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Research Article

Growth and metabolism of a freshwater chlorophycean alga *Chlorella vulgaris*: Toxic effects and ecological implications of two textile dyes

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Abstract

The present study aimed to evaluate the toxicity of two textile dyes, Drimarene red and Lanasyn olive, towards a ubiquitous freshwater alga *Chlorella vulgaris*. The parameters such as specific growth rate, protein, and pigments (total chlorophyll and carotenoid) were monitored. The study showed considerable inhibition of growth, pigments (total chlorophyll and carotenoid) and protein content in the algal populations with increasing concentrations of the dyes. More than 50% reduction in pigment level was observed after exposure to graded concentrations of the dyes. Further, the contents of vital elements (C, H, N, S) of the alga were analyzed after exposure for 96 h to understand the metabolic unrest in the cells. According to the analysis, the content of all four elements (C, H, N, S) decreased due to dye toxicity, indicating changes in the biochemical processes due to the altered elemental profile. From the results, it is evident that the dyes influenced the growth and metabolism of *C. vulgaris* in a concentration dependent manner and between these two dyes, Drimarene red was found to be more toxic. Based on the findings, an effective guideline for the treatment of textile effluents before discharge is imperative for preventing ecological damage to the aquatic ecosystems due to dye pollution.

Key words: *Chlorella vulgaris*, Elemental content, Growth, Pigments, Textile dyes

Highlights

- Studied the toxic effect of 2 textile dyes on the growth and metabolism of *C. vulgaris*.
- Considerable inhibition in growth, pigments and protein content of alga were noticed.
- 50% reduction in pigment level (total chlorophyll and carotenoid) of alga was observed.
- Four elements (C, H, N, S) of alga were decreased due to dye toxicity.

INTRODUCTION

Textile dyes are chemical stuff used to give colour to fabrics, and its industry has become one of the profitable ventures worldwide (Salinas *et al*., 2018). As per an estimate, 10-50% of the dyes are lost during the colouring process, finding its way to the natural water bodies (Lellis *et al*., 2019). Wastewater discharged from the textile-dyeing industry is a heterogeneous and complex mixture of various pollutants containing large amounts of dyes, disinfectants, surfactants, and other recalcitrant organic substances (Punzi *et al*., 2015; Liang *et al*., 2017; Pazdzior *et al*., 2017). The ecology of receiving water bodies is negatively influenced because of the decrease in primary production due to a decreased light penetration, oxygen deficiency, and loss of downstream beneficial uses of ground and surface water, including recreational, drinking, agricultural and aquacultural applications (Ibrahim *et al.,*1996). Synthetic textile dyes are toxic substances causing deleterious effects on human health and aquatic organisms (Arlt *et al*., 2002) and posing threats worldwide, either due to their recalcitrant properties or due to the formation of degradation products which are even more harmful than the dyes (Baek *et al*., 2010).

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Lanasyn olive is an acid dye, and Drimarene red is a reactive dye used in the textile industry. To the authors' knowledge, information about the toxicity of Lanasyn olive is not available. However, an assessment of the toxicity of Drimarene red R-7B (used in the present investigation) by Worksafe Australia (Worksafe Australia, Approved Criteria for Classifying Hazardous Substances, Australian Government Publishing Service, Canberra, March 1994) shows that the dye exposure of rats resulted in low toxicity via the oral route [Lethal Dose 50 (LD_{50}) >2000 mg/kg] and dermal (LD_{50}>2000 mg/kg) route. Exposure to the above doses for 28 days suggested that the kidney and stomach were the target organs. The dye caused skin sensitization up to 70% in rabbits at a concentration of 2%. The ecotoxicological study by Work safe Australia shows that the aquatic species *viz.* Carp (*Cyprinus carpio*), *Daphnia magna*, *Scenedesmuss ubspicatus* (alga), and bacteria from aerobic wastewater exhibited median lethal concentration (LC_{50}) values 24, 26, 16.3 and > 100 mg/L, respectively.

The toxicity levels of various wastewaters/ textile dyes can be characterized directly through the biological analyses of different organisms, such as algae (Petala *et al*., 2009; Raptis *et al*., 2014). Algae are important primary producers in aquatic ecosystems, and any disturbances in their dynamics can affect the balance of the entire ecosystem (Silva *et al*., 2009). Algae are sensitive to environmental changes and are commonly considered as indicator organisms to measure the effect of pollutants in water (Qian *et al*., 2008). Algae are also used as test organisms to assess the toxicity of many substances including textile dyes (Vinitnantharat *et al*., 2008). *Chlorella vulgaris* (family Chlorophyta) was used in this study as a test organism as it can be cultivated easily and provides highly reproducible results, their abundance in the aquatic ecosystem and high sensitivity to pollutants (Ramadass *et al*., 2016).

Based on the above background, this study aimed to evaluate the toxic effect of commonly used textile dyes in wool, cotton and linen, *viz.* acid dye (Lanasyn olive) and reactive dye (Drimarene red) in freshwater chlorophycean alga *Chlorella vulgaris*. It hopes to provide some useful information and baseline data for controlling ecological risks of industrial discharged dye wastewater.

MATERIALS AND METHODS

Dyes and chemicals

The dyes (Drimarene red and Lanasyn olive) were obtained from local Achroma Limited (Mumbai, India). Working standards of 10, 20, 30, 40 and 50 ppm were prepared after a range-finding experiment following the guidelines of OECD (1984). BG-11 media (Allen, 1968) was used as a nutrient medium for the culture of *C. vulgaris.*

Cultural methods for *Chlorella vulgaris*

The unialgal populations of *C. vulgaris* were maintained in the Micro-Algal laboratory, Aquatic Environmental Management, ICAR-CIFE Mumbai. The culture was sub-cultured in a BG-11 medium under photoautotrophic conditions (Light intensity: 3500±100 lux; photoperiod: 12:12 h light and dark periods; temperature: 24±2°C). To avoid clump formation, the culture was manually shaken twice a day thoroughly.

Toxicity assays

Ninety-six-hour toxicity test was conducted as per the OECD guidelines 201 (OECD, 1984) with a modification of extended exposure time of 96 h instead of 72 h. The exponentially growing algal populations were used, and a uniform culture density was maintained at $3x10^5$ cells mL^{-1}. For the treatments with graded concentrations of the dyes, the algal cells harvested through centrifugation were resuspended in 10, 20, 30, 40 and 50 ppm solutions prepared using the BG-11 medium. The culture was mixed thoroughly by manual shaking (twice a day). Algal samples were collected at 24 h intervals. The pellets obtained after centrifugation (3000 g, 15 minutes) were washed thoroughly with deionised water to remove the adsorbed dye molecules. The washed biomass was resuspended in the same

volume of medium for the measurement of turbidity at 650 nm against the blank (BG-11 medium).

The direct optical density of the suspension was measured by a double-beam spectrophotometer (UV plus model, Motras Scientific Ltd, India) operated with Scanalyse software. The spectrophotometer was equipped with Tungsten - Halogen lamps, a dual silicon photodiode detector, and blazed holographic grating in Czerny-Turner mounting in the monochromator. The equipment was calibrated using potassium dichromate solution prepared by solubilizing 57.0 mg of the compound in 0.005 M sulphuric acid and then maintaining the volume to 1 litre. The values of absorbance (A) at the wavelengths 235, 257, 313 and 350 nm were measured to determine that the values were accurate and within the acceptable range.

After the measurement of turbidity for specific growth rate calculation, the algal suspension was centrifuged again (3000 g, 15 min.), and the pellet obtained was used for pigment and protein analysis. The dried biomass was used for elemental analysis. All the measurements were recorded in triplicates.

Analytical procedures

Growth rate: Growth rate was determined by calculating the specific growth rate (*K*) and generation time (*x*) as per the equation given by Kratz and Myers (1955).

Specific growth rate,

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

N_0: initial OD at 650 nm at time '0' (T_0) and N_t: final OD at time 't' (T_t), when culture is in log phase.

Generation time, *x* (in days) $= \frac{In(2)}{k} = \frac{0.693}{k}$

Protein content: Protein content was determined by the method given by Lowry *et al*. (1951) using standard protein (Bovine serum albumin).

Pigment content: Fifteen mL algal culture was used for the determination of pigment content after exposure (96 h) to graded dye concentrations. The algal suspension was centrifuged for 15 min at 3000xg, and the supernatant (containing the dye) was discarded. Algal pellets were washed thoroughly with sterile double-distilled water to remove the adsorbed dye on algal cells. Further, N, N-dimethyl formamide (DMF) (15 mL) was added to the pellets and incubated for 24 h in dark conditions at room temperature. The suspension was centrifuged again for 15 min at 3000xg, the resulting supernatant was collected and optical densities were measured at the prescribed wavelength for the estimation of total chlorophyll and carotenoids. Total chlorophyll and carotenoids were estimated using the formula given by Moran (1982) and Chamovitz *et al*. (1993).

Estimation of EC_{50} of textile dyes

The selected graded concentration of dyes used in the present study was based on a range finding test, and the selected organism was exposed to a graded concentration of 5-200 ppm. Then, 96 h EC_{50} values were calculated for individual dyes using the cell number of *C. vulgaris* using probit analysis (Finney, 1971).

Effect of dyes on the elemental composition

After exposure of algal cells for 96 h, at the concentrations corresponding to EC_{50} values of the dyes, changes in the elemental composition of the organism were observed. Algal cells were centrifuged and washed thoroughly with deionised water and centrifuged again (3000xg, 15 min) to obtain the pellet. The pellets were oven-dried at 60ºC overnight, and powdered algal biomass was analysed using the CHNS Analyser (Elementar, Vario MICRO, India). All four elements (carbon, nitrogen, hydrogen, and sulphur) were estimated for both control and treatment. The conditions for CHNS analysis were: Thermal conductivity detector (TCD) temperature 59-60°C, reduction tube

temperature 850°C; combustion tube temperature 1150°C; carrier gas: helium (flow rate 200±10 mL.min^{-1}); combustion gas-oxygen: flow rate 10±2°C (no combustion), 30±2°C (combustion).

Statistical analysis

Data were analyzed using SPSS 22.0 by subjecting to one-way analysis of variance (ANOVA). Results are presented as a mean ± standard error. An Independent t-test was done to analyze the percentage elemental composition of the algae after exposure to dyes for 96 h.

RESULTS

A survey of local dye house in Mumbai showed that the concentration of the dye discharged into aquatic environments varied in a wide range (20-200 ppm) due to variations in the processes involved in the dying of the fabric. The quantity discharged in the environment depends mainly on the type of fabric and the number of cycles involved in the complete process. The EC_{50} of the dyes used in the present study were: 28.33 ppm (Drimarene red) and 29.81 ppm (Lanasyn olive). Considering the value of $EC_{50,}$ graded concentrations of 10-50 ppm were selected for the experiment. The spectrophotometric method used for the quantification of the dyes showed an excellent sensitivity up to 0.01 ppm. Other techniques, such as GC-MS and HPLC, provide higher sensitivity, but the spectrophotometric method is widely used for quantification of dyes owing to their distinct colour development properties in the water, even in trace amounts (Moosvi *et al.*, 2005; Yaseen and Scholz, 2018a; 2018b).

Effect of dyes on growth rate and generation time of algae: When the algal cells are exposed to different dye concentrations, it was observed that SGR gradually decreased with an increase in dye concentration after treatment with both dyes. A corresponding increase in generation time was also noticed with decreasing value of SGR for both the dyes (Fig. 1). A linear increase in cell density was observed with time up to 20 ppm; however, growth was arrested at concentrations above 20 ppm for Drimarene red. Whereas, for Lanasyn olive, there was comparatively less inhibitory as the algae showed higher tolerance towards this dye, which grows up to 30 ppm and further growth was suppressed at 40 ppm.

The percentage growth inhibition of *C. vulgaris* was calculated from the SGR. From the result, it was noticed that there was a significant difference ($p<0.05$) in percentage growth inhibition for *C. vulgaris* at various concentrations (10-50 ppm) of both dyes (Fig. 1). The highest percentage inhibition of 65% (Drimarene red) and 64% (Lanasyn olive) was recorded at 20 ppm that indicates that vital metabolic processes were destabilised due to toxicity (Fig. 1).

Effect of dyes on protein content of algae: The percentage protein inhibition in both dyes showed a significant increase with the dye concentrations (Fig. 2). For Drimarene red, the highest inhibition of 66% was recorded at 50 ppm. Whereas, 31% inhibition was observed for Lanasyn olive at the highest concentration. The experiments reveal that Drimarene red had a pronounced negative effect on protein content as compared to Lanasyn olive.

Effect of dyes on pigment content of algae: Total chlorophyll and carotenoids were estimated in the algal cultures exposed to both dyes. The findings confirm a significant reduction in pigments with an increase in the dye concentration. A decrease of 70.7% (Drimarene red) and 54.8% (Lanasyn olive) was noticed at 50 ppm dye concentration for total chlorophyll. The carotenoids also decreased by 61% (Drimarene red) and 59.6% (Lanasyn olive) in the algal samples exposed to 50 ppm dye concentration (Fig. 3).

Effect of dyes on elemental composition (C, H, N, S): A change in the elemental

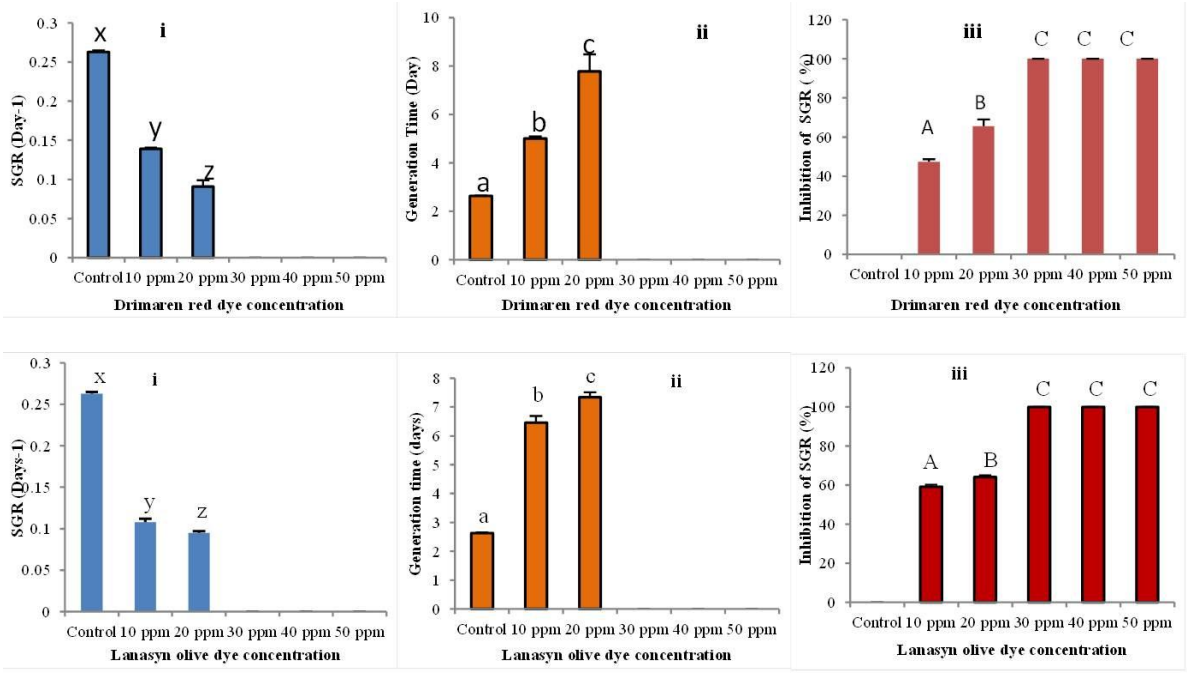

Fig. 1. Effect of various concentrations of Drimarene red (above) and Lanasyn Olive (below) dye on specific growth rate (i), generation time (ii) and % inhibition of SGR (iii) of *C. vulgaris*. Data are represented in mean ± SE, n=3. The data labels with different superscripts in alphabetical symbols are significantly (P≤0.05) different from each other

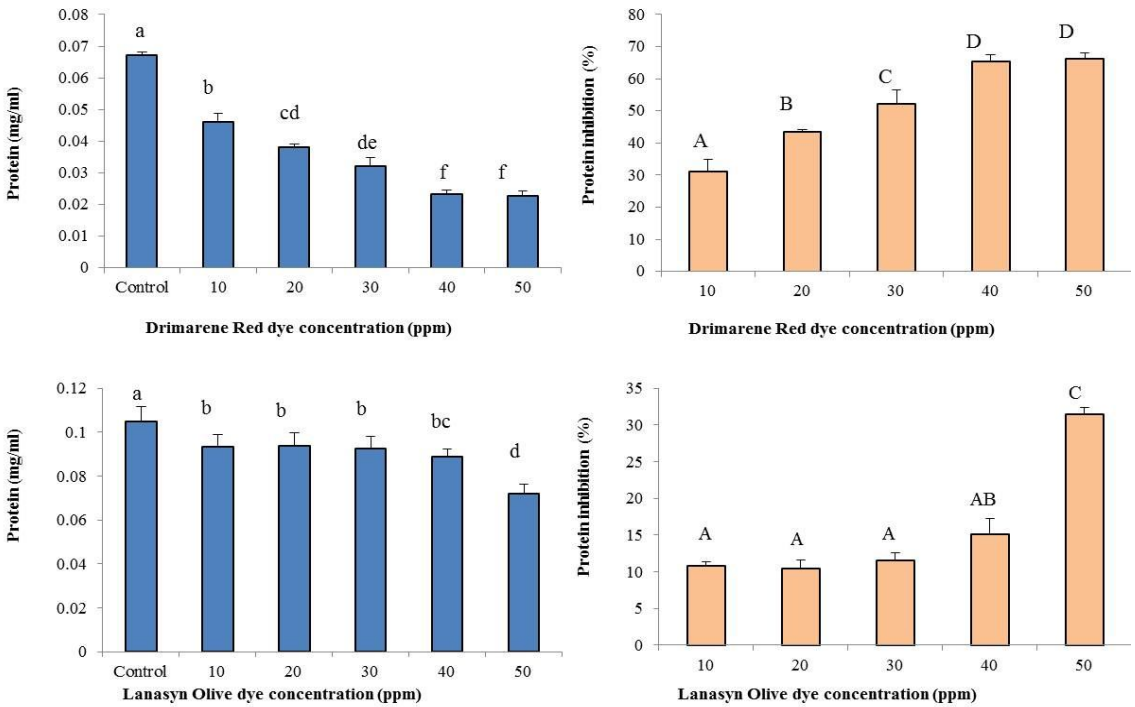

Fig. 2. Effect of various concentrations of Drimarene red (above) and Lanasyn olive (below) dye on the protein content (mg/mL) and protein inhibition (%) of *C. vulgaris*. Data are represented in mean ± SE, n=3. The data labels with different superscripts are significantly (P≤0.05) different from each other

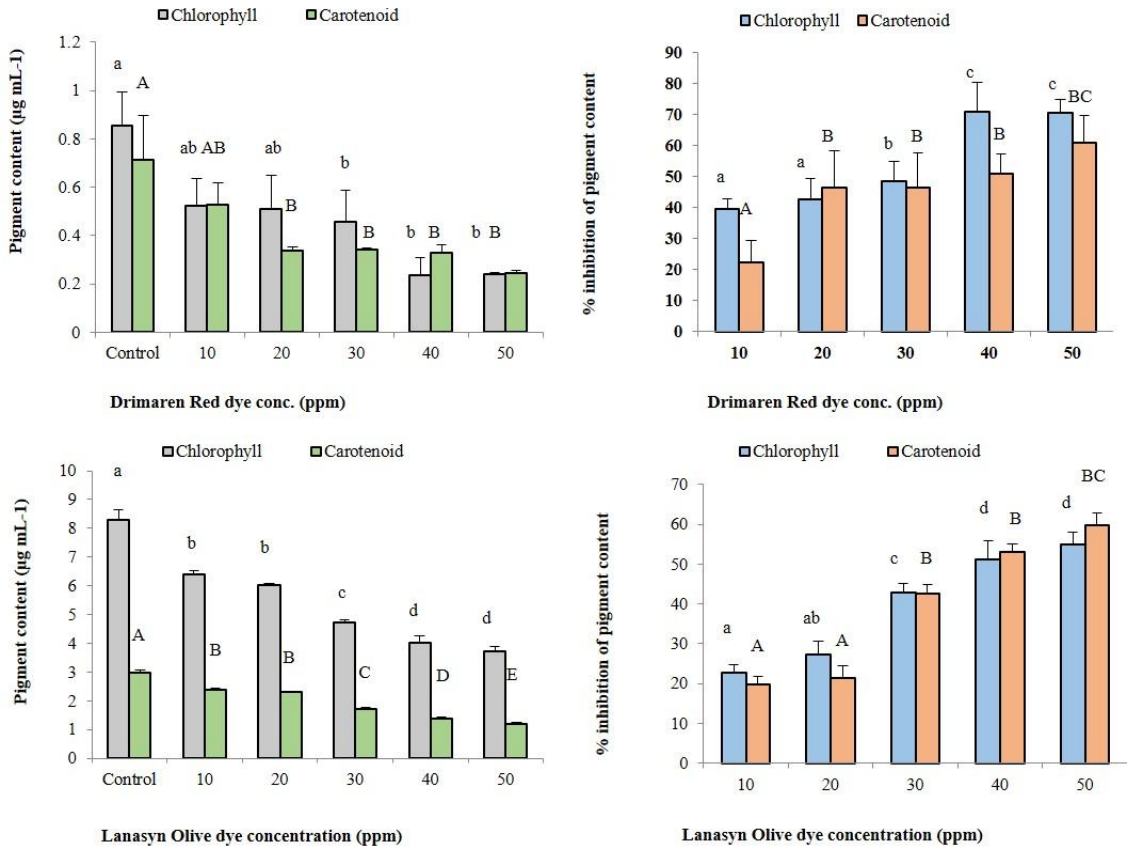

Fig. 3. Effect of various concentrations of Drimarene red (above) and Lanasyn Olive (below) dye on the pigment content and % inhibition of pigment of *C. vulgaris*. Data are represented in mean ± SE, n=3. The data labels with different superscripts are significantly (P≤0.05) different from each other

Table 1. Effect of textile dye on major elements (percentage content) of *C. vulgaris* (data are represented in mean ± SE, n=3)

Elements	Controls	Drimarene red	Lanasyn olive
Nitrogen	8.603^{A}±0.494	8.146^{A}±0.151	8.146^{A}±0.151
Carbon	43.826^{A}±0.182	42.694^{B}±0.234	42.694^{B}±0.234
Hydrogen	4.987^{A}±0.145	3.507^{B}±0.268	3.507^{B}±0.268
Sulphur	1.223^{A}±0.154	0.000^{B}±0.000	0.000^{B}±0.000

Mean values with different superscripts are significantly (P≤0.05) different from each other

composition of *C. vulgaris* after exposure to various concentrations of dyes is tabulated in Table 1. There was a reduction in all four elements (C, H, N, S) as compared to the control for both dyes. But significant reduction ($p<0.05$) was observed only in the carbon, hydrogen and sulphur for Drimarene red, and carbon and sulphur in case of Lanasyn olive.

DISCUSSION

The toxicity of the dyes was evaluated by monitoring the effects on specific growth rate

(SGR), generation time, protein content, pigments content and elemental composition of *C. vulgaris* after exposure to graded concentrations.

A significant ($p<0.05$) decrease in the specific growth rate and increase in the generation time of *C. vulgaris* was observed due to the toxic effect of both dyes. Recently similar work on different dyes was reported by Gita *et al*. (2019) on textile dyes toxicity towards microalgae *Chlorella* sp., and was noticed that toxicity increases with the dye concentration. Significant inhibition of growth of *C. vulgaris* was recorded in this experiment for both the dye exposer. Recently, dye toxicity was reported in *Scenedesmus obliquus* by Cai *et al*. (2020), who observed an increase in the growth inhibition rate of algae with time (from 3 to 15 days) and noticed a positive correlation between algal growth inhibition rate and superoxide dismutase (SOD) activity inhibition rate.

In this study, dye exposure significantly reduced the protein content of *C. vulgaris.* A similar phenomenon was reported by Dwivedi (2013), who noticed that an increase in the dye (congo red) concentration resulted in the suppression of protein content in *S. platensis.* The reduction in protein content was due to the reduction in the growth of the alga, and protein content was found to be the lowest (33.87 µg/mL) at the highest concentration of the dye (100 mg/L). Further, Gita *et al*. (2019) and Hu and Wu (2001) also noticed that an increase in dye concentration, growth and protein content of the microalgae were inhibited. Therefore, the present findings and data validate the earlier reports on the toxicity of textile dyes towards microalgae.

From this observation, it is evident that chlorophyll and carotenoids content of *C. vulgaris* showed susceptibility towards both dyes. Therefore, the study revealed that an increase in dye concentration resulted in a progressive decrease in pigment content in both the dye exposer. The effects of the dyes on photosynthesis may be direct through inhibition of the synthesis of the pigments, or it may be indirectly through the inhibition of PS-II (Photosystem II) activity either by limiting the light availability or due to interference in the electron transport chain (Shukla *et al*., 1992). The cumulative toxic effect on these pigments results in a significantly lower IC-50 value for O_2 evolution. The presence of high concentration of dye in water might also affect the light transmittance through water and ultimately jeopardize the photosynthesis and growth of algae (Hu and Wu, 2001). It is hypothesized that the complex physico-chemical properties of the dye effluents would also be responsible for the overall toxicity rather than the dye component alone. Presence of metals used as mordant to fix the dye, such as cadmium, chromium and copper in textile effluents, can exhibit various interactive toxic effects on aquatic biota (Mathur *et al*., 2012). So, the cumulative toxic effects of the dye effluent may be synergistic, additive, or antagonistic as a function of the different effluent constituents and not the dye alone (Mahalakshmi *et al*., 2015).

Till now, very few reports on the effects of dyes on the elemental composition of algae are available. One such study was published by Gita *et al*. (2019) on *Chlorella* sp. on different dyes. A significant decrease in the percentage value of carbon, nitrogen, hydrogen and sulphur of *C. vulgaris* after exposure to the dyes was reported by the above author. In an earlier investigation, chemical such as paraquat and atrazine was also tested on green algae, *Chlamydomonas moewusii* and *C. reinhardtii* and noticed a significant decrease in the carbon-nitrogen ratio in treatments compared to control (Fernandez-Naveira *et al*., 2016). This decrease in C/N ratio was due to decrease in C content than N content in the dry biomass. Due to the inhibition of photosynthesis, especially PS-II, algal cells do not have enough energy to fix the CO_2, which leads to a decrease in C content in the cultures. From the present investigation, it is apparent that sulphur content was strongly reduced in the algal cells after exposure to both dyes. This might be the result of reduction in

protein content as proteins are the building block of the cells, and sulphur limitation may decrease the synthesis of sulphur-containing amino acids.

In conclusion, it can be summarized that the growth rate, protein, and pigment concentrations are an effective way to assess dye toxicity against the alga *C. vulgaris*. The concentration of the vital elements C, H, N and S are also useful markers for the assessment of the toxicity of textile dyes. Given the key role of microalgae in aquatic ecosystems, a better understanding of the toxicity mechanisms is a prerequisite for baseline information for further studies on diverse species. Overall, the findings of this investigation establish that dye toxicity influences the key metabolic processes such as pigment and protein synthesis and also through the negative effects on the ratio of the vital elements (C, H, N, S) in the dye-exposed algae. The findings show the apparent toxic effects on microalgae that form the most important trophic level in aquatic ecosystems. The reduced biomass of microalgae due to dye toxicity has wide ranging ecological disturbances, including the altered trophic structure and transfer of vital nutrients along the trophic levels.

Conflict of interest: Authors have no conflict of interest in this study.

Author's contribution: SG: Analysis, acquisition of data, interpretation of the results, manuscript drafting; SPS: Supervision, conception, design, funding; manuscript editing; GD: Supervision, conception, design, manuscript editing; TGC: Performed statistical analysis of data.

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