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Interaction between a Synthetic Dye Bath and Selenium in Their Toxicity to *Dunaliella tertiolecta* under Two Light Intensities

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Azo dyes are the largest chemical class of dyes used regularly for textile dying and paper printing. During textile processing, as much as 2%–50% of the dyestuff applied may be lost to the wastewater (Novotny et al., 2006). As a result of their slow biological degradation, dyes pass untreated through various stages of wastewater treatment plants and are subsequently released into the environment. Although many studies have been conducted on the impacts of azo dyes on the ecosystem (Wang et al., 2002) and their toxicities to fresh water green algae (Novotny et al., 2006), little is known about the toxicity of dyes alone, and in the presence of other chemicals, to marine algae. The use of algal species such as *Dunaliella tertiolecta* as a test organism may be extremely important for the assessment of environmental impact in particular aquatic systems in some countries such as Turkey, which is surrounded on three sides by seas and has a high production capacity related to the modernization of the textile sector.

Toxic concentrations of organics in the environment can be greatly influenced by the presence of other chemicals (Wong et al., 1997). Of these chemicals, the role of selenium (Se) in the biosphere, both beneficial and deleterious, is gradually being determined (Li et al., 2003, Lemly, 2004). Agricultural drain water, sewage sludge, fly ash from coal-fired power plants, oil refineries, and mining of phosphates and metals are all sources of selenium contamination of the aquatic environment. In the aquatic environment, Se can exist in inorganic form (selenite, selenate, elemental Se, metal selenides) and in organic forms with direct Se-C bonds (Köbl, 1995). Although the total Se levels in natural water are in the range 0.1–400 μg L^{-1} (Akl et al., 2006), the reported Se content in seawater in Turkey is limited (0.14 ± 0.09 μg L^{-1}, Saçan and Balcıoğlu, 2001). Selenium is the primary element that is required for the normal growth of many marine phytoplankton; however, it becomes toxic to algae at elevated concentrations by interfering with their sulfur-metabolism (Li et al., 2003). The concentrations at which toxicity is observed vary with the organisms. Additionally, Se leads to substantial bioaccumulation in fish tissue (Wright and Welbourne, 2002), algae (Saçan and Balcıoğlu, 2001), and the foodweb (Lemly, 2004). On the other hand, beneficial effects have been reported on primary producers because of its protective role against harmful the effects of certain toxic heavy metals (Wang et al., 2004). Therefore, it is important to examine the interaction between Se and azo dyes. In this study, the toxicity of azo dyes to the marine alga *Dunaliella tertiolecta* was investigated in order to test whether the effect of Se on algal growth may mask the effect of azo dyes. The objective of this study was to determine the impact of a synthetic dye bath containing six azo dyes on the growth of *D. tertiolecta* in the presence and absence of Se in a batch culture by using a light intensity of 76.8 $\mu E\ m^{-2}s^{-1}$, which is in the light intensity range recommended by international guidelines (Cleuvers et al., 2002) for test protocols. Another objective of this study was to investigate the impact of a different photon flux density (38.4 $\mu E\ m^{-2}s^{-1}$) on the response of algae to the synthetic dye bath in the presence and absence of Se, with the knowledge that colored substances can inhibit algal growth, not as a result of their toxic action, but by absorbing the light necessary for growth (Cleuvers et al., 2002).

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Materials and Methods

Acute toxicity tests were performed with *D. tertiolecta* in batch cultures according to the standard procedure (Method 8111, APHA, 1998). *D. tertiolecta* was cultured in a 500-mL Erlenmeyer flask in a controlled growth chamber (WTCB Binder Model KBF 240, Germany) under a continuous light regime, and was maintained at 18 ± 2°C. The inoculum was prepared with *D. tertiolecta* harvested from a four-to-eight-day old stock culture. Each milliliter of inoculum contains approximately 10^4 cells mL^{-1} (±10%). Experimental cultures were carried out in natural filtered (GF/C Glass microfiber Whatman filters, England) seawater and were enriched with modified f/2 medium (Saçan and Balcıoğlu, 2006). Seawater was taken from the coast of Samatya in Istanbul and was stored in a freezer at –24°C in a plastic container after filtration. Natural seawater was characterized by measurements of pH (8.41 ± 0.01), salinity (22.2 ± 0.2‰), conductivity (32.7 ± 0.2 mS cm^{-1}), chloride (11.93 ± 0.13 g L^{-1}), alkalinity (178.0 ± 0.1 mg L^{-1} $CaCO_3$), nitrate (1.22 ± 0.09 mg L^{-1}), total Kjeldahl nitrogen (15.0 ± 0.5 mg L^{-1}), and phosphate (51.4 ± 0.9 mg L^{-1}) based on standard procedures (APHA, 1998). For toxicity experiments, cultures of *D. tertiolecta* in the log phase were exposed to three concentrations of Se (0.5, 1.0, and 1.5 mg L^{-1}) as H_2SeO_3 (Riedel-de Haën, Germany), $Na_2SeO_3.5H_2O$ (Riedel-de Haën, Germany), and Na_2SeO_4 (SIGMA, Switzerland). All chemicals were reagent grade. Since selenite had the greatest stimulatory effect on the growth of algae among the studied forms of Se, the growth of *D. tertiolecta* exposed to the dye bath in the presence and absence of selenite was determined at two light intensities: 38.4 μE $m^{-2}s^{-1}$ and 76.8 μE $m^{-2}s^{-1}$. The growth response of *D. tertiolecta* exposed to each of the studied chemicals was determined by daily measurements of optical density at 750 nm (OD_{750}) in a 1-cm cuvette with a spectrophotometer (Shimadzu UV-1208, Japan) over 8 d. The recipe of the synthetic dye bath solution is shown in Table 1. It was prepared in deionized water and was diluted 40-fold to represent the effluent after the washing and rinsing stages (Arslan, 2000), and it was filter-sterilized before addition to the sterile culture medium. A concentration series of 20%, 40%, 80%, and 100% of the 40-fold diluted synthetic dye bath solution was prepared and was enriched with modified f/2 medium. Control experiments were performed with associated chemicals. All experiments were performed in triplicate. IC_{25} values (the concentration of the tested substance that decreases the growth by 25%) were determined by using (Table 1) linear interpolation combined with bootstrapping as outlined in USEPA (1993). The SC_{20} value (stimulatory concentration) describing the concentration, which increases growth 20% above that of the control population, was used for growth stimulation. The area under the growth curve was calculated by using the equation of LeBlond and Duffy (2001). Percent inhibition/stimulation (Ig %) was calculated from the equation used by Saçan and Balcıoğlu (2006). In order to obtain a confidence interval for the SC_{20} value, each I_g value per concentration was plotted against each concentration, and curve-fitting analysis was carried out directly on the data by using SPSS 11.0 software. Fisher statistic (F) and p-values were taken into consideration in testing the quality of the curve-fitting. The joint toxicity of mixtures is generally evaluated with method based on empirical toxic unit (TU) concepts (Moreau et al., 1999). Toxic unit is the ratio of $100/Y$, where Y corresponds to either IC_{25} or SC_{20}. In this study, TU was calculated by using both the SC_{20} and IC_{25} values obtained from optical density measurements. Paired t-test was used to determine significant differences among the concentrations, the Se species, and the two light intensities. Results were considered to be significantly different at the level $p \leq 0.05$.

Table 1 The concentration of the chemical components present in the synthetic dye bath (Arslan, 2000)

Dyestuff	Reactive group	Concentration (g L^{-1})
Reactive black 5	Sulphatoethylsulphonate	0.583
Reactive red 23	Sulphatoethylsulphonate	0.084
Reactive yellow 37	Sulphatoethylsulphonate	0.126
Reactive orange 69	Monochlorodifluoropyrimidine	0.167
Reactive red 171	Monochlorodifluoropyrimidine	0.076
Reactive blue 209	Monochlorodifluoropyrimidine	0.092
Associated chemicals	Function	Concentration (g L^{-1})
Urea	Increase solubility of the dyestuff	3.0
NaCl	Transfer dyestuff to fabric	70.0
Na_2CO_3 (soda ash)	pH buffer	5.0
NaOH	Produce covalent bonds between dyestuff and fabric	4.0

Table 2 Changes in OD_{750} of *Dunaliella tertiolecta* exposed to selenium under illuminations of 76.8 μE $m^{-2}s^{-1}$ and 38.4 μE $m^{-2}s^{-1}$

Chemicals	Light Intensity						
	76.8 μE $m^{-2}s^{-1}$					38.4 μE m^{-2} s^{-1}	
	Concentration (mg L^{-1})						
		Control	0.5	1.0	1.5	Control	0.5
H_2SeO_3 selenious acid	AUC[a]	0.89	1.77	1.39	1.39	0.22	0.41
	CV	3.25	6.91	8.51	1.38	12.74	12.07
	Ig (%)[b]		–98	–57	–56		–85
$Na_2SeO_3.5H_2O$ sodium selenite	AUC	0.89	1.61	1.25	1.32	0.22	0.36
	CV	3.25	18.67	7.10	11.44	12.74	1.49
	Ig (%)		–81	–40	–48		–62
Na_2SeO_4 sodium selenate	AUC	0.89	1.11	1.31	0.98	0.22	0.20
	CV	3.25	9.94	6.24	5.42	12.74	7.77
	Ig (%)		–25	–47	–11		9

[a] All AUC are mean values from repeated tests ($n = 3$)

[b] *Ig* (%) = % growth inhibition/stimulation; negative values show stimulation of growth with respect to control

CV, coefficient of variation

Results and Discussion

The area under the growth curves (AUC) and growth stimulation/inhibition (*I*g) appear in Table 2, together with the coefficient of variations for the two light intensities (76.8 μE $m^{-2}s^{-1}$ and 38.4 μE $m^{-2}s^{-1}$). As can be seen from Table 2, Se (H_2SeO_3, $Na_2SeO_3.5H_2O$, and Na_2SeO_4) had a stimulatory effect on the growth of algae, which has also been reported previously by Wheeler et al. (1982). The experiment was repeated and yielded the same results. At 76.8 μE $s^{-1}m^{-2}$, no significant difference was observed between selenious acid and sodium selenite in terms of their effect on the growth of the alga. It is likely that cations (H^+ and Na^+) have no effect on the uptake of Se by algae within the studied concentration range. However, a significant difference occurred between selenite (H_2SeO_3, $Na_2SeO_3.5H_2O$) and selenate (Na_2SeO_4) at concentrations of 0.5 mg L^{-1} and 1.5 mg L^{-1}. The maximal stimulatory effect was observed for 0.5 mg L^{-1} of selenite; however, the effect decreased at the higher test concentration and then stayed constant. On the other hand, the highest stimulatory effect of selenate was observed at 1.0 mg L^{-1}. These results reflected that algal response to selenite and selenate is different.

Since the lowest concentration at which we observe a significant difference ($p < 0.05$), only between the growth response of algae to selenite and selenate at 0.5 mg L^{-1}, and this concentration is close to the maximum measured environmental Se concentration (Akl et al., 2006), we repeated the same experiment for only 0.5 mg L^{-1} for the three forms of Se at half the light intensity. Figures 1(a) and 1(b) represent the growth curves of *D. tertiolecta* in the presence of 0.5 mg L^{-1} Se under illuminations of 76.8 and 38.4 μE $m^{-2}s^{-1}$, respectively. As the light intensity decreased, the stimulatory effects of all forms of Se decreased. There was again no significant difference between the two forms of selenite in terms of *I*g (%) values. At 38.4 μE $s^{-1}m^{-2}$, although selenate did not influence the algal response as compared to control, selenite stimulated the growth of algae. This stimulation effect of selenite at low light intensity can be attributed to the adaptation of *Dunaliella* to the darker environment in the presence of selenite, and this effect is linked to energy-dependent processes as stated by Gorbi et al. (2001). The algal growth response to a higher concentration of Se species was not determined, because it is well known that the growth of some species of marine microalgae could be affected by a high concentration of selenite and selenate (Wong and Luis, 1991). The concentrations at which toxicity is observed vary with the organisms: algae 0.01–80 mg Se L^{-1}, invertebrates 0.07–200 mg Se L^{-1}, vertebrates 0.09–82 mg Se L^{-1} (Li et al., 2003).

The stimulatory response of *D. tertiolecta*, evaluated with the help of the 8-d SC_{20} values, is listed in Table 3 with their best-fit equations together with the 8-d IC_{25} and toxic unit (TU). There was a significant difference ($p < 0.05$) from the control at all studied dilutions (% v/v). Paired *t*-tests showed that there was a significant difference between both the 8-d IC_{25} and SC_{20} values of *D. tertiolecta* exposed to the dye bath with and without Se at two light intensities. Although a stimulatory effect was observed in low doses [20–40% (v/v)], an inhibitory effect was observed at high doses [80–100% (v/v)] under the illumination of 76.8 μE $m^{-2}s^{-1}$. Table 3 shows that

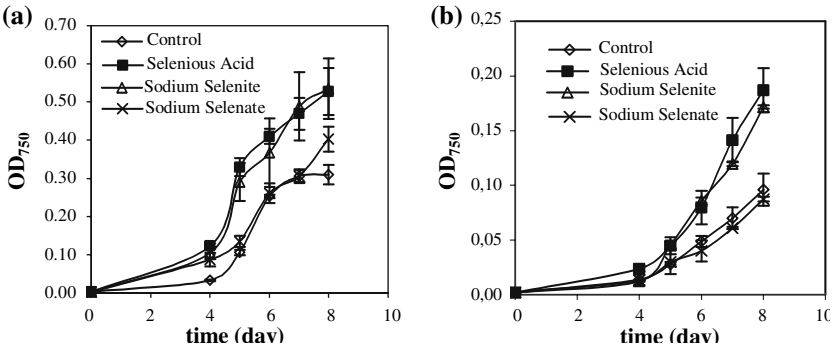

Fig. 1 Growth curves of *D. tertiolecta* exposed to 0.5 mg L^{-1} Se in the form of H_2SeO_3, $Na_2SeO_3.5H_2O$, and Na_2SeO_4 at light intensities of (a) 76.8 μE $m^{-2}s^{-1}$, and (b) 38.4 μE $m^{-2}s^{-1}$

Table 3 Variation in optical density at 750 nm and the 8-d IC_{25} and SC_{20} of *D. tertiolecta* exposed to the dye bath in the presence and absence of Se under the illumination of 76.8 μE $m^{-2}s^{-1}$ and 38.4 μE $m^{-2}s^{-1}$

Spectrophotometric method	Control	% Dilution (v/v)				IC_{25} % dilution (v/v)	TU[c]	Equation (SC_{20} = alnX*+b)	*p*-value	*F*	SC_{20} % dilution (v/v)	TU
		20	40	80	100							
Light intensity = 76.8 μE $m^{-2}s^{-1}$												
Dye bath												
AUC[a]	0.88	1.16	0.95	0.46	0.01	52.01 ± 4.64	1.92	–75.90lnX + 370.38	0.05	17.45	27.12 ± 0.74	3.68
CV	16.71	2.70	2.03	0.70	5.59							
Ig (%)[b]		–31	–8	48	99							
Dye bath and selenium												
AUC	0.88	1.40	1.15	0.46	0.01	60.11 ± 1.17	1.66	–95.59lnX + 460.53	0.04	24.41	35.15 ± 0.58	2.84
CV	16.71	0.84	6.31	8.99	15.75							
Ig (%)		–60	–30	47	99							
Light intensity = 38.4 μE $m^{-2}s^{-1}$												
Dye bath												
AUC	0.29	0.40	0.33	0.06	0.09	53.05 ± 0.25	1.89	–75.61lnX + 371.99	0.05	18.54	28.13 ± 1.61	3.55
CV	5.04	1.79	9.05	8.50	23.47							
Ig (%)		–37	–13	80	67							
Dye bath and selenium												
AUC	0.29	0.35	0.29	0.04	0.09	46.87 ± 2.45	2.13	–66.21lnX + 324.16	0.05	17.93	22.55 ± 1.62	4.43
CV	5.04	2.80	5.31	17.41	4.27							
Ig (%)		–20	5	86	70							

[a] All AUC are mean values from repeated tests (n = 3)

[b] Ig (%) = % growth inhibition/stimulation; negative values show stimulation of growth with respect to control

[c] TU, toxic unit in terms of IC_{25}; TU, toxic unit in terms of SC_{20} ; *X, % dilution of the dye bath; CV, coefficient of variation

the addition of 0.5 mg Se L^{-1} in the form of H_2SeO_3 significantly increased the stimulatory effect of the dye bath at low doses. Since the stimulatory effect of Se decreased with increasing dye bath concentration, it can be concluded the dye bath–Se interaction on algal growth is concentration-dependent. The average 8-d IC_{25} values for *D. tertiolecta* increased from 52.01 ± 4.64% to 60.11 ± 1.17% (v/v) with the addition of Se. Parallel to the significant increase in the 8-d IC_{25} value, a decrease from 1.92 to 1.66 was observed in the TU value with the addition of Se. Considering the TU value calculated on the basis of 8-d IC_{25}, the dye bath was moderately toxic to *D. tertiolecta*, either alone or in combination with Se (TU > 1.0, Villegas-Navarro et al., 2001). When the results were compared in terms of the 8-d SC_{20} values, a significant increase ($p < 0.05$) from 27.12 ± 0.74% (v/v) to 35.15 ± 0.58% (v/v) was observed. Considering the TU values calculated on the basis of the 8-d SC_{20} value, the dye bath alone was very toxic (TU ≥ 3.0, Villegas-Navarro et al., 2001) to *D. tertiolecta*, whereas it was moderately toxic (TU > 1.0, Villegas-Navarro et al., 2001) to *D. tertiolecta* in the presence of Se. These observations suggest that selenium increased the resistance of algae toward dye bath constituents.

At 38.4 μE $m^{-2}s^{-1}$, a stimulatory effect was observed when the synthetic dye bath dilution was in the range of 20%–40% with and without Se, but an inhibitory effect appeared above this concentration range (Table 3) that is similar to the results obtained under the illumination of 76.8 μE $m^{-2}s^{-1}$. There was no significant difference ($p > 0.05$) between the 8-d IC_{25} values of dye bath at the two light intensities. This similarity shows that the toxicity of the dye bath to algae is not caused by the absorption of light by the colored dye bath itself. Although Se increased the stimulatory effect of the dye bath at 76.8 μE $m^{-2}s^{-1}$, it decreased the stimulatory effect of the dye bath at 38.4 μE $m^{-2}s^{-1}$. These effects are probably due to a reduced Se uptake caused by reduced photosynthesis. The decrease in algal growth with the addition of Se is clear, when the 8-d IC_{25} and SC_{20} values are considered. TU values calculated on the basis of 8-d IC_{25} showed that the dye bath was moderately toxic with or without Se (TU > 1.0, Villegas-Navarro et al., 2001). When TU values were evaluated on the basis of 8-d SC_{20}, they were 4.43 and 3.55 for the dye bath with and without Se, respectively. These results reflect that the dye bath was very toxic to *D. tertiolecta* either alone or in combination with Se (TU ≥ 3.0, Villegas-Navarro et al., 2001). The IC_{50} values of several azo dyes for fresh water alga were reported (Novotny et al., 2006). However, the toxicity of the dye bath and selenium separately and in combination has never been assessed for *D. tertiolecta*; therefore, we could not compare our results with the literature values. Although environmental factors such as pH and suspended sediments may enhance/decrease the acute or chronic toxicity of dye bath formulations, at low concentrations, the release of the dye bath or Se to the environment separately or in combination can lead to uncontrolled algal growth and, consequently, can exacerbate the eutrophication problem. Both stimulation and inhibition of algal growth influence the entire aquatic ecosystem by changing algal populations. Moreover, if algal growth is affected, the biomass at higher tropic levels is impacted as well. Additionally, long-term effects of continuous low-level exposure to chemicals and their metabolites are not well understood.

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