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ABSTRACT

In this study, the interactions between assisting chemicals, dye bath, three azo dyes and selenium in their toxicity to *Dunaliella tertiolecta* and *Vibrio fischeri* were determined. Three response parameters, including optical density at 750 nm, *in vivo* chlorophyll fluorescence and cell number were used to monitor the growth of algae exposed to the tested substances in batch culture. The bioluminescence was measured in a luminometer (ToxAlert® by Merck).

In order to understand whether the toxicity is due to the light absorption of azo dye or the dye itself, the preliminary experiments were carried out at two light intensities (37.4 µE $m^{-2}s^{-1}$ and 76.8 µE $m^{-2}s^{-1}$). Algal growth was also examined with different forms and concentrations of selenium under two different light regimes. Selenite and selenate had different effects on the growth of alga. When the light intensity was lowered, the stimulatory effects of all forms of selenium decreased. However, when algae were exposed to dye bath, no significant decrease was observed in the algal growth. The results reflect that selenium is linked to energy-dependent processes and toxicity of dye bath to algae is not due to the light absorption of colored dye bath itself.

D. tertiolecta was found to be more sensitive than *V. fischeri* to the tested chemicals, since for all the tested chemicals with and without selenium, hormetic response was observed on the growth of *D. tertiolecta*, however, only inhibitory effect was observed on *V. fischeri*. Considering the average 8-d IC_{25}, SC_{20} and 30-min EC_{25} values expressed as per cent dilution (v/v) the order of toxicity was found as Dye Bath= Remazol Black 5>Reactive Yellow 37>Reactive Orange 69=Assisting chemicals. For algae, selenium increased the stimulatory effect of all the tested chemicals at low concentration range, whereas it decreased the inhibitory effect at high concentration range, except assisting chemicals and Reactive Orange 69. On the other hand, selenium did not change the inhibitory effect of the tested chemicals on *V. fischeri.* Additionally, the influences of stimulation and inhibition of algal growth on aquatic ecosystem were discussed.

ÖZET

Bu çalışmada, yardımcı kimyasalların, boya banyosu ve üç azo boya çözeltisinin selenyum ile birlikte *Dunaliella tertiolecta* ve *Vibrio fischeri* üzerindeki toksisitesine olan etkisi araştırıldı. Test edilen maddelere maruz bırakılan algin büyümesi, kesikli kültürde 750 nm'de optik yoğunluk, klorofil floresans ve hücre sayısı olmak üzere üç parametre kullanılarak izlendi. Bioışıması luminometre (ToxAlert® 100 (Merck)) kullanılarak ölçüldü.

Toksisitenin azo boyalarının ışığı absorblamasından mı yoksa boyanın kendisinden mi kaynaklandığını anlamak için ilk deneyler iki ışık yoğunluğu (37.4 µE $m^{-2}s^{-1}$ ve 76.8 µE $m^{-2}s^{-1}$) kullanılarak yapıldı. Algin büyümesi ayrıca selenyumun değişik bileşik form ve konsantrasyonlarıyla iki farklı ışık şiddeti kullanılarak incelendi. Selenit ve selenatın algin büyümesine farklı etkileri olduğu görüldü. Işık şiddetinin yarıya düşürülmesiyle selenyumun çalışılan tüm bileşiklerinde görülen stimulan etkinin düştüğü gözlenirken, aynı şartlarda alg boya banyosuna maruz bırakıldığında, algin büyümesinde önemli bir düşüş gözlenmedi. Bu sonuçlar, selenyumun enerjiye bağlı proseslerle ilişkili olduğu ve boya banyosunun alg üzerindeki toksisitesinin ışığı absorblamasından değil, kendisinden kaynaklandığı şeklinde yorumlandı.

Selenyumlu ve selenyumsuz olarak test edilen tüm kimyasalların, *D. tertiolecta*'nın büyümesi üzerinde hormetik etki göstermesine karşılık, *V. fischeri* üzerinde yalnızca inhibisyon etkisi göstermesi, *D. tertiolecta*'nın *V. fischeri*'ye göre çalışılan kimyasallara karşı daha duyarlı bir organizma olduğunu göstermiştir. 8 günlük IC_{25} ve SC_{20} ile 30 dakikalık EC_{25} değerlerinin seyreltme oranı bazında hesaplanan ortalama değerleri göz önüne alındığında, toksisite sıralaması Boya banyosu=Remazol Siyah 5>Reaktif Sarı 37>Reaktif Turuncu 69=yardımcı kimyasallar şeklinde bulundu. Alg için, selenyumun yardımcı kimyasal ve Reaktif Sarı 37 dışında test edilen tüm kimyasalların düşük konsantrasyon aralığında stimulan etkisini artırdığı ve yüksek konsantrasyonlarda ise inhibisyon etkisini azalttığı görüldü. Ancak, selenyumun test edilen kimyasalların *V. fischeri*'ye olan inhibisyon etkisini değiştirmediği görüldü. Ayrıca algin büyümesinde görülen stimulan ve inhibisyonun su ekosistemine olan etkileri tartışıldı.

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SYMBOLS AND ABBREVIATIONS

Symbol	Explanation	Unit used
AC	Assisting Chemicals	
AC and Se	Assisting Chemicals and Selenium	
AUC	Area Under the Curve	
BOD_5	5-d Biochemical Oxygen Demand	mg L^{-1}
C.I.	Color Index	
COD	Chemical Oxygen Demand	mg L^{-1}
CV	Coefficient of Variation	
d	Day	
DB	Dye Bath	
DB and Se	Dye Bath and Selenium	
DHAP	Dihydroxyacetone Phosphate	
DHAPR	Dihydroxyacetone Phosphate Reductase	
EC_{20}	The Effective Concentration of a Test Sample That Causes a 20% Reduction in Survival	
EC_{25}	The Effective Concentration of a Test Sample That Causes a 25% Reduction in Survival	
EC_{50}	The Effective Concentration of a Test Sample That Causes a 50% Reduction in Survival	
EC_{100}	The Effective Concentration of a Test Sample That Causes a 100% Reduction in Survival	
F	Fischer Statistics	
GLM	General Linear Model	
GSHPx	Glutathione Peroxides	
h	hour	
IC_{25}	The Concentration of the Tested Substance That Decrease the Growth by 25 %	

IC_{50}	The Concentration of the Tested Substance That Decrease the Growth by 50 %	
Ig (%)	Per cent Growth Inhibition	
LC_{50}	The Concentration That Causes Mortality in 50% of the Organisms	
LD_{50}	The Dose That Causes Mortality in 50% of the Organisms	
LOEC	Lowest Observed Effective Concentration	
min	minute	
OD_{750}	Optical Density at 750 nanometer	
PAH	Polycyclic Aromatic Hydrocarbons	
RB	Remazol Black 5	
RB and Se	Remazol Black 5 and Selenium	
RO 69	Reactive Orange 69	
RO 69 and Se	Reactive Orange 69 and Selenium	
RY	Reactive Yellow 37	
RY and Se	Reactive Yellow 37 and Selenium	
Se	Selenium	
S.D.	Standard Deviation	
SC_{20}	The Concentration of the Tested Substance That Increase the Growth by 20%	
TKN	Total Kjeldahl Nitrogen	
TU	Toxic Unit	
USEPA	United States Environmental Protection Agency	
X	Dilution (% v/v)	
Y	Mean value of Area Under the Growth	
Y’	Lowest Observed Effective Concentration	

1. INTRODUCTION

The textile industry is an important contributor to many national economies, encompassing both small- and large- scale operations worldwide. Considering both the volume discharged, physical and chemical properties of its effluent, the wastewater generated by the textile industry is rated as the most polluting among all industrial sectors. Azo, triphenylmethane and heteraocyclic/polymeric dyes are extensively used dyes in the textile industry. These dyes are not readily biodegradable when discharged to the environment. They are therefore persistent and many of them are toxic or contain carcinogenic compounds with long turnover times (Pointing et al., 2000). In 1991, the world production of dyes was estimated at 668,000 tones of which an estimated 70% were azo dyes (Vijaya and Sandhya, 2003). Toxic concentrations of azo dyes in the environment can be greatly influenced by the presence of other chemicals. Of the chemicals, selenium is an interest as it is classified as an essential element for all plants and animals. Many microorganisms are known to be able to use selenium in their metabolism. However, at higher concentrations it can be deleterious to algae and other aquatic biota. (Price et al., 1987). Selenium becomes toxic for marine organisms at seawater concentrations higher than required to provide the essential dose. On the other hand, concentration range of 0.5-40 mg L^{-1} was reported to be suitable for culture enhancement of *S. platensis* (Li et al., 2003). It is reasonable to examine the effects of dye bath in the presence of selenium on the growth of algae since a stimulation of algal growth due to selenium may mask the effect of toxic substances.

Throughout the world, where the industrial effluents and hazardous wastes are posing a growing problem, a number of biological assays have been developed and evaluated for aquatic toxicity testing. Of the test assays, the animals do not respond to all wastes whereas algae respond to all wastes by stimulation, inhibition or both (Weyers and Vollmer, 2000). For example, test assays on various effluents from municipalities and industries have demonstrated greater sensitivity to algae than animal species (Wanberg et al., 1995; Lewis et al., 1993; Walsh, 1983). Algae have a high ecological relevance because of their role as primary producers in the aquatic food web, thus being of great scientific importance.

Mainly freshwater species have been proposed in the past for the evaluation of ecotoxicity of anthropogenic compounds on aquatic environments. However, the proposal of seawater species as test organism was scarce which may be extremely important for the assessment of environmental impact in particular aquatic systems in some countries like Turkey, which is surrounded on three sides by sea. Salt content of textile effluents are high. In general, the quantity varies from 5 to 60 g L^{-1} (Muthukumar and Selvakumar, 2004). Therefore the selection of the unicellular marine algal species, *Dunaliella tertiolecta*, as a test organism for toxicity testing seems to be logical, since it fulfils most of the criteria for a bioassay organism (DeWitt et al., 1989) and can grow under a wide range of saline habitats and seashore pools (Browitzka and Brown, 1974). It has also been proposed as a standard organism for seawater toxicity tests (APHA, 1998). Furthermore, little is known about the toxicity of dyes singly and in combination with metals to marine water algae (Manu and Chaudhari, 2003).

Algae may be quantified by various methods such as visual observation; microscopically by cell counting; photometrically by absorbance measurement; and by ATP quantification, fluorometrically or electronically, using counting devices. Enumeration of algal cells with an electronic particle counter or microscopic chamber (hemacytometer or blood cell counter) is time consuming and many environmental laboratories do not have the resources to purchase an electronic particle counter. Whereas, absorbance and fluorescence, for which affordable instruments can be used to determine biomass for algae tests (USEPA, 1993). In this study, cell counting, optical density and *in vivo* chlorophyll fluorescence methods were used to monitor the algal growth response to the exposed chemicals.

Even though, the algal growth inhibition test is an established tool for the assessment of the phytotoxicity of xenobiotic, with light absorbing substances, such as dyes, some problems arise. Since algae convert light energy to food through photosynthesis, light is a limiting factor in alga test and is as essential as growth media. Any decrease in the quantal flux in the photosynthetically active spectral region will cause a reduction of algal growth, which is independent of a potential toxicity of the test substance. In other words, due to light absorption of colored substances, availability of light for the algae is diminished; this will inevitably result in reduced algal growth (Cleuvers et al., 2002). Thus, beneath the

primary factor of toxicity, a secondary factor, namely the light absorption of a colored substance by inhibiting the metabolism of algal cells, will confound the analysis of the results (Oswald, 1988). Up to now, there has been no international agreement on how to handle light absorbing substances in the algal growth inhibition test. In this study, in order to understand whether light absorption of dyes will effect the algal growth or not, the experiments are planned to be carried out under the two light intensities (38.4 μE $m^{-2}s^{-1}$ and 76.8 μE $m^{-2}s^{-1}$).

There are some environmental and experimental factors such as test duration, temperature, nutrients, pH, and composition of wastewater, test medium, test conditions and the choice of response variables which influence test results (Saçan and Balcıoğlu, 2004) as well as light. An exposure period of 72-96 h is used in most toxicity tests; however, in this study 8-d test was used to see the potential effect of effluents on growth of algae since toxicity lost was reported after the sixth and seventh day (Walsh et al., 1982).

Of the bioassays bacterial screening test is also used for assessing toxicity of industrial wastewater. Bacterial screening tests have been based on the measurement of the reduction in light emission of the luminescent bacteria. They are rapid, reproducible, simple to use, unambiguous, cause no ethical problems and are cost-effective (Farre et al., 2001). Different organisms can be used as a test species to evaluate the toxic potential of an effluent. In the present work, we use the bioluminescence inhibition of *Vibrio fischeri*, because this organism is internationally used and standardized. Microtox® and ToxAlert® 100 are the two examples used for bioluminescence inhibition tests. In this study, ToxAlert® 100 by Merck was used to screen the toxicity of dye bath, assisting chemicals and three hydrolyzed dyes with and without selenium.

The main objective of this investigation is to evaluate growth responses of algae to assisting chemicals, dye bath, and the three hydrolyzed dyes having the highest concentrations in the synthetic dye bath recipe namely- Remazol Black 5, Reactive Orange 69, and Reactive Yellow 37- using various response parameters such as cell number, optical density at 750 nm and *in vivo* chlorophyll fluorescence during 8-d. Determination of the impact of different forms and concentrations of selenium on the growth of algae is another aim of this study knowing that the presence of other chemicals such as metals can

greatly influence the toxicity of dyes (Wong et al., 1997). Selenium is selected for this study because in one hand, it has become the primary element being required for normal growth and exerted a protection mechanism against harmful effects of certain toxic heavy metals; on the other hand, it becomes toxic at elevated concentrations to algae by interfering with sulfur-metabolism (Price et al., 1987). In order to decide which form of selenium is effective on the growth of *D. tertiolecta*, we aim to study the growth response of algae to the three forms of selenium including H_2SeO_3, $Na_2SeO_3.5H_2O$ and Na_2SeO_4. To examine the impact of a reduced light condition, performing additional tests using two different light intensities (38.4 µE $m^{-2}s^{-1}$ and 76.8 µE $m^{-2}s^{-1}$) is the third aim of this study. Finally the fourth aim is to evaluate the application potential of the marine water luminescent bacterium, *V. fischeri,* as a toxicity screening tool for the synthetic dye bath and three hydrolyzed dyes with and without selenium and to compare the results of this bioassay with those of algal assay.

2. THEORETICAL BACKGROUND

2.1. Textile Industry

Textile industry constitute the World's second largest industry, and textile processes have considerable impact on water quality, given the large volume of wastewater they generate and the physical and chemical properties of their effluents (USEPA, 1997). Textile wastewaters are complex waste products containing dyes, sizing agents and dyeing aids that are characterized by their deep blue color and high concentration of environmental pollutants (Banat et al., 1996).

Colored wastewater is a consequence of batch processes both in the dye manufacturing industries and in the dye-consuming industries. Dyes are synthetic in nature and generally have complex chemical structure hence persisting in nature. Some of these dyes and/or product of dye degradation are proven to be carcinogens and mutagens. Apart from the aesthetic deterioration of natural water bodies, dyes also cause harm to the flora and fauna in natural environment (Manu and Chaudhari, 2003).

2.1.1. Dyestuff used in textile industry

Dyes may be classified in several ways, according to chemical constitution, application class and end use. The primary classification of dyes is based on the fibers to which they can be applied and the chemical nature of each dye. Table 2.1 lists the major dye classes, fixation rates and the types of fibers for which they have an affinity.

The reactive and direct types for cotton dyeing and disperse types for polyester dyeing are commonly used in nowadays. Azo dyes are among the reactive dyes (Table 2.1) that are extensively used for dying cotton fabrics. Azo dyes are the most important of the dye classes, with the largest range of colors. All azo dyes contain at least one "-N=N-" group usually attached to two radicals of which at least one but usually both aromatic groups (Figure 2.1) (Zissi and Lyberatos, 1996; Beydilli et al., 1998). Reactive azo dyes occur in textile dyehouse wastewater in concentration ranging from 5 to 1500 mg L^{-1} due

to their poor fixation to fabrics (Pierce, 1994). Usually, dyestuffs with low biodegradability pass through the wastewater treatment plant and are released into the environment because of their high solubility in water. Their low BOD_5:COD ratio values (less than 0.1) indicate their resistance to conventional biological treatment. Effluents containing compound of azo dyes are more recalcitrant towards environment (Lopez et al., 1998).

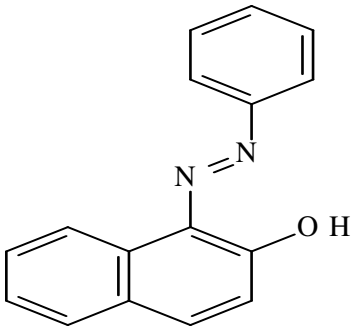


Figure 2.1. Chemical Structure of Azo Dyes (www.uncwil.edu/chem/chmlab212martin/212exp30.ppt)

2.1.2. Studies on the Toxicity of Dyes

There are several studies on toxicity of the dyes and their impact on the ecosystem. Some of these studies about the toxicity of dyes can be summarized as follow;

Ademoroti et al. (1992) found that residual water from textile facilities carried highly concentrated pollutants. Toxic heavy metals also promote the depletion of dissolved oxygen and destabilize the ability of water to reduce microbial loads and, thus, its ability for autopurificaiton.

Rutherford et al. (1992) determined the toxicity of wastewater from three textile mills to several organisms, and proved that the wastewaters were toxic to all organisms due to the large amounts of chemicals used by textile facilities.

Laughton et al. (1994) conducted toxicity bioassays in textile facilities in New Brunswick, Canada, using *Salmo gairdneri* and *Daphnia magna*. They demonstrated that wastewater remained toxic despite having been treated.

Table 2.1. Typical characteristics of dyes used in textile dyeing operations (USEPA, 1995; Snoweden-Swan, 1995).OOKPPKPKPLOPOPOĞ

Dye Class	**Characteristics**	**Associated Fibers**	**Typical Fixation (%)**	**Typical Pollutants Associated with Various Dyes**
Acid	Water-soluble anionic	Wool, nylon	80-93	Color; organic acids; unfixed dyes
Basic	Water-soluble cationic, very bright	Acrylic some polyesters	97-98	N/A
Direct	Water-soluble, anionic, poor wet fastness	Cotton, rayon, other cellulosics	70-95	Color; salt; unfixed dye; cationic fixing agents; surfactant; defoamer; leveling and redarding agents; finish; diluents
Disperse	Colloidal dispersion, low water solubility, good wet fastness	Polyester, acetate	80-92	Color; organic acid; carriers; leveling agents; phosohates; defoamers; lubricants; dispersants; delustrants; diluents
Reactive	Water-soluble, anionic, good wet fastness	Other synthetics	60-90	Color; salt; alkali; unfixed dye; surfactants; defoamers; diluents; finish
Sulfur	Organic compounds containing sulfur or sodium sulfide	Cotton, other cellulosics	60-70	Color; alkali; oxidizing agent; reducing agent; unfixed dye
Vat	Water-soluble, anionic, good wet fastness	Cotton, other cellulosics	80-95	Color; alkali; oxidizing agents; reducing agents

Villegas-Navarro et al. (1999) used *Daphnia magna* as the sensor organism and LC_{50} as a criterion to measure the toxicity of textile wastewater, both treated and non-treated. They found that the textile industry produced toxic non-treated water, and the treated water was also toxic.

In the study performed by Slabbert and Venter (1999) stream water containing the discharges of paper mill and the textile industry that flowed into a dam was reported to be very toxic. This toxicity seriously affected the protozoan (inhibition 9%), algae (inhibition 51%), bacteria (inhibition 71%) and toad embryos (lethality 36%, deformation 54%) in this river.

Galassi and Benfenati (2000) studied the acute toxicity of leachate from an industrial landfill and a textile effluent and their fractions with *Daphnia magna*. They found that in both cases the toxic effects due to xenobiotics were high.

Sharma and Sobti (2000) reported a large number of dyes to be toxic, genotoxic, mutagenic, teratogenic and/or carcinogenic. They determined the genotoxicity of four textile dyes (commonly used in India namely Sulfur Red Brown 360, Jade Green 2G, Reactofix Turquoise Blue 5GFL and Direct Scarlet 4BS) by *Bacillus subtilis* spore Rec assay. All these dyes were found to be toxic at higher doses.

Farre et al. (2001) used ToxAlert® 100 to evaluate various samples from influents and effluents of wastewater treatment work and also untreated wastewater from textile industries. They found that samples with high contents of industrial wastes from the textile and tannery industries were the most toxic samples.

Comparative toxicological profiles for bacteria (*Vibrio fischeri*), algae (*Scenedesmus subspicatus*), daphnia (*Daphnia magna*), fish (*Poecilia reticulata*), and plants (soybean-*Glycine max*, rice-*Oryza* sativa and wheat - *Triticum aestivum*) and as genotoxic effects (*Vicia faba*), were presented for both raw and ozonated textile effluents by Rosa et al (2001). They found that the relative sensitivity of bioassays in decreasing order was plant enzymes > bacteria > algae ≈ dapnids ≈plant biomass ≈ germination rate > fish. Also, they reported that both raw and treated textile effluents they tested had no significant genotoxic

effect. Furthermore, they observed that textile effluents were a stimulant to algal growth, mainly for treated effluents at higher concentrations (25 and 50%).

Wang et al. (2002) studied the toxicity of 11 dye stuffs from a textile dyeing mill in Ayazağa, Istanbul, Turkey. They found that the inhibition effects of numerous dyestuffs to luminescent bacteria differed considerably with the concentration.

Gottlieb et al. (2003) determined the toxicity of C.I. Reactive Black 5 and three procion dyes, as found in textile effluents using the bioluminescent bacterium *V. fischeri*. They found that hydrolyzed Reactive Black 5 had a slightly greater toxicity than the parent form.

2.2. Selenium

2.2.1. Chemistry of Selenium

John Jacob Berzelius, a chemistry teacher in Stockholm first identified selenium in 1817. Berzelius and his colleague Johann Gottlieb Gahn were studying a method of producing sulfuric acid in lead cameras when they observed residues of a substance with a very intense scent in the bottom of the camera. At first, it was thought as tellurium. However, a more careful analysis revealed that there were no residues of this element, in spite of its identical properties. To this new substance was given the name selenium, term that derives from the Greek *Selene* (Moon) (http://nautilus.fis.uc.pt/st2.5/ scenes-e/elem/e03400.html).

Selenium (Se) is classified as metalloid that has an atomic number 34, belongs to the VIA group elements. Selenium closely resembles sulphur in chemical properties with respect to atomic size, bond energies, ionization potential and electron affinities. Differences between these two elements are that selenium exists as reduced quadrivalent form whereas sulfur occurs as oxidized quadrivalent form and there is also difference in acid strengths between these two elements (Tinggi, 2003).

2.2.2. Sources and Impact of Selenium Contaminations

Selenium is a naturally occurring trace element. The abundance of selenium in the lithosphere is about 8×10^{-5} % or 800 mg metricton^{-1}. Selenium is widely dispersed in igneous rocks, probably as selenides; in sulfides, where it is isomorphous with sulfur; in hydrothermal deposits, where it is associated with silver, gold, antimony, and mercury; and in sulfide and porphyry copper deposits, where it occurs in large quantities but in small concentration. It is estimated concentration in the oceans is more than 0.09 ng mL^{-1}; here it occurs mostly as selenate ion SeO_4^{2-} (Kirk et al., 1972).

Industrial and agricultural activities have hastened the release of selenium from geologic sources and made them available to fish and wildlife in aquatic and terrestrial ecosystems around globe. Sources of selenium can be summarized as follows:

2.2.2.1. Coal Mining and Combustion. One of the primary sources of selenium mobilization in the environment is the procurement, processing, and combustion of coal for electric power production. All categories of solid waste and liquid effluents from the power industry are highly enriched with selenium as compared to the earth crust and surface water. Enrichment factors for selenium in coal can exceed 65 and are among the highest of all trace elements. Furthermore, when coal is burned to produce electricity, the ash that remains is enriched with selenium. The most serious impacts to aquatic life have occurred as a result of this type of selenium contamination (Lemly, 2004).

2.2.2.2. Gold, Silver, and Nickel Mining. Selenium is an important elemental component of the mineral matrix of ore deposits. Although present in low concentrations relative to other constituents, it has the potential to rapidly affect aquatic life because of its propensity to bioaccumulate and increase in concentration as it moves up the food chain (Lemly, 2004).

2.2.2.3. Metal Smelting. Metal ores contain selenium, and the physical/chemical treatment of this ore to extract the desired metal often releases selenium and other constituents into the process water and solid waste that is left. These wastes can contaminate local aquatic habitats (Lemy, 1994). Selenium can be easily volatilized and emitted into the air as a

vapor when a heating process is applied. Once released, this selenium cools and coalesces or adheres to atmospheric dust particles, subsequently reaching terrestrial and aquatic system by either wet or dry deposition (Germani et. al., 1981, Small et al., 1981).

2.2.2.4. Municipal Landfills. Municipal landfills can generate leach water contains elevated concentrations of selenium (5-50 µg Se L^{-1}) (Lemly, 2004). The source material for selenium in landfills involves large amount of photoelectronic components such as rectifiers, capacitors, photocopy printer/toner products, etc., which require selenium to operate properly (Sharma and Singh, 1983).

2.2.2.5. Oil transport, Refining, and Utilization. Similarly to the coal industry, procurement and refining oil produces a variety of selenium-laden wastes. However, crude oil contains much higher concentrations of selenium than coal (500-2000 µg Se L^{-1} vs. 0.4-24 µg Se g^{-1}), thus the potential for hazardous amounts to be released in process waters and effluents is relatively high (Lemly, 2004).

2.2.2.6. Agricultural Irrigation. When subsurface irrigation drainage is discharged into surface waters a variety of serious biological effects can take place. The immediate impact is degradation of surface and groundwater quality through salinization and contamination with toxic or potentially toxic trace elements (e.g., selenium, arsenic, boron, molybdenum, chromium, etc.) (Lemly, 2004).

2.2.3. Selenium essentiality

Biological importance of selenium for human and animal organisms was recognized over 40 years ago. Selenium is a normal component of some enzymes, proteins and aminoacryl derivatives of nuclei acid. Lately selenium has been found to be incorporated in 21st amino acid, selenocysteine, which plays a unique part in reading genetic information during synthesis of proteins. Trace elements are known to play an important part in oxygen metabolism. Some of them e.g. Fe and Cu form free hydroxyl radicals while others, such as Zn and Se, can reduce the harmful effect of radicals on the organism Selenium owe this property to its being incorporated in glutathione peroxides (GSHPx) (Mosulishvilli et al., 2002). This GSHPx assists in intercellular defense mechanism against oxidative damage by

preventing the production of active oxygen (Ursini and Bindoli, 1987). Selenium bioeffects are mainly involved in immune function, reproduction, mood, thyroid function, cardiovascular disease, viral infection, metal toxicity and photosensitivity of the eye (Jr and Gray, 1998, Rayman, 2000, Li et al., 2003).

In the aquatic environment selenium can exist in inorganic form (selenite, selenate, elemental selenium, metal selenides and in organic forms with direct Se-C bonds) (Köbl, 1994). Selenate and selenite are the most common inorganic forms of selenium. In aerobic environments selenate should be predominant form. In freshwater organisms, approximately 36 % of total selenium is present as selenate, the rest as selenite and selenide (Eisler, 1985). In other words, selenate coexists with selenite in many aquatic systems (Robberecht and Van Grieken, 1983). The amount of selenium in various species of *Dunaliella* was reported as varying from species to species (Yamaoka et al, 1990). A review of marine literature indicates that selenium occurs in seawater as selenite ions (SeO_3^{2-}) with a reported average of 0.2 μg L^{-1} (Riley and Skirrow, 1975). Although selenium is essential element, required for normal growth, at elevated concentrations it has toxic effect to algae by interfering with sulfur-metabolism (Price et al., 1987).

2.2.4. Selenium toxicity

In recent years the physiological role of selenium as a trace element has created considerable speculation and some controversy. Selenium has been reported as having carcinogenic as well as toxic properties; other authorities have presented evidence that selenium is highly beneficial as an essential nutrient. It has three levels of biological activity *(1)* trace concentrations are required for normal growth and development; *(2)* moderate concentrations can be stored and homeostatic functions maintained; and *(3)* elevated concentration can result in toxic effects.

Selenium toxicity causes disorders known as “alkali disease” and “blind staggers” on animal with respect to feeding with plants which accumulate selenium (Magg and Glen, 1967). Environmental toxicity of selenium in humans rare, however, the effects of selenium toxicity have been reported to cause hypochromic anemia and leucopenia, gastrointestinal disturbances (vomiting, diarrhea), hair and nail changes, and neurological

manifestations including acroparesthesias, weakness, convulsions, and decrease cognitive function (Clark et al., 1996; Gasmi et al., 1997). Selenium becomes toxic for marine organisms at seawater concentrations higher than required to provide the essential dose. 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} (Kölbl, 1994).

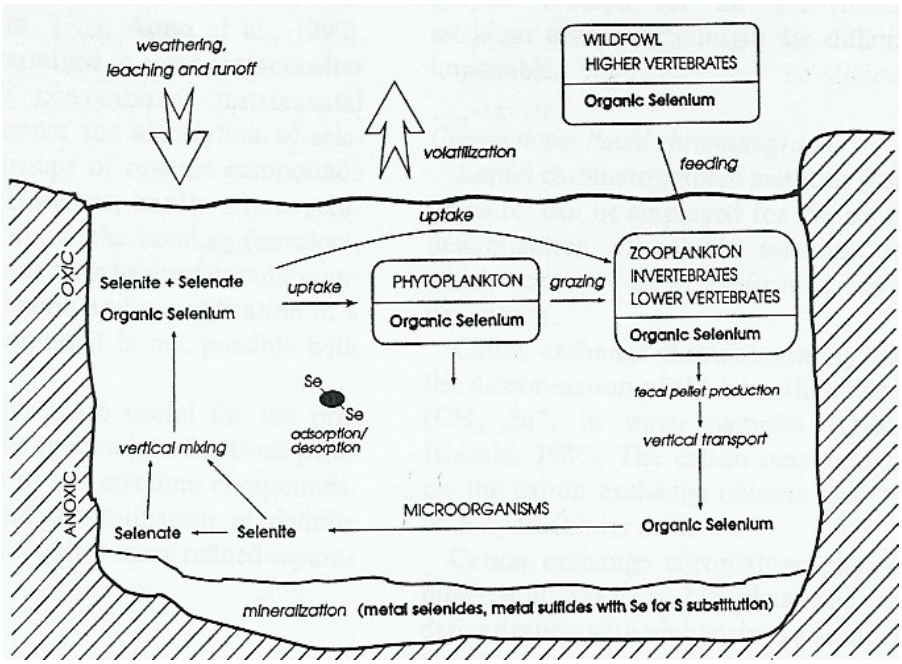


Figure 2.2. Biogeochemical cycling of selenium in the aquatic environment (Kölbl, 1994).

Several factors complicate the establishment of aquatic toxicological thresholds for selenium. These factors are associated with the complex biochemistry of selenium in aquatic ecosystems; first, selenium occurs in different oxidation states in aquatic environment including oxidized selenates (Se^{+6}), selenites (Se^{+4}), elemental selenium (Se^{0}), and reduced selenides (Se^{-2}). Each form is known to differ in bioavailability and toxicity to aquatic organisms. Second, selenium can undergo biotransformation between inorganic and organic forms as a result of biotic and abiotic processes, which are not well characterized. Third, selenium also has been shown to bioaccumulate in aquatic food webs to the extent that dietary exposure to selenium becomes a critical exposure pathway for top predatory aquatic and aquatic-dependent organisms (Sappington, 2002). The margin of safety for selenium between normal waterborne concentrations and those that lead to substantial bioaccumulation is extremely small (Lemly, 1994). It may possible produce an antagonistic or synergistic effect.

2.2.5. Studies on Selenium Bioaccumulation and Toxicity

There are several studies on toxicity and bioaccumulation of selenium and their impact on the aquatic system and algae. Some of these studies can be summarized as follow;

The toxicity and bioaccumulation of selenium in 4^+ and 6^+ oxidation states were investigated in a marine unicellular alga *Cricosphaera elongata* in culture. Selenite was found more toxic than selenate (Boisson et al., 1995).

The bioaccumulation of selenium by *D. tertiolecta* has been reported by Saçan et al. (1999) and they found that 0.5 mg L^{-1} selenium in the form of selenate prolonged the exponential phase of *D. tertiolecta*.

Takayanagi (2001) reported acute toxicity of Se (IV), Se (VI), to red seabream, *Pargus major*, an important commercial saltwater fish. He found that the different oxidation states of selenium caused diverse effects on the test fish. *Pargus major* appeared to be more sensitive to Se (IV) than to Se (VI). Se (IV), the lower oxidation state, was about seven times more toxic than Se (VI). Similarly, Hamilton and Buhl (1990) compared the relative sensitivity of *Chinook salmon* and *Coho salmon* to two oxidation state of Se, and they reported that Se (IV) was more toxic. Niimi and LaHam (1976) also reported that Se (IV) was 4 to 10 times more toxic than Se (VI) to the freshwater species (*Brachydanio rerio*) they studied.

Hamilton et al. (2002), was determined the effects on razorback sucker larvae (*Xyrauchen texanus*) exposed to waterborne and dietary selenium and arsenic, and zooplankton collected from sites adjacent to the Colorado. They found that selenium concentration of $\geq$ 4.6 μg g^{-1} in food organisms adversely affected the survival of razorback sucker larvae and elevated arsenic in one food source seemed to interact with selenium to reduce the toxic effect of selenium in mammals. Additionally, mortality was slightly delayed in larvae exposed to the highest selenium concentration due to the interaction between selenium and other inorganic elements.

Selenium is an essential trace element at low concentrations, as well as a toxicant at high concentration, to *Spirulina platensis*, which is consistent with other trace elements. *Spirulina platensis* is able to resist selenite through accumulation in cells at a low concentration of selenite and reduce selenite to elemental selenium at a higher concentration since Se^0 is poorly soluble and thus less toxic than selenite. The toxicity decrease of higher concentration selenite in the presence of sulfite suggested that there is competition between selenium and sulfur metabolism in *Spirulina platensis. Spirulina platensis* could bioaccumulate Se efficiently during the culture and accumulation increased with selenite concentration (Li et al., 2003).

Although most of the toxicity data on acute waterborne selenium toxicity is for the selenite form, we worked with the three forms of selenium. These are selenious acid (H_2SeO_3), sodium selenite ($Na_2SeO_3.5H_2O$) and sodium selenate (Na_2SeO_4).

2.3. Effect of Light Intensity

Light is an important environmental variables that can directly effect the growth of algal populations and influence the behavior of contaminants in aquatic system so, it is clear that the ecotoxicological testing of light absorbing substances such as dyes, cause specific problems in algal growth inhibition tests. Any decrease in quantal flux in the photosynthetically active spectral region will cause a reduction of algal growth, which is independent of a chemical toxicity of the test substance (Newsted, 2004; Cleuvers and Weyers, 2003). Bearing this in mind during the toxicity tests light needs to be controlled. The recommended light intensity is usually between 60 to 120 $\mu E\ m^{-2}s^{-1}$ and is less for freshwater blue green algae and marine algae than for freshwater green species (Mayer et al., 1998).

2.3.1. Studies on the Algal Response to Different Light Intensities

The studies about the effect of light intensity on the growth of algae can be summarized as follows;

Wangberg and Blanck (1988) generated 100% effective concentration (EC_{100}) values for three different algae and 19 chemicals at two light intensities (2 and 10 W m^{-2}). However, no general light-dependent toxicity was observed.

Dominguez-Bocanegra et al. (2004) analyzed impact of environmental factors such as light intensity, aeration and nutrients on the growth and astaxanthin production of unicellular clear water microalgae *Haematococcus pluvialis*. Maximum growth of *Haematococcus pluvialis* obtained at 28°C under continuous illumination (177 µmol photon $m^{-2}s^{-1}$) of white fluorescent light.

2.4. Response Parameters

Algae may be quantified by various methods such as cell counting, spectrophotometrically, or fluorometrically. For many years, electronically performed with an electronic particle counter or manually performed with a hemacytometer has been the reference methods used in order to determine cell density. Measurement of cell density electronically is precise and sensitive but the equipment costs are high. Further, microscopic counting with hemacytometer is time consuming and less accurate (Mayer et al., 1997; Markle et al., 2000).

Today phytoplankton biomass typically is estimated by measuring the fluorescence of chlorophyll *a* either *in vivo* or *in vitro* the principle photosynthetic pigment present in all algae. Measurement by fluorescence, the absorption of light energy at one wavelength and its instantaneous mission at a longer wavelength, is a highly specific, convenient, and sensitive analytic technique. Measurement of chlorophyll fluorescence *in vitro* is based on its extraction from disintegrated cells in an organic solvent such as methanol, ethanol, or acetone and on its subsequent determination by spectrophotometry and fluorometry, or chromatography (Jacobsen, 1978; Otsuki and Takamura, 1987). Acetone is typically used as an extraction solvent and the most commonly used extractive method developed by Strickland and Parsons in 1972. However, it has some disadvantages such as: being time consuming, requiring a standard sampling procedure, a necessity of a large volume of sampling and exposures possible quantitative changes during sample storage (Gregor and Marsalek, 2004). *In vivo* fluorescence is re-radiation of light absorbed by algal pigments in

intact cell. *In vivo* analysis is the direct measurement of chlorophyll in algal cells without extraction or chemical treatment. These measurements can be taken using discrete samples or continuous-flow. The obvious advantage of *in vivo* analysis is rapid, on-the-spot measurement eliminating the delays for extraction and laboratory measurement. For qualitative analysis, *in vivo* measurement alone may answer the analyst's questions. For quantitative determinations, the *in vivo* data are compared with other measurements, including fluorometric extractive data.

Quantification of algae by photometrically with absorbance measurement is inexpensive and a frequently used method but it offers lower precision and sensitivity than that of fluorescence. Various absorbance wavelengths have been suggested for determination of algal biomass. USEPA states 750 nm, the wavelength used for turbidity correction in the calculation of chlorophyll (USEPA, 1994; Mayer et al., 1997; Geis et al., 2000).

Comparisons of different techniques indicate that absorbance and fluorescence are better at accounting for total chlorophyll content and are also easier and less time consuming in the laboratories. Among the fluorometry and spectrophotometry; fluorometry is at least 1000 times more sensitive than spectrophotometry (Geis et al., 2000).

2.5. Test Organisms

Biological toxicity testing is applied to organisms at different tropic levels. The test organisms incorporated in these assays include microorganisms, plants, and invertebrates and fish. Most of the aquatic plants including algae are more sensitive than animals to a variety of potential toxicants and also algae are the dominant primary producers in the aquatic food chain. Test assays on various effluents from municipalities and industries have demonstrated greater sensitivity to algae than animal species. Algae and duckweed were more sensitive than invertebrates and fish to several detergent surfactants, textile effluents, acridine, dyes, synfuels, herbicides, and a variety of other phytotoxic compounds (Lewis, 1993; Walsh et al., 1982).

2.5.1. *Dunaliella tertiolecta*

D. tertiolecta is described by Butcher (1959) as an ovoid to ellipsoidal cell 9-11 µm long and 5.5-7 µm wide with an acute apex, tapering anter or, rounded posterior and a thin firm, hyaline periplast. It is bioflagellata unicellular green algae with a cell structure typical of the members of the order volvocales (class *Chlorophyceae*) though lacking a cell wall (http://www.marinebiology.edu/Phytoplankton/Profiles/Dunaliella-tertiolecta.htm).

D. tertiolecta is a naked, unicellular green alga which is well known for its extraordinarily high tolerance to salt stress, high light and relatively high temperatures (Berges and Falkowski, 1998). Although the optimum salinity for *D. tertiolecta* is 0.05 mol L^{-1} which is native to the marine environment it can grow in salinities ranging from 0.05 to 3.0 mol L^{-1}NaCl (Jahnke and White, 2003). Other *Dunaliella* species (*bardawil, parva* and *salina*) grew at higher salinities (5 mol L^{-1}NaCl), but could not acclimate to salinities less than 0.2–0.35 mol L^{-1}. *D. tertiolecta* grow in media with high concentration of $MgSO_4$ where as this species does not grow in media containing $MgCl_2$, $Mg(NO_3)_2$ and LiCl (Fujii, 1991). Members of the genus *Dunaliella* can be found in Dead Sea, the Great Salt Lake in Utah, some lakes in Wadi Natman in Egypt and many other sites (Oren et al., 1995). The culture of *D. tertiolecta* used in this study was isolated from the Sea of Marmara by the research group from the Scientific and Technical Research Council of Turkey Marmara Research Center (TÜBİTAK-MAM).

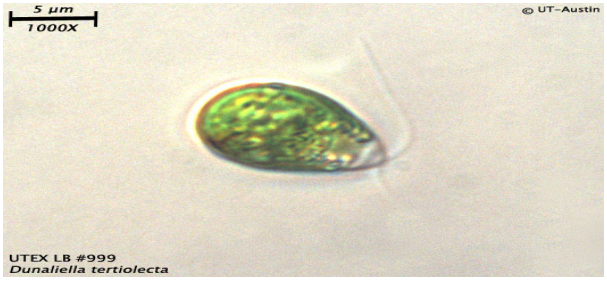


Figure 2.3. A cell of *D. tertiolecta* (http://www.bio.utexas.edu/research/utex/photogallery/Dunaliella_tertiolecta_999.htm)

2.5.2. *Vibrio fischeri*

V. fischeri is a gram-negative heterotrophic bacterium belonging to the *Vibrionaceae*, a large family within the gamma-proteobacteria, consisting of many species that are characterized by both cooperative and pathogenic interactions with animal tissue. *V. fischeri* has a worldwide distribution, principally in temperate and subtropical waters, where it occupies a variety of niches. In addition to being a light-organ symbiont of several species of squids and fish, this bacterium occurs as a member of the enteric consortia of many marine animals, as a pathogen of certain invertebrates, and as a 'free-living' saprophyte growing on dissolved and particulate organic matter. In locations where it forms light-organ symbioses with animals, free-living *V. fischeri* cells in seawater serve as the inoculums for the juvenile animal host (http://ergo.integratedgenomics.com/ Genomes/VFI/index.html).

The marine bacterium *V. fischeri* exists naturally either in a free-living planktonic state or as a symbiotic of certain luminescent fish or squid (Ruby and Nealson, 1976; Ruby and McFall-Ngai, 1999). The bacteria colonies specialized light organs in fish or squid, which cause them to bioluminescence. Luminescence in fish or squid is thought to be involved in the attraction of prey or even as camouflage. An ideal bacterial subject for studies is *V. fischeri*, a luminous marine species that lives as plankton in seawater and can also enter into benign associations with a number of marine organisms. Unlike many symbiotic bacteria, *V. fischeri* has a well-described environmental biology, has genes (e.g., lux) that have been useful in biotechnological applications, is closely related to bacteria of aquacultural importance, and has been studied using advanced molecular genetics.

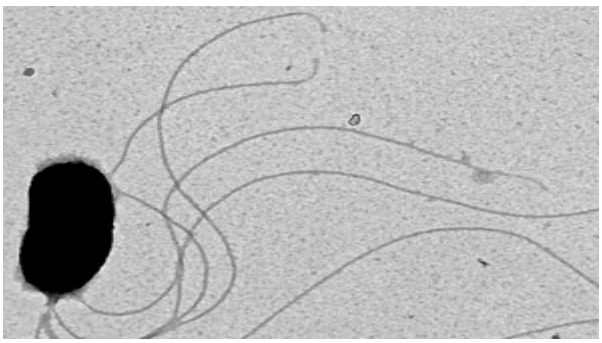


Figure 2.4. A cell of *V. fischeri tertiolecta* (http://ergo.integratedgenomics.com/ Genomes /VFI/index.html).

2.6. Studies on *Dunaliella tertiolecta*

There are limited studies on the toxicity of textile effluents to *D. tertiolecta*. Therefore, a few toxicity studies on *D. tertiolecta* can be summarized as follows:

The potential ecotoxicity of resuspended sediment arising from the dredging work along the coastal waters of Hong Kong was determined by Cheung et al. (2003). They demonstrated that the release of tributyltin from two contaminated sites during dredging was unlikely to attain the effective or lethal concentrations on the marine microalga (*D. tertiolecta*) and benthic invertebrate (*Melita koreana*).

The algal growth response to the raw and treated effluents of an aluminum plating plant and a pharmaceutical plant was investigated by Saçan et al. (2004) with *D. tertiolecta*. They found that all wastes affected algal growth either by inhibition only or by stimulation at low concentrations and inhibition at high concentrations.

Weiner et al. (2004) examined both atrazine uptake and cellular characteristics of microalgae to predict algal sensitivity. They listed the test species in order of increasing sensitivity as: *Isochrysis galbana*, *D. tertiolecta*, *Phaeodactylum tricornutum*, *Pseudokirchneriella subcapitata*, and *Synechococcus* sp.

Two commercially available, self-polishing antifouling paints were examined by Löschau and Kratke (2005) in order to get information on their antifouling properties and toxicological potential. They found that effects of a water-soluble paint were more pronounced in larvae of *Balanus amphitrite, Artemia salina* and in green algae *D. tertiolecta*. They also found that embryos of the freshwater species *Danio rerio* and *V. fisheri* were more affected by an organic solvent-based paint.

2.7. Toxicity Studies on *Vibrio fischeri*

The studies about the determination of the toxicity of textile effluents on *V. fischeri* were mentioned in section 2.1.2. There are also other toxicity studies on *V. fischeri*.

Two miniaturized growth inhibition assays with *V. fischeri* and *Pseudomonas putida* were used in order to determine the toxicity of heavy metals and some reference substances by Schmitz et al. (1998).

Choi and Meier (2001) monitored the toxic effect of metal plating wastes with using Microtox® assay which employs the marine bacterium *V. fischeri* and the results of the Microtox® assay were compared with data derived from conventional whole effluent toxicity testing method with *Daphnia magna*, *Ceriodaphnia dubia*, and *Pimephales promelas*. They found that Microtox® assay correlated better with the acute fish and daphnid toxicity test with metal-rich water.

Acute toxicity of pesticides in water was assessed by Fernández-Alba et al. (2001) singly and in mixtures using various responses of the luminescent bacterium *V. fischeri*, the aquatic invertebrate *Daphnia magna*, and the MitoScanTM assay. They found that *D. magna* assay was the most sensitive and best able to detect toxic interactions of mixtures.

Polycyclic aromatic hydrocarbons (PAHs) compounds to sediments of the Niger Delta, Nigeria were assessed by Olajire et al. (2005) using combined chemical analysis and toxicity bioassay techniques with *V. fischeri* and *Lemna minor*. The toxicity bioassays indicated that the sample collected from the Warri Refinery Area was the most toxic to *V. fischeri* and samples from Ogunu and Warri Refinery area showed high toxicity to *Lemna minor*. The total PAH concentrations showed no correlation with toxicity bioassays, and thereby implied that chemical analysis of PAHs cannot be an indicator of sediment toxicity.

LeBlond and Duffy (2001) found that the Microtox® assay is able to overcome many of the complications seen in the algal growth assay. Purchasing a freeze-dried stock solution of organisms may be the greatest advantage, as this eliminate a great deal of time and labor and greatly reduces the genetic variance one would expect from a laboratory grown solution.

2.8. Algal Culture Methods

There are several methods for culturing algae. Of the culturing methods, only “batch" and "continuous” cultures were included in the following section.

2.8.1. Batch Culture

The most common culture system is the batch culture, due to its simplicity and low cost. This is a closed system in which there is no input or output of materials. The algal population cell density increases constantly until the exhaustion of some limiting factor, while other nutrient components of the culture medium decrease over time. Any products produced by the cells during growth also increase in concentration in the culture medium. Batch culture systems are highly dynamic.

A significant advantage of batch culture systems is their operational simplicity. It was also found that the accuracy of growth rate determination was highest in an artificial medium as compared to cells grown in natural surface water. This would make suspect the use of batch culture as a bioassay technique for the determination of toxic substances in natural waters. If, however, the toxic substance is added at known concentrations to the artificial growth medium, better results should be obtained. A significant advantage of batch culture systems is their operational simplicity.

In this method algal cells are allowed to grow and reproduce in a closed container. They have a finite amount of nutrient, and when that is exhausted, their growth stops and eventually they die. These types of cultures typically last for about one week. After that, sub-culturing must be made by adding some cells from the old culture into a flask containing fresh growth medium. This type of culture is one way to illustrate some of the most important concepts in ecology, namely the effects of limited resources on population growth, i.e. "carrying capacity" of an environment (http://io.uwinnipeg.ca/~simmons/ysesp/iso5.htm).

2.8.2. Continuous Culture

Continuous culture systems have been widely used to culture microbes for industrial and research purposes. This method of culturing algae differs from the batch culture method in that fresh medium is added to the culture at a constant rate and old media (and some of the algae cells) is removed at the same rate. The culture therefore never runs out of nutrients. The glassware and tubes for this type of system are much more complex then the simple batch culture.

Batch and continuous culture systems differ in that in a continuous culture system, nutrients are supplied to the cell culture at a constant rate, and in order to maintain a constant volume, an equal volume of cell culture is removed. This allows the cell population to reach a "steady state" (i.e. growth and cell division where the growth rate and the total number of cells per milliliter of culture remains constant). Fogg and Thake (1987) make the distinction between "turbidostat" and "chemostat" continuous culture systems. In the turbidostat system, fresh medium is delivered only when the cell density of the culture reaches some predetermined point, as measured by the extinction of light passing through the culture. At this point, fresh medium is added to the culture and an equal volume of culture is removed. The diluted culture increases in cell density until the process is repeated. In a chemostat, the medium is delivered at a constant rate, which ultimately determines growth rate and cell density. The principal advantage of continuous culture is that the rate of dilution controls the rate of microbial growth via the concentration of the growth-limiting nutrient in the medium (http://io.uwinnipeg.ca/ ~simmons/ysesp/iso5.htm).

2.9. Toxicity Tests and Dose-Response Relationships

The USEPA (1994) defines toxicity testing as "the means to determine the toxicity of a chemical or effluent using living organism. A toxicity test measures the degree of response of an exposed test organism to a specific chemical or effluent." There are many forms and methodologies for a toxicity test; the two most widely used forms of toxicity testing are the acute and chronic toxicity tests performed using both freshwater and marine organisms. Acute toxicity tests are short-term tests designed to measure the effects of

pollutants on species during a short part of their life span. The tests, which typically run for between 48 and 96 h, usually measure the effects of pollutants on the survival of a species. The results of these tests are often reported as an 'EC_{50}', which is the effective concentration of a test sample that causes a specific effect (here a 50% reduction in survival). However, chronic tests are used for low-level pollutants and high-risk situations. These are long-term tests relative to the life span of the species, and are designed to measure larval development or growth, and reproductive success or failure (Donald, 1998).

The concept of a dose-response (or concentration response) relationship is a fundamental one in toxicology. This concept assumes that there is a casual relationship between the dose of a toxicant (or concentration for toxicants in a solution) and a measured response. The classical concentration-response relationship is depicted as a sigmoidal shaped (s-shaped) curve; however the particular shape of concentration-response curve may differ for each coupled toxicant and response pair (Calabrese, 2002). In general more severe responses occur at higher concentrations of toxicant, and less severe responses occur at lower concentrations. Two parameters of this curve are used to describe it: (1) the concentration or dose that results in 50% of the measured effect and (2) the slope of the linear part of the curve that passes through the midpoint. The midpoint is commonly referred to as a LD_{50}, LC_{50}, EC_{50}, and IC_{50}. The definitions of these parameters can be described as follows (Landis and Yu, 1999):

LD_{50} : The dose that causes mortality in 50% of the organisms tested estimated by graphical or computational means.

LC_{50} : The concentration that causes mortality in 50% of the organisms tested estimated by graphical or computational means.

EC_{50} : The concentration that has an effect on 50% of the organisms tested estimated by graphical or computational means.

IC_{50} : Inhibitory concentration that reduces the normal response of an organism by 50% estimated by graphical or computational means. Growth rates of algae, bacteria, and other organisms are often measured as an IC_{50}.

Generally the concentration-response concept is the basis for the determination of inhibition concentration in toxicity. Inhibition concentration is a point estimate of the toxicant concentration that would cause a given per cent reduction in a non-lethal, non-

quantal biological measurement, such as growth. For example, an IC_{25} is the estimated concentration of a toxicant that would cause a 25% reduction in average growth. Inhibition concentrations may be calculated using a linear interpolation method such as EPA's Bootstrap Procedure (http://www.casaweb.org/Glossary/Glossary.pdf).

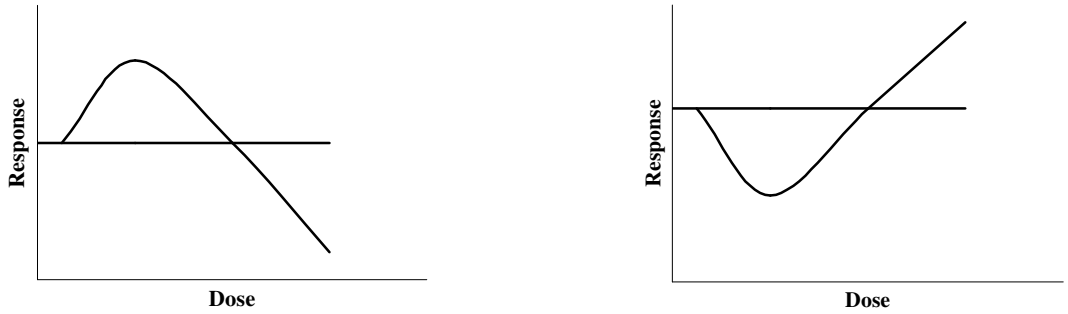
There is much debate over the fundamental shape of the dose response curve in the low-dose zone. The field of toxicology has been dominated by the use of two dose-response models, the threshold model and the linear model (Figure 2.5). Although there has been much controversy over the nature of dose-response (i.e. is it threshold or linear), a re-invigorated hypothesis has emerged that the most fundamental shape of the dose-response is neither linear nor threshold, but rather biphasic, that is U-shaped. Such U-shaped dose-response relationships are often referred to as being examples of the phenomenon of hormesis that is characterized as a dose response with stimulation at low and inhibition at high dose. Furthermore, hormesis is characterized by either U-shaped or an inverted U-shaped dose response depending on the end-point measured (Figure 2.6). For instance, the dose-response would be U-shaped if the end point were carcinogenesis, mutagenesis, and disease incidence however; the inverted U-shaped (J-shaped) could occur if longevity, growth or fecundity had been measured (Calabrese, 2004).

The occurrence of hormesis has been suggested in various biological, toxicological, pharmacological and medical investigations. Furthermore, although most of the experimental data relevant to the concept of the hormesis has been performed with single agents over a broad range of doses, hormetic effects have been reported in studies dealing with complex mixtures of petroleum and with wastewater effluents (Calabrese and Baldwin, 2002).

The quantitative features of the hormetic dose response is a modest stimulation at low doses where the maximum stimulation is typically 30-60% greater than the control, and a range of stimulation that can be variable but is typically less than 10-20 fold, although approximately 5-7% of hormetic dose responses can exceed 100-fold. In fact, so routinely was the hormetic response observed that the investigator proposed the creation of the term SC_{20} (i.e. the stimulation concentration for 20% increase above the controls) to describe the stimulatory response in the low concentration range (Walsh et al., 1982).

(a) (b)

Figure 2.5. Stylized curves illustrating (a) linear, (b) threshold model (Calabrese, 2002).



(a) (b)

Figure 2.6. The most common form of hormetic dose response curves (a) inverted U-shaped, (b) U-shaped (Calabrese, 2002).

3. MATERIALS AND METHODS

3.1. The Synthetic Reactive Dye Bath and Single Dye Stock Solutions

In this study synthetically prepared dye bath and single dye solutions according to the procedure obtained from local integrated textile manufacture plant were used. In the exhausted dye bath 15% dyestuff (in their hydrolyzed form) and 100% of all assisting chemicals remain. The synthetic dye bath contained six reactive dyestuffs widely used in textile industry and proper amounts of assisting chemicals. The concentration of the reactive dyes and assisting chemicals in the synthetic stock solutions are summarized in Table 3.1. In order to guarantee that all dyestuffs in the prepared mixture were 100% hydrolyzed, they were dissolved in boiling deionized water together with their assisting chemicals and stirred for 4 hour and then the dye bath and single dye stock solution was kept in a cool and dark conditions for 12 hour before use (Arslan, 2000).

3.2. Preparation of Algal Culture

In this study, a unicellular green algae "*D. tertiolecta*" had been used throughout the experiments. Culture of the green algae, *D. tertiolecta*, was supplied by (TÜBİTAK). *D. tertiolecta* was cultured in 500 mL Erlenmeyer flask in a controlled growth chamber (WTCB Binder Model KBF 240) under a continuous light regime, and maintained at 18±2°C. Although we carried out experiments at two light intensities (38.4 μE $m^{-2}s^{-1}$and 76.8 μE $m^{-2}s^{-1}$) for dye bath with and without selenium, for other tests, we used 76 μE $m^{-2}s^{-1}$ as a "standard light intensity" since 60 to 120 μE $m^{-2}s^{-1}$ is recommended in international guidelines.

The inoculum was prepared using *D. tertiolecta* harvested from four- to-eight day old stock culture. Each milliliter of inoculums must contain enough cells to provide an initial cell density approximately 10,000 cells mL^{-1} (±10%) in the test chambers. Experimental cultures were carried out in natural filtered (GF/C Glass microfiber Whatman filters) seawater and enriched with f/2 medium (Okay et al., 1994) described in Table 3.2.

Table 3.1. The concentration of the chemical components present in the synthetic dye bath and their functions in the dyeing process (Arslan, 2000).

Dyestuff (color index)	**Company**	**Reactive Group**	**Concentration(g L^{-1})**
Remazol Black SB 133% (Reactive Black 5)	Dystar (Hoechst)	Sulphatoethylsulphonate	0.583
Remazol Red 3B (Reactive Red 23)[a]	Dystar (Hoechst)	Sulphatoethylsulphonate	0.084
Remazol Brilliant Yellow GL (Reactive Yellow 37)	Dystar (Hoechst)	Sulphatoethylsulphonate	0.126
Drimarene Orange K-GL (Reactive Orange 69)	Clariant (Sandoz)	Monochlorodifluoropyrimidine	0.167
Drimarene Rubinol R/K-5BL (Reactive Red 171)	Clariant (Sandoz)	Monochlorodifluoropyrimidine	0.076
Drimarene Blue K-2RL (Reactive Blue 209)	Clariant (Sandoz)	Monochlorodifluoropyrimidine	0.092
Assisting 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

[a] Contain 1.6% Copper in Complexed Form

Seawater was taken in May 2003 from the coast of Samatya in Istanbul and stored in deep freeze after filtration at –24ºC in a plastic container. Natural seawater was characterized by measurement of pH, temperature, salinity, conductivity, chloride, alkalinity, nitrate, total kjeldahl nitrogen and phosphate based on standard procedures (APHA, 1998) (Table 3.3).

Each treatment had a minimum of three replicates plus at least one control. Two types of control were disposed: flasks with microalgae but no azo dyes and/or selenium (growing controls) and flasks without alga or azo dyes and/or selenium (background controls). Flasks were hand shaken twice daily to prevent settling of the cells. All glassware were washed with nitric acid and rinsed thoroughly with tap water. Deionized water was used for the final rinse. All glassware was sterilized at 180ºC for two hours in a sterilizer. Seawater and nutrient solutions were sterilized by standard autoclaving procedure.

3.3. Test Medium

Batch experiments were carried out 500 mL conical flask and each treatment (including the control) had three replicates. In order to examine the impact of different concentrations and forms of selenium on the growth of *D. tertiolecta*, cultures of *D. tertiolecta* in the lag phase exposed to increasing selenite and selenate concentration ranging from 0.5-1.5 mg L^{-1} in the form of H_2SeO_3 (Riedel-de Haën), $Na_2SeO_3.5H_2O$ (Riedel-de Haën) and Na_2SeO_4 (Sigma). The subsequent growth rate was recorded by measuring optical density at 750 nm (OD_{750}) with a spectrophotometer (Shimadzu UV-1208) for 8-d.

Since we observed that, selenite (SeO_3^{2-}) has the most stimulatory effect on the growth of algae, *D. tertiolecta* exposed to dye bath singly and in combination with 0.5 mg L^{-1} selenium in the form of selenious acid (H_2SeO_3) under continuous illuminations of 38.4 µE $m^{-2}s^{-1}$ and 76.8 µE $m^{-2}s^{-1}$.

Table 3.2. Final concentration of nutrients in culture medium (f/2) and origin of chemical substances used (Okay et al., 1994).

Nutrient	Amount (mg L^{-1})	Concentration (μM)	Origin
$NaH_2PO_4.2H_2O$	75	882.35	Merck
$NaH_2PO_4.2H_2O$	5	23.82	Merck
$Na_2SiO_3.5H_2O$	12.9	60.85	Merck
$CuSO_4.5H_2O$	0.01	0.04	Merck
$ZnSO_4.7H_2O$	0.022	0.09	Merck
$CoCl_2.6\ H_2O$	0.01	0.04	Panreac
$MnCl_2.4\ H_2O$	0.18	1.11	Merck
$FeCl_3.6\ H_2O$	3.15	11.65	Merck
$Na_2Mo_4.2\ H_2O$	0.6	0.13	Merck

Table 3.3. Characterization of seawater.

Parameters	Unit	Value
pH		8.4
Temperature	°C	21.1
Salinity	‰	22.2
Conductivity	mS cm^{-1}	32.7
Cl^-	g L^{-1}	11.93
NO_3^-N	mg L^{-1}	1.2
TKN	mg L^{-1}	15
Alkalinity	mg $L^{-1}CaCO_3$	178
PO_4^{3-}	mg L^{-1}	51.4

Determination of the growth response of assisting chemicals, dye bath, and three hydrolyzed dyes alone and in combination with selenium on *D. tertiolecta* was carried out using three different response parameters; cell number, OD_{750}, and *in vivo* chlorophyll fluorescence during 8-d. Synthetic dye bath and single dye stock solution made up 40-fold concentrated in deionized water together with assisting chemicals and were filter sterilized prior to addition to the sterile culture medium. They were diluted with natural seawater to give a concentration series of 20, 40, 80, and 100% and enriched with modified f/2 medium (Okay et al., 1994).

3.4. Measurement of Algal Response

Algal response was measured with three different methods: *(1)* microscopically with cell counting by a Thoma hemacytometer under binoculor light microscope. Algae were fixed by formaldehyde before cell counting, *(2) in vivo* chlorophyll fluorescence intensities with a fluorimeter (PerkinElmer LS55 Luminescence Spectrophotometer) and *(3)* optical density (OD_{750}) with a spectrophotometer (Shimadzu UV-1208). Direct fluorescence was measured from a 2-mL aliquot with a 430-nm excitation filter and 663-nm emission filter. A low/high fluorescence level compared to control indicated a reduced/increased level of photosynthetic pigments and therefore toxicity to the photosystems.

The coefficient of variation (CV) was calculated by dividing the standard deviation by the mean and multiplying by 100 for the purpose of comparison of endpoint variability as follows:

$$\mathrm{CV} = \left(\frac{S.D.}{Y} \right) \times 100 \tag{3.1}$$

where *S.D.* is the standard deviation and *Y* is the mean. Although no significant differences between response variables could be observed, cell count gave higher coefficient of variation (CV) in Dye bath, Remazol Black 5 and Reactive Yellow 37 than those based on *in vivo* chlorophyll fluorescence. A calibration curve of *in vivo* chlorophyll fluorescence versus cell number was used for these cases (See Appendix A).

3.5. Data Analysis

Growth curves were constructed and the area under the growth curve for individual test vessels was calculated with the following equation (LeBlond and Duffy, 2001):

$$AUC = \frac{(N_1 - N_0)}{2} \times t_1 + \frac{N_1 + N_2 - 2N_0}{2} \times (t_2 - t_1) + ... + \frac{N_{n-1} + N_n - 2N_0}{2} \times (t_n - t_{n-1}) \tag{3.2}$$

where,

AUC = area under the growth curve,

N_0 = absorbance (750 nm)/fluorescence intensity at the beginning of the test (relative units),

N_1 = absorbance (750 nm)/fluorescence intensity at the selected time interval (relative units),

N_n = absorbance (750 nm)/fluorescence intensity at the end of the selected time interval (relative units),

t_1 = the first selected time, and

t_n = the n times selected times.

Per cent inhibition/stimulation of the growth area of individual test vessels to the mean value for control in each experiment was calculated using the following equation:

$$I_g(\%) = \frac{AUC_c - AUC_t}{AUC_c} \times 100 \quad (3.3)$$

where,

I_g = per cent inhibition/stimulation of the growth area of an individual test vessel,

AUC_t = area of each test vessel, and

AUC_c = mean area of the controls in each experiment.

(+) Positive percentage indicates inhibition and (-) negative percentage indicates a stimulating effect as the algal density in the waste containing sample is higher than that of the control.

3.6. Toxicity Measurement Using *V. fischeri*

Toxicity was determined by using *V. fischeri* and the following standard operational procedure. The bioluminescence was measured in a luminometer (ToxAlert® 100 (Merck), by Leyla Tolun, Earth and Marine Sciences Research Institute, Scientific and Technical Research Council of Turkey-Marmara Research Center (TÜBİTAK-MAM).

V. fischeri is a marine bacterium that requires an environment with an adequate salt content. All samples were prepared in aqueous solution containing 2% NaCl solution prior to measurement. All samples were tested in duplicates. The reconstitution time for the dried bacterial reagent was 15-min. Then, the bioluminescence of each vial was measured. The vials were incubated at 15°C for a fixed time. After this period of incubation, the 30-min bioluminescence of each vial was measured. Finally, the percentage of inhibition for each vial was obtained by the software incorporated into the device.

The inhibition of natural luminescence of photo-bacteria is regarded as toxicity endpoint. For the tested chemicals two toxic designations for each sample were utilized namely EC_{25} and EC_{50}. The EC_{25} and EC_{50} is the amount of a substance or a sample that produces 25% and 50% bioluminescence inhibition, respectively.

3.7. Statistical Analysis

Dunnett's post-hoc test was employed to determine if treatments were significantly different than the control using the General Linear Model (GLM), univariate procedure in SPSS 11.0 software. The most common parameter used in algal toxicity assays is the IC_{50} or IC_{25}, *i.e.*, the concentration of the tested substance that decreases the growth by 50% or 25%, respectively. IC_{25} values were determined using linear interpolation combined with bootstrapping (known as the IC_p method) as outlined in USEPA (1993) and Noberg-King (1993) (See Appendix B). In order to obtain the 8-d SC_{20} value, each I_g value per concentration was plotted against each concentration and curve fitting analysis was applied directly on the data using SPSS-PC+ software (SPSS Inc.). The best fitting model was selected based on the maximum *F* (Fischer statistic) and minimum *p*-values. From the developed curve fitting equations the 8-d SC_{20} values were found using Scientific Workplace-PC+ software. Data were statistically analyzed by the paired *t*-test ($p<0.05$).

For *V. fischeri* The *EC*-values were calculated by plotting growth inhibition value against the concentration and curve fitting analysis was applied directly on the data using SPSS-PC+ software (SPSS Inc.). The best fitting model was selected from the developed curve fitting equations. The 30-min EC_{25} and EC_{50} values were found using Scientific Workplace-PC+ software. Statistical analyses were done by using paired *t*-test ($p<0.05$).

The joint toxicity of mixtures is generally evaluated with method based on empirical toxic unit (TU) concepts (Moreau et al., 1999). TU corresponds to the ratio of 100/Y, where Y' corresponds to the lowest observed effective concentration (LOEC). In this study, TU (% v/v) was calculated using both the 8-d IC_{25}, SC_{20} and EC_{25} values obtained from the three response parameters.

4. RESULTS AND DISCUSSION

4.1. Effect of Selenium on the Growth of *Dunaliella tertiolecta*

A typical growth pattern for *D. tertiolecta* growing in f/2 medium with selenious acid, sodium selenite and sodium selenate for the concentration range of 0.5 mg L^{-1}-1.5 mg L^{-1} under the continuous illumination of 76.8 μE $m^{-2}s^{-1}$ is shown in Figures 4.1(a), (b) and (c), respectively. Error bars show the mean standard deviation between triplicates. 0.5 mg L^{-1} selenious acid and sodium selenite had the highest stimulatory effect on the growth of algae among the studied concentrations. However, sodium selenate had the highest stimulatory effect at 1 mg L^{-1} compared to the control. These results reflected that algal response to selenite and selenate is different. Stimulatory effect of selenium was also reported in the literature by Lindström and Rhode (1978), Wheeler et al. (1982) and Wehr and Brown (1985).

Algae depend on light for their growth, however; colored substances such as dyes can cause some problems in algal toxicity tests. Due to their light absorption, colored substances can inhibit algal growth, which is not the result of a toxic action. If light saturation is not achieved, then a decrease in the quantal flux in the photosynthetically active spectral region will inevitably result in reduced algal growth, independent from toxicity of the test substance. This shading effect can confound measures of chemical toxicity (Cleuvers and Weyers, 2003; Cleuvers et al., 2002). Thus, in order to see if this case was true or not in our study we decreased the light intensity by one half. Since we planned to determine the effect of synthetic dye bath on the growth of *D. tertiolecta* in the presence and absence of selenium at two light intensities, at first we needed to study how algae respond to selenium species by changing the light intensity. There is no available information on this topic in the literature. Calculated area under the growth curves and growth inhibitions were tabulated in Table 4.1 together with the coefficient of variations for two light intensities (76.8 μE $m^{-2}s^{-1}$ and 38.4 μE $m^{-2}s^{-1}$).

As it can be seen from Table 4.1 and Figure 4.2 all forms of selenium (H_2SeO_3, $Na_2SeO_3.5H_2O$ and Na_2SeO_4) tested with *D. tertiolecta* were effective on the growth under continuous illumination of 76.8 μE $m^{-2}s^{-1}$. Stimulation effect of selenium on the growth

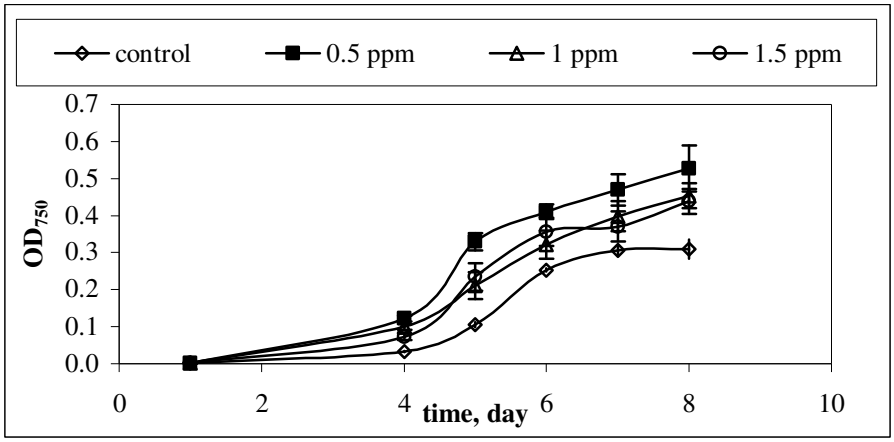
response of algae was observed compared to control within the studied concentration level ranging from 0.5-1.5 mg L^{-1} (Figure 4.2). The maximal stimulation effect was observed for 0.5 mg L^{-1} of H_2SeO_3 and $Na_2SeO_3.5H_2O$. However, it decreased at the higher test concentrations and then stayed constant. At 76.8 µE $s^{-1}m^{-2}$, no significant difference was observed between selenious acid and sodium selenite on the growth of alga. However, significant difference was observed between selenite and selenate on the growth of algae at the lowest studied concentration (0.5 mg L^{-1}). Concentration dependent growth stimulation was observed for the selenite. As the concentration of selenite increased stimulatory effect decreased.

Table 4.1. Changes in optical density at 750 nm of *D. tertiolecta* exposed to 0.5- 1.5 mg Se L^{-1} under illumination of 76.8µE $m^{-2}s^{-1}$ and 38.4µE m^{-2} s^{-1}.

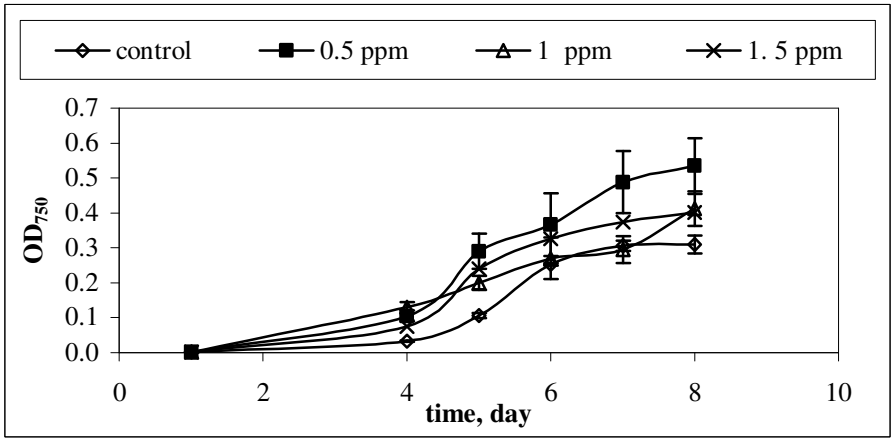
Light Intensity							
	76.8 µE $m^{-2}s^{-1}$					**38.4µE m^{-2} s^{-1}**	
Chemicals	**Concentration (mg L^{-1})**						
		Control	0.5	1.0	1.5	Control	0.5
H_2SeO_3	AUC[a]	0.89	1.77	1.39	1.39	0.22	0.43
	CV	3.25	6.91	8.51	1.38	12.74	15.39
	Ig (%)[b]		-98	-57	-56		-94
$Na_2SeO_3.5H_2O$	AUC	0.89	1.61	1.25	1.32	0.22	0.34
	CV	3.25	18.67	7.10	11.44	12.74	3.32
	Ig (%)		-81	-40	-48		-55
Na_2SeO_4	AUC	0.89	1.11	1.31	0.98	0.22	0.20
	CV	3.25	9.94	6.24	5.42	12.74	7.93
	Ig (%)		-25	-47	-11		8

[a] All AUC are mean values from repeated tests (*n*=3); CV = coefficient of variation.
[b] Ig (%) = Per cent growth inhibition/stimulation; negative values show stimulation of growth with respect to control

The low-dose stimulatory effect was clearly observed for all forms of selenium. The algal growth response to higher concentration of selenium species was not determined, since it is well known that the growth of some species of marine micro algae could be affected by a high concentration of selenite and selenate (Wong and Luis, 1991). Selenium becomes toxic for marine organisms at seawater concentrations higher than required to provide the essential dose. 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).



(a)



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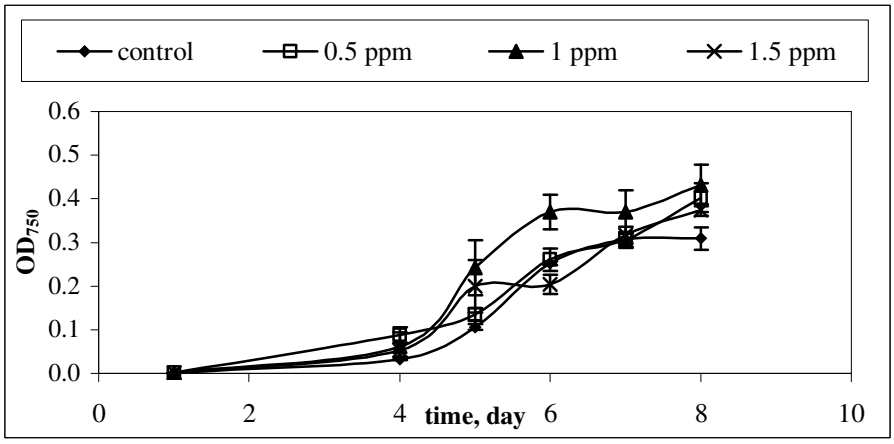


(c)

Figure 4.1. Growth curves of *D. tertiolecta* exposed to different concentrations of (a) selenious acid, (b) sodium selenite and (c) sodium selenate.

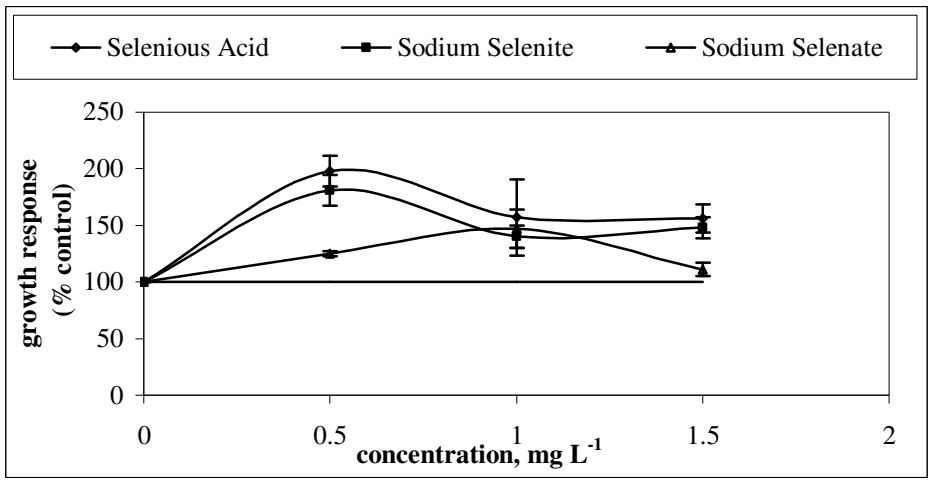
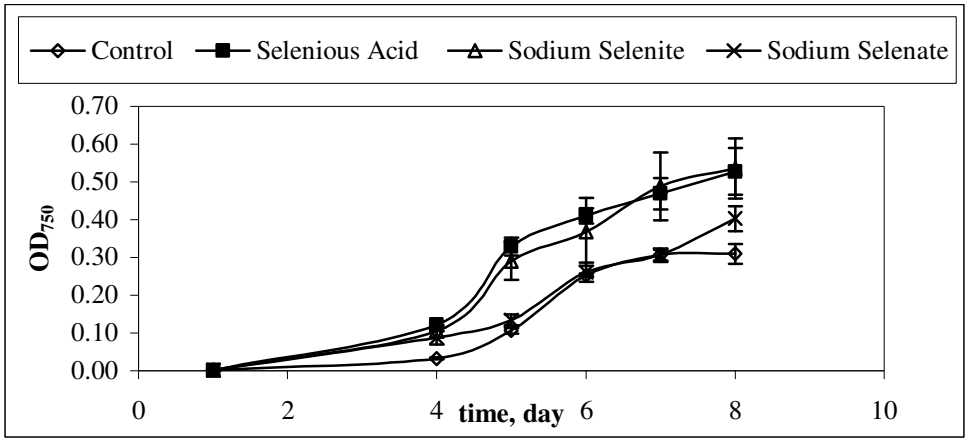
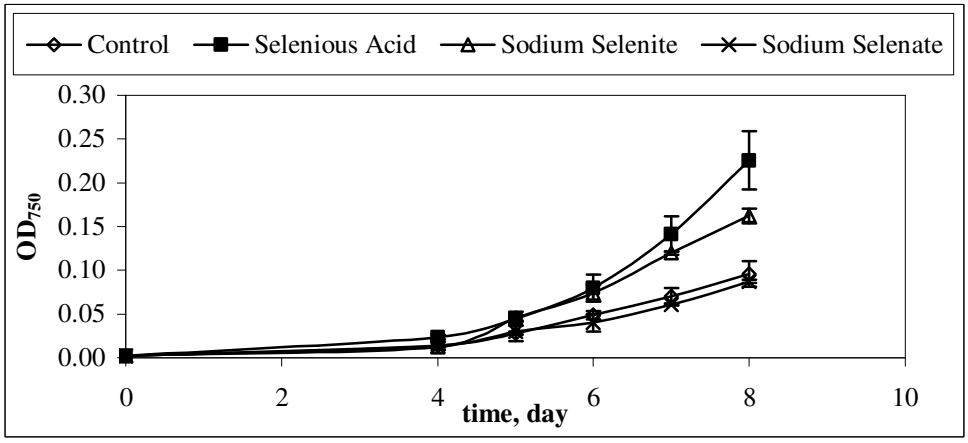


Figure 4.2. Representative dose-response curves of algae exposed to H_2SeO_3, $Na_2SeO_3.5H_2O$ and Na_2SeO_4 obtained from the measurement of optical density at 750 nm under the illumination of 76.8 µE $m^{-2}s^{-1}$.

Since we observed significant difference only between the growth response of algae to selenite and selenate at 0.5 mg L^{-1}, we repeated the same experiment for only 0.5 mg L^{-1} for the three forms of selenium by lowering the light intensity one-half. As the light intensity decreased by one-half (from 76.8 µE $m^{-2}s^{-1}$ to 38.4 µE $m^{-2}s^{-1}$), the stimulatory effects of all forms of selenium decreased. Figures 4.3(a) and 4.3(b) represent the growth curves of *D. tertiolecta* in the presence of 0.5 mg L^{-1} selenium under the illuminations of 76.8 µE $m^{-2}s^{-1}$ and 38.4 µE $m^{-2}s^{-1}$, respectively. It can be seen that the lag phase of *D. tertiolecta* was prolonged when the light regime was decreased (Figure 4.3. (b)). At low light intensity, there was again no significant difference between the algal response exposed to selenious acid and sodium selenite. Selenate did not influence the algal response at 38.4 µE $s^{-1}m^{-2}$ compared to control. In contrast to selenate, selenite stimulated the growth of algae at 38.4 µE $s^{-1}m^{-2}$ although per cent growth stimulation values for selenite were remarkably lower than that of the illumination of 76.8 µE $s^{-1}m^{-2}$. This stimulation effect of selenite at low light level can be attributed to the adoption of *Dunaliella* in darker environment in the presence of selenite. Although we can not explain why a decrease in stimulatory effect of selenium was observed at lower light intensity with the information we gather from our study, it can be suggested that it is linked to energy-dependent processes as stated by Gorbi et al. (2001).



(a)



(b)

Figure 4.3. Growth curves of *D. tertiolecta* exposed to 0.5 mg L^{-1} selenium 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 $m^{-2}s^{-1}$.

The stimulating effect of selenite on the growth of *S. plantesis* was reported to be derived from the increased level of Se enzyme resulting in the more effective removal of free radicals and consequently a reduction in the rate of algal cell decline (Li et al., 2003). It was also reported by the same authors that selenite is either reduced to elemental selenium which is poorly soluble and less toxic than selenite or majority of the inorganic selenium was changed to inorganic selenium by integrating with the algal bioligands such as protein, lipids, polysaccharides, and other algal components, or both during the bioaccumulation of selenite in cells of *S. plantesis*. The research of Bottino et al. (1984) on *Dunaliella primolecta, Chlorella sp.*, and *Porhydrium cruentum* indicated there were both free- and protein-bound seleno-amino acids in these microalgae. Usually, Se may follow

sulfur metabolism when it is being incorporated into amino acids and protein. In addition to protein, lipids and polysaccharides were also involved in the accumulation of Se as stated above. Gennity et al. (1984) has suggested that lipid-associated selenium was not metabolically incorporated. Selenium was mainly bound with polar-lipids indicating that selenium was probably noncovalently bound to the lipids (Li et al., 2003). In case of polysaccharides, according to the distribution of selenium in the different parts of polysaccharides, selenium is perhaps mainly integrated with those polysaccharides near the algal cell wall, e.g. peptidoglikan and other components. Uptake and distribution of selenium in *D. tertiolecta* needs to be studied in more detail, however, it is beyond the scope of this work.

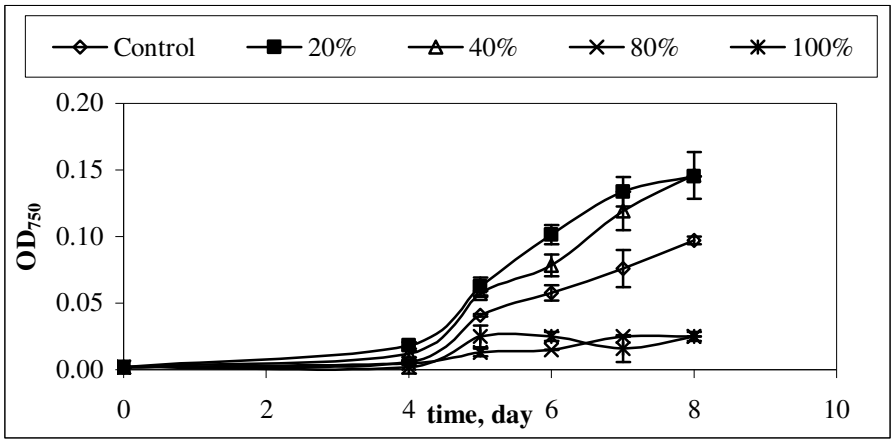
4.2. Effect of Synthetic Dye bath on the Growth of *Dunaliella tertiolecta* in the Presence and Absence of Selenium at 38.4 µE $m^{-2}s^{-1}$

Growth curves of *D. tertiolecta* exposed to synthetic dye bath singly and in combination with selenium for 8-d under the illumination of 38.4 µE $m^{-2}s^{-1}$ were represented in Figure 4.4 (a) and (b), respectively. Hormetic response was observed in dye bath with and without selenium. Hormesis is a dose-response phenomenon characterized by low-dose stimulation, high-dose inhibition. Hormetic-like biphasic dose-response routinely observed by USEPA scientists**.** Hormetic effects have also been reported in studies dealing with complex mixtures of petroleum (Mommaerts 1973, Dunstan 1975, Delistrary 1986, Nicoletti and Egli, 1998) and with wastewater effluent (Srivastava and Sahai 1987; Joy 1990).

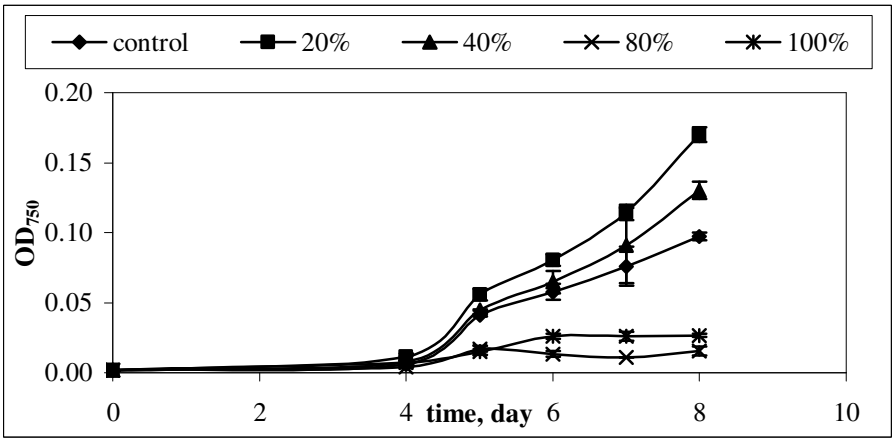
The area under the growth curve (AUC) of *D. tertiolecta* exposed to synthetic dye bath with and without selenium calculated from the optical density at 750 nm for 8-d under the illumination of 38.4 µE $m^{-2}s^{-1}$ was tabulated in Table 4.2 together with the 8-d IC_{25} and toxic unit (TU). Stimulatory response of *D. tertiolecta* evaluated with the help of the 8-d SC_{20} values was also listed in Table 4.2 with their best fitted equations.

Significant difference (p>0.05) was observed between the 8-d IC_{25} and SC_{20} values of *D. tertiolecta* exposed to dye bath in the presence and the absence of selenium. The decrease in algal growth with the addition of selenium is clear considering the 8-d IC_{25} and

SC_{20} values. When the 8-d IC_{25} value decreased from 53.05% (v/v) to 46.87% (v/v), the 8-d SC_{20} value decreased from 28.13% (v/v) to 22.55% (v/v). Parallel to these results toxic unit in terms of 8-d IC_{25} was increased from 1.88 to 2.13.



(a)



(b)

Figure 4.4. Growth curves of *D. tertiolecta* exposed to synthetic dye bath under the illumination of 38.4 µE $m^{-2}s^{-1}$ obtained from the measurement of optical density at 750 nm (a) in the presence of selenium (b) in the absence of selenium.

Table 4.2. Variation in optical density at 750 nm and the 8-d IC_{25} and SC_{20} of *D. tertiolecta* exposed to dye bath in the presence and absence of selenium under the illumination of 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**							
DYE BATH												
AUC[a]	0.29	0.40	0.33	0.06	0.09							
CV	5.04	1.79	9.05	8.50	23.47	53.05±0.25	1.89	-75.61LnX+371.99	0.05	18.54	28.13±1.61	3.55
Ig (%)[b]		-37	-13	80	67							
DYE BATH and SELENIUM												
AUC	0.29	0.35	0.29	0.04	0.09							
CV	5.04	2.80	5.31	17.41	4.27	46.87±2.45	2.13	-66.21 LnX +324.16	0.05	17.93	22.55±1.62	4.43
Ig (%)		-20	5	86	70							

[a] All AUC are mean values from repeated tests (n=3); CV = coefficient of variation.
[b] Ig (%) = Per cent 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= Per cent dilution of the dye bath

Stimulatory effects occurred if synthetic dye bath with and without selenium are in the range of 20-40%, but inhibitory effect appeared beyond this concentration range (Table 4.2). Addition of 0.5 mg L^{-1} selenium decreased the stimulatory effect of dye bath significantly ($p<0.05$). The 8-d SC_{20} values were calculated using linear regression analysis of transformed dye bath dilution percentage as natural logarithm data versus percentage inhibition. Concentration-response relationship for *D. tertiolecta* exposed to dye bath with and without selenium is presented in Figure 4.5.

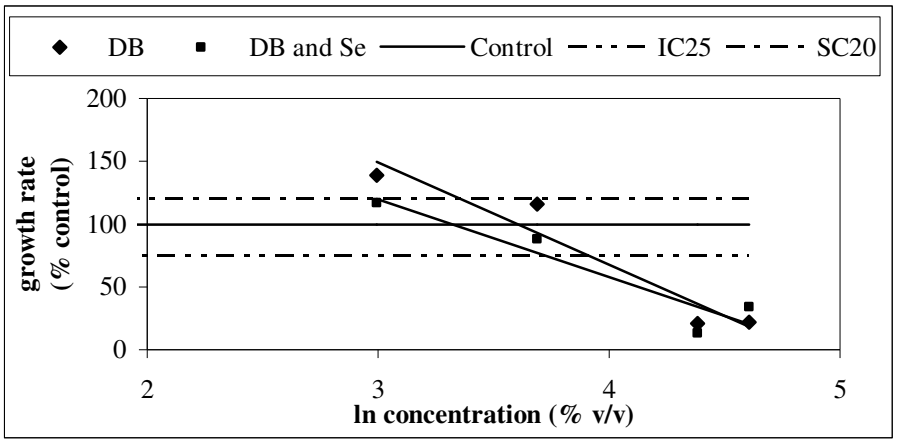


Figure 4.5. Representative dose-response curves of *D. tertiolecta* exposed to dye bath with and without selenium obtained by the measurement of optical density at 750 nm under the illumination of 38.4 µE $m^{-2}s^{-1}$.

TU values calculated on the basis of 8-d IC_{25} showed that dye bath with and without selenium were moderately toxic (TU > 1.0, Villegas-Navarro et al., 2001). TU values of dye bath with and without selenium were 4.43 and 3.55, respectively when TU values were evaluated on the basis of 8-d SC_{20}. Dye bath either singly or combined with selenium was very toxic (TU ≥ 3.0, Villegas-Navarro et al., 2001) to *D. tertiolecta*. This results indicated that they may cause adverse effects in aquatic environment by stimulation of algal growth at low concentrations since the entire aquatic ecosystem may be influenced by changes in algal populations. If the biomass of algae becomes too high or if certain species become abundant, water quality may be negatively impacted. Decreased water transparency and oxygen consumption in bottom waters after settling are two principal consequences of algal overproductivity. Decreases in water transparency may effect growth and survival of higher order, vascular aquatic plants and cause change in fish populations. Additionally,

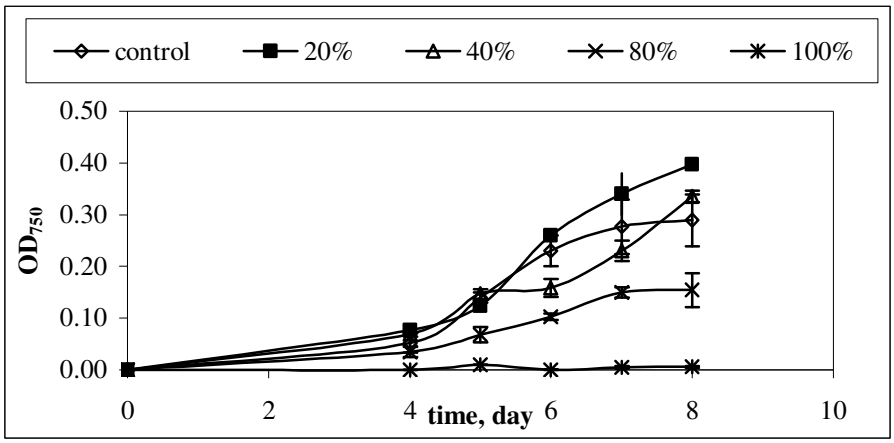
long term effects of continuous low-level exposure to chemicals and their metabolites are not well understood.

4.3. Effects of a Synthetic Dye Bath on the Growth of *Dunaliella tertiolecta* in the Presence and Absence of Selenium at 76.8 µE $m^{-2}s^{-1}$

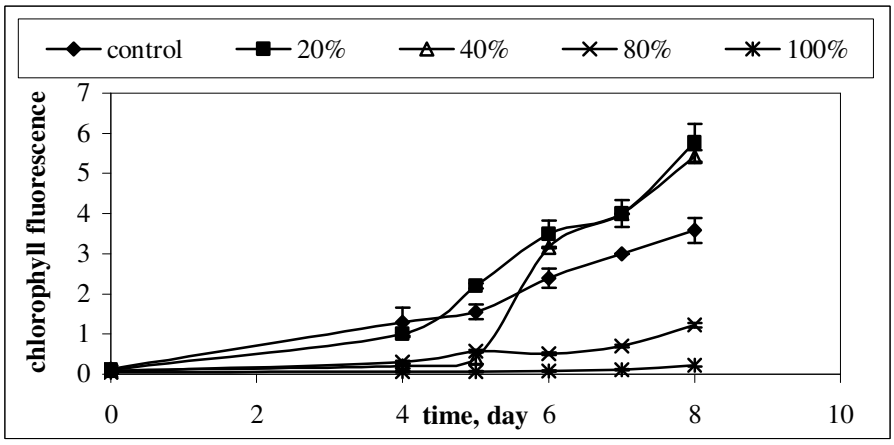
Toxicity of synthetic dye bath, including assisting chemicals- urea, NaCl, Na_2CO_3 and NaOH- and six hydrolyzed dyes was tested singly and combined with selenium using three different response parameters –OD_{750}, *in vivo* chlorophyll fluorescence and cell number- under the illumination of 76.8 µE $m^{-2}s^{-1}$. Figure 4.6 and Figure 4.7 show the growth curves of *D. tertiolecta* exposed to different dilution ratios of dye bath with and without selenium for the three response variables, respectively. Error bars show the mean standard deviation between triplicates. All studied dilutions (% v/v) revealed a significant difference from the control ($p < 0.05$).

Calculated area under the growth curves (AUC) from optical density, *in vivo* chlorophyll fluorescence and manual cell counting were tabulated in Table 4.3 together with the 8-d IC_{25}, SC_{20} and TU values. Stimulatory effects on the growth of alga were observed at lower percentage and an inhibitory effect was observed at higher dilution percentage both in the presence and in the absence of selenium for each response parameter. The high molecular weight compound may undergo photochemical degradation into more labile low molecular weight compounds which are then directly utilized by plankton as a substrate (Joseph and Joseph, 2001) leading to stimulation for the dye bath.

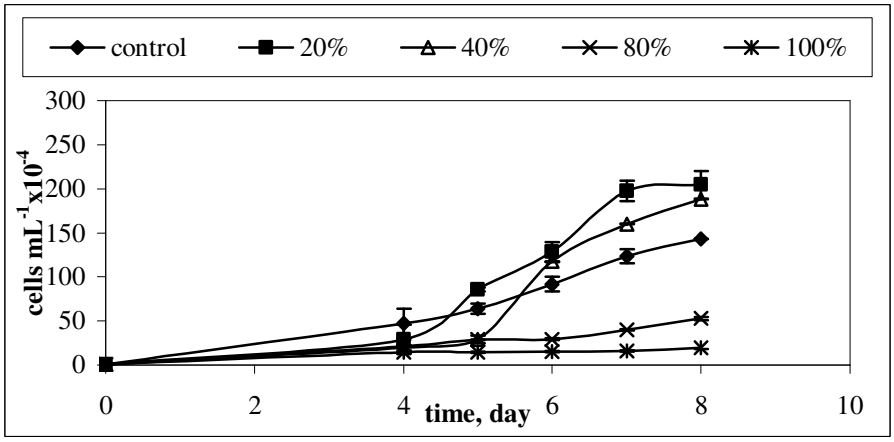
The average values of the 8-d IC_{25} and SC_{20} were used in discussions since they are not significantly ($p>0.05$) different based on the measured response parameters. Stimulatory effect was observed in the dye bath concentration range of 20-53% (v/v). In contrast, inhibitory effect was observed at higher dilution percentages (>53 %). Dye bath was toxic to algae with the average 8-d IC_{25} of 53.83±2.08% (v/v). The average 8-d SC_{20} values of dye bath was 27.90±0.81% (v/v) which was calculated using linear regression analysis of transformed dye bath dilution percentage as natural logarithm data versus percentage inhibition from the measured three response parameters.



(a)

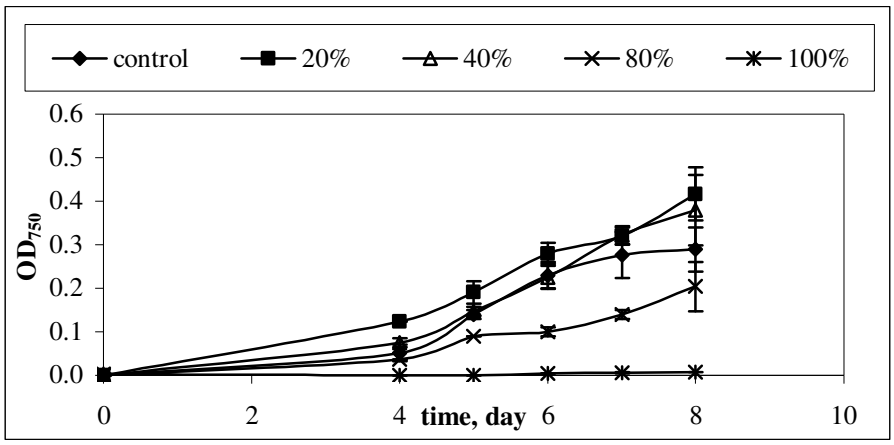


(b)

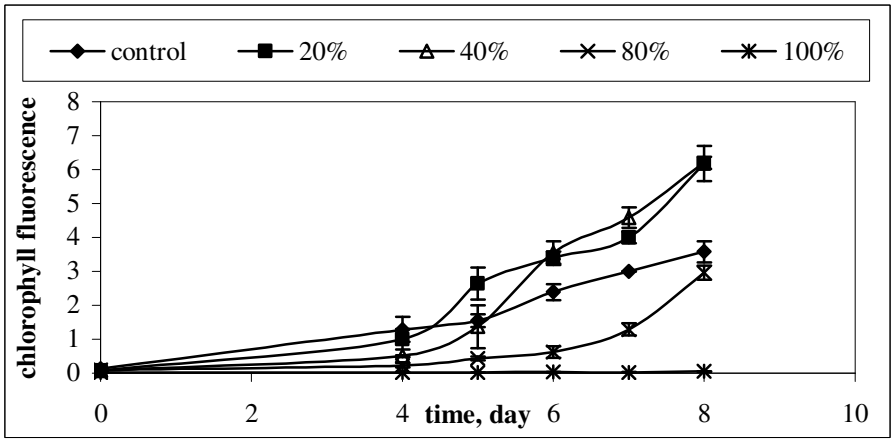


(c)

Figure 4.6. Growth curves of *D. tertiolecta* exposed to dye bath obtained from the measurement of (a) optical density at 750 nm (b) *in vivo* chlorophyll fluorescence and (c) cell count at 76.8 μE $m^{-2}s^{-1}$.



(a)



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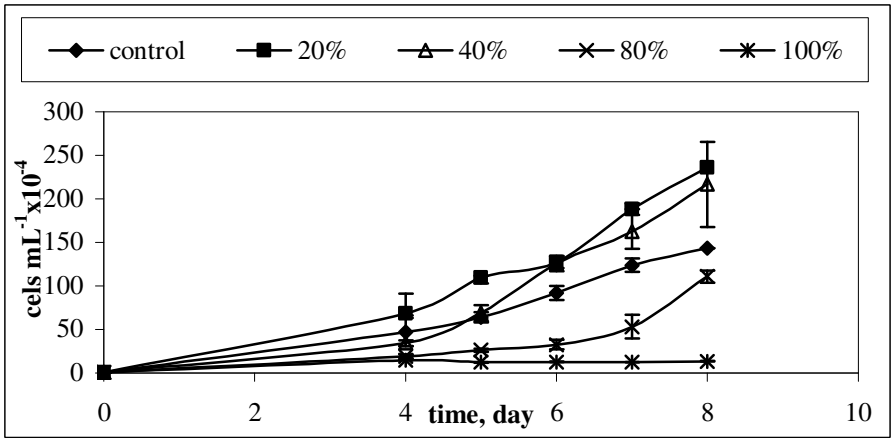


(c)

Figure 4.7. Growth curves of *D. tertiolecta* exposed to dye bath in the presence of selenium obtained from the measurement of (a) optical density at 750 nm (b) *in vivo* chlorophyll fluorescence and (c) cell count at 76.8 µE $m^{-2}s^{-1}$.

Table 4.3. Variation in optical density at 750 nm, *in vivo* chlorophyll fluorescence, cell number and toxicity parameters of *D. tertiolecta* exposed to dye bath in the presence and absence of selenium under the illumination of 76.8 µE $m^{-2}s^{-1}$.

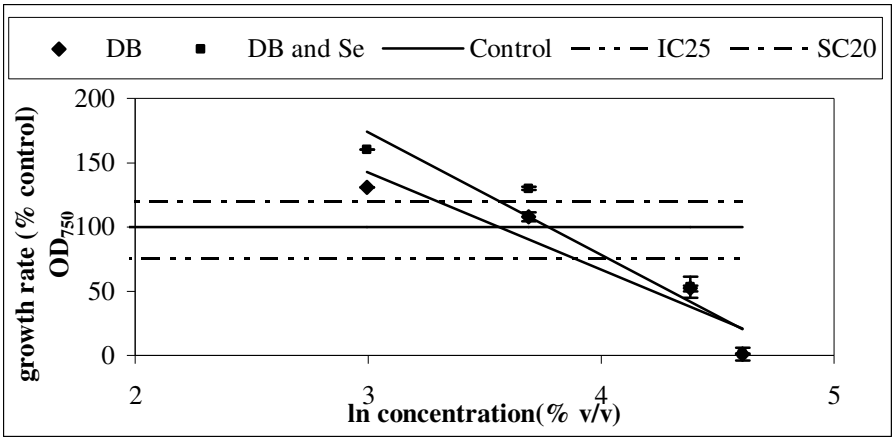
Method		% dilution (v/v)					IC_{25} % dilution (v/v)	TU[c]	Equation (SC_{20}=aLnX[*]+b)	*p*-value	*F*	SC_{20} % dilution (v/v)	TU[•]
		Control	20	40	80	100							
DYE BATH													
Spectrophotometric	AUC[a]	0.88	1.16	0.95	0.46	0.01	52.01±4.64	1.92	-75.90LnX+370.38	0.05	17.45	27.12±0.74	3.68
	CV	16.71	2.70	2.03	0.70	5.59							
	Ig (%)[b]		-31	-8	48	99							
Spectrofluorometric	AUC	11.20	16.97	10.86	3.28	0.27	53.73±0.79	1.86	-92.77LnX +433.48	0.01	403.06	29.29±1.13	3.41
	CV	19.13	2.23	5.72	4.80	23.13							
	Ig (%)		-52	3	71	98							
Cell number	AUCx10^4	463.04	648.57	445.01	178.18	85.89	55.67±0.80	1.80	-75.70LnX+369.84	0.002	418.01	27.28±0.57	3.67
	CV	3.71	1.02	0.17	5.60	2.82							
	Ig (%)		-40	4	62	81							
DYE BATH and SELENIUM													
Spectrophotometric	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							
Spectrofluorometric	AUC	11.20	18.79	14.98	4.69	0.05	58.33±1.13	1.71	-104.45LnX+495.23	0.03	34.47	35.66±1.59	2.80
	CV	19.13	3.75	15.79	3.09	79.38							
	Ig (%)		-68	-34	58	100							
Cell number	AUCx10^4	463.04	750.49	590.35	232.78	75.33	59.48±1.36	1.68	-90.72LnX+444.17	0.02	49.11	37.71±0.66	2.65
	CV	3.71	0.96	3.00	3.36	1.41							
	Ig (%)		-62	-27	50	84							

[a] All AUC are mean values from repeated tests (n=3); CV = coefficient of variation
[b] Ig (%) = Per cent 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= Per cent dilution of the dye bath

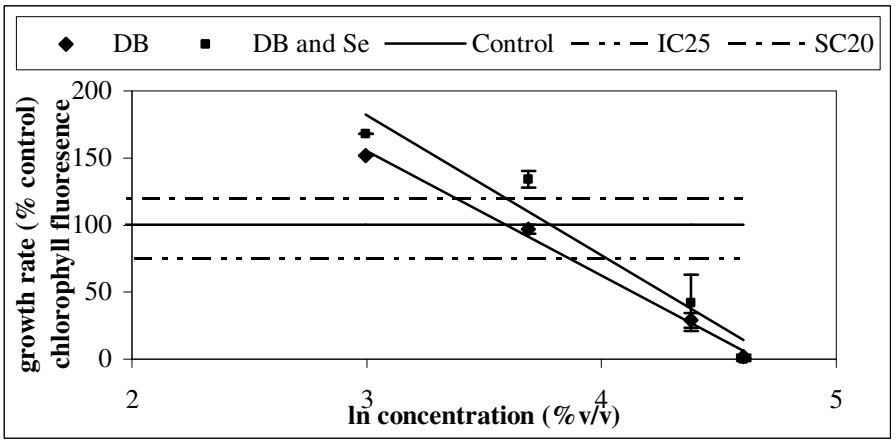
Addition of 0.5 mg Se L^{-1} in the form of H_2SeO_3 significantly increased the growth of *D. tertiolecta*. Representative dose response curves of dye bath were shown in Figure 4.8 for the measured three endpoints. These curves represent that the addition of selenium significantly increased the stimulatory effect at low doses. Since the stimulatory effect of selenium decreased with the increase of dye bath concentration, it can be concluded the dye bath-selenium interactions on algal growth was concentration dependent. The average 8-d IC_{25} values of *D. tertiolecta* increased to 59.30±1.22% (v/v) with the addition of 0.5 mg Se L^{-1}. This increase of the 8-d IC_{20} values with the addition of selenium may be due to the detoxification mechanism of algae. It is known that in algal species, selenium may be incorporated in enzymes (glutathione peroxidase) with Se-amino acids as their functional groups (Mattoo et al., 1988, Price and Harrison, 1988) which remove toxic peroxide thus preventing membrane damage (Shrift 1954a; 1954b; Wheeler et al., 1982). The decrease in inhibitory effects of dye bath with the addition of selenium can also be attributed to a capacity of regulating the influx and efflux of selenite or to detoxify it intercellularly as stated by Boisson et al., (1995).

When the results were compared in terms of the average 8-d SC_{20} values, an increase from 27.90±0.81% (v/v) to 36.17±0.94% (v/v) was observed with the addition of selenium to the synthetic dye bath. 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 dye bath with and without selenium. Parallel to the increase in the 8-d IC_{25} value, an average decrease from 1.86 to 1.68 was observed in TU values with the addition of 0.5 mg Se L^{-1}. Considering the TU values calculated on the basis of 8-d IC_{25}, dye bath either singly or combined with selenium was moderately toxic to *D. tertiolecta* (TU > 1.0, Villegas-Navarro et al., 2001). However, when TU was calculated on the basis of 8-d SC_{20} value, dye bath was very toxic (TU ≥ 3.0, Villegas-Navarro et al., 2001) to *D. tertiolecta* whereas it was moderately toxic (TU > 1.0) to *D. tertiolecta* in the presence of selenium.

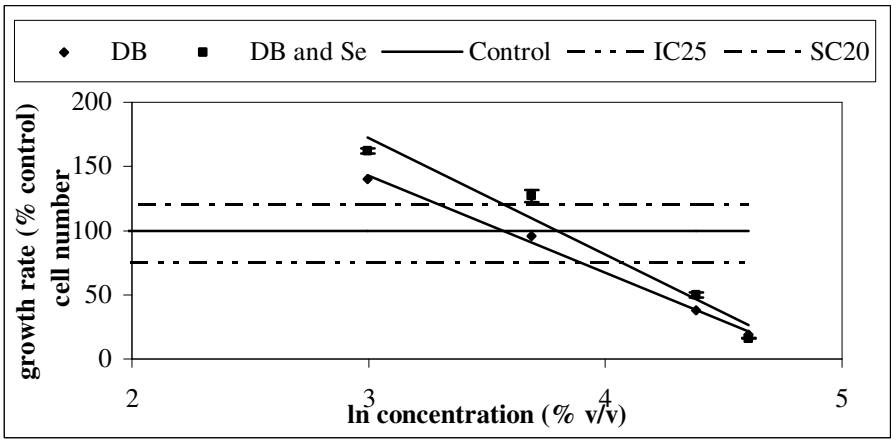
Although environmental factors such as pH and suspended sediments may enhance/decrease the acute or chronic toxicity of dye bath formulations, at low concentrations the release of dye bath singly or combined with selenium to the environment can lead to uncontrolled algal growth and consequently exacerbate eutrophication problem.



(a)



(b)



(c)

Figure 4.8. Representative dose-response curves of *D. tertiolecta* exposed to dye bath in the presence and absence of selenium obtained by the measurement of (a) optical density at 750 nm (b) *in vivo* chlorophyll fluorescence and (c) cell count.

4.4. Effect of Assisting Chemicals on the Growth *Dunaliella tertiolecta* in the Presence and Absence of Selenium

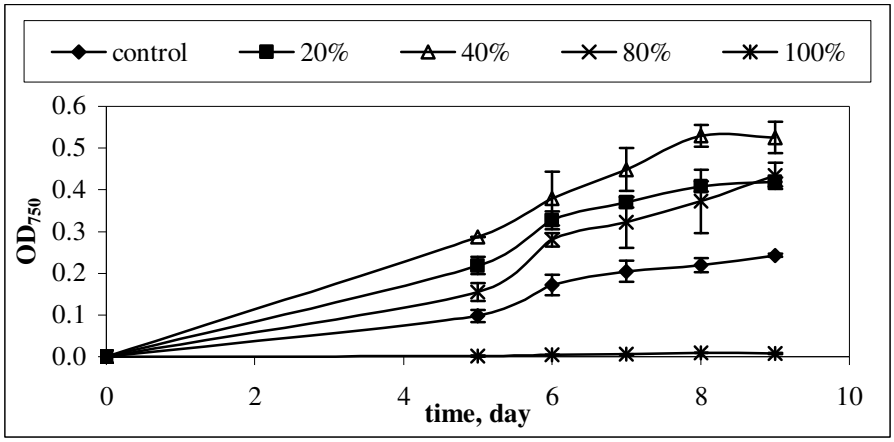
As presented in Table 3.1 the dye bath recipe contains four assisting chemicals -urea, NaCl, Na_2CO_3 and NaOH. Figure 4.9 and 4.10 show the effects of assisting chemicals with and without selenium on the growth response of algae measured by OD_{750}, *in vivo* chlorophyll fluorescence and cell counts. Table 4.4 shows the area under the growth curves calculated from OD_{750}, *in vivo* chlorophyll fluorescence and cell counts when *D. tertiolecta* exposed to different concentrations of assisting chemicals alone and combined with 0.5 mg Se L^{-1} together with the 8-day IC_{25}, SC_{20} and TU values.

Significant difference was observed between all studied dilutions (% v/v) and control. Stimulation effect on the growth of *D. tertiolecta* was observed both in the presence and absence of selenium. Cultures enriched with assisting chemicals have higher optical density, chlorophyll fluorescence and number of cells compared to control. In other words, growth in the concentration range of 20-80% (v/v) was stimulatory compared to cultures having no assisting chemicals. Growth of algae in the highest studied dilution percentage (100%) decreased by approximately 88% compared to control. This decrease may be due to reduction of ions present in seawater, since seawater was not used in 100% dilution. Although the toxicity of single dyes has been studied in the past (Sharma and Sobti, 2000; Rajaguru et al., 2001; Wang et al., 2002; Chen, 2002 and Gottlieb et al., 2003), assisting chemicals are not studied from the toxicity point of view. The present study clearly shows that assisting chemicals are stimulatory to *D. tertiolecta* in the absence of azo dyes. Therefore, studies with dyes and assisting chemicals may over or underestimate the stimulatory/inhibitory effects of dyes. Stimulatory effect of assisting chemicals to algal growth can be attributed to salt tolerance of alga. *D. tertiolecta* is a marine alga and will grow in salinities ranging from 0.05 to 3.0 mol L^{-1} NaCl (Jahnke and White, 2003). Salinity extremes will produce osmotic and/or ionic effects in photosynthetic organisms. *Dunaliella* is a photosynthetic unicellular green algae adapted to extreme environments such as salinity and has an enzyme unique to *Dunaliella* called the osmoregulatory isoform of dihydroxyacetone phosphate (DHAP) reductase (Osm-DHAPR). It is involved in the synthesis of free glycerol for osmoregulation in extreme environments. This extraordinarily high tolerance to salt stress may be the reason of this

high stimulatory effect. However, we can not attribute the toxicity of assisting chemicals to the high salt content of the solution, because the sodium chloride concentration in the studied dilutions is between 0.006-0.030 mol L^{-1} which is a suitable environment for *D. tertiolecta.*

Quadratic correlation gave the best fit for dose-response relationship considering the three response variables. Therefore, two intersection points were determined from the best fitted equation (Table 4.4). The three different response parameters used to assess the toxicity of assisting chemicals reflected no significant difference (p>0.05). The difference between the average 8-day SC_{20} values of *D. tertiolecta* exposed to assisting chemicals with and without selenium and obtained from the three response parameters was not statistically significant (p>0.05) both in the lower and upper values. However, the stimulatory concentration calculated for the upper limit was very close to the inhibitory concentration calculated from IC_p method (Table 4.4). Therefore, we also calculated the 8-d IC_{25} values from the best fitted equations.

Assisting chemicals had stimulatory effect in the range of 5.32-78.03% (v/v) and inhibitory effect with the average 8-d IC_{25} value of 88.95±1.64% (v/v) calculated from the curve fitting. On the other hand, the average 8-d IC_{25} value of assisting chemicals was 79.33±6.47% (v/v) which was calculated from linear interpolation combined with bootstrapping known as IC_p method. In the presence of selenium, stimulatory effect range was 4.45-81.55% (v/v) while the 8-d IC_{25} value was 92.16±3.14% (v/v) calculated from the curve fitting. On the other hand, the average 8-d IC_{25} value of assisting chemicals was 76.85±6.82% (v/v) which was calculated from linear interpolation. When the results were evaluated in terms of TU calculated on the basis of 8-d IC_{25}, assisting chemicals was moderately toxic to *D. tertiolecta* (TU > 1.0, Villegas-Navarro et al., 2001). However, when TU was calculated using lower limit of SC_{20} (assuming that lower limit of SC_{20} is equal to LOEC) assisting chemicals was very toxic to *D. tertiolecta* (TU ≥ 3.0, Villegas-Navarro et al., 2001).



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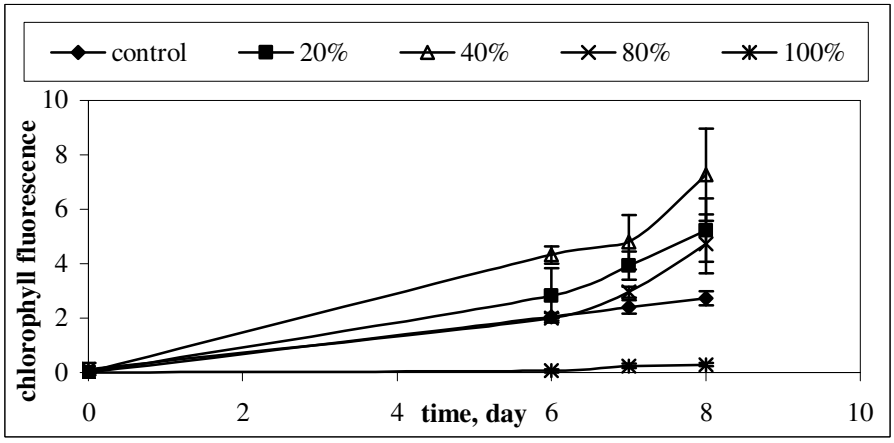

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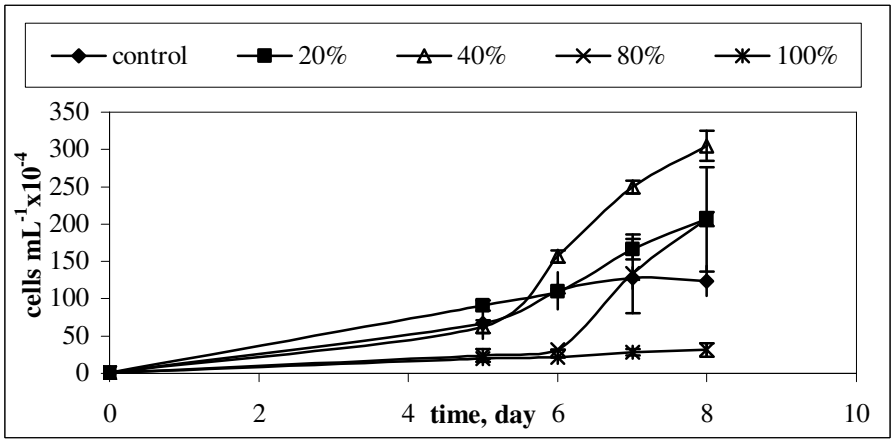
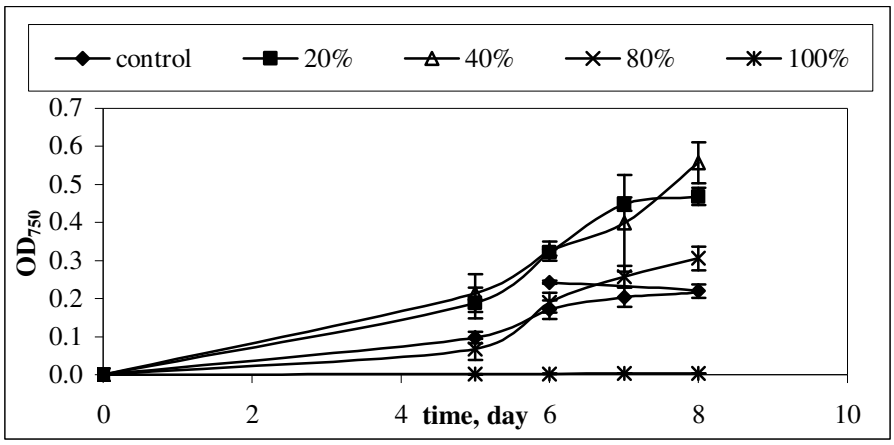
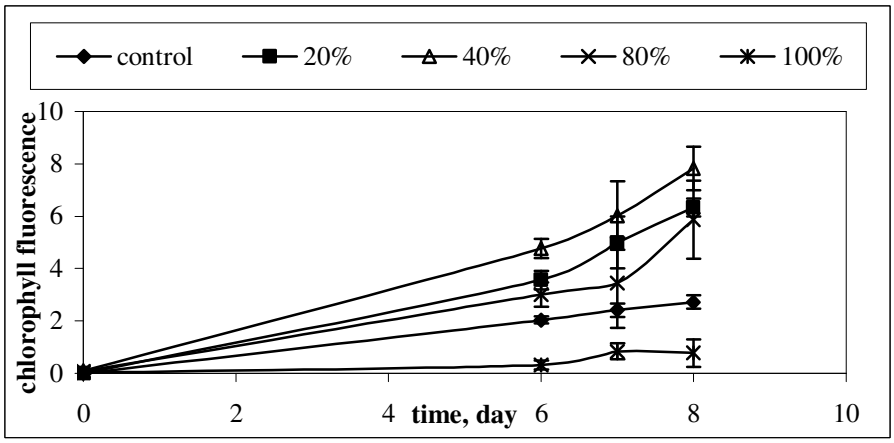


(c)

Figure 4.9. Growth curves of *D. tertiolecta* exposed to assisting chemicals obtained by the measurement of (a) optical density at 750 nm (b) *in vivo* chlorophyll fluorescence and (c) cell count.



(a)



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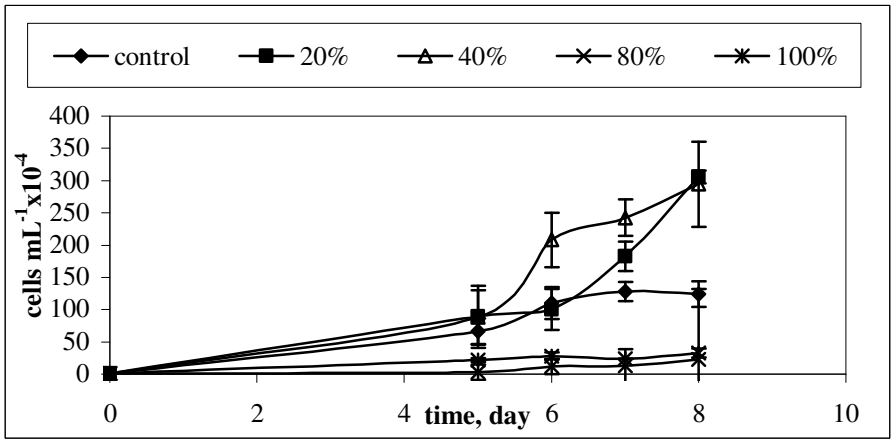


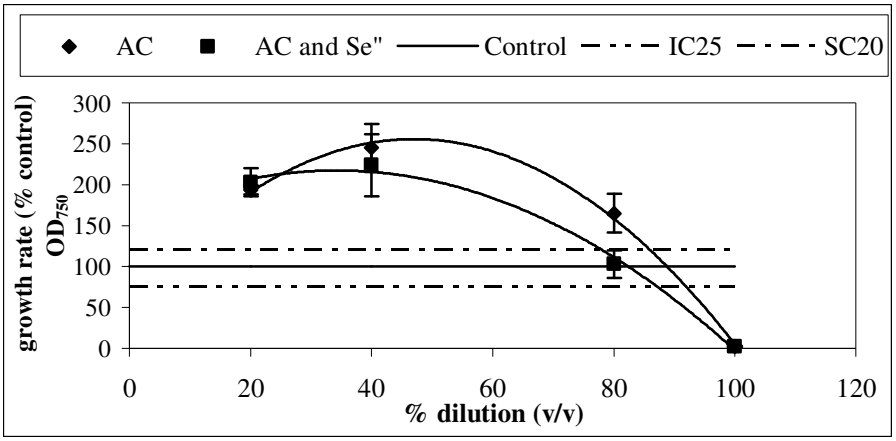
(c)

Figure 4.10. Growth curves of *D. tertiolecta* exposed to assisting chemicals in the presence of selenium obtained by the measurement of (a) optical density at 750 nm (b) *in vivo* chlorophyll fluorescence and (c) cell count.

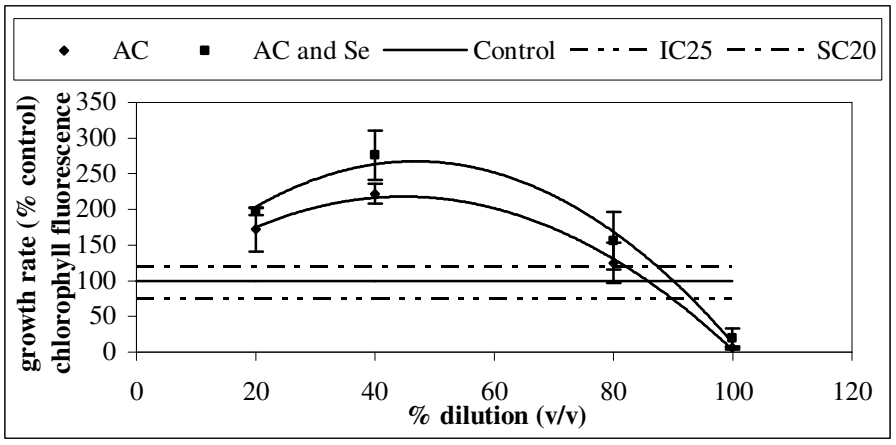
Table 4.4. Variation in optical density at 750 nm, *in vivo* chlorophyll fluorescence, cell number and toxicity parameters of *D. tertiolecta* exposed to assisting chemicals in the presence and absence of selenium under the illumination of 76.8 µE $m^{-2}s^{-1}$.

Method		Control	% dilution (v/v)				IC_{25} % dilution (v/v)	TU[c]	Equation (SC_{20}= $aX^{*2}+bX+c$)	*p*-value	*F*	SC_{20} % dilution (v/v)	TU•
			20	40	80	100							
ASSISTING CHEMICALS													
Spectrophotometric	AUC[a]	1.01	1.96	2.47	1.67	0.02	85.04†±0.11	1.18	$-0.08C^2+6.99C+93.99$	0.01	99.69	3.90±0.49	25.64
	CV	1061	2.78	11.94	14.43	32.88	91.87‡±1.12					85.26±1.54	1.17
	Ig (%)[b]		-95	-145	-65	98							
Spectrofluorometric	AUC	10.92	18.75	24.22	13.61	0.62	84.94±0.25	1.17	$-0.06C^2+5.52C+96.262$	0.01	167.62	4.56±2.51	21.93
	CV	5.61	17.92	6.25	22.84	37.88	89.58±1.32					81.20±0.94	1.23
	Ig (%)		-72	-122	-25	94							
Cell number	AUCx10^4	478.86	699.79	725.03	448.41	128.18	68.03±19.06	1.47	$-0.03C^2+2.63C+102.19$	0.01	282.18	7.52±2.61	13.30
	CV	4.35	11.55	4.73	13.61	11.54	85.41±2.47					67.63±5.44	1.48
	Ig (%)		-46	-51	6	73							
ASSISTING CHEMICALS and SELENIUM													
Spectrophotometric	AUC	0.78	1.58	1.74	0.80	0.01	80.94±3.08	1.24	$-0.07C^2+4.44C+108.43$	0.02	48.02	2.27±1.40	44.05
	CV	12.85	8.52	17.00	16.43	31.75	92.57±4.70					84.50±3.85	1.18
	Ig (%)		-103	-124	-3	98							
Spectrofluorometric	AUC	10.92	19.77	27.77	15.70	2.01	84.50±1.69	1.18	$-0.08C^2+6.56C+94.81$	0.02	60.20	3.91±0.49	25.57
	CV	5.61	2.83	13.47	28.19	73.70	92.03±1.45					84.77±3.33	1.18
	Ig (%)		-81	-154	-44	82							
Cell number	AUCx10^4	478.86	733.48	822.37	429.43	136.93	65.13±15.68	1.54	$-0.04C^2+3.14C+103.40$	0.01	52.21	7.19±4.72	13.91
	CV	4.35	22.17	23.20	14.33	13.99	91.87±4.66					75.37±4.74	1.33
	Ig (%)		-53	-72	10	71							

[a] All AUC are mean values from repeated tests (n=3); CV = coefficient of variation
[b] Ig (%) = Per cent growth inhibition/stimulation; negative values show stimulation of growth with respect to control
† IC_{25} = 25% Inhibition concentration calculated from *IC*p method
‡ IC_{25} = 25% Inhibition concentration calculated from SPSS curve fitting
[c] TU= Toxic Unit in terms of IC_{25} • TU= Toxic Unit in terms of SC_{20}
* X= Per cent dilution of the assisting chemicals



(a)



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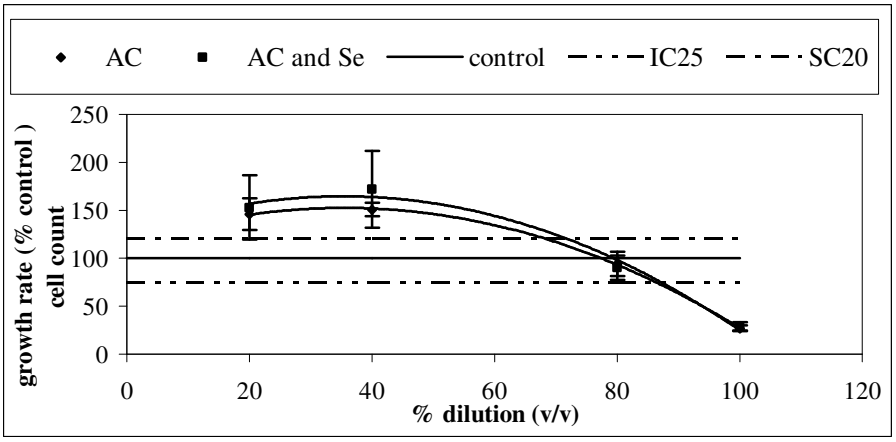


(c)

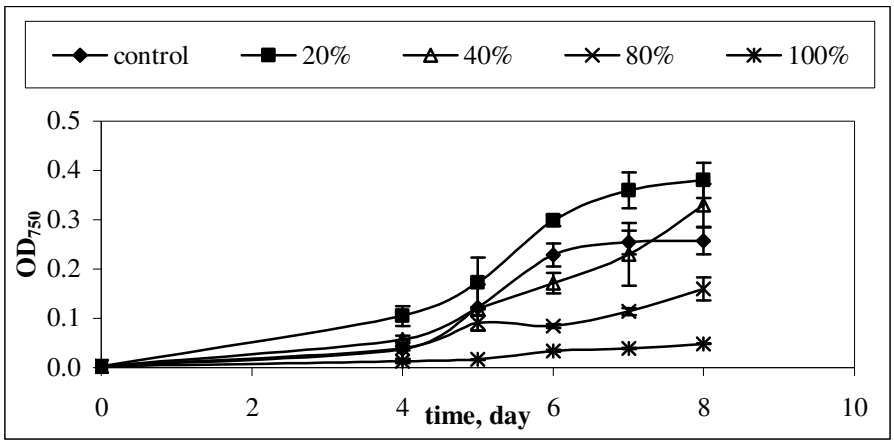
Figure 4.11. Representative dose-response curves of *D. tertiolecta* exposed to assisting chemicals in the presence and absence of selenium obtained by the measurement of (a) optical density at 750 nm (b) *in vivo* chlorophyll fluorescence and (c) cell count.

4.5. Effect of Remazol Black 5 on the Growth of *Dunaliella tertiolecta* in the Presence and Absence of Selenium

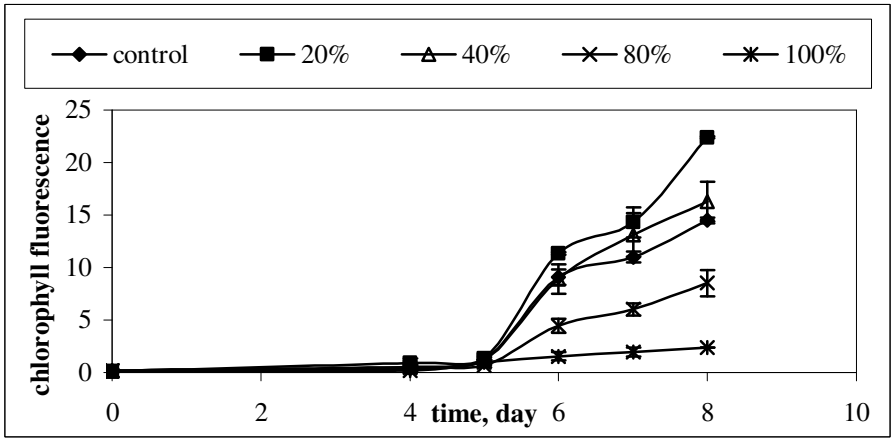
Remazol Black 5 with a sulphatoethylsulphonate reactive group has the highest concentration among the six azo dyes present in the dye bath recipe. Toxicity of Remazol Black 5 was tested in the presence and absence of selenium using three different response parameters, namely OD_{750}, *in vivo* chlorophyll fluorescence and cell number. Figure 4.12 and Figure 4.13 show the growth curves of *D. tertiolecta* exposed to different dilution ratios of Remazol Black 5 singly and combined with selenium for the three response variables. Significant difference ($p<0.05$) was observed between control and all studied dilutions (% v/v). AUC calculated from the three response parameters were tabulated in Table 4.5 together with the 8-d IC_{25}, SC_{20} and TU values. The three different response parameters used to assess the toxicity of Remazol Black 5 reflected no significant difference ($p>0.05$).

Although growth of algae was inhibitory in the concentration range of 40-100% (v/v) it was stimulatory in the concentration range of 20-40% (v/v). Remazol Black 5 had stimulatory effect with the average 8-d SC_{20} of 33.23±3.81% (v/v) whereas it had inhibitory effect with the average 8-d IC_{25} of 55.84±4.50% (v/v) on the growth of *D. tertiolecta*. Addition of 0.5 mg Se L^{-1} significantly changed both the inhibitory and stimulatory effects of Remazol Black 5 on *D. tertiolecta*. In the presence of selenium, a significant increase in the average 8-d IC_{25} value up to 66.89±2.45% (v/v) was obtained.

Figure 4.14 shows the representative dose response curves of *D. tertiolecta* exposed to Remazol Black 5 in the presence and absence of selenium. Dose response curves were constituted using linear regression analysis of transformed dye bath dilution percentage as natural logarithm data versus percentage inhibition from the measured three response parameters. All correlation coefficients were ≥ 0.92. Hormetic response was observed in Remazol Black 5 with and without selenium. Stimulatory and inhibitory effects of chemicals on the aquatic environment were discussed in the previous sections. A significant difference was observed between 8-d IC_{25} and SC_{20} values of Remazol Black 5 added singly and in combination with selenium (Table 4.5 and Figure 4.14).



(a)



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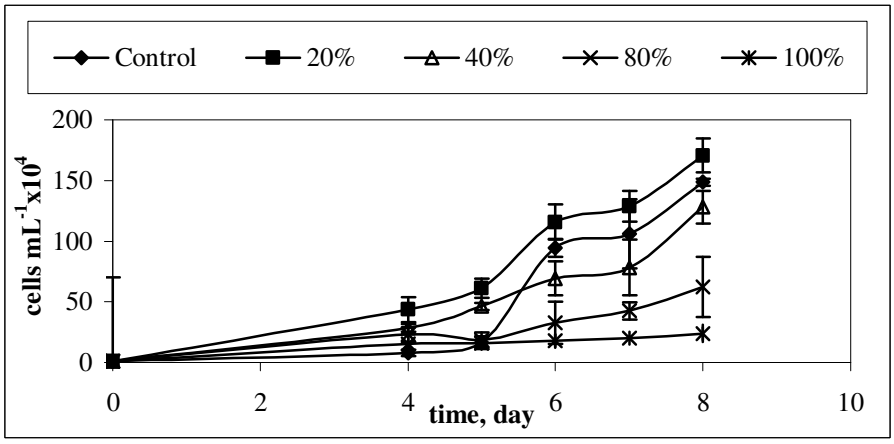
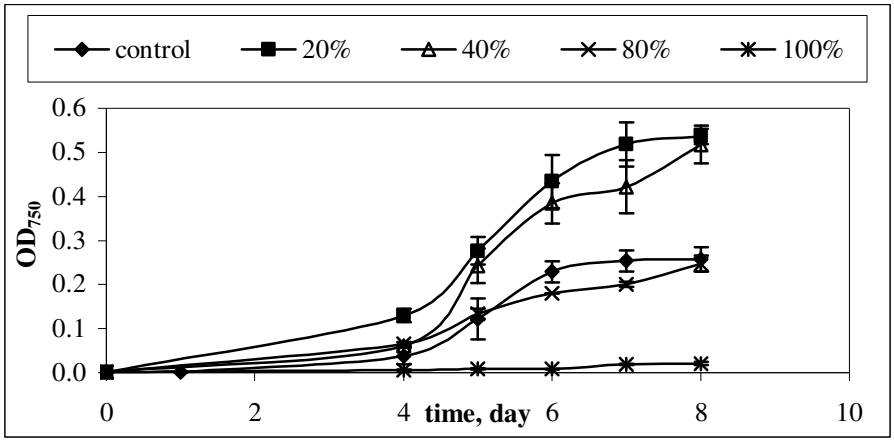
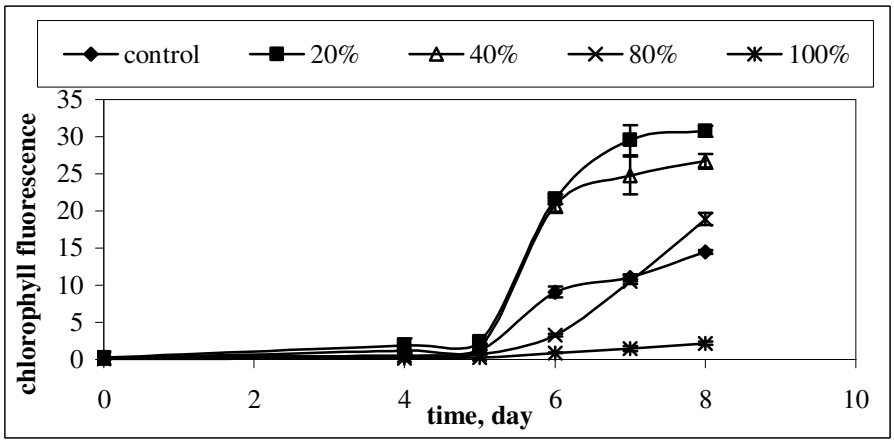


(c)

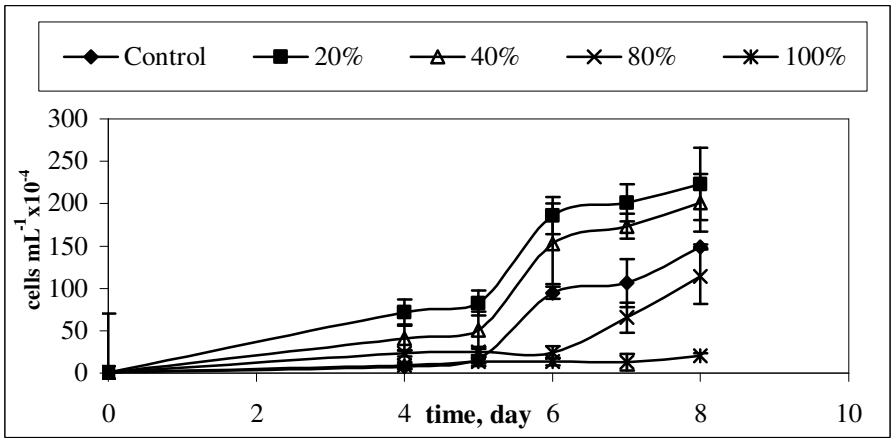
Figure 4.12. Growth curves of *D. tertiolecta* exposed to Remazol Black 5 obtained by the measurement of (a) optical density at 750 nm (b) *in vivo* chlorophyll fluorescence and (c) cell count.



(a)



(b)



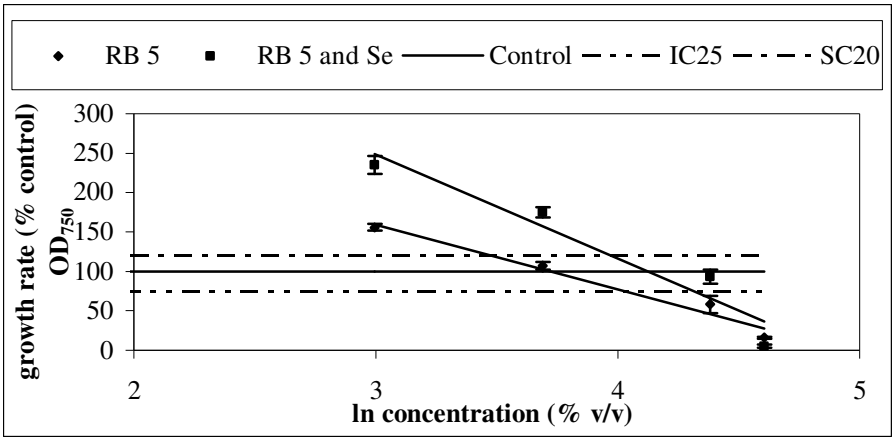
(c)

Figure 4.13. Growth curves of *D. tertiolecta* exposed to Remazol Black 5 in the presence of selenium obtained by the measurement of (a) optical density at 750 nm (b) *in vivo* chlorophyll fluorescence and (c) cell count.

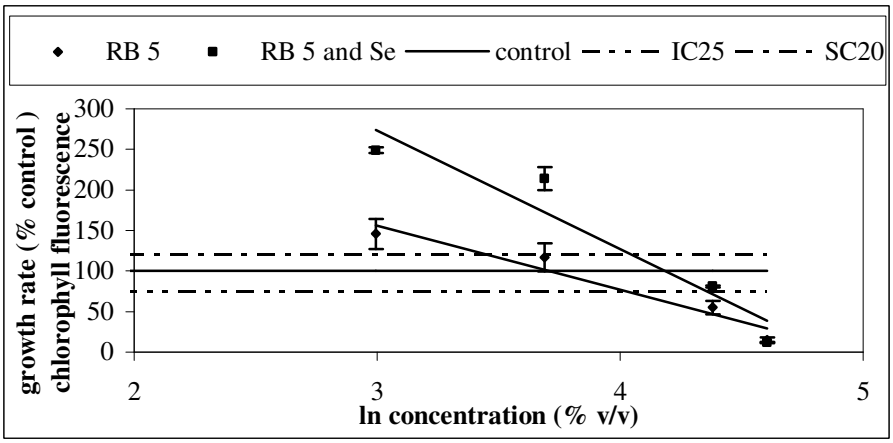
Table 4.5. Variation in optical density at 750 nm, *in vivo* chlorophyll fluorescence, cell number and toxicity parameters of *D. tertiolecta* exposed to Remazol Black 5 in the presence and absence of selenium under the illumination of 76.8 µE $m^{-2}s^{-1}$.

Method		**Control**	**% dilution (v/v)**				IC_{25} **% dilution (v/v)**	**TU**[c]	**Equation (SC_{20}=aLnX*+b)**	***p*-value**	***F***	**SC_{20}**	**TU**▪
			20	**40**	**80**	**100**							
REMAZOL BLACK 5													
Spectrophotometric	AUC[a]	0.83	1.30	0.89	0.49	0.14							
	CV	11.77	2.85	4.21	18.23	8.31	48.65±10.38	2.05	-82.02LnX+405.61	0.01	68.68	32.47±0.63	3.08
	Ig (%)[b]		-56	-7	42	84							
Spectrofluorometric	AUC	29.06	42.51	34.04	16.06	4.30							
	CV	5.37	12.68	15.02	14.67	21.11	61.24±1.11	1.63	-78.97LnX +392.66	0.03	32.38	31.63±4.99	3.16
	Ig (%)		-46	-17	45	85							
Cell number	AUCx10^4	326.58	547.06	353.11	194.00	102.89							
	CV	7.16	6.29	10.06	32.15	21.31	57.63±2.00	1.73	-81.92LnX+412.71	0.00	459.25	35.59±5.82	2.81
	Ig (%)		-68		41	68							
REMAZOL BLACK 5 and SELENIUM													
Spectrophotometric	AUC	0.83	1.96	1.46	0.77	0.04							
	CV	11.77	4.77	3.68	9.49	38.41	65.65±2.10	1.52	-132.16lnX +644.80	0.04	24.96	53.06±2.10	1.88
	Ig (%)		-135	-75	7	95							
Spectrofluorometric	AUC	29.06	72.23	62.13	23.54	3.53							
	CV	5.37	1.53	6.78	1.40	5.12	68.36±0.65	1.46	-146.37LnX +712.5	0.04	21.70	57.28±1.60	1.76
	Ig (%)		-149	-114	19	88							
Cell number	AUCx10^4	326.58	728.22	518.61	205.90	67.78							
	CV	7.16	18.43	2.38	10.09	35.70	66.65±4.60	1.50	-124.74LnX+605.22	0.01	129.70	48.40±5.82	2.07
	Ig (%)		-123	-59	37	79							

[a] All AUC are mean values from repeated tests (n=3); CV = coefficient of variation.
[b] Ig (%) = Per cent 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= Per cent dilution of Remazol Black 5



(a)



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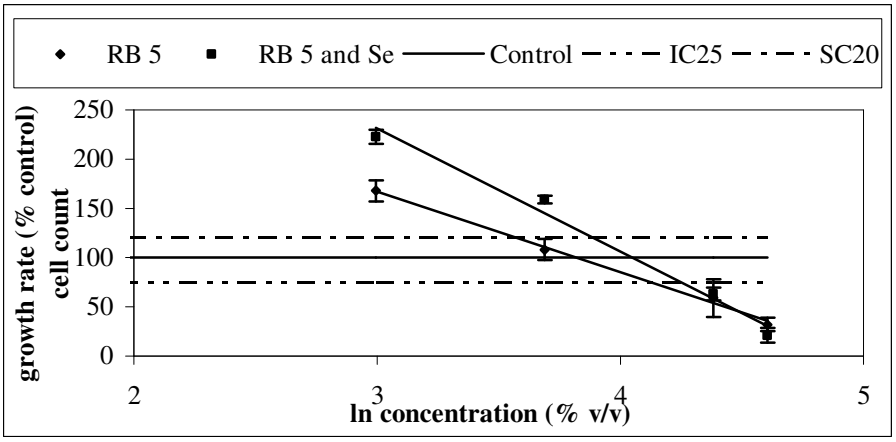


(c)

Figure 4.14. Representative dose-response curves of *D. tertiolecta* exposed to Remazol Black 5 in the presence and absence of selenium obtained by the measurement of (a) optical density at 750 nm (b) *in vivo* chlorophyll fluorescence and (c) cell count.

The tested selenium concentration reduced the inhibitory effect of Remazol Black 5 on the growth of algae (Table 4.5). The stimulatory effect was more remarkable at the dilution percentages less than the 8-d IC_{25} values. Although at lower Remazol Black 5 dilutions (20% v/v) a combination of selenium and Remazol Black 5 appeared to have a stronger stimulatory effect on the growth of algae than that of a single dye, the stimulatory contribution of selenium to a single dye decreases at a higher dilution percentage reflecting that the stimulatory contribution of selenium to a single dye is composition-dependent.

The average 8-d SC_{20} value obtained from the three response parameters -OD_{750}, chlorophyll fluorescence and cell number- increased from 33.23±3.81% (v/v) to 52.91±3.17% (v/v) with the addition of selenium. The average TU in term of 8-d IC_{25} decreased from 1.80 to 1.49; similarly, a decrease from 3.02 to 1.80 was observed when the average toxic unit was evaluated in term of the average 8-d SC_{20} with the addition of selenium. Considering the TU values calculated on the basis of 8-d IC_{25}, Remazol Black 5 either singly or combined with selenium was moderately toxic to *D. tertiolecta* (TU > 1.0, Villegas-Navarro et al., 2001). However, when TU was calculated on the basis of 8-d SC_{20} value, Remazol Black 5 was very toxic (TU ≥ 3.0, Villegas-Navarro et al., 2001) to *D. tertiolecta* whereas it was moderately toxic (TU > 1.0) to *D. tertiolecta* in the presence of selenium.

The average corresponding concentrations inhibiting 25% of growth was 9.75±0.36 mg L^{-1} and 8.14±0.65 mg L^{-1} in the presence and absence of selenium, respectively. The average corresponding concentrations for the 8-d SC_{20} of Remazol Black 5 obtained from the three response parameters were increased from 4.85±0.56 mg L^{-1} to 7.71±0.46 mg L^{-1} with the addition of selenium.

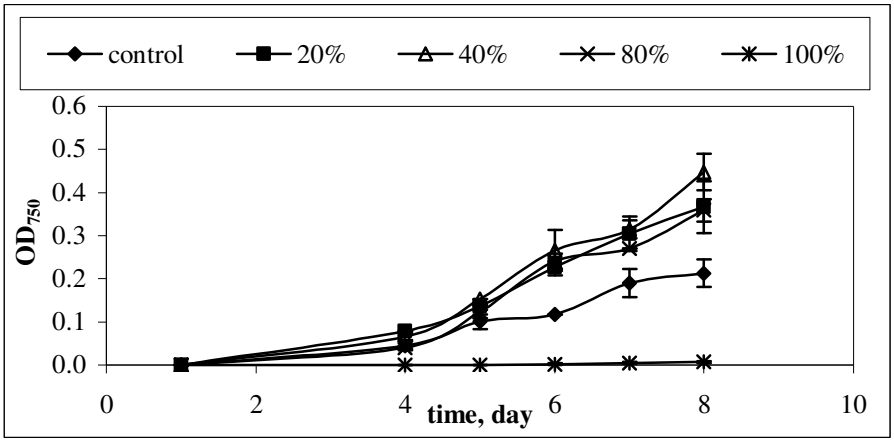
4.6. Effects of Reactive Orange 69 on the Growth of *Dunaliella tertiolecta* in the Presence and Absence of Selenium

Reactive Orange 69 has monochlorodifluoropyrimidine as a reactive group and has the second highest concentration in the dye bath recipe given in Table 3.1. Toxicity evaluation of Reactive Orange 69 was done by using the three different endpoint measurements. Growth curves of *D. tertiolecta* exposed to Reactive Orange 69 singly and

in combination with selenium for OD_{750}, *in vivo* chlorophyll fluorescence and cell number were presented in Figure 4.15 and 4.16, respectively. Significant difference was observed between all studied dilutions (% v/v) and control. Table 4.6 shows the calculated AUC and the growth inhibition per cents from the three response parameters of *D. tertiolecta* exposed to different concentration of Reactive Orange 69 singly and combined with selenium.

Quadratic correlation gave the best fit for dose-response relationship considering the three response variables. Therefore, two intersection points were determined from the best fitted equation (Table 4.6). All correlation coefficients were > 0.96. The three different response parameters used to assess the toxicity of Reactive Orange 69 reflected no significant difference (p>0.05). The difference between the average 8-day SC_{20} values of *D. tertiolecta* exposed to Reactive Orange 69 with and without selenium and obtained from the three response parameters was not statistically significant (p>0.05) both in the lower and upper values. However, the stimulatory concentration calculated for the upper limit was very close to the inhibitory concentration calculated from IC_p method (Table 4.6). Therefore, we also calculated the 8-d IC_{25} values from the best fitted equations.

Reactive Orange 69 had stimulatory effect in the range of 6.40-81.89% (v/v) and inhibitory effect with the average 8-d IC_{25} value of 87.49±5.57% (v/v) calculated from the curve fitting. On the other hand, the average 8-d IC_{25} value of Reactive Orange 69 was 83.99±1.39% (v/v) which was calculated from linear interpolation combined with bootstrapping known as IC_p method. In the presence of selenium, stimulatory effect range was 1.95-88.07% (v/v) while the 8-d IC_{25} value was 93.90±4.45% (v/v). The increase in stimulatory response was not uniform at all concentrations but was most pronounced at 20-40% dilutions. Although within the same concentration range, Reactive Orange 69 with selenium appeared to have a stronger stimulatory effect on the growth of *D. tertiolecta* than that of Reactive Orange 69 added singly (Figure 4.17), this is not reflected in the 8-d SC_{20} values. The stimulatory contribution of selenium to Reactive Orange 69 not reflected in the 8-d SC_{20} values indicates that the stimulatory contribution of selenium to single dye is composition-dependent.



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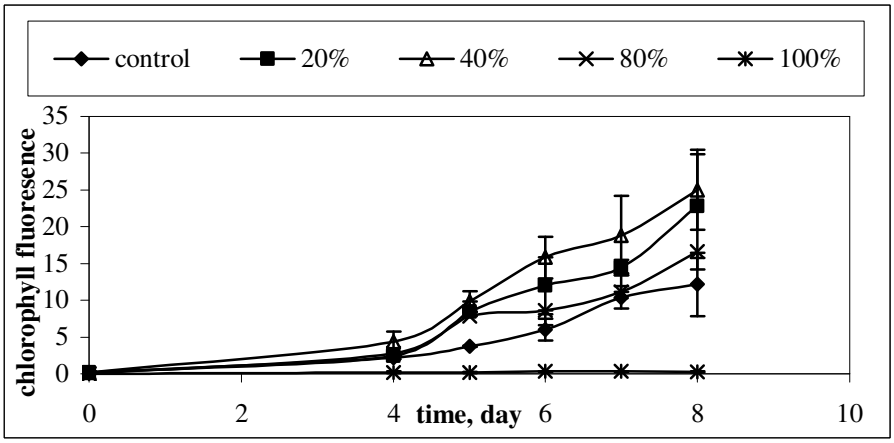

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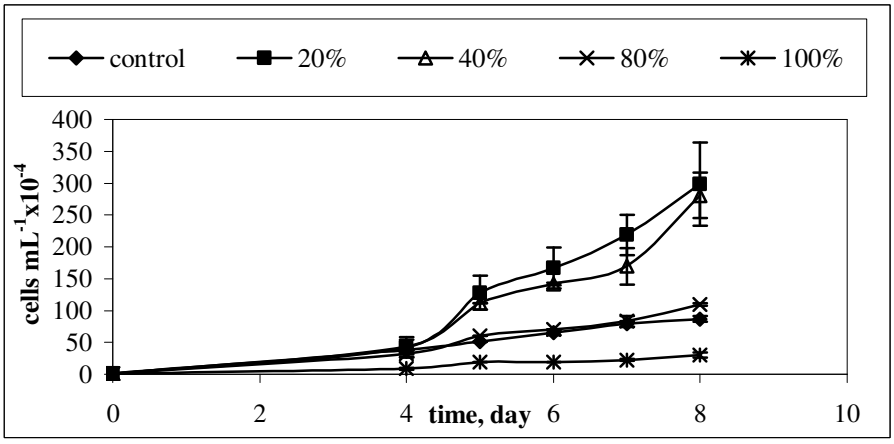
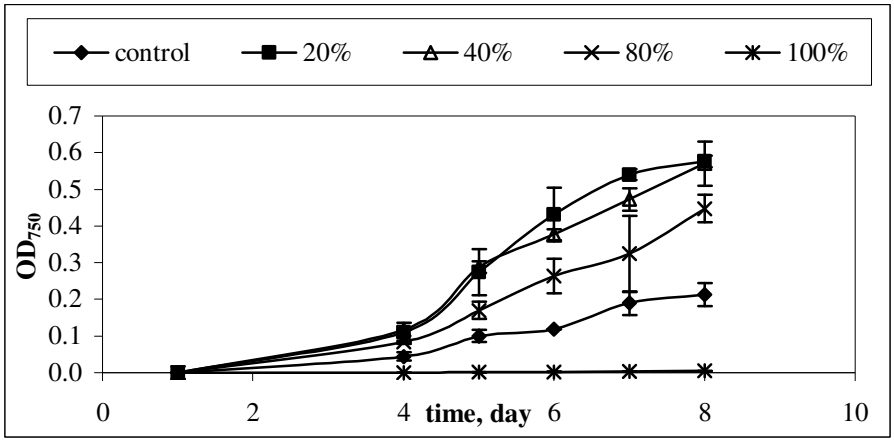
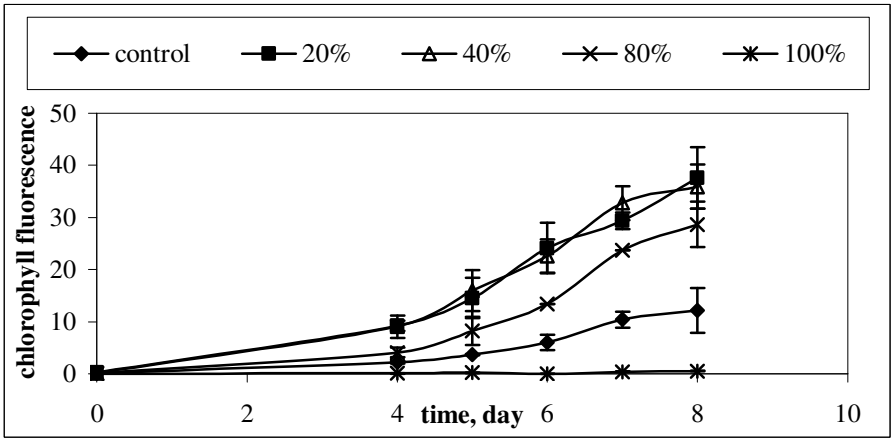


(c)

Figure 4.15. Growth curves of *D. tertiolecta* exposed to Reactive Orange 69 obtained by the measurement of (a) optical density at 750 nm (b) *in vivo* chlorophyll fluorescence and (c) cell count.



(a)



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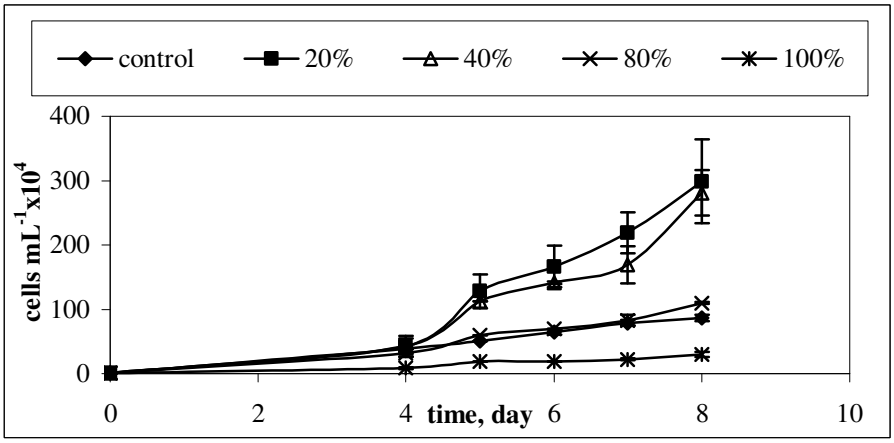


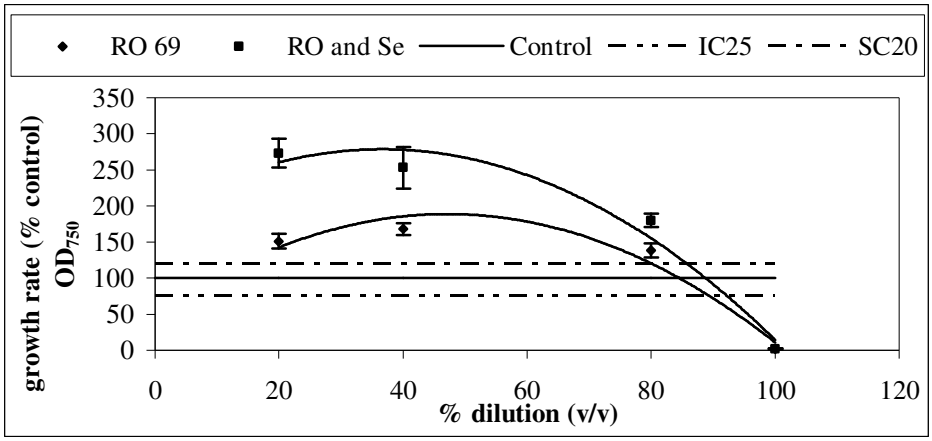
(c)

Figure 4.16. Growth curves of *D. tertiolecta* exposed to Reactive Orange 69 in the presence of selenium obtained by the measurement of (a) optical density at 750 nm (b) *in vivo* chlorophyll fluorescence and (c) cell count.

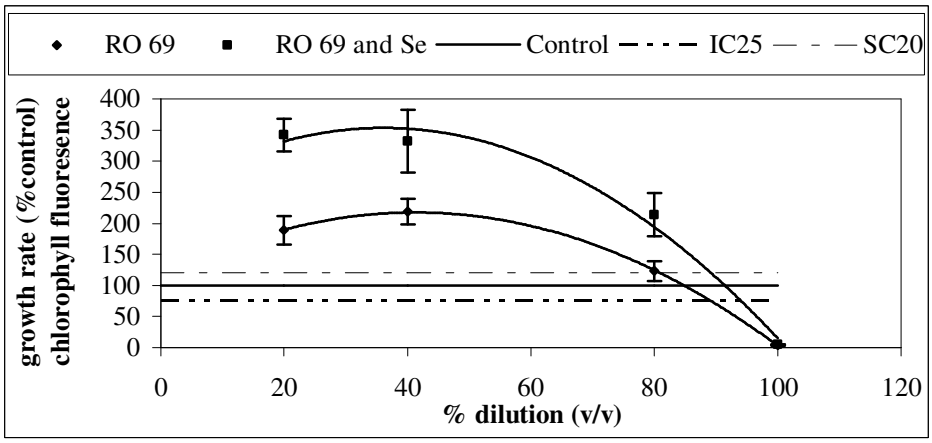
Table 4.6. Variation in optical density at 750 nm, *in vivo* chlorophyll fluorescence, cell number and toxicity parameters of *D. tertiolecta* exposed to Reactive Orange 69 in the presence and absence of selenium under an illumination of 76.8 µE $m^{-2}s^{-1}$.

Method		Control	% dilution (v/v)				IC_{25} % dilution (v/v)	TU[c]	Equation (SC_{20}= $aX^{*2}+bX+c$)	*p*-value	*F*	SC_{20}	TU[▪]
			20	40	80	100							
REACTIVE ORANGE 69													
Spectrophotometric	AUC[a] CV Ig (%)[b]	0.65 17.42	1.09 5.85 -68	1.21 4.64 -86	0.94 6.68 -45	0.01 66.77 98	84.72[†]±0.65 91.39[‡]±7.47	2.05	$-0.06X^2+4.92X+94.56$	0.04	22.78	5.52±0.76 82.07±7.48	18.11 1.22
Spectrofluorometric	AUC CV Ig (%)	32.43 14.92	61.28 12.26 -89	71.17 9.22 -119	40.00 12.99 -23	1.39 28.85 96	83.32±2.35 87.09±6.61	1.63	$-0.07X^2+5.56X+101.56$	0.00	1448.59	3.76±0.62 78.88±7.86	26.59 1.27
Cell number	AUCx10^4 CV Ig (%)	335.26 1.89	544.38 5.67 -66	551.30 2.94 -69	342.61 3.19 -2	48.22 3.19 86	83.93±1.17 96.92±2.64	1.73	$-0.04X^2+3.59X+103.33$	0.01	121.33	9.93±0.81 84.71±3.07	10.07 1.18
REACTIVE ORANGE 69 and SELENIUM													
Spectrophotometric	AUC CV Ig (%)	0.65 17.42	1.71 7.60 -163	1.88 10.05 -189	1.27 4.76 -96	0.01 14.79 98	85.04±0.01 94.00±3.35	1.18	$-0.10X^2+9.06X+104.92$	0.01	76.75	1.72±0.70 89.12±3.31	58.13 1.12
Spectrofluorometric	AUC CV Ig (%)	32.43 14.92	108.85 7.71 -242	89.02 15.08 -232	56.40 16.41 -114	1.46 3.39 95	83.99±1.98 94.64±3.15	1.19	$-0.12X^2+10.91X+121.22$	0.05	19.91	2.43±0.48 90.23±3.77	41.15 1.11
Cell number	AUCx10^4 CV Ig (%)	335.26 1.89	670.00 3.32 -100	722.39 5.62 -115	332.12 2.69 1	86.80 6.12 74	75.32±6.75 93.57±6.85	1.33	$-0.06X^2+4.75X+111.52$	0.05	18.90	1.69±2.30 84.86±6.97	59.17 1.18

[a] All AUC are mean values from repeated tests (n=3); CV = coefficient of variation.
[b] Ig (%) = Per cent growth inhibition/stimulation; negative values show stimulation of growth with respect to control
[†] IC_{25} = 25% Inhibition concentration calculated from ICp method
[‡] IC_{25} = 25% Inhibition concentration calculated from SPSS curve fitting
[c] TU= Toxic Unit in terms of IC_{25} [▪]TU=Toxic Unit in terms of SC_{20}
[*] X= Per cent dilution of Reactive Orange 69



(a)



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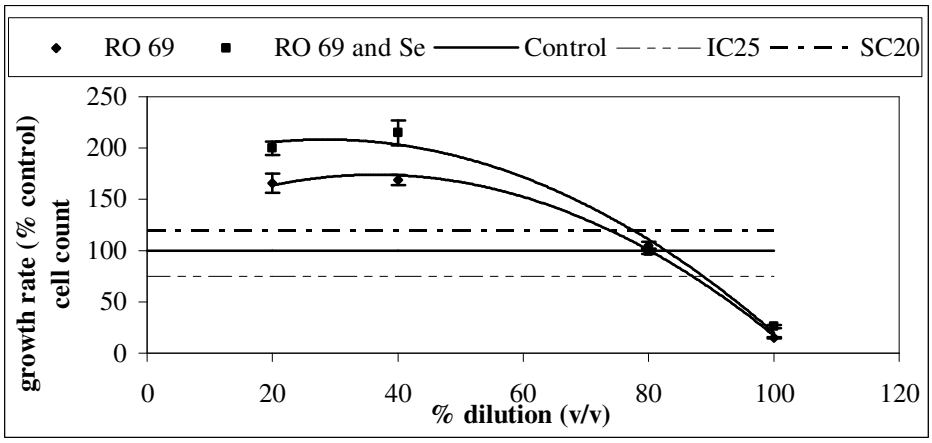


(c)

Figure 4.17. Representative dose-response curves of Reactive Orange 69 in the presence and absence of selenium obtained by the measurement of (a) optical density at 750 nm (b) *in vivo* chlorophyll fluorescence and (c) cell count.

Neither the 8-d IC_{25} nor 8-d SC_{20} value of Reactive Orange 69 in the presence and absence of selenium was not significantly different (p>0.05). This situation is similar to the response of assisting chemicals. Considering the 8-d IC_{25} values of Reactive Orange 69 and assisting chemicals, we couldn't differentiate between the toxicity of Reactive Orange 69 and assisting chemicals since they are very close to each other. The average 8-d IC_{25} values expressed as mg L^{-1-} were 3.40±0.12 mg L^{-1} and 3.51±0.06 mg L^{-1} with and without selenium, respectively. The average stimulant concentration ranges were 0.27-3.41 mg L^{-1} and 0.08-3.68 mg L^{-1} singly and combined with selenium. When the results were evaluated in terms of TU calculated on the basis of 8-d IC_{25}, Reactive Orange 69 and Reactive Orange 69 with selenium were moderately toxic to *D. tertiolecta* (TU > 1.0, Villegas-Navarro et al., 2001). On the other hand, when TU was calculated using lower limit of SC_{20} (assuming that lower value of SC_{20} is equal to LOEC) the dye was very toxic to *D. tertiolecta* (TU ≥ 3.0, Villegas-Navarro et al., 2001) both in the presence and absence of selenium.

4.7. Effects of Reactive Yellow 37 on the Growth of *Dunaliella tertiolecta* in the Presence and Absence of Selenium

Reactive Yellow 37 is the second azo dye present in the dye bath recipe having sulphatoethylsulphonate as a reactive group. Also, it has the third highest concentration among the six azo dyes present in the dye bath recipe. Figure 4.18 and 4.19 show the effects of Reactive Orange 69 added singly and in combination with selenium on the growth response of algae measured by OD_{750}, *in vivo* chlorophyll fluorescence and cell counts. Significant difference was observed between all studied dilutions (% v/v) and control. The results were denoted in Table 4.7 with the 8-day IC_{25}, SC_{20} and TU. The test sensitivities based on different response parameters reflected no significant difference. Curve fitting analysis applied directly on the data was carried out to calculate SC_{20} values, whereas IC_{25} values were determined using linear interpolation combined with bootstrapping known as the IC_p method.

Stimulatory effect was observed on the growth of *D. tertiolecta* exposed to both Reactive Yellow 37 and Reactive Yellow 37 with selenium. Figure 4.20 represented the dose-response curves of Reactive Yellow 37 singly and in combination with selenium

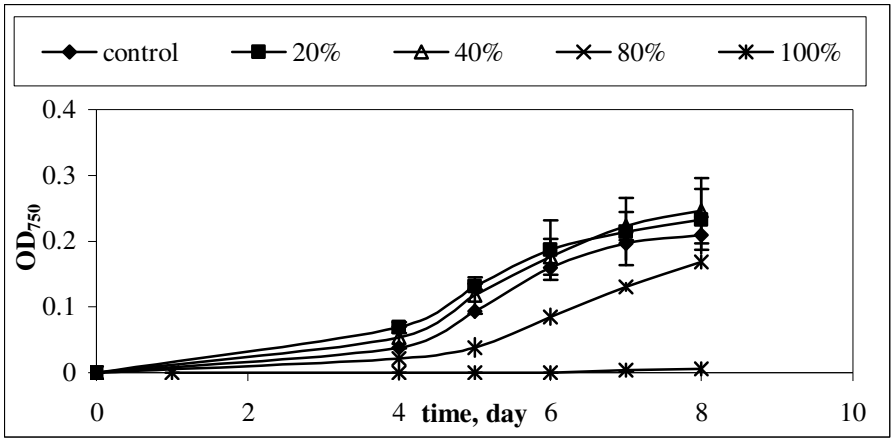
obtained from the measurement of the three endpoints. There was a significant difference (p<0.05) between Reactive Yellow 37 and Reactive Yellow 37 with selenium in all response parameters. Growth inhibition range was 80-100% (v/v) when *D. tertiolecta* exposed to only Reactive Yellow 37. The average 8-d IC_{25} value of *D. tertiolecta* exposed to Reactive Yellow 37 was 77.38±3.74% (v/v). The average 8-d IC_{25} value of *D. tertiolecta* exposed to Reactive Yellow 37-selenium combination increased to 84.66±0.74% (v/v) reflecting that resistance of algae towards dye with the addition of selenium increased.

Increase in stimulatory effect with the addition of selenium was evident in 8-d SC_{20} values. The range of the average SC_{20} values changed from 7.67-64.45% (v/v) to 4.03-82.70% (v/v) with the addition of selenium. Considering the TU calculated on the basis of 8-d IC_{25}, Reactive Yellow 37 with and without selenium was moderately toxic to *D. tertiolecta* (TU > 1.0, Villegas-Navarro et al., 2001). On the other hand, when TU was calculated on the basis of the lower limit of SC_{20} (assuming that lower limit of SC_{20} is equal to LOEC) Reactive Yellow 37 was very toxic to *D. tertiolecta* (TU ≥ 3.0, Villegas-Navarro et al., 2001) both in the presence and absence of selenium.

When the results were criticized on the basis of concentration the average 8-d IC_{25} increased from 2.43±0.12 mg L^{-1} to 2.66±0.02 mg L^{-1} with addition of selenium. The average stimulant concentration ranges were changed from 0.26-2.03 mg L^{-1} to 0.13-2.6 mg L^{-1} with the addition of selenium.

4.8. Comparison of the Effect of the Three Azo Dyes on the Growth of *Dunaliella tertiolecta*

The toxic concentration of studied chemicals both in terms of the 8-d IC_{25} and SC_{20} were tabulated in Table 4.8 for the three response parameters. Since dye bath is a mixture of ten chemicals and assisting chemicals is a mixture of four chemicals, only per cent dilution (v/v) values were used to express their 8-d IC_{25} and SC_{20} values instead of concentration.



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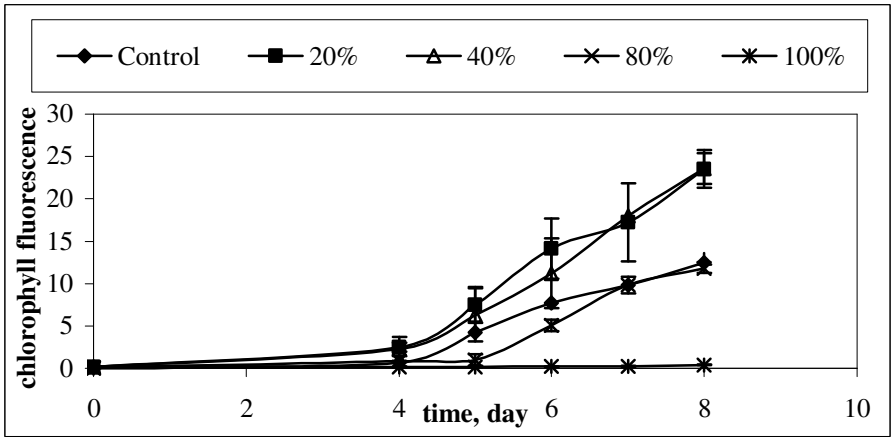
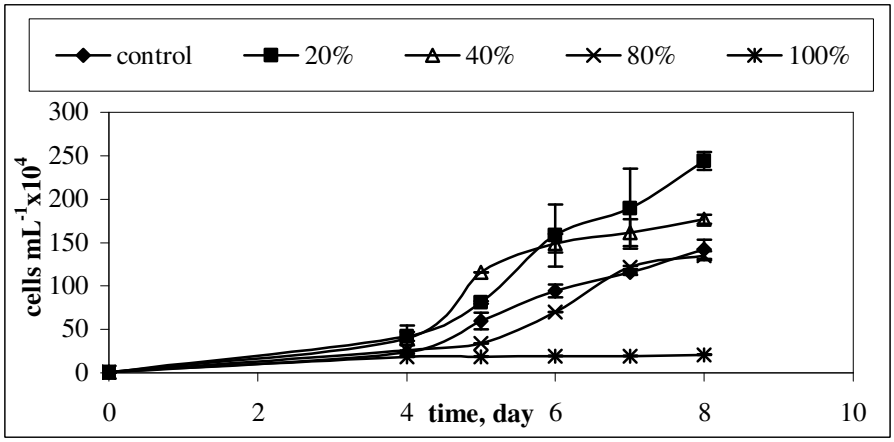
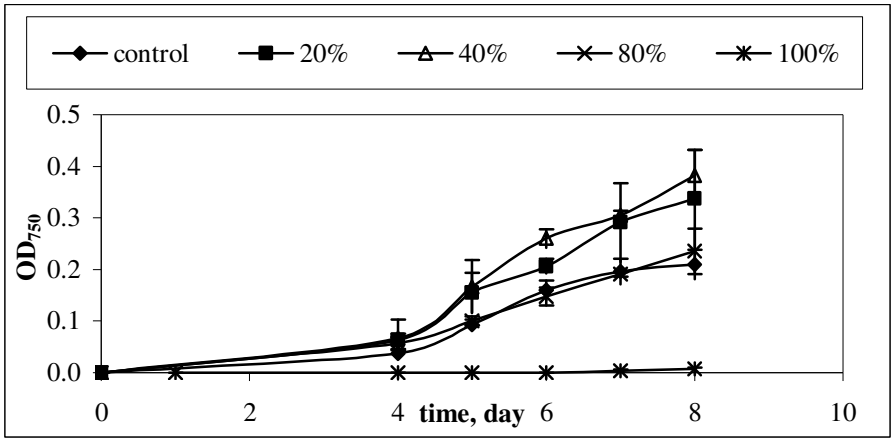


(b)



(c)

Figure 4.18. Growth curves of *D. tertiolecta* exposed to Reactive Yellow 37 obtained by the measurement of (a) optical density at 750 nm (b) *in vivo* chlorophyll fluorescence and (c) cell count.



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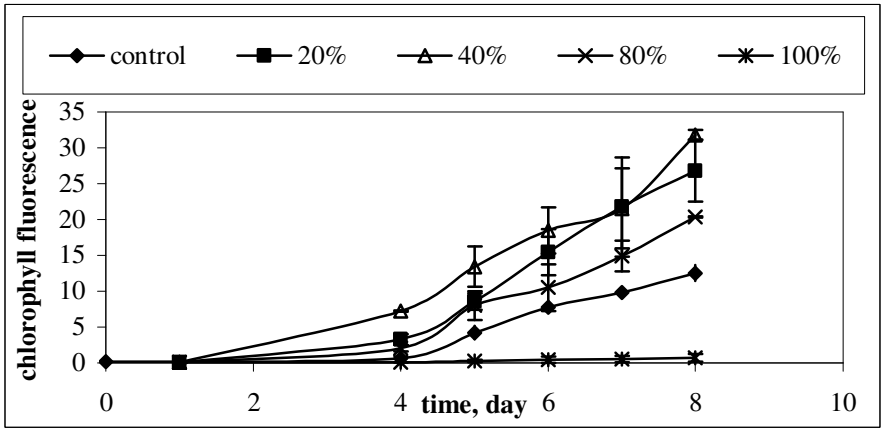

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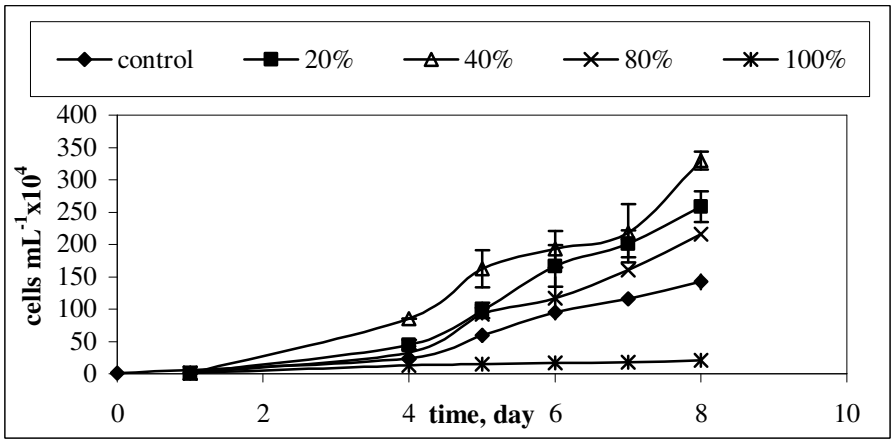


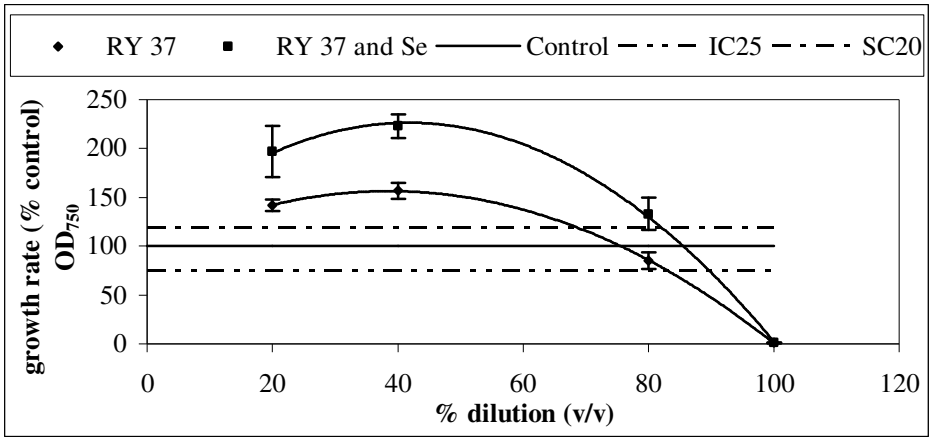
(c)

Figure 4.19. Growth curves of *D. tertiolecta* exposed to Reactive Yellow 37 in presence of selenium obtained by the measurement of (a) optical density at 750 nm (b) *in vivo* chlorophyll fluorescence and (c) cell count.

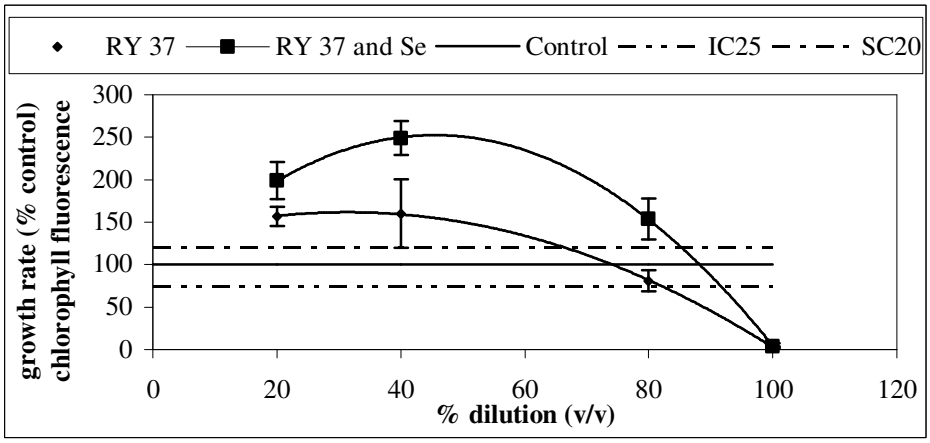
Table 4.7. Variation in optical density at 750 nm, *in vivo* chlorophyll fluorescence, cell number and toxicity parameters of *D. tertiolecta* exposed to Reactive Yellow 37 in the presence and absence of selenium under an illumination of 76.8 µE $m^{-2}s^{-1}$.

Method		Control	% dilution (v/v)				IC_{25} % dilution (v/v)	TU[c]	Equation (SC_{20}= aX^{*2}+bX+-c)	*p*-value	*F*	SC_{20}	TU▪
			20	40	80	100							
REACTIVE YELLOW 37													
Spectrophotometric	AUC[a]	0.60	0.85	0.94	0.51	0.01						7.64±0.30	13.08
	CV	13.01	4.35	5.23	10.19	48.04	82.32±0.58	1.21	$-0.04X^2+3.02X+99.30$	0.00	3513.57	67.04±4.35	1.49
	Ig (%)[b]		-42	-57	15	99							
Spectrofluorometric	AUC	35.72	56.23	57.20	28.25	1.09						6.11±2.62	16.37
	CV	6.38	7.22	25.13	15.28	26.82	76.76±3.63	1.30	$-0.04X^2+3.08X+104.18$	0.01	136.22	66.69±10.70	1.50
	Ig (%)		-57	-60	19	97							
Cell number	AUCx10^4	443.94	624.17	601.22	327.10	107.97						9.26±2.72	10.80
	CV	4.41	10.74	5.99	5.75	0.54	73.06±7.01	1.37	$-0.03X^2+1.89X+105.02$	0.02	51.78	59.62±1.86	1.68
	Ig (%)		-41	-35	26	76							
REACTIVE YELLOW 37 and SELENIUM													
Spectrophotometric	AUC	0.60	1.18	1.34	0.80	0.01						3.30±1.43	30.30
	CV	13.01	13.30	5.3	5.72	43.75	84.54±0.44	1.18	$-0.07X+5.89X+101.74$	0.00	660.23	83.65±1.69	1.20
	Ig (%)		-97	-123	-33	99							
Spectrofluorometric	AUC	35.72	71.09	89.01	50.13	1.52						3.50±1.38	18.87
	CV	6.38	7.85	7.08	3.80	1.19	84.91±0.56	1.18	$-0.08X^2+6.91X+97.12$	0.00	603.08	84.95±1.40	1.18
	Ig (%)		-99	-149	-54	96							
Cell number	AUCx10^4	443.94	718.70	874.63	504.15	85.13						5.30±1.03	18.87
	CV	4.41	7.48	11.47	21.97	14.64	84.52±1.30	1.18	$-0.05X^2+4.50X+98.13$	0.00	446.95	79.50±3.87	1.26
	Ig (%)		-62	-97	-19	81							

[a] All AUC are mean values from repeated tests (*n*=3); CV = coefficient of variation.
[b] Ig (%) = Per cent 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= Per cent dilution of Reactive Yellow 37



(a)



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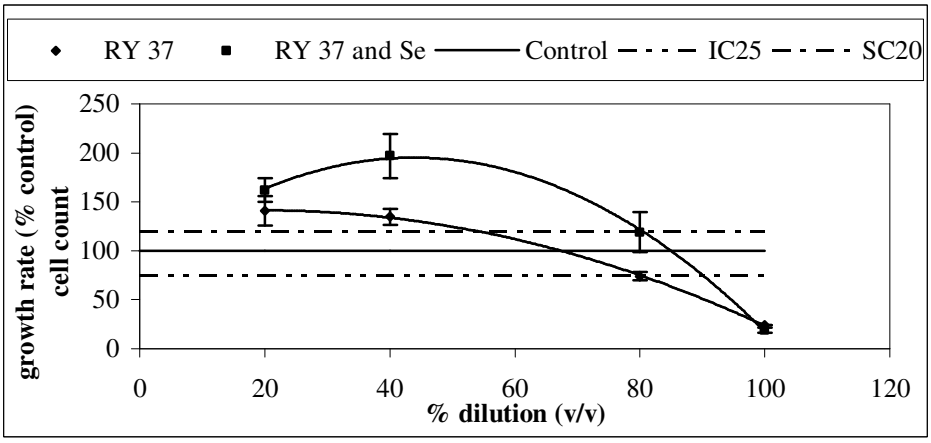


(c)

Figure 4.20. Representative dose-response curves of Reactive Yellow 37 in the presence and absence of selenium obtained by the measurement of (a) optical density at 750 nm (b) *in vivo* chlorophyll fluorescence and (c) cell count.

Among the three azo dyes investigated, Remazol Black 5 exhibited the highest toxicity with 8-d IC_{25} values of 55.84±4.50% (v/v). The average 8-d IC_{25} levels based on azo dye dilutions (% v/v) indicate the toxicity sequence as Remazol Black 5 > Reactive Yellow 37 > Reactive Orange 69. The toxicity of the dye bath on the basis of the 8-d IC_{25} value was found to be approximately equal to that of Remazol Black 5 whereas the toxicity of assisting chemicals was equal to the Reactive Orange 69. In the presence of selenium the sequence was the same as described above; Dye bath = Remazol Black 5 > Reactive Yellow 37 > Reactive Orange 69 = Assisting chemicals. The same sequence was obtained when the comparison was done considering the 8-d SC_{20} values both in the presence and absence of selenium. Although selenium decreased the inhibitory effect of Remazol Black 5 and Reactive Yellow 37, these two dyes were still toxic to *D. tertiolecta* as stated in the relevant sections.

For the case of toxicity expressed on a concentration basis, Reactive Yellow 37 was the most toxic with a concentration of 2.43±0.12 mg L^{-1} followed by Reactive Orange 69 with a concentration of 3.51±0.06 and then Remazol Black 5 with a concentration of 8.14±0.65 mg L^{-1}. In the presence of selenium, the same sequence was observed with concentrations of 2.66±0.02 mg L^{-1}, 3.40±0.12 mg L^{-1} and 9.75±0.36 mg L^{-1} for Reactive Yellow 37, Reactive Orange 69 and Remazol Black 5, respectively. When the stimulatory effect of studied chemicals expressed on a concentration basis, Remazol Black 5 had the lowest stimulatory effect with the average concentrations of 4.85±0.56 mg L^{-1} and 7.71±0.46 mg L^{-1} singly and combined with selenium, respectively (Table 4.8). Since the 8-d SC_{20} values of Reactive Orange 69 and Reactive Yellow 37 obtained from quadratic relations, we had two 8-d SC_{20} values. Between these two reported 8-d SC_{20} values, the stimulatory effect was much more than 20 % as indicated in Figures 4.17 and 4.20. Remazol Black 5 was followed by Reactive Orange 69 with the average concentration ranges of 0.27-3.41 mg L^{-1} and 0.08-3.68 mg L^{-1} singly and combined with selenium, respectively. Reactive Yellow 37 had the highest stimulatory effect among the tested three azo dyes. The average stimulant concentrations were in the range of 0.26-2.03 mg L^{-1} for Reactive Yellow 37 and 0.13-3.06 mg L^{-1} for Reactive Yellow 37-selenium combination. The contribution of assisting chemicals to the toxicity of azo dyes must be taken into consideration when the toxic concentration of azo dyes used.

Table 4.8. The 8-d IC_{25} and SC_{20} values of the three azo dyes expressed on a concentration basis in the presence and absence of selenium.

Method	Chemical					
	Remazol Black 5		**Reactive Orange 69**		**Reactive Yellow 37**	
	***IC*$_{25}$ (mg /L)**	***SC*$_{20}$ (mg/L)**	***IC*$_{25}$ (mg /L)**	***SC*$_{20}$ (mg/L)**	***IC*$_{25}$ (mg/L)**	***SC*$_{20}$ (mg/L)**
Spectrophotometric	7.09±1.51	4.73±0.09	3.54±0.03	0.23±0.03 3.42±0.31	2.59±0.02	0.24±0.01 2.11±0.14
Spectrofluorometric	8.93±0.16	4.61±0.73	3.48±0.10	0.17±0.03 3.29±0.33	2.42±0.11	0.26±0.08 2.10±0.34
Cell number	8.40±0.29	5.18±0.85	3.50±0.05	0.41±0.03 3.53±0.13	2.30±0.22	0.29±0.09 1.87±0.06
	8.14*±0.65	4.84*±0.56	3.51*±0.06	0.27*±0.03 3.41*±0.26	2.44*±0.12	0.26*±0.06 2.02*±0.18
	Remazol Black 5 and Selenium		**Reactive Orange 69 and Selenium**		**Reactive Yellow 37 and Selenium**	
Spectrophotometric	9.57±0.31	7.73±0.31	3.55±0.00	0.07±0.03 3.72±0.14	2.66±0.01	0.10±0.04 2.63±0.05
Spectrofluorometric	9.96±0.09	8.35±0.23	3.51±0.08	0.10±0.02 3.77±0.16	2.67±0.02	0.11±0.04 2.67±0.04
Cell number	9.71±0.67	7.05±0.85	3.14±0.28	0.07±0.10 3.54±0.29	2.66±0.04	0.17±0.03 2.50±0.12
	9.75*±0.36	7.71*±0.46	3.40*±0.12	0.08*±0.05 3.68*±0.20	2.66*±0.02	0.13*±0.04 2.60*±0.07

* Average values

4.9. Toxicity of Synthetic Dye Bath in the Presence and Absence of Selenium to *Vibrio fischeri*

Toxicity of synthetic dye bath including assisting chemicals and six azo dyes listed in Table 3.1 in the presence and absence of selenium was determined using bioluminescence inhibition assay, ToxAlert® 100 (Merck). Calculated growth inhibition percentages were tabulated in Table 4.9 together with the 30-min EC_{25} and EC_{50}, and TU values in the presence and absence of selenium.

All studied dilutions (% v/v) had inhibitory effect on *V. fischeri*. Synthetic dye bath was toxic with the 30-min EC_{25} value of 22.99±0.25% (v/v). Similarly, the synthetic dye bath with 0.5 mg Se L^{-1} in the form of H_2SeO_3 had toxic effect with the 30-min EC_{25} value of 24.04 ±0.32% (v/v) on *V. fischeri*. The 30-min EC_{50} value of the synthetic dye bath increased from 42.14±0.21% (v/v) to 44.01±0.58% (v/v) with the addition of selenium. However, no significant difference (p>0.05) was observed between the 30-min EC_{25} and EC_{50} values of dye bath with and without selenium. Although TU decreased from 4.35 to 4.15 with the addition of selenium dye bath either singly or in combination with selenium was indicated that the dye bath was very toxic to *V. fischeri* (TU ≥ 3.0, Villegas-Navarro et al., 2001). Figure 4.21 representing the dose response curve of the synthetic dye bath in the presence and absence of selenium also proves these results. Error bars show the mean standard deviation between duplicate.

4.10. Toxicity of Assisting Chemicals in the Presence and Absence of Selenium to *Vibrio fischeri*

The toxicity of assisting chemicals in the presence and absence of selenium was evaluated by using *V. fischeri*. The toxicity results were tabulated in Table 4.10 together with the 30-min EC_{25}, EC_{50} and TU values. All studied dilutions showed inhibitory effect on *V. fischeri*. The 30-min EC_{25} and EC_{50} values of assisting chemicals to *V. fischeri* was 26.52±0.70% (v/v) and 46.80±0.51% (v/v), respectively and calculated TU was 3.77. With the addition of selenium the 30-min EC_{25} and EC_{50} values of assisting chemicals increased to 28.63±1.46% (v/v) and 49.90±1.30% (v/v), respectively and the TU decreased to 3.49.

No significant difference (p>0.05) was observed between the 30-min EC_{25} and EC_{50} values of assisting chemicals with and without selenium. TU of the assisting chemicals both in the presence and absence of selenium indicated that the assisting chemicals were toxic to *V. fischeri* (TU ≥ 3.0, Villegas-Navarro et al., 2001). Representative dose response curves of assisting chemicals singly and in combination with selenium were shown in Figure 4.22. When the toxicity of assisting chemicals was compared with the toxicity of the dye bath, a significant difference (p<0.05) was observed between the 30-min EC_{25} and EC_{50} values of the dye bath and assisting chemicals both in the presence and absence of selenium.

Table 4.9. Variation in toxicity parameters of *V. fischeri* exposed to the dye bath in the presence and absence of selenium.

	% dilution (v/v)					**Equation (EC_{25}/EC_{50} =aX*+b)**	**R^2**	**F**	***p*- value**	**EC_{25} % dilution (v/v)**	**TU[b] (% v/v)**	**EC_{50} % dilution (v/v)**
	6.25	**12.5**	**25**	**50**	**80**							
DYE BATH												
Ig (%)[a]	3.8	9.65	30.3	60.6	99.05	-1.30X+104.50	0.99	1878.11	0	22.99±0.25	4.35	42.14±0.21
CV	-	3.66	-	-	0.21							
DYE BATH and SELENIUM												
Ig (%)[a]	5.6	9.8	23.95	56.25	96.45	-1.25X+105.10	0.99	996.31	0	24.04±0.32	4.15	44.01±0.58
CV	0.10	5.77	0.89	0.38	0.22							

[a] Ig (%) = Per cent growth inhibition/stimulation; negative values show stimulation of growth with respect to control
[b] TU= Toxic Unit in terms of EC_{25}
* X= Per cent dilution (v/v) of dye bath

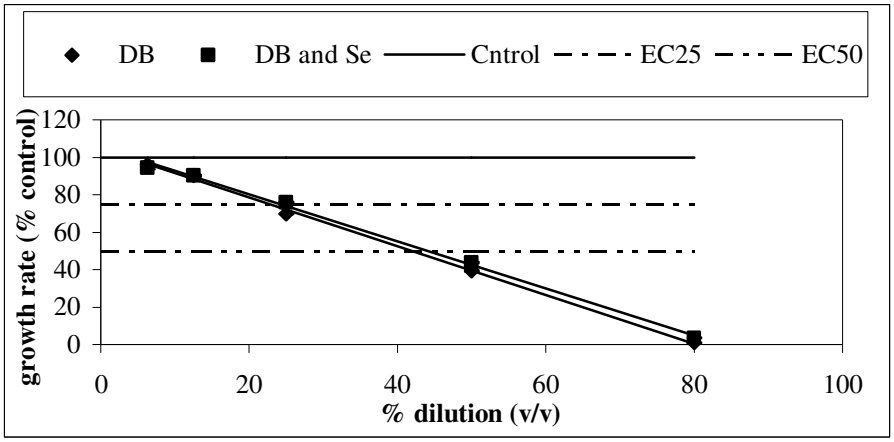


Figure 4.21. Representative dose-response curve of *V. fischeri* exposed to the synthetic dye bath in the presence and absence of selenium.

4.11. Toxicity of Remazol Black 5 in the Presence and Absence of Selenium to *Vibrio fischeri*

Toxicity of Remazol Black 5 having the highest concentration in the dye bath recipe given in Table 3.1 to *V. fischeri* was evaluated in the presence and absence of selenium. Five different dilutions were prepared in duplicate and the growth inhibition percentages were listed in Table 4.11 together with the 30-min EC_{25} and EC_{50}, and TU values.

The 30-min EC_{25} and EC_{50} values of Remazol Black 5 were 23.33±0.33% (v/v) and 43.48±0.21% (v/v), respectively. Both the 30-min EC_{25} and EC_{50} values slightly increased with the addition of 0.5 mg Se L^{-1}. In the presence of selenium the 30-min EC_{25} and EC_{50} values of *V. fischeri* were 24.08±0.27% (v/v) and 44.65±0.33% (v/v), respectively. There was no significant difference (p<0.05) between the 30-min EC_{25} and EC_{50} values of Remazol Black 5 with and without selenium. Representative dose-response curves of *V. fischeri* exposed to Remazol Black 5 in the presence and absence of selenium was shown in Figure 4.23. With the addition of selenium TU decreased from 4.29 to 4.15 indicating that Remazol Black 5 with and without selenium was very toxic to *D. tertiolecta* (TU ≥ 3.0, Villegas-Navarro et al., 2001).

Table 4.10. Variation in toxicity parameters of *V. fischeri* exposed to the assisting chemicals in the presence and absence of selenium.

	% dilution (v/v)					**Equation (EC_{25}/EC_{50} =aX*+b)**	**R^2**	**F**	***p*- value**	**EC_{25} % dilution (v/v)**	**TU[b] (% v/v)**	**EC_{50} % dilution (v/v)**
	6.25	**12.5**	**25**	**50**	**80**							
ASSISTING CHEMICALS												
Ig (%)[a]	7.70	10.60	15.30	46.90	97.00	-1.22X+106.96	0.99	77.05	$31x10^{-4}$	26.52±0.70	3.77	46.80±0.51
CV	6.53	-	10.17	2.41	0.44							
ASSISTING CHEMICALS and SELENIUM												
Ig (%)[a]	3.7	11.9	12.00	40.7	92.7	-1.17X+107.48	0.99	54.91	$51x10^{-4}$	28.63±1.46	3.49	49.90±1.50
CV	1.56	3.07	1.11	6.25	1.53							

[a] Ig (%) = Per cent growth inhibition/stimulation; negative values show stimulation of growth with respect to control
[b] TU= Toxic Unit in terms of EC_{25}
* X= Per cent dilution (v/v) of assisting chemicals

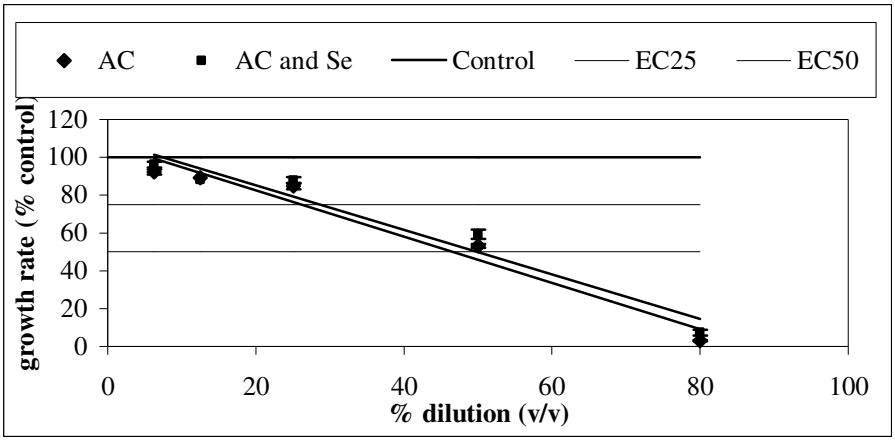


Figure 4.22. Representative dose-response curve of *V. fischeri* exposed to the assisting chemicals in the presence and absence of selenium.

The corresponding concentrations inhibiting 25% of the growth was 3.51±0.04 mg L^{-1} and 3.40±0.05 mg L^{-1} in the presence and absence of selenium, respectively. The corresponding concentration inhibiting 50% of growth was increased from 6.33±0.03 mg L^{-1} to 6.51±0.05 mg L^{-1} with the addition of selenium. However, these increases in the 30-min EC_{25} and EC_{50} values of assisting chemicals with and without selenium were not significant (p>0.05).

The toxicity of Remazol Black on the bioluminescence bacteria compared with assisting chemicals and dye bath both in terms of 30-min EC_{25} and EC_{50}. Significant difference (p<0.05) was observed between 30-min EC_{25} and EC_{50} values of assisting chemicals and Remazol Black 5 both in the presence and absence of selenium. However, no significant difference (p>0.05) was found between 30- min EC_{25} and EC_{50} values of dye bath and Remazol Black 5. This result reflects that the toxicity of dye bath is mainly governed by Remazol Black 5 having the highest azo dye concentration in dye bath recipe.

Table 4.11. Variation in toxicity parameters of *V. fischeri* exposed to Remazol Black 5 in the presence and absence of selenium

	% dilution (v/v)					Equation (EC_{25}/EC_{50} =aX*+b)	R^2	F	p- value	EC_{25}		TU[b] (% v/v)	EC_{50}	
	6.25	12.5	25	50	80					% dilution (v/v)	mg L^{-1}		% dilution (v/v)	mg L^{-1}
REMAZOL BLACK 5														
Ig (%)[a]	6.8	9.8	26.65	55.45	97.15	-1.24X+103.95	0.99	753.35	$1x10^{-4}$	23.33±0.33	3.40±0.05	4.29	43.48±0.21	6.33±0.03
CV	2.48	8.66	5.04	1.91	0.80									
REMAZOL BLACK 5 and SELENIUM														
Ig (%)[a]	7.85	11.05	21.55	53.55	95.85	-1.21X+104.25	0.99	279.43	$5x10^{-4}$	24.08±0.27	3.51±0.04	4.15	44.65±0.33	6.51±0.05
CV	2.70	1.92	0.98	1.98	0.07									

[a] Ig (%) = Per cent growth inhibition/stimulation; negative values show stimulation of growth with respect to control
[b] TU= Toxic Unit in terms of EC_{25}
* X= Per cent dilution of Remazol Black 5

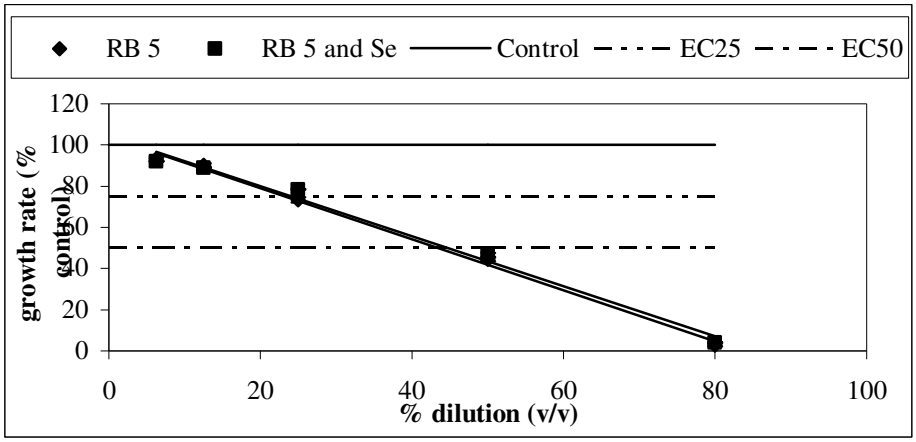


Figure 4.23. Representative dose-response curve of *V. fischeri* exposed to Remazol Black 5 in the presence and absence of selenium.

Literature toxicity data for *V. fischeri* exposed to Remazol Black 5 reported by Wang et al. (2002). They studied the toxicity of dyestuff, auxiliaries and selected effluents in textile finishing industry to luminescent bacteria, *V. fischeri*. Remazol Black 5 was evaluated moderately toxic to *V. fischeri* in their study. Its EC_{20} value after the 15 minute exposure was reported to be greater than 125 mg L^{-1}. However, in this study the determined corresponding concentration inhibiting 25% of growth after 30-minute exposure was 3.40±0.05 mg L^{-1}. Due to the scarce information on the preparation of their azo dye solution (either contains assisting chemicals or not) and the differences between the test duration (15 min vs 30 min) and the luminometer they used (LUMIStox), it is difficult to compare the obtained results.

4.12. Toxicity of Reactive Orange 69 in the Presence and Absence of Selenium to *Vibrio fischeri*

Toxicity evaluation of Reactive Orange 69 both singly and in combination with selenium, was done using bioluminescence inhibition assay, ToxAlert® 100 by Merck. Reactive Orange 69 is the only studied dye having monochlorodifluoropyrimidine as a reactive group. Calculated growth inhibition per cents were tabulated in Table 4.12 together with 30-min EC_{25} and EC_{50}, and TU values.

The 30-min EC_{25} and EC_{50} values of Reactive Orange 69 were 29.76±0.99% (v/v) and 49.12±0.87% (v/v) respectively. The 30-min EC_{25} and EC_{50} values of Reactive Orange 69 with selenium were 28.13±0.20% (v/v) and 48.19±0.12% (v/v). No significant difference (p>0.05) was observed between the 30-min EC_{25} and EC_{50} values of Reactive Orange 69 with and without selenium. TU values calculated on the basis of the 30-min EC_{25} value were 3.55 and 3.36 with and without selenium, respectively. TU values indicated that Reactive Orange 69 and Reactive Orange 69 with selenium were very toxic to *V. fischeri* (TU ≥ 3.0, Villegas-Navarro et al., 2001). Considering the concentration, a change from 1.24±0.04 to 1.17±0.01 mg L^{-1} and from 2.05±0.04 to 2.01±0.01 mg L^{-1} was observed in the 30-min EC_{25} and EC_{50} values of Reactive Orange 69 with the addition of selenium, respectively. Representative dose-response curves of *V. fischeri* exposed to Reactive Orange 69 in the presence and absence of selenium was shown in Figure 4.24.

No significant difference (p>0.05) was observed between the 30-min EC_{25} and EC_{50} values of Reactive Orange 69 and assisting chemicals both in the presence and absence of selenium. This result indicated that the toxicity of Reactive Orange 69 may not be its own toxicity; but due to the toxicity of assisting chemicals. On the other hand, there was a significant difference (p<0.05) between the 30-min EC_{25} and EC_{50} values of Reactive Orange 69 and dye bath both in the presence and absence of selenium.

4.13. Toxicity of Reactive Yellow 37 in the Presence and Absence of Selenium to *Vibrio fischeri*

Reactive Yellow 37 is the dyestuff having the lowest concentration among the tested dyes. It has the same reactive group –sulphatoethylsulphonate- with Remazol Black 5. Toxicity of Reactive Yellow 37 was evaluated in the presence and absence of selenium by using luminescent bacteria *V. fischeri*. The calculated growth inhibitions of *V. fischeri* were tabulated in Table 4.12 together with the 30-min EC_{25}, EC_{50} values and TU.

Table 4.12. Variation in toxicity parameters of *V. fischeri* exposed to Reactive Orange 69 in the presence and absence of selenium.

	% dilution (v/v)					Equation (EC_{25}/EC_{50} =aX*+b)	R^2	*F*	*p*- value	EC_{25}		TU[b] (% v/v)	EC_{50}	
	6.25	12.5	25	50	80					% dilution (v/v)	mg L^{-1}		% dilution (v/v)	mg L^{-1}
REACTIVE ORANGE 69														
Ig (%)[a]	2.1	5.45	10.45	42.00	97.20	-1.23X+109.48	0.96	68.03	$37x10^{-4}$	29.76±0.99	1.24±0.04	3.36	49.12±0.12	2.05±0.04
CV	0.81	9.84	0.68	6.40	0.29									
REACTIVE ORANGE 69 and SELENIUM														
Ig (%)[a]	4.05	4.55	14.95	50.2	92.5	-1.24X+110.05	0.96	188.38	$8x10^{-4}$	28.13±0.20	1.17±0.01	3.55	48.19±0.12	2.02±0.01
CV	1.77	1.55	2.84	0.85	0.46									

[a] Ig (%) = Per cent growth inhibition/stimulation; negative values show stimulation of growth with respect to control
[b] TU= Toxic Unit in terms of EC_{25}
* X= Per cent dilution of Reactive Orange 69

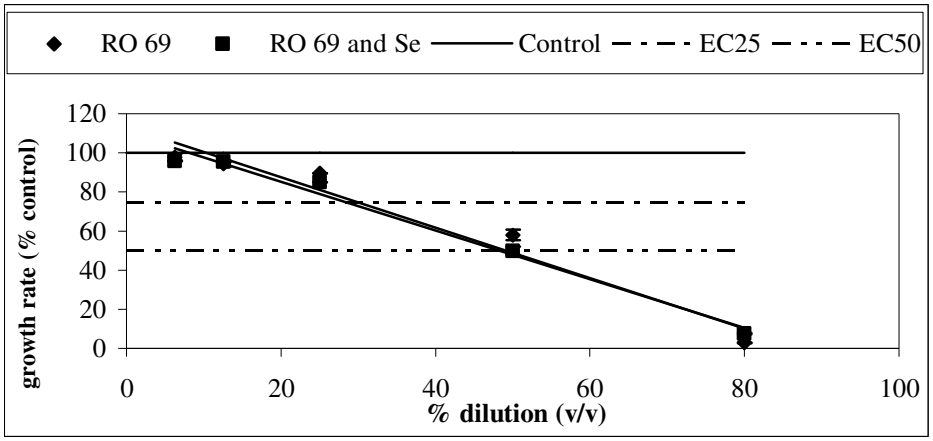


Figure 4.24. Representative dose-response curve of *V. fischeri* exposed to Reactive Orange 69 in the presence and absence of selenium.

Reactive Yellow 37 had an inhibitory effect on *V. fischeri* in the range of 25-80% (v/v). In the presence of selenium this range changed to 12.5-80% (v/v) (Table 4.12). Inhibitory effect could not be determined at lower dilution percentage both in the presence and absence of selenium. There was no significant difference (p>0.05) between the 30-min EC_{25} and EC_{50} values of Reactive Yellow 37 and Reactive Yellow 37-selenium combination. The 30-min EC_{25} and EC_{50} values of Reactive Yellow 37 were 33.49±0.67% (v/v) and 53.45±0.72% (v/v), respectively. A change from 33.49±0.67% (v/v) to 33.34±0.16% (v/v) and from 53.45±0.72% (v/v) to 53.89±0.78% (v/v) was observed in the 30-min EC_{25} and EC_{50}, respectively with the addition of 0.5 mg Se L^{-1}. TU values calculated on the basis of the 30-min EC_{25} in the presence and absence of selenium were 3.00 and 2.99, respectively. TU values indicated that the dye was toxic to *V. fischeri* both in the presence and absence of selenium (TU ≥ 3.0, Villegas-Navarro et al., 2001).

Reactive Yellow 37 itself was toxic to *V. fischeri* at 1.06±0.02 mg L^{-1} and at 1.05±0.01 with selenium. The corresponding concentrations inhibiting 50% of growth was 1.68±0.02 mg L^{-1} and 1.70±0.02 mg L^{-1} in the presence and absence of selenium, respectively. Representative dose-response curves of *V. fischeri* exposed to Yellow 37 in the presence and absence of selenium was shown in Figure 4.16. Selenium did not have any significant effect on the toxicity of Reactive Yellow 37.

Table 4.13. Variation in toxicity parameters of *V. fischeri* exposed to Reactive Yellow 37 in the presence and absence of selenium.

	% dilution (v/v)					Equation (EC_{25}/EC_{50} =aX*+b)	R^2	F	p- value	EC_{25}		TU[b] (% v/v)	EC_{50}	
	6.25	12.5	25	50	80					% dilution (v/v)	mg L^{-1}		% dilution (v/v)	mg L^{-1}
REACTIVE YELLOW 37														
Ig (%)[a]	0.00	0.00	9.75	28.80	94.35	-1.25X+116.96	0.96	33.73	$102x10^{-4}$	33.49±0.67	1.06±0.02	2.99	53.45±0.72	1.68±0.02
CV	-	-	0.73	2.46	0.67									
REACTIVE YELLOW 37 and SELENIUM														
Ig (%)[a]	4.05	4.55	14.95	50.2	92.5	-1.21X+115.55	0.96	30.41	$313x10^{-4}$	33.34±0.16	1.05±0.01	3.00	53.89±0.78	1.70±0.02
CV	1.77	1.55	2.84	0.85	0.46									

[a] Ig (%) = Per cent growth inhibition/stimulation; negative values show stimulation of growth with respect to control
[b] TU= Toxic Unit in terms of EC_{25}
* X= Per cent dilution of Reactive Yellow 37

There was a significant difference (p<0.05) between the 30-min EC_{25} and EC_{50} values of Reactive Yellow 37 and the dye bath both in the presence and absence of selenium. The comparison of Reactive Yellow 37 with assisting chemicals indicated that there was also a significant difference (p<0.05) between the 30-min EC_{25} and EC_{50} values both in the presence and absence of selenium.

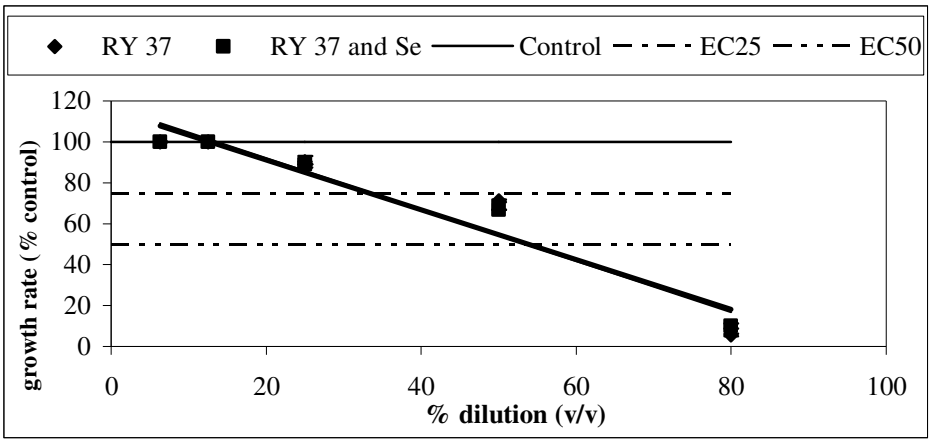


Figure 4.25. Representative dose-response curve of *V. fischeri* exposed to Reactive Yellow 37 in the presence and absence of selenium.

4.14. Comparison of the Toxicity of Synthetic Dye Bath, Assisting Chemicals, Remazol Black 5, Reactive Yellow 37, Reactive Orange 69 to *Vibrio fischeri*

The 30-min EC_{25} levels based on per cent dilutions (v/v) indicated the toxicity sequence was dye bath=Remazol Black 5>assisting chemicals=Reactive Orange 69>Reactive Yellow 37. The sequence did not change with the addition of selenium. When toxicity expressed in terms of EC_{25} (mg L^{-1}) Reactive Yellow 37 was the most toxic with a concentration of 1.05±0.02 mg L^{-1}, followed by Reactive Orange 69 with a concentration of 1.24±0.04 mg L^{-1} and then by Remazol Black 5 with a concentration of 3.40±0.05 mg L^{-1}. In the presence of selenium, the same sequence was observed with concentrations of 1.05±0.01 mg L^{-1}, 1.17±0.01 mg L^{-1} and 3.51±0.04 mg L^{-1} for Reactive Yellow 37, Reactive Orange 69 and Remazol Black 5, respectively.

Among the three investigated hydrolyzed dyes, Reactive Yellow 37 exhibited the highest toxicity with 30-min EC_{50} values of 1.68±0.02 mg L^{-1}. It was followed by Reactive

Orange 69 with a concentration 2.05±0.04 mg L^{-1} and Remazol Black 5 had the lowest toxicity with a concentration of 6.33±0.03 mg L^{-1}. The sequence did not change with the addition of selenium with concentrations 1.70±0.02 mg L^{-1}, 2.02±0.01 mg L^{-1} and 6.51±0.05 mg L^{-1} for Reactive Yellow 37, Reactive Orange 69 and Remazol Black 5 respectively. Selenium did not influence the inhibitory effect of the studied chemicals on *V. fischeri*. TU values of all the tested chemicals indicated that all of them were very toxic to *V. fischeri* both in the presence and absence of selenium.

4.15. Toxicity of Synthetic Dye Bath, Assisting Chemicals, Remazol Black 5, Reactive Yellow 37, Reactive Orange 69 to *Dunaliella tertiolecta* and *Vibrio fischeri*

Although both test organisms –*D. tertiolecta* and *V. fischeri*- are marine organisms, synthetic dye bath, assisting chemicals, Remazol Black 5, Reactive Yellow 37, and Reactive Orange 69 had different toxicity level on these organisms. The 8-d IC_{25} and 30-min EC_{25} values were compared (Figure 4.26) in order to understand the differences between the tolerances of two marine microorganisms to the tested chemicals. Although different notations were used in order to express the toxicity, both of them expressed the concentration of the tested substance that decreases the growth by 25%. Generally IC_{50} or IC_{25} is the most common parameter used in algal toxicity assays and EC_{50} or EC_{25} is the most common parameter used in bioluminescence assays.

Although all of the studied chemicals had stimulatory effect at low doses and inhibitory effect at high doses (hormetic behavior) on the growth of marine alga, *D. tertiolecta*, ToxAlert® 100 (Merck) assay detected only an inhibitory effect on *V. fischeri* for all the tested substances. For all of the studied chemicals, selenium increased the stimulatory effect at low concentration range and decreased the inhibitory effect on *D. tertiolecta*, except assisting chemicals and Reactive Orange 69. The reason for these cases was discussed in relevant sections. On the other hand, selenium did not influence the inhibitory effect of the studied chemicals on marine bacterium *V. fischeri.*

The synthetic dye bath was the most toxic among the tested chemicals to both *D. tertiolecta* and *V. fischeri*. The average 8-d IC_{25} values obtained from the three response parameters of *D. tertiolecta* exposed to the dye bath was 53.80±2.08 % (v/v) whereas the

30-min EC_{25} value of *V. fischeri* was 22.99 ±0.25% (v/v). In the presence of selenium, the average 8-d IC_{25} and 30-min EC_{25} values were 59.30±1.22% (v/v) and 24.04±0.32% (v/v) for *D. tertiolecta* and *V. fischeri*, respectively.

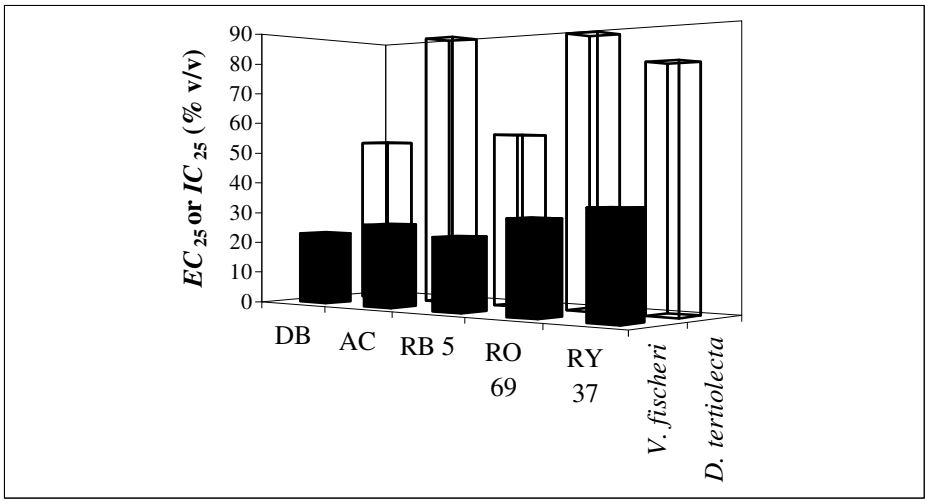
For both of the organisms selenium had no significant change in 8-d IC_{25} and 30-min EC_{25} values of assisting chemicals. An average change from 88.95±1.64% (v/v) to 92.16±3.14% (v/v) was observed in 8-d IC_{25} values of *D. tertiolecta* and a change from 26.52±0.70% (v/v) to 28.63±1.46% (v/v) was observed in 30-min EC_{25} values of *V. fischeri* with the addition of selenium. However, these changes were not significant (p>0.05).

Remazol Black 5 had an inhibitory effect with the average 8-d IC_{25} of 55.84±4.50% (v/v) on the growth of *D. tertiolecta*. In the presence of selenium, a significant increase in the average 8-d IC_{25} value up to 66.89±2.45 % (v/v) was obtained. On the other hand, it had inhibitory effect on *V. fischeri* with the 30-min EC_{25} value of 23.33±0.33 % (v/v). In the presence of selenium, the 30-min EC_{25} value increased to 24.08±0.27 % (v/v) although this increase was not significant (p>0.05). Selenium decreased the inhibitory effect of Remazol Black 5 on marine algae.

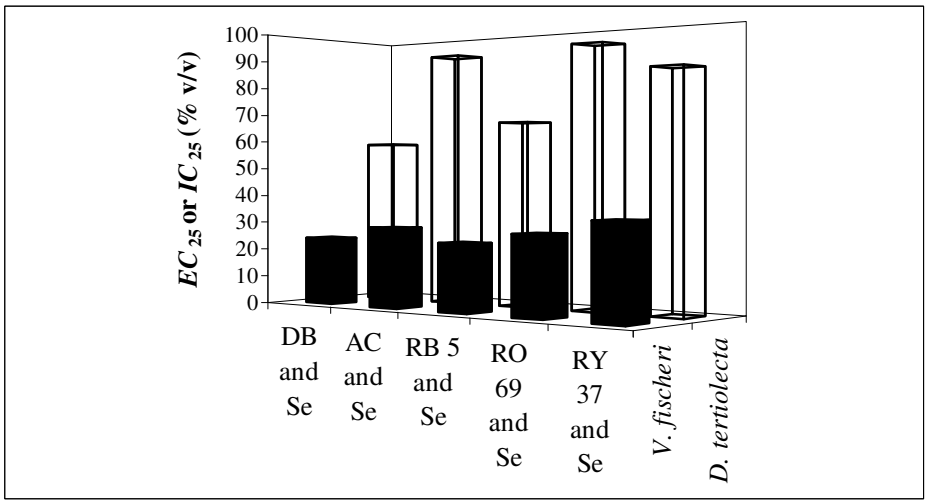
The 30-min EC_{25} (% v/v) values of Reactive Orange 69 were 28.13±0.20% (v/v) and 29.76±0.99 % (v/v) for *V. fischeri* whereas the average 8-d IC_{25} (% v/v) of *D. tertiolecta* were 93.90±4.45% (v/v) and 87.49±5.57% (v/v) with and without selenium, respectively. However, these changes were not significant. In other words, the responses of both organisms to the exposure of Reactive Orange 69-selenium combination were similar considering the influences of selenium.

For *V. fischeri*, the 30-min EC_{25} (% v/v) values of Reactive Yellow 37 was not affected by the addition of selenium, whereas the average 8-d IC_{25} (% v/v) value of Reactive Yellow 37 increased from 77.38±3.74% (v/v) to 84.66±0.74% (v/v) for *D. tertiolecta.* Selenium decreased the inhibitory effect of Reactive Yellow 37 on *D. tertiolecta*, whereas it did not affect the response of *V. fischeri* exposed to the same dye.

Our data show that *D. tertiolecta* was more sensitive to the tested chemicals, since it responded both by stimulation and inhibition, whereas *V. fischeri* responded only by inhibition.



(a)



(b)

Figure 4.26. Comparison of the average 8-d IC_{25} and 30-min EC_{25} of tested chemicals (a) singly (b) in combination with selenium for *D. tertiolecta* and *V. fischeri*.

5. CONCLUSIONS

The individual and combined effects of dye bath, assisting chemicals and three hydrolyzed dyes with selenium on the growth of *D. tertiolecta* and *V. fischeri* were examined using three response parameters namely- OD_{750}, *in vivo* chlorophyll fluorescence and cell number. It could be observed that the parameters based on optical density and *in vivo* chlorophyll fluorescence were more sensitive than those related to cell number for almost all tested chemicals considering the CV values. On the other hand, there was no significant difference (p>0.05) between response parameters.

Algal growth was examined with different forms and concentrations of selenium and under two different light intensities Nutrient like behavior of selenium was observed for the studied forms and concentrations of selenium. Selenite and selenate had different effect on the growth of alga. Selenite in the form of selenious acid and sodium selenite have maximal stimulation effect. When the light intensity lowered by one-half the stimulatory effect of all forms of selenium decreased. Conversely, no significant decrease in the algal growth was observed when algae exposed to dye bath. The results reflect that selenium is linked to energy-dependent processes and toxicity of dye bath to algae is not due to the light absorption of colored dye-bath itself. Selenate did not influence the growth response at low light intensity compared to control.

The results presented here have shown that all the tested chemicals with and without selenium are stimulatory at low doses and inhibitory at high doses (hormetic response). Azo dyes were significantly toxic to *D. tertiolecta* than were the azo dye-selenium combination, except Reactive Orange 69. The regression of dose-response was quadratic for all the tested chemicals except dye-bath and Remazol Black 5. When dose-response curves were represented by quadratic equations two stimulatory doses (SC_{20}) were calculated. Between these two stimulatory doses, the stimulatory effect was much more than 20%. Assisting chemicals had a high stimulatory effect on the growth of algae. This is probably due to the tolerance of *D. tertiolecta* to the wide range of salt concentrations (e.g. from 0.05 to 3.0 mol L^{-1} NaCl). As a consequence of salt tolerance of algae, toxicity of azo dyes was underestimated. When algal growth stimulation occurred, it was at a

relatively low concentration, suggesting that symptom of eutrophication may be expected in the vicinity of the outfall. Stimulation/inhibition of algal growth influences the entire aquatic ecosystem by changing algal populations.

Considering the average 8-d IC_{25}, and SC_{20} expressed as per cent dilution (v/v) the order of toxicity was found as Dye Bath= Remazol Black 5>Reactive Yellow 37>Reactive Orange 69=Assisting chemicals. For algae, selenium increased the stimulatory effect at low concentrations and decreased the inhibitory effect of the tested chemicals except assisting chemicals and Reactive Orange 69. Considering the average 30-min EC_{25} expressed as per cent dilution (v/v) the order of toxicity was found as Dye Bath= Remazol Black 5>Reactive Orange 69=Assisting chemicals>Reactive Yellow 37. Only inhibitory effect was observed for *V. fischeri* exposed to each of the studied chemicals. In contrast to *D. tertiolecta*, addition of selenium did not influence the toxicity of tested substances. Nevertheless, the order of toxicity stayed the same in the presence of selenium for both organisms. When the toxicity parameters (8-d IC_{25}, SC_{20} and 30-min EC_{25}) expressed as concentration basis (mg/L) for the three azo dyes tested the order of toxicity was found as Reactive Yellow 37> Reactive Orange 69> Remazol Black 5. The sequence did not change with the addition of selenium.

The toxicity of Reactive Orange 69 may be due to the toxicity of assisting chemicals since there was no significant difference between 8-d IC_{25}, SC_{20} and 30-min EC_{25} values of assisting chemicals and Reactive Orange 69 both in the presence and absence of selenium. From these results, it is obvious that dyes having sulphatoethylsulphonate as a reactive group (Remazol Black 5 and Reactive Yellow 37) were significantly more toxic to algae than the dye having monofluorodichloropyrimidine as a reactive group (Reactive Orange 69).

The results indicated that *D. tertiolecta* had higher sensitivity than *V. fischeri* to the tested chemicals since all the tested chemicals had both stimulatory and inhibitory effect on the growth of *D. tertiolecta* whereas only an inhibitory response was observed in *V. fischeri*. The other important result was at the same dilution percentage of studied substances, *D. tertiolecta* responded by stimulation whereas *V. fischeri* responded by inhibition.

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APPENDIX A

Calibration Curves of Dye Bath, Remazol Black 5 and Reactive Yellow 37

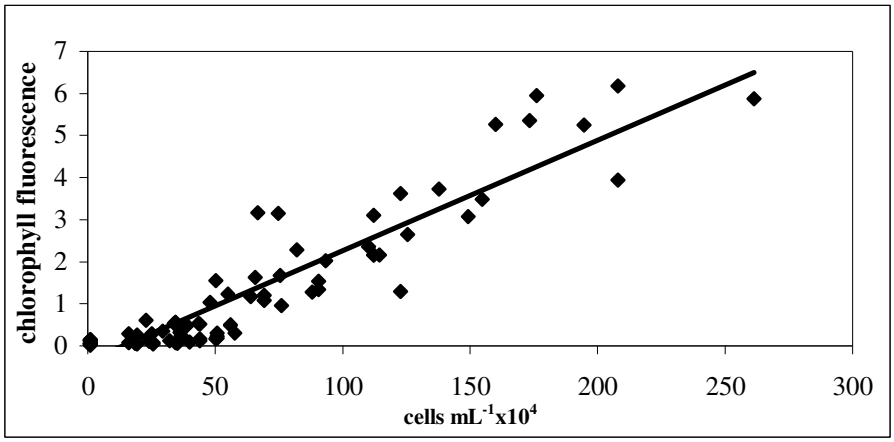

Figure A.1. Plot of chlorophyll fluorescence versus cell mL^{-1} for the dye bath ($y^{**} = 0.0263x^{\dagger\dagger} - 0.3675$ with R^2=0.856).

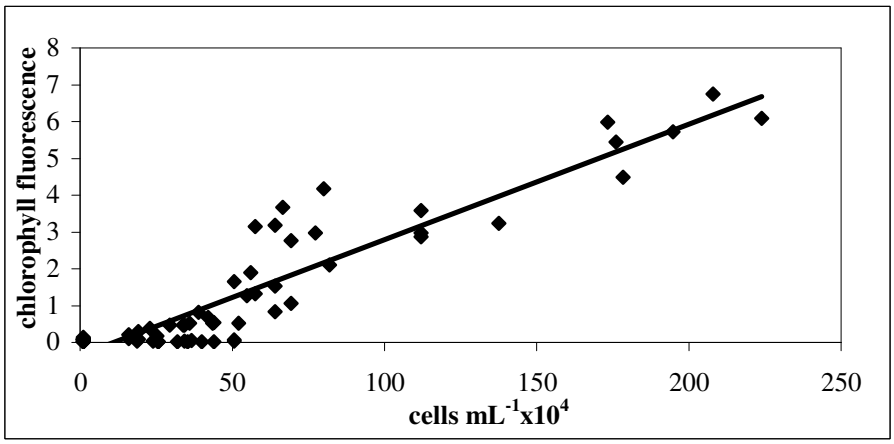

Figure A.2. Plot of chlorophyll fluorescence versus cell mL^{-1} for the dye bath and selenium ($y^{**}=0.0314x^{\dagger\dagger}-0.3498$ with R^2=0.861).

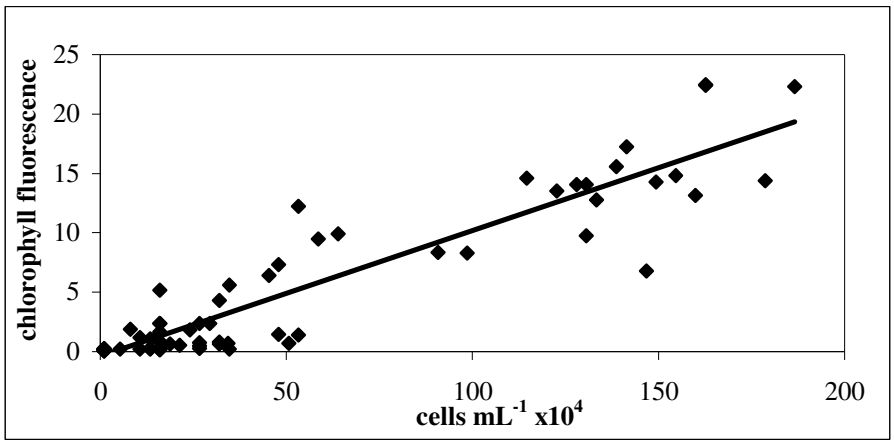

Figure A.3. Plot of chlorophyll fluorescence versus cell mL^{-1} for Remazol Black 5 ($y^{**} = 0.1056x^{\dagger\dagger} - 0.3925$ with $R^2 = 0.858$).

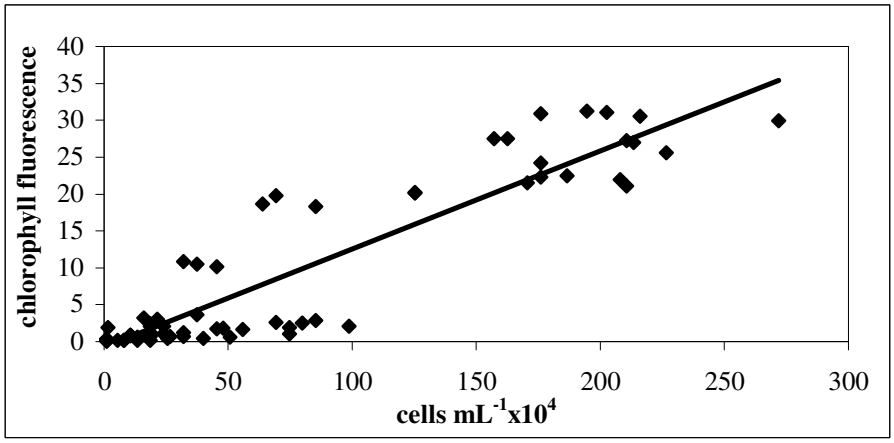

Figure A.4. Plot of chlorophyll fluorescence versus cell mL^{-1} for the Remazol Black 5 and selenium ($y^{**} = 0.133x^{\dagger\dagger} - 0.7335$ with $R^2 = 0.856$).

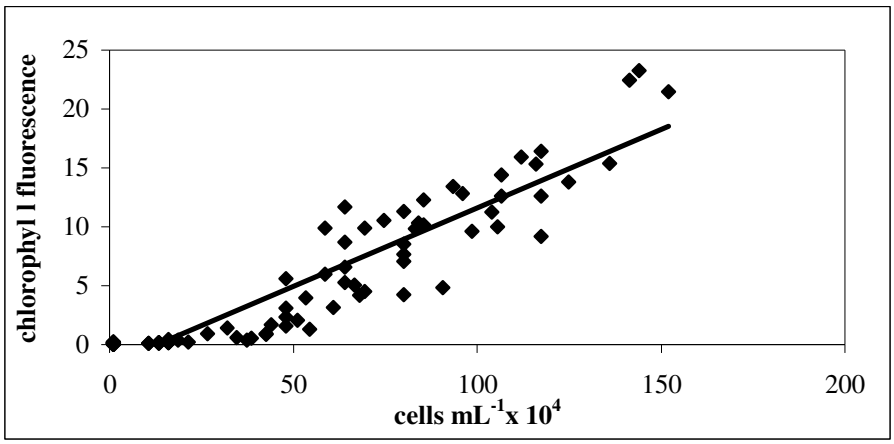

Figure A.5. Plot of chlorophyll fluorescence versus cell mL^{-1} for Reactive Yellow 37 ($y^{**} = 0.1331x^{\dagger\dagger} - 1.7156$ with $R^2 = 0.859$).

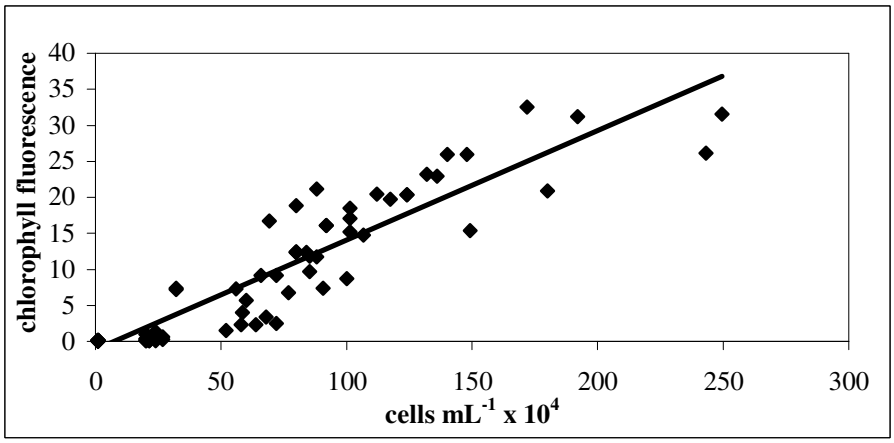

Figure A.6. Plot chlorophyll fluorescence versus cell mL^{-1} for Reactive Yellow 37 and Selenium ($y^{**} = 0.152x^{\dagger\dagger} - 1.1659$ with $R^2 = 0.856$).

** y=Chlorophyll fluorescence
†† x=Cells mL^{-1}

APENDIX B

Output of *IC*p Method for Assisting Chemicals

Conc. ID	1	2	3	4	5
Conc. Tested	0	20	40	80	100
Response 1	0.001	0	0	0	0
Response 2	0	0.202	0.305	0.160	0.003
Response 3	0	0.351	0.390	0.292	0.005
Response 4	0.085	0.355	0.482	0.366	0.007
Response 5	0.095	0.394		0.452	0.009
Response 6	0.113	0.425			0.009
Response 7	0.145				
Response 8	0.179				
Response 9	0.192				
Response 10	0.175				
Response 11	0.220				
Response 12	0.218				
Response 13	0.200				
Response 14	0.229				
Response 15	0.230				
Response 16	0.246				
Response 17	0.244				
Response 18	0.239				

*** Inhibition Concentration Percentage Estimate ***

Toxicant/Effluent: add sp1

Test Start Date: Test Ending Date:

Test Species: *Dunaliella tertiolecta*

Test Duration: 8 days

DATA FILE: Assisting chemicals spectrophotometric

OUTPUT FILE: Assisting chemicals spectrophotometric

Conc. ID	Number Replicates	Concentration Means	Response	Std. Dev.	Pooled Response Means
1	18	0.000	0.156	0.087	0.212
2	6	20.000	0.288	0.161	0.212
3	4	40.000	0.294	0.209	0.212
4	5	80.000	0.254	0.178	0.212
5	6	100.000	0.006	0.004	0.006

The Linear Interpolation Estimate: 85.1334 Entered P Value: 25

Number of Resamplings: 80

The Bootstrap Estimates Mean: 83.9493 Standard Deviation: 2.7421

Original Confidence Limits: Lower: 74.7303 Upper: 85.2148

Expanded Confidence Limits: Lower: 72.6496 Upper: 85.2311

Resampling time in Seconds: 0.00 Random_Seed: 10044807