents the energy transfer from chl-*a* pigments to PS reaction centers (Lutz *et al*., 1998). In the present study fluorescence emissions from PS II was taken as the parameter to evaluate the combination effects of dimethoate and atrazine on *A. doliolum*. Nine different combinations, applying the concentration addition model, were taken and the predicted EC_{50}s were calculated. The predicted EC_{50} ranged from 12.76 – 28.16 µM and increased with increase in the concentration of dimethoate in the combination. From the PS II fluorescence yield the observed EC_{50}s were in the range from 12.62 ±0.93 µM to 28.74± 1.39 µM (Table 2). Like the predicted ones the observed EC_{50}s also increased with increase in the concentration of dimethoate in the combination. With higher dimethoate content in the mixture

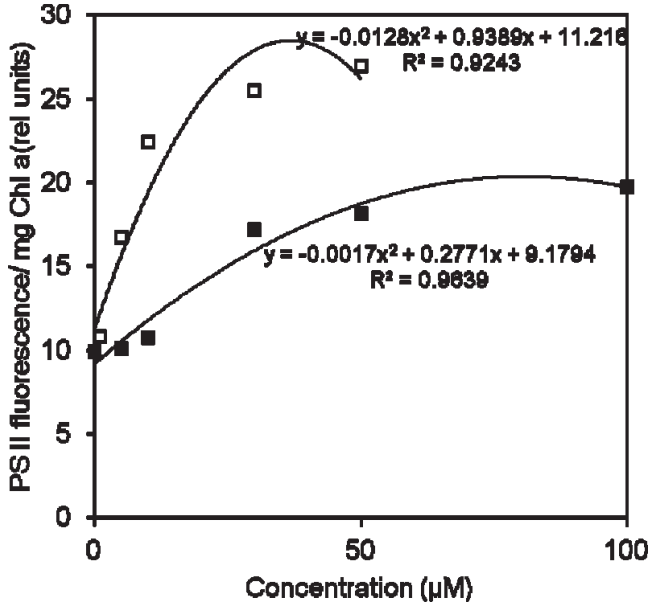

Fig. 1. Effect of dimethoate (■) and atrazine (□) on PS II fluorescence of *Anabaena doliolum*. The regression lines are the result of first order polynomial relation between the fluorescence yield and the concentrations.

Table 1
Culture absorbance (Abs; 678 nm), Chl-*a* content (µg/10^7 cells) and pigment fluorescence (rel. units) of *Anabaena doliolum* treated separately with dimethoate and atrazine

Conc (µM)	Abs	Chl *a*	PBS	PS II	PBS/PS II	PS II/mg Chl	PS I	PS II	PS I/PS II
Dimethoate									
0	0.316	2.384	47.39	23.72	1.998	9.950	1.726	3.168	0.545
5	0.311	2.375	47.64	23.98	1.987	10.097	1.765	3.394	0.520
10	0.293	2.296	48.97	24.65	1.987	10.736	1.932	4.216	0.458
30	0.159	1.897	50.42	32.58	1.548	17.174	2.024	4.799	0.422
50	0.083	1.284	50.26	23.33	2.154	18.170	1.687	4.018	0.420
100	0.009	0.095	3.402	1.879	1.811	19.779	0.085	0.212	0.401
Atrazine									
0	0.326	2.379	47.86	23.65	2.024	9.941	1.732	3.179	0.545
1	0.307	2.368	49.77	25.62	1.943	10.819	1.839	4.176	0.440
5	0.257	2.286	53.65	38.34	1.399	16.772	2.287	6.719	0.340
10	0.185	1.995	54.82	44.71	1.226	22.411	2.465	7.129	0.346
30	0.043	1.748	51.63	44.58	1.158	25.503	2.315	6.932	0.334
50	0.006	0.165	6.64	4.45	1.492	26.970	0.217	0.642	0.338

Table 2
Effect of combination of dimethoate and atrazine on PS II fluorescence of *Anabaena doliolum* measured after 4 days. The values of the pesticides given in parentheses are the % of the chemical in the combination. Observed EC50 have been calculated from the PS II fluorescence yield. The t values given in the last column are the results of independent comparison

Dimethoate (µM)(%)	Atrazine (µM)(%)	Predicted (P) EC50 (µM)	Obereved PS II /mg Chl a	Observed (O) EC50 (µM)	O/P ratio	t value
3.317(10)	10.746(90)	12.76	23.74	12.62± 0.93	0.989	1.62
6.634(20)	9.552(80)	13.69	22.88	13.29± 0.89	0.971	1.19
9.950(30)	8.358(70)	14.78	22.13	15.11±0.82	1.022	1.83
13.267(40)	7.164(60)	16.05	21.66	16.14±0.76	1.006	1.08
16.584(50)	5.970(50)	17.56	20.72	17.74±1.04	1.010	0.94
19.901(60)	4.776(40)	19.38	20.04	19.56±1.12	1.009	0.83
23.218(70)	3.582(30)	21.63	19.55	21.42±1.38	0.990	1.22
26.534(80)	2.388(20)	24.47	18.27	24.84±1.76	1.015	1.36
29.851(90)	1.194(10)	28.16	17.98	28.74±1.39	1.021	1.21

the observed EC_{50}s were insignificantly higher than that of the predicted ones whereas the reverse was noted when the concentration of atrazine was high in the combination. The t-values showed that in none of the combinations there was significant difference between the predicted and observed EC_{50}s, thus confirming the fact that the combination effect of dimethoate and atrazine in the cyanobacterium is purely additive.

Researches have shown that OP insecticides primarily cause neurotoxicity via the inhibition of acetylcholinesterase (Cometa *et al.,* 2007). Atrazine treatment significantly increased the toxicity of dimethoate, chlorpyrifos malathion and parathion when applied in combination or the target organisms were preexposed to atrazine before OP treatments (Choung *et al.,* 2011; Fu *et al.,* 2013; Xing *et al.,* 2013). Fu *et al.,* (2013) reported that atrazine treatment significantly enhanced toxicity of chlorpyrifos but inclusion of a recovery period after atrazine exposure eliminated the synergism. In *Chironomus tentans* atrazine alone up to 1000 µg/l did not show significant toxicity to the midges in a 48-h bioassay.

However, atrazine concentrations as low as 1 µg/l in combination with dimethoate, 10 µg/l in combination with, demeton-S-methyl and 100 µg/l in combination with disulfoton (all at EC_{25}) significantly enhanced the toxicity of each organophosphate insecticide (Anderson and Zhu, 2004; Choung *et al.,* 2011). Similarly addition of atrazine (10 µg/l) significantly increased the toxicity of terbufos to *Ceriodaphnia cf dubia* (Choung *et al.,* 2011). This indicated that synergism of atrazine with OP insecticides is common in invertebrates and fish though antagonism is observed with some pesticides (Cometa *et al.*, 2007; Choung *et al.,* 2011, Fu *et al.,* 2013). However, in the present observation in *Anabaena doliolum* such synergism was not seen when photosynthesis was taken as a parameter. It was instead additive effects which may be attributed to the same site and similar mechanism of action of both the pesticides.

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