Introduction

Industrial wastewaters containing dyestuff (textile, leather, food, paper, chemical and petrochemical industries) have a variety of characteristic features [1]. The wastewater composition consists of organic and inorganic compounds, chemicals and dyes [2]. Due to its complex structure, namely aromatic rings that afford highly optical and physicochemical stability, dyes are known to be recalcitrant toxic pollutants with carcinogenic and mutagenic potential [3-4]. Generally, dyestuffs with low biodegradability pass through the wastewater treatment plant and are released into the environment [5]. In addition to traditional treatment methods, advanced treatment methods (membrane-separating technology, adsorption, ozone oxidation, electrochemical oxidation, photo-Fenton process, and UV/H₂O₂) have been needed for dye removal [5-7], although a by-product that may be produced during treatment methods can be more toxic than dyes [8].

Many complex and aromatic dyes due to their incomplete degradation and accumulation display toxic effects in aquatic ecosystems and public health [3]. It is important to evaluate the dyes considering the ecotoxic effects on aquatic organisms [9], although determination of toxic effects takes a longer time than physicochemical parameters. However, the acute toxicity test should be as rapid as possible and result
within seconds. In recent years we have seen that acute toxicity tests with bioluminescent bacteria, a rapid-screening acute toxicity test, can give results within minutes compared to the other acute toxicities [10-13].

In the acute toxicity test carried out with bioluminescent bacteria on colored wastewaters as well as some natural samples, the main thing is that color and turbidity scatter the light and are being seen as toxicity [14]. When the bacteria are exposed to toxic substances, bacterial luciferase is inhibited and light is rapidly reduced [15]. Although humic acid compounds are nontoxic, they raise secondary problems when determining toxicity [16]. If the color originates from turbidity, suspended solids or emulsified matters, it effects the optical emission from the bioluminescent bacteria since it absorbs or reflects the light and causes interaction in the test results. In accordance with ISO 11348-3 [17], it is stated that the sample should be especially filtrated if it is turbid [18]. In addition, if the toxicity is directly associated with the particles, this conducted pretreatment may cause a decrease in toxicity [19].

The Microtox (M500) analyzer is a laboratory-based photometer that measures acute toxicity, the interaction caused by the color and turbidity has been attempted to be prevented through the color correction procedure prepared by the producing company [15]. In the following years, in order to prevent the interaction caused by the color and turbidity, Flash Assay developed by Lappalainen et al. [18] has been accepted as ISO 21338:2010 standard [20]. Unfortunately, the Microtox (M500) analyzer is not a suitable device to carry out flash assay because the device is only intended to measure the luminescence of the bacterial population manually set at the specified times (5, 15 and 30). It cannot measure a continuous luminescence at a given time interval (e.g., 5-10 minutes) and at the same time does not have the mixing feature. It only conducts a mathematical correction equivalent to the absorbance values of the samples at a wavelength of 490 nm for color correction. So, researchers studying with different analyzers have also preferred the different color correction procedure for solving this problem [21-24].

The aim of this study was to examine the change of acute toxicity values of wastewaters containing dyestuff depending on time and color parameters. To this end, toxic/non-toxic synthetic samples were prepared, in particular in accordance with the scale of the color parameter of the textile industry wastewaters. Acute toxicity tests with bioluminescent bacteria were performed by taking into account the effect of the color parameter on toxicity. In this study, an acute toxicity test was conducted with 30 seconds, which is shorter than the time specified in the original Microtox® test protocol. It was named Microtox Basic Test-30 seconds (sBT). Thus, the results of the original Microtox Basic Test (BT), Microtox Color Correction Procedure recommended by the manufacturer (BT-CC), and the Microtox Basic Test-30 seconds (sBT) were compared, taking into account the effect of time and color parameters.

### Material and Methods

The studied dyes were selected according to the color of wastewater containing dyestuff in the project report prepared by Turkish Ministry of Environment and Urbanization and TUBITAK [25]. It used dyes with blue and purple wavelengths and which were included in the scale of the color parameter of the textile industry wastewaters as to the report. Samples were prepared with different dyes and sets were formed. These sets were tested via a Microtox Model 500 analyser and the acute toxicity results were given as EC50 (mg/L). Sets and methods used in the conducted study were explained in the following headings.

#### Preparation of Dye Sets

In order to define the effect of color parameters, sets prepared with different chemicals consisting of food dye, methylene blue, crystal violet and formaldehyde were used. Methylene blue, crystal violet and formaldehyde were prepared by diluting with distilled water at concentrations which their toxicity could be analyzed. The concentration of food dye was prepared according to the instructions for use. According to this, the Brilliant Blue Food Dye (KRK- Brilliant Blue - E133 CAS No: 6104-59-2) is non-toxic. Methylene blue (Merck-Methylene Blue CAS No: 122965-43-9) has the same color but is toxic. In order to reveal the effect of color apart from blue, crystal violet (Merck - Crystal violet CAS No: 548-62-9) was included in this study. Another chemical used in the study is formaldehyde (Merck-Formaldehyde 37% Pure CAS No: 1.040.021.000). In addition, the synthetic toxicity was formed by adding formaldehyde, which is colorless and toxic, to the non-toxic food dye. Besides, the synthetic extreme toxicity was formed by adding formaldehyde, which is colorless and toxic, to the toxic methylene blue and crystal violet. Thus, it was aimed to the toxic effects of the possible interactions depending on the color parameter. Acute toxicity values of four different chemicals, which were defined as Set X, were measured via Microtox. In order to determine the correlation between color and luminescence; the analyses were studied with various concentrations of methylene blue in Set Y. All samples were studied as 2 parallels with control sample; calculations were made over the average of toxicity values. All of the studied dye sets were summarized in Table 1.

#### Change of Acute Toxicity Tests with Bioluminescent Bacteria

The Microtox acute toxicity test is based on luminescence inhibition of the marine gram negative bacteria. Lyophilized Vibrio fischeri (NRLRB-11177) was used as test organism; the test was carried out in standard producer protocol [15] and ISO 11348-3:2007.
Bacterial suspension was added to the sample osmotically arranged with 2% NaCl and to the sample dilutions. After the bacteria were exposed to the sample, photometry was carried out periodically. Acute toxicity tests were carried out in the Microtox Model 500 analyzer. Moreover, formaldehyde (volume rate of 0.08%), known to be colorless and toxic, was preferred as a control chemical in toxicity tests [26].

**Basic Test (45%) (BT)**

The basic test (45%) recommended by the manufacturer for samples that have unknown toxicity and wastewater was applied. 5, 15 and 30 minutes were selected as exposure times and EC \(_{50}\) values were found.

**Basic Test (45%) – Color Correction Procedure (BT-CC)**

After the results of the Basic Test (45%) were obtained, the Microtox Color Correction Procedure (BT-CC) was applied to the test results. The procedure, which is based on a mathematical correction, was applied to determine the effect of color on toxicity. According to this procedure, spectrophotometer readings were made at a wavelength of 490 nm. It recorded the percentage absorbance (ABSx) for each of the concentrations. EC \(_{50}\) values were calculated by Microtox Omni software for all exposure times (5, 15 and 30 minutes) [15].

**Basic Test (45%) – 30 seconds (sBT)**

30 seconds as exposure time, which is shorter than the exposure time specified in the original Microtox test protocol, was studied. The mixture of the sample and the bacteria suspension were mixed in vortex and the bacterial luminescence was measured at the end of 30 seconds. sBT is a toxicity test that has been investigated at 30 seconds.

**Statistical Analysis**

Repeated measure ANOVA Test- SPSS 17.0 was applied in order to determine the statistical significance of the differences in the results of the acute toxicity tests.
the color parameter) increased, the acute toxicity also increased. The toxicity values obtained at concentrations of 25 mg/L and 45 mg/L attracted notice. These methylene blue concentrations were found to be less toxic than their previous methylene blue concentrations.

Comparison of sBT and BT

The luminescence of bacteria was measured before and after exposure to the samples in Set X. The luminescence changes of two tests were given in Fig. 2 in order to understand the difference between the original test (BT) and the rapid test (sBT). We found that the lowest luminescence change was observed in control samples (dye is not added, containing only NaCl) of sBT. When the life stage of the bacteria participates in the account, the longer the exposure time, the more likely the reduction in the luminescence in the control. As the exposure time increased, the difference between the luminescence increased generally. The lowest luminescence change of different dilutions (5.63%, 11.25%, 22.50%, 45.00%) of the samples was determined in the sBT.

Methylene blue concentrations were analyzed for all exposure times. The results were presented in Table 3 among BT results on behalf of the comparison of luminescence at the shortest (30 sec) and the longest (30 min) exposure times. It was found that the lowest luminescence change was observed at 30 seconds (sBT). Since the exposure time was as short as 30 seconds, it could be assumed that no color interaction occurred. When the control samples in Table 3 were examined, the luminescence change at the 30th minute was the highest value (25.945). There were also control samples in which the luminescence change did not happen at 30 sec. In other words, there was no luminescence decrease due to the short exposure time. Also, at different dilutions of the methylene blue concentrations, sBT was seen as the test with the lowest luminescence change. This change could result from both exposure time and color. In this sense, examining the results of...
the control samples was meaningful for the change of luminescence. However, it was not possible to see the effect of the color parameter. While acute toxicity has been calculated by Microtox Omni software, the change in the control sample has been considered. Thus, the error contributed by luminescence decrease caused by the length of exposure time has been minimized. For this reason, the difference of EC$_{50}$ values of methylene blue concentrations has been thought to result from the change in color parameters.

According to the analysis on the correlation between BT and sBT, it was found that there was a linear and strong relationship ($R^2 = 0.954; R^2 = 0.919; R^2 = 0.945; p<0.01$) (Fig. 3). Firstly, it could be said that sBT and BT were in concordance with each other. However, the difference between EC$_{50}$ was also remarkable. This case associated that sBT, which was compatible with BT, had higher sensitivity.

### Comparison of Acute Toxicity Tests with Each Other

The BT-CC has been based on the abs value of the sample read with 490 nm wavelength and the correction on the toxicity result. The luminescence was not change based on the exposure time as sBT. BT-CC was applied to the samples in Sets X and Y. Thereupon, it was compared before and after color correction regardless of set distinction. The correlation between BT and BT-CC was found to be a linear function as presented in Fig. 4. The highest correlation was detected at 15 min ($R^2 = 0.988; p<0.01$); the lowest correlation was found at 30 min ($R^2 = 0.907; p<0.01$). Literature about bioluminescence bacteria predicting toxicity of dyestuffs about color interaction is scant. In the study of Fungaro et al. [27], Microtox color correction was performed in order to prevent the hard blue color observed in the samples. There were other studies [30-31] using Microtox Color Correction, but the toxicity values after and before color correction were not compared. In another study conducted by Tsiridis et al. [32] for the toxicity detection of humic acid, a reading was made at 490 nm wavelength and color correction was performed. At the end of the color correction, it was found that the effect of the color in a given unit on the acute toxicity was at a negligible level and did not change the toxicity result. In another study conducted by Ashworth et al. [33], acute toxicity values of different products before and after color correction were found; no effective change was found on the acute toxicity results of products such as tea, coffee, soybean, etc. It was found that the color correction performed in accordance with the protocol at 490 nm wavelength had no effect. The results obtained

<table>
<thead>
<tr>
<th>Luminescence Change at Exposure Time</th>
<th>$\Delta I_s$</th>
<th>$\Delta I_{10}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (mg/L)</td>
<td>Dilution (%)</td>
<td>0.00</td>
</tr>
<tr>
<td>5</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>10</td>
<td>0.000</td>
<td>3.488</td>
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<tr>
<td>15</td>
<td>0.000</td>
<td>9.392</td>
</tr>
<tr>
<td>25</td>
<td>0.000</td>
<td>15.116</td>
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<tr>
<td>30</td>
<td>2.174</td>
<td>17.021</td>
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<tr>
<td>35</td>
<td>0.000</td>
<td>10.000</td>
</tr>
<tr>
<td>40</td>
<td>3.740</td>
<td>18.868</td>
</tr>
<tr>
<td>45</td>
<td>0.000</td>
<td>26.596</td>
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<tr>
<td>50</td>
<td>5.825</td>
<td>15.385</td>
</tr>
</tbody>
</table>

($\Delta I_s =$ Luminescence change at 30 sec. $\Delta I_{10} =$ Luminescence change at 30 min)
The analysis on the correlation between sBT and BT-CC has indicated a linear function and high correlation with $R^2 = 0.980$ at 5 min. ($p<0.01$) (Fig. 5). This relationship is stronger than between BT and BT-CC at 5 min ($R^2 = 0.976$, $p<0.01$). An alternative assay incorporating color and turbidity correction, referred to as kinetic/flash assay, was first proposed by Lappalainen et al. [18] using a commercial test kit. To this end, sBT was developed on the basis of the rapid time (30 sec.) inspired by Lappalainen et al. [18]. Furthermore, the Color Correction Procedure (BT-CC) recommended by the producer company has been applied in this study. When the results are compared, it can be seen that the tests are compatible with each other. Furthermore, statistically sBT has a significant difference, especially according to the results obtained from BT (5, 15 and 30 min) and BT-CC (30 min). This significant difference also investigates when the luminescence change is examined.

The kinetic bioluminescence inhibition assay-flash-assay (but not the Microtox test format) was conducted [34-36]. In a study conducted by Kurvet et al. [34], it was studied 30 seconds, 15 and 30 minutes as exposure time, it was found that the results varied according to the chemical characterization of the studied sample. In this case, the toxicity value of Cu$^{2+}$ was the highest in 30 minutes, while it was seen that the toxicity value was decreased as the exposure time increased in other samples (3,5 - DCA and aniline). Toxicity was measured with ToxAlert 100 and Flash in the study of Kovats et al. [37]. It was found that the luminescent bacterium test (Microtox/ToxAlert) turbidity and color of the sample might cause a significant decrease of light output due to physical effects, creating the potential for false-positives. It was determined that fundamental differences between the protocols/results of Microtox/ToxAlert and flash assay. The results of this study have overlapped with conclusions from other studies.

There is no study of Microtox acute toxicity test-30 seconds in literature, but there are many studies about the kinetic method, and suitable analyzers have been used. For this reason, no comparison could be made on numerical values. The lack of a kinetic method of measurement of Microtox was a limiting aspect of this study. However, the shortest possible time (30 sec.) has been studied for the present Microtox toxicity test. In order to avoid color interference and to determine the faster feasibility of the test, studies with shorter exposure times should be carried out.

### Statistical Analysis

Repeated Measure ANOVA Test, SPSS 17.0 was used. A significant difference was found between the administered acute toxicity tests ($F = 4.919$; $p<0.01$). The most important data has been that there was significant difference among sBT and BT (all exposure times). Moreover, it was found that there was significant difference among sBT and BT-CC (30 min). Although the results obtained have been numerically close to each other, the difference has been statistically significant.

### Conclusions

The dyes that color our globe have become marketing strategies in the world thanks to their vivid colors as a result of chemical structures of dyes becoming brighter and more resistant to sunlight. However, these properties make them more recalcitrant and more toxic than other pollutants. The presence of these toxic substances in micro-levels in the receiving environment can be enough to change the balance in aquatic ecosystems. For this reason, just as with other parameters, it is important to estimate the toxicity correctly. The use and development of bioluminescence bacteria has increased due to the rapid acute toxicity test. However, more reliable color
and turbidity corrections are needed. Provided that it is preferred sBT for determining acute toxicity of dyestuff wastewater, the results can be obtained more rapid and more sensitively. sBT might be used as a pre-screening test in routine monitoring for determining dyestuff wastewater toxicity.

Acknowledgements

This work was supported by the Research Fund of Istanbul University (BAP YL- Project No. 3153).

Conflict of Interest

The authors declare no conflict of interest.

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