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Evaluation of Toxicity of a Textile Dye (Optilan Red) towards a Green Microalga *Chlorella vulgaris*

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A B S T R A C T

In this study, the effect of textile dye (Optilan Red) toward microalgae *Chlorella vulgaris* was monitored for a period of 96 hours. The parameters such as specific growth rate (SGR), EC_{50}, protein, chlorophyll, carotenoid and elemental composition of the microalgae were recorded. Results showed that SGR for *C. vulgaris* decreased with increase in concentration of dye and a significant decrease was noticed upto 20 ppm however, further increase in concentration showed no growth of the organism. EC_{50} for Optilan Red was found to be 23.16 ppm. The protein level of treated *C. vulgaris* showed significant reduction in all the concentrations. Further significant increase in percentage protein inhibition was noticed with increase in dye concentrations. More than 50% reduction in level of both pigments evident at 50 ppm. Maximum percentage inhibition was 66.6% and 79.4% for total chlorophyll and carotenoid, respectively (at 50 ppm). It was noticed that elemental composition of *C. vulgaris* was reduced significantly as compared to the control. This study emphasizes that assessment of toxicity of textile dyes towards photoautotrophic organisms including micro-algae is of prime importance to assess the impacts of dye pollution in aquatic environment.

Introduction

Dyes may be defined as substances providing color to the substrate (Kirk-Othmer, 2004; Bafana *et al*., 2011; Drumond Chequer *et al*., 2013). Such substances with considerable colouring capacity are widely employed in the textile, pharmaceutical, food, cosmetics, plastics, photographic and paper industries (Bafana *et al*., 2011; Rocha *et al*., 2016). In the textile industry, up to 200,000 tons of these dyes are lost to effluents every year during the dyeing and finishing operations, due to the inefficiency of the dyeing process (Ogugbue and Sawidis, 2011). In addition, the increased demand for textile products and the proportional increase in their production, and the use of synthetic dyes have together

contributed to dye wastewater becoming one of the substantial sources of severe pollution problems in current times (Ogugbue and Sawidis, 2011; Dos *et al*., 2007). Unfortunately, most of these dyes escape from textile industry persist in the environment as a result of their high stability to light, temperature, water, detergents, chemicals, soap and other parameters such as bleach and perspiration (Couto, 2009). The wastewater from textile plants is classified as the most polluting of all the industrial sectors, considering the volume generated as well as the effluent composition (Sen and Demirer, 2003; Ben *et al*., 2012). Textile wastewaters are characterized by extreme fluctuations in many parameters such as chemical oxygen demand, biochemical oxygen demand, pH, color and salinity. This wastewater contains mixture of natural and synthetic dye including azo, methane, nitro, and carbonyl that recalcitrant to the degradation (Syafalni *et al*., 2012). These discharges of synthetic dye into the aquatic system have generated much concern due to its reported genotoxic, mutagenic, teratogenic and carcinogenic effects (Srivastava *et al*., 2004; Chowdhury and Saha, 2010). In addition, the effects caused by other pollutants in textile wastewater, and the presence of very small amounts of dyes (<1 mg/L for some dyes) in the water, which are nevertheless highly visible, seriously affects the aesthetic quality and transparency of water bodies such as lakes, rivers and others, leading to damage to the aquatic environment (Ibrahim *et al*., 1996; Wijetunga *et al*., 2010; Gita *et al*., 2017a).

Microalgae are microscopic unicellular organisms capable of converting solar energy to chemical energy via photosynthesis. However, there is much utilization of microalgae in aquaculture which mainly related to nutrition, the basis of the energy flow through the aquatic grazing food chain (De and Persoon, 1988). But due to discharge of synthetic dye in aquatic environment from the textile factories found toxic to various aquatic microorganism. In general, dyes have low toxicity in mammals and aquatic organisms (O'Neill *et al*., 1999), but products formed by their biodegradation, mainly aromatic amines from the anaerobic reduction of dyes, can be harmful (Razo *et al*., 1997; Pinheiro *et al*., 2004). It hinders growth, pigment content, protein content and also disturb the photosynthetic activity. Some dyes are highly toxic and mutagenic that it decreases light penetration and causing oxygen deficiency and limiting downstream beneficial uses such as recreation, drinking water and irrigation (Forgacs *et al*., 2004; Przystaś *et al*., 2012; Hubbe *et al*., 2012). During the recent past year few studies has been carried out and reported on effect of various dyes in microalgae.

In earlier study on the toxicity of textile dye (Metomega Chrome Orange GL) towards a diazotrophic cyanobacteruim *Nostoc muscorum* found that protein, chlorophyll-a, phytocyanin and carotenoid content showed a progressive decreased with concentration of the dye and decreased in photosynthetic evolution also observed by 75, 81, and 92% (Shukla *et al*., 1992). Another study on toxicity assessment was performed using *Selenastrum capricornutum* chronic toxicity test at 1 mg/L of the active colorant in the dyes. All except two of the dyes examined have anionic colorants and many of which are reactive and metal complexes. Only the two cationic dyes demonstrated toxicity (Greene *et al*., 1996). In 2005, a studied on comparative use of bacterial, algal and protozoan tests to study toxicity of azo- and anthraquinone dyes. Among these, *S. capricornutum* algal test was the most sensitive to evaluate toxicity of the dyes (Novotny *et al*., 2006). Further, most of the toxicity studies were carried out in microalgae were mainly focused on cyanobacteria and other microalgae. Based on

the above context, evaluation of toxicity of textile dye, Optilan Red towards *Chlorella vulgaris* has been carried out to examine the toxic effect of dye on *C. vulgaris*.

Materials and Methods

Culture of *Chlorella vulgaris*

Algal sample was obtained from Micro- Algal laboratory, Aquatic Environmental Management, ICAR-CIFE. The unialgal populations of *C. vulgaris* were cultured under prescribed photoautotrophic conditions (Ripka *et al.*, 1979) in BG-11 (Allen, 1968) selective media and maintained in Micro- Algal laboratory of Aquatic Environmental Management, ICAR-CIFE. The pure culture of *C. vulgaris* was sub-cultured in sterile BG-11 medium under photoautotrophic conditions in plant growth chamber with an illumination of 3500 ± 100 lux using compact fluorescent lamps (Philips, 23 W).

The photoperiod was fixed at 16:8 hour light and dark periods and temperature was maintained at 24 ± 2^{o} C. The cultures were shaken twice a day to ensure the proper mixing of the algal suspension. The culture was transferred to 20 L capacity glass aspirator bottle for the continuous culture of alga. The cultures were aerated using stoneaerator which supplied air at the bottom of the vessel, and the air-flow was adjusted to a level that ensured proper mixing of the culture.

Preparation of textile dyes solution

Textile dye optilan red was supplied free of cost by Achroma limited (Mumbai, India). From the 50 ppm stock solution, working solutions of 10, 20, 30, 40, 50 ppm were prepared by dissolving required quantity of dye powder in deionised water as per (Gita *et al*., 2017b).

Toxicity testing

Toxicity experiment was conducted according to OECD guidelines 201 (OECD 1984) with certain modifications when necessary. An inoculum of *Chlorella vulgaris* was prepared for the experiment in BG-11 medium two days before the test to ensure that the algal cells exposed to dyes are in exponential phase. Exponentially growing algal culture was harvested by centrifugation and re-suspended in the dyes solution of graded concentrations in medium. The culture density for all the experiments was maintained at 3 x 10^5 cells mL^{-1}.

Three replicates at each test concentration including control were incubated in plant growth chamber for 96 hr under the following photoautotrophic conditions, temperature: 24 ± 2 ^{0}C; light intensity: 3500 ± 100 lux and photoperiod was fixed at 16:8 hour light and dark periods. The cultures were manually shaken twice a day to re-suspend any settled cells. Samples were analysed every 24 hrs time interval by measuring the direct optical density at 650 nm using a Double beam UV-visible spectrophotometer (MOTRAS Scientific, New Delhi) and calculate the specific growth rate and generation time, protein content and pigment content.

Growth rate or doubling time

The specific growth rate (K) of the alga was calculated by using the formula given by Kartz and Myers (1955):

$$K\ (day^{-1}) = \frac{2.303\ log N_t - log N_0}{T_t - T_0}.$$

where, N_0 is the initial optical density at 650 nm at time T_0 and N_t is the final number optical density at time T_t when culture is in exponential phase.

The generation time or mean generation time (days) was calculated using the formula:

$$x = \frac{In\ (2)}{k} = \frac{0.693}{k}$$

Where,K is the specific growth rate

Pigment content

After 96 hr exposure of algal cells in graded concentration of optilan red dye, a volume of 15 ml algal cells were taken in a 15ml centrifuged tube and centrifuged (Eltek Microprocessor High-speed Research refrigerated centrifuge, MP 400 R, India) at 5000 rpm for 10min. The supernatant containing the dye was discardedand algal pellets were washed three times with sterilized double-distilled water to remove the dye molecules adsorbed on the surface of cells and 15 mL of N, N-dimethylformamide (DMF) was added to the remaining pellets and kept for 24-h for incubation at the room temperature. Then, centrifuged for 10min at 5000rpm and supernatants were collected in centrifuged tube and optical densities were measured at precribed wavelength (461nm and 664nm) and total chlorophyll and carotenoid were estimated.

The pigments Chlorophyll (Moran, 1982) and carotenoid (Chromavitz, 1993) was estimated by the following formula:

Total chlorophyll = (µg/ml) =$O.D._{664}$×11.92

Carotenoid (µg/ml) = [O.D.461 – (0.046×O.D.664] × 4

Protein content

For estimation of protein content for *C. vulgaris*1 mL volume of sample were taken at 24-h intervals during the test period and protein content was estimated in each sample using bovine serum albumin (BSA) as a standard protein by applying the method given by Lowry method (Lowry, 1951).

Estimation of EC_{50}

96-h EC_{50} of Optilan Red dye for *C. vulgaris* was calculated using probit analysis, SPSS 22.0. EC_{50} is the concentration of the test substance that results in 50% reduction in growth or algal cells within the stated exposure period.

CHNS analysis

After 96-h of exposure, algal cells inoculated in BG-11 medium having concentration of EC_{50} value of Optilan Red dyes was centrifuged and washed with distilled water to remove the dye completely from the algal cells. Algal sample was oven dried at 60ºC overnight and prepare into powdered form. The powdered algal samples was analysed using CHNS Analyzer (Elementar, VarioMICRO, India). Then, carbon, nitrogen, hydrogen, and sulfur content were estimated.

Results and Discussion

Growth curve of *Chlorella vulgaris*

Algal growth was measured for every 24 hr for the culture period of 10 days. The specific growth rate and generation time of alga was recorded as 0.295 day^{-1} and 2.35 days, respectively.

Algal growth inhibition test

The effects of the various concentrations of Optilan Red dyes on the specific growth rate, EC_{50}, protein content and pigment content for *C. vulgaris* was studied for the exposure period of 96 hours. The concentration range taken for the algal growth inhibition test was determined on the basis of results from range-finding tests.

Specific growth rate

After exposing *C. vulgaris* to the graded concentrations of Optilan Red dye, it was observed that with an increase in the concentration of dye, the specific growth rate (SGR) of *C. vulgaris* was decreases upto 20 ppm and no growth was detected from 30 ppm. A significant ($p<0.05$) decreased in the specific growth rate and increase in the generation time of *C. vulgaris* for all the concentrations of the dye was recorded (Table 1).

Percentage growth inhibition

Based on the optical densities of cells in the controls and treatments, percentage inhibition of SGR was calculated post 96-h of the experiment. A significant difference ($p<0.05$) in percentage SGR inhibition of *C. vulgaris* was observed among the various concentrations of OptilanRed dye. The highest percent inhibition for Optilan Red (82%) was occurred at 20 ppm concentration (Figure 1).

Estimation of median effective concentration (EC_{50}): Post 96-h EC_{50} of Optilan Red dye for *C. vulgaris*was found to be 23.16 ppm at 95% confidence limit.

Protein content inhibition

The protein content of *C. vulgaris* exposed to various concentrations of Optilan Red dye was measured after 96hrs exposure. The protein level of treated *C. vulgaris* showed significant reduction in all the concentration. Further significant increase in percentage protein inhibition was noticed with increased in dye concentrations (Figure 2).

Pigment content inhibition

The content and composition of pigments in *C. vulgaris* were measured after 96-h of exposure to dye. The pigments, chlorophyll and carotenoids were measured and showed significant inhibition with increased in concentration of dye.

Table.1 Effect of various concentrations of Optilan Red (ppm) on SGR and Generation time of *C. vulgaris* (Data are represented in mean ± SE, n=3)

Dye (Optilan Red) Concentration	Average SGR (day^{-1})	Generation time (days)
Control	0.105 ± 0.01	6.695 ± 0.619
10 ppm	0.038 ± 0.001	18.197 ± 0.61
20 ppm	0.022 ± 0.000	31.236 ± 0.000
30 ppm	NGD	NGD
40 ppm	NGD	NGD
50 ppm	NGD	NGD

[NGD: No growth detected; The data are significant difference ($p<0.05$) from each other]

Table.2 Effect of Optilan Red on major elements composition of *C. vulgaris* (Data are represented in mean ± SE, n=3)

Elements	Percent content[#]	
	Controls	**Optilan red**
Nitrogen	8.603^{A}±0.494	8.231^{A}±0.131
Carbon	43.826^{A}±0.182	43.425^{B}±0.050
Hydrogen	4.987^{A}±0.145	4.866^{A} ±0.144
Sulfur	1.223^{A}±0.154	0.000^{B}±0.000

[#]A and B denote the significant ($p<0.05$) difference in between the treatment and corresponding control tested by independent t-test.

Fig.1 Effect of various concentrations Optilan Red (ppm) on the growth of *C. vulgaris*. Data are represented in mean±SE, n=3

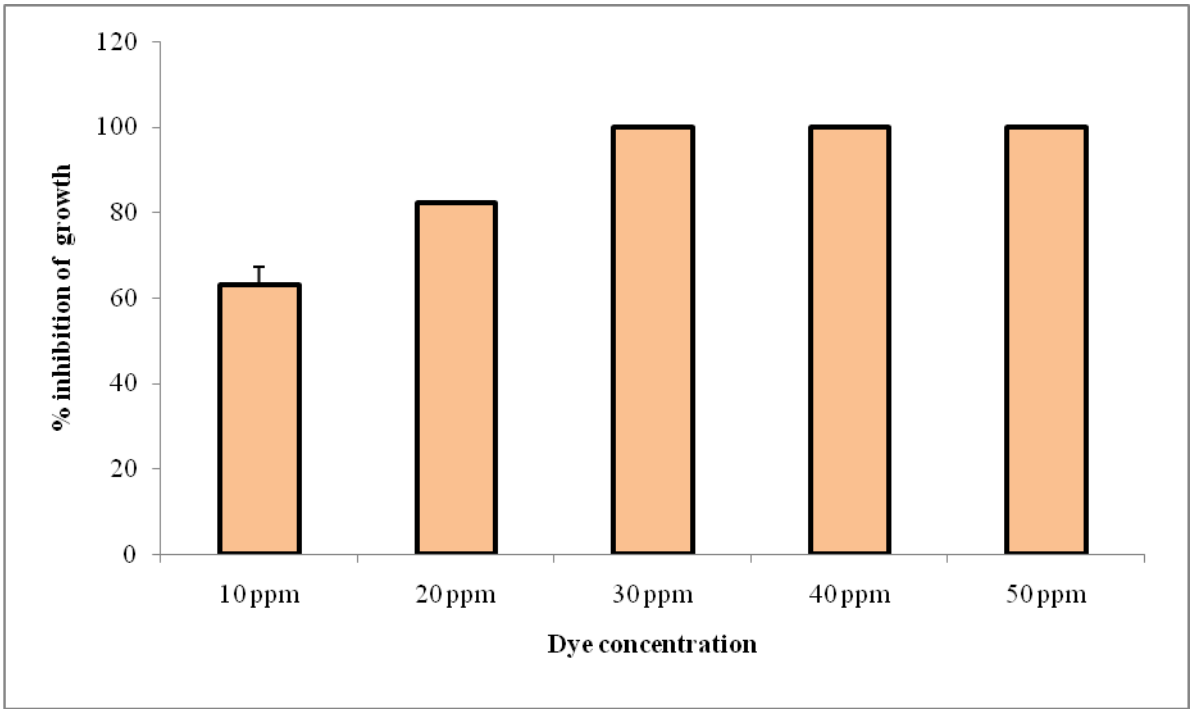


Fig.2 Effect of various concentrations of Optilan Red on the protein content of *C. vulgaris* after 96 h. Data are represented in mean±SE, n=3. The data are significant difference ($p<0.05$) from each other

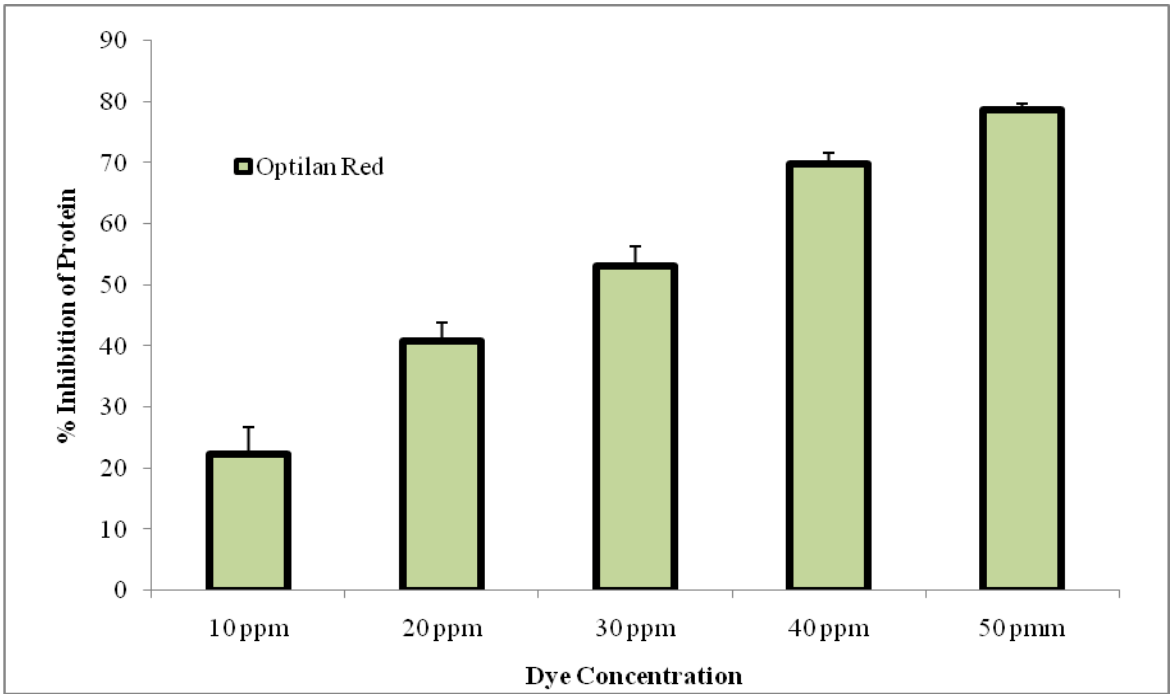
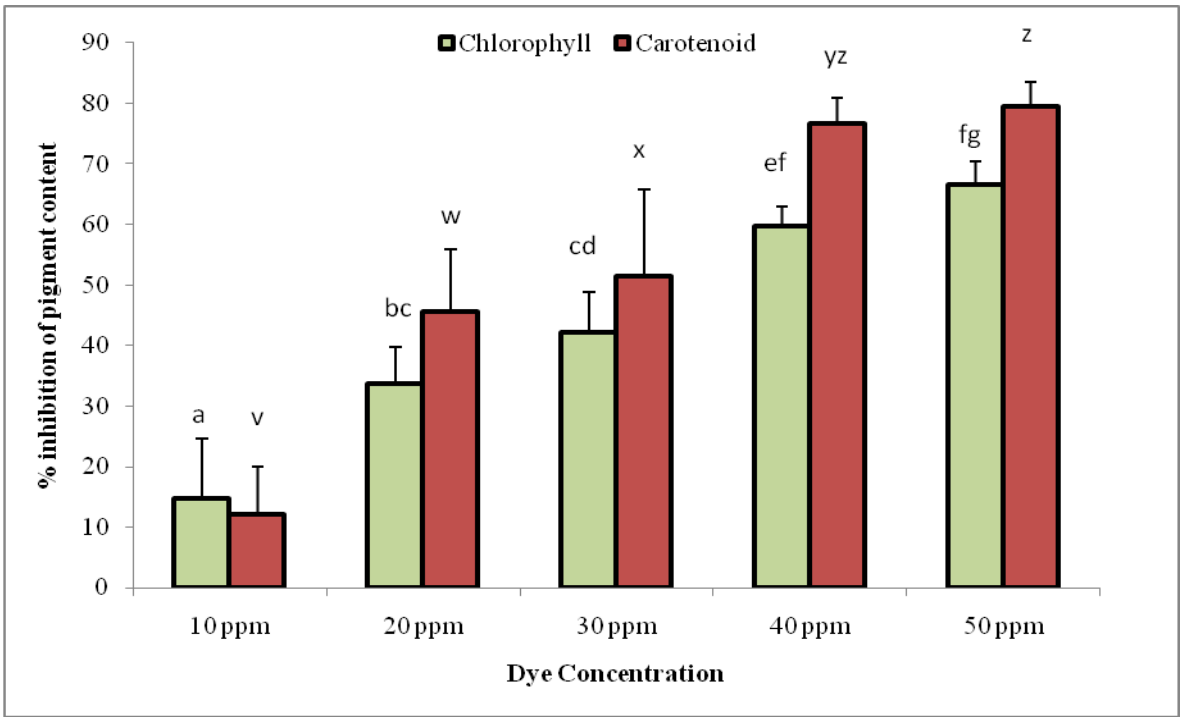


Fig.3 Effect of various concentrations of Optilan Red on the pigment content of *C. vulgaris*. Data are represented in mean±SE, n=3. The data labels represent the significant difference ($p<0.05$)



More than 50% reduction in level of both pigments was found at 50 ppm as compared to control. For the pigment chlorophyll, maximum percentage inhibition of 66.6% was obtained in 50 ppm concentration. Whereas for carotenoids, maximum percentage inhibition of 79.4% was found in 50 ppm concentration. A significant ($p<0.05$) difference in pigment inhibition for different treatment groups was observed (Figure 3).

Effect of textile dyes on elemental composition of *C. vulgaris*

Carbon, nitrogen, hydrogen and sulfur content were estimated from the EC_{50}value of the dye exposed to *C. vulgaris* cells for 96-h for both treatments and control. EC_{50} values for Optilan Red dye were found to be 23.16 ppm. On applying t test, it was noticed that the percent value of carbon and sulfur content was significantly different from control whereas hydrogen and nitrogen were not significantly different from control (Table 2).

In the present study, the growth of *C. vulgaris* was reported upto 20 ppm after which no growth was detected. In case of protein content, there was significant reduction in level of protein was detected with increase in concentration. Shukla *et al*., (1992) also reported decreased in level of protein of microalgae *Nostoc muscorum* with toxic effect of textile dye Metomega Chrome Orange GL. Whereas pigment chlorophyll and carotenoid was also found decreased with increase in concentration. For chlorophyll, maximum percentage inhibition of 66.6% was observed and 79.4% for carotenoid was observed. Shukla *et al*., (1992) also reported the decreased content of pigments for both chlorophyll and carotenoid in microalgae when exposed to textile dye.

The elemental composition of *C. vulgaris* was also found significantly different as compared to control for carbon and sulphur content. The significant reduced in the content of sulfur may be due to decrease in amino acid of protein of the microalgae due to toxicity.

From the above data, it shows that Optlian Red dye is toxic to microalgae *C. vulgaris* in term of growth, protein content, pigments content and also in elemental composition. Therefore, assessment of toxicity of textile dye is of prime important in order to protect the aquatic organism as well as from the pollution of dye in aquatic environment.

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