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Comparative Studies on the Acute Toxicities of Auxiliary Chemicals Used in Textile Finishing Industry by Bioluminescence Test and Neutral Red Test

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The textile finishing industry is an important contributor to many national economies. Due to the rapid change in customers' demands, the textile finishing industry is challenged to use high quantities of dyes and auxiliaries. Because of the high cost of conventional animal tests, most of the chemicals involved in the present manufacturing technology have been poorly tested for their toxicity (Grossblatt et al. 1984). The lack of adequate information on toxicity makes human risk assessment and management of the chemicals impossible. Although the amount of auxiliaries consumed in wet dyeing processes is much higher than dyes, much less is known about the auxiliaries in aquatic environment.

Toxicity is a complex biological phenomenon, only biotests can fully distinguish between what is or is not harmful to living organisms. The utility of biotests is well established for environmental hazard assessment of chemicals and chemical products (Baun and Nyholm 1996). A serious limitation is that toxicity is not an unequivical chemical property but a function of both the test organism and the test conditions. No single test or species of living organism shows uniform sensitivity to all chemical compounds. To detect chemicals with specific modes of action, it is therefore necessary to use a battery of biotests with different classes of organisms in order to be able to detect such specific toxicity with a reasonably large probablility. Therefore, the battery of biotests with different sensitivity profiles is often recommended and used to assure adequate evaluation of the ecotoxicological situation (Blaise 1991). Various test methods, criteria and procedures have been developed to assess the toxicity of chemicals. The most advantageous use of the tests is in battery form, so that tests can complement each other.

The bioluminescence test is based on reconstituted freeze-dried (viable) luminescent bacteria and where the reduction of light output of luminescent bacteria after contact with chemicals is used as toxicity endpoint. It is a time-saving and cost-effective test and is widely used as a reproducible and sensitive screening method to determine the acute toxicity of different type of samples (Asami et al. 1996; Bulich 1979; 1982; Kahru et al. 1996). It is often chosen as the first test in a battery of biotest.

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The neutral red (NR) cytotoxicity assay is a cell survival/viability chemosensitivity assay, based on the ability of viable cells to incorporate and bind neutral red, a supravital dye. The standardized test can be used to score cell injury as well as to determine the number of remaining cells after toxic insult. The main application of the NR assay has been the testing of chemicals for their cytotoxic potentials, with decreased cell numbers, either due to cytostasis or death, as the desired endpoint. The assay has been used to determine and screen the relative acute cytotoxocity of a broad spectrum of chemical test agents due to its simplicity, speed, reproducibility, sensitivity and economy (Babich and Borenfreund 1990). The NR assay was designed specifically to meet the needs of environmental, industrial, pharmaceutical and other testing laboratories concerned with acute toxicity testing.

In the present study, bioluminescence test and the neutral red test were used to evaluate the toxicity of 8 auxiliaries used in textile finishing industry to understand the comparativity of relative acute toxicity of chemicals and the comparativity of two biotests. The 50% effective concentration named as EC_{50} and NR_{50} were calculated, respectively. The toxicity rankings of the 8 auxilaiaries obtained by the two bioassays were compared.

MATERIALS AND METHODS

The auxiliary chemicals investigated were provided by a textile dyeing and finishing mill in Ayazaga, Istanbul, Turkey. Water used was purified using a Milli-Q (Milli-pore, Watford, UK) (18.2 MΩcm).

In this study a comparable bacterial toxicity test, available commercially in Germany through Dr. Bruno Lange GmbH, known as the Lumistox test was used. Luminescent bacteria (*Vibrio fischeri*) and all reagents required for the bioluminescence test were obtained commercially from Dr. Bruno Lange GmbH Berlin (Germany). NaCl was extra pure grade purchased from Merck Co., Germany. All chemical samples were prepared in aqueous solution containing 2% NaCl prior to measurement. The bacteria was first reactivated and kept in 15±1°C. The decrease in bioluminescence, indicating a toxic effect by the test substance, was measured after 15 minutes contact with samples, according to DIN 38412 L34 and L341 and the international standard ISO DIS 11348, on DR LANGE LUMIStox 300 photometer (Dr. Bruno Lange GmbH Berlin, Germany). Analyses were carried out at 15±1°C. All samples were tested in duplicate. The inhibition of natural luminescence of photobacteria is regarded as a toxicity endpoint. EC_{50} (sample concentrations required to cause 50% bioluminescence inhibition) were calculated by LUMISsoft Version 3.01.

The H4-IIE rat hepatoma cell line was obtained from the American Type Culture Collection and used as cell model to test the cytotoxicity of chemicals. Neutral red (NR, 3-amino-m-dimethylamino-2-methyl-phenazine hydrochloride) was purchased from Sigma Co., Dulbecco's MEM (DMEM, containing 1.0g/L D-

Glucose, 3.7g/L $NaHCO_3$ and 1.0289g/L N-Acetyl-L-alanyl-L-glutamine, Berlin, Germany) was supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100μg/mL streptomycin and used as cell culture mudium. Stock cultures of H4-II E cells were trypsinized with trypsin-versene mixture (Contains 200 mg versene and 500 mg trypsin per liter in balanced salt solution without Ca^{2+} and Mg^{2+}, Whittaker bioproducts), count and dilute into growth medium to achieve the desired number of cells. 2.5×10^3 cells were seeded into individual wells of a 96-well microtitre tissue culture plate for 24 hours (Coulter counter was used to count the cell munbers). The medium was then replaced with fresh medium, with or without the addition of various concentrations of test agent, using 8-well lane per concentration (samples used in neutral red test were filter sterilized (0.22μm)).

The plate is incubated for three days at 37°C, 5% CO_2 incubator (Heraeus instrument). An 0.4% aqueous stock solution of NR was prepared and an aliquot added to complete DEME medium to a final concentration of 50μg/mL. 1M HEPES buffer (Sigma Co.) was added at the same time to complete the medium to a final concentration of 20μM. The medium was then removed and replaced with NR and HEPES-containing medium and, after incubation for an additional 2-4 hrs to allow for uptake of the dye, the cells are rapidly fixed with a solution of 1% formaldehyde:1% $CaCl_2$ and then washed twice with PBS. The dye is then extracted from the intact, viable, i. e., those cells surviving the exposure to the test agent, with a solution of 1% acetic acid:50%ethanol. The plate was agitated on a microtiter plate shaker for 10-20 min at room temperature. The absorbance of solubilized dye which was found to be linear with the number of surviving, viable cells (Babich and Borenfreund 1990) was then determined using a Microplate reader (SLT LABINSTRUMENTS, Easy Reader, EAR 340 AT, Australia) at 550 nm. The readings in 8 replication were averaged and the results expressed as absorbance observed as % of control cultures. Midpoint cytotoxicity, NR50 was calculated from the concnetration-response cytotoxicity curve to a value representing 50% of the absorbance observed with control cells. The first part of the assay consisted of the screening of a broad range of concentration of the test agents followed by a narrow range.

RESULTS AND DISCUSSION

The ranking of chemicals according to one toxicity potency is of limited value unless such ranking can be correlated with other toxicity data. In this study, two bioassays were comparatively employed to assess the toxicities of 8 auxiliaries used in textile finishing industry. Luminescent bacteria are very suitable test organisms for range finding and/or screening tests, mainly due to the very convenient toxicity endpoint, short exposure time and cost-efficiency of a test. Figure 1 presents the relationship between auxiliaries concentration and luminescence inhibition. EC_{50} (sample concentrations required to cause 50% bioluminescence inhibition) were calculated by LUMISsoft Version 3.01 and listed in Table 1.

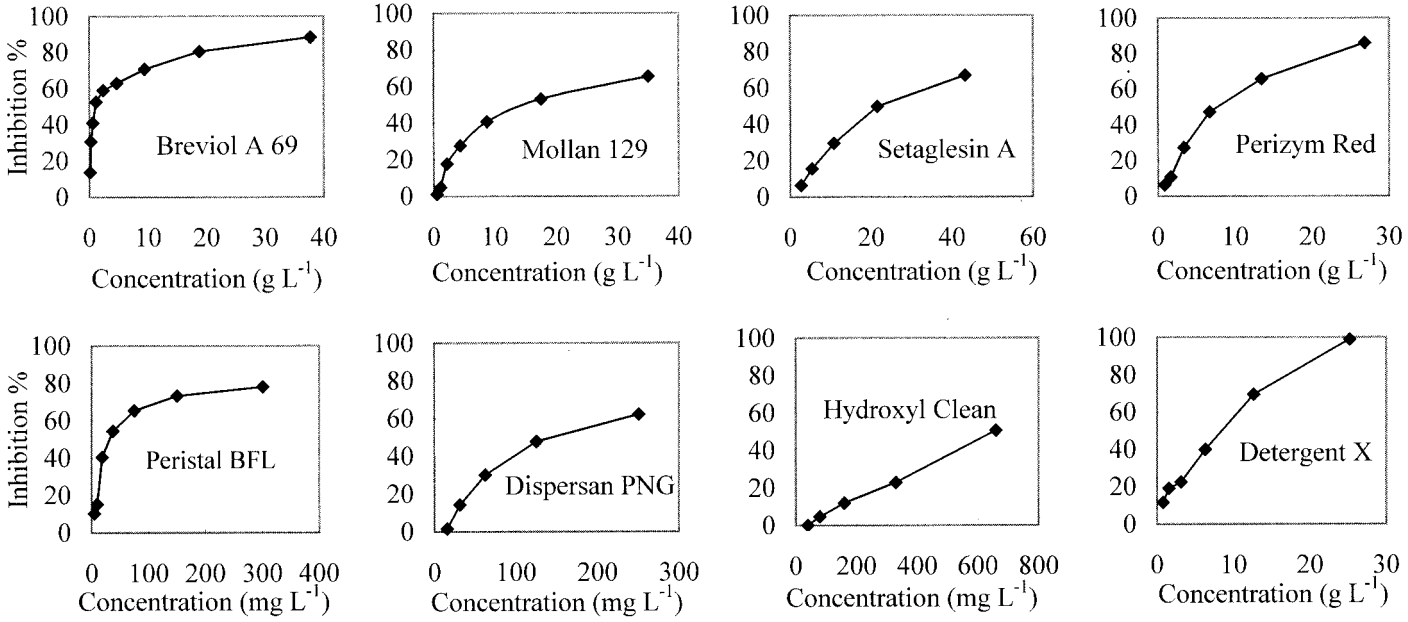

Figure 1. The luminescence inhibition to photobacteria (*Vibrio fischeri*) of auxilary chemicals used in textile industry

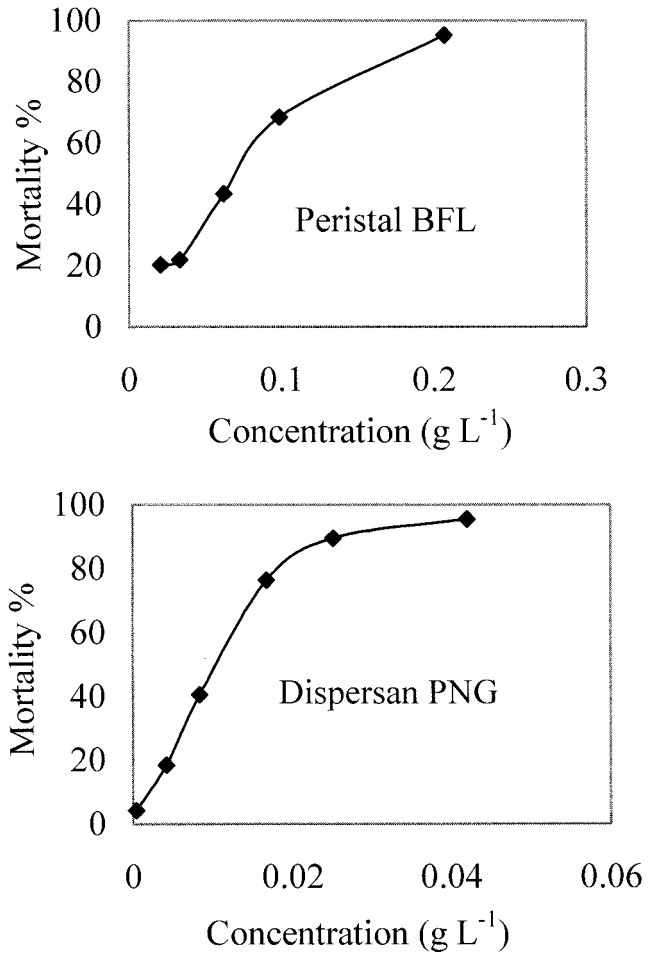

Figure 2. Relationship between the mortality (%) of rat hepatoma cell and concentrations of auxiliary chemicals used in textile industry

The data for construction of the concentration-response cytotoxicity curve as shown in Figure 2 taking two tested auxiliaries as example, generated with the NR assay procedure are used to evaluate statistically the mid-point cytotoxicity value, or NR50. The NR_{50} value is tabulated also.

It has been demonstrated, from Figures 1 and 2, a positive correlation, but not a linear, between the concentration of auxiliaries and an inhibition to *Vibrio fischeri* and the mortality of rat hepatoma cell, respectively.

Table 1. EC_{50} and NR_{50} values (g L^{-1}) for auxiliaries used in textile industry by Bioluminescence test and Neutral Red test

Test agents	Comment	EC_{50}	NR_{50}
Setaglesin A	dissolvent	22.91	0.73
Mollan 129	ionacceptor	15.03	1.55
Perizym Red	antiperoxide	7.48	3.51
Breviol A69	dispersent	1.57	0.70
Levelgal FTS	anti-creasing	0.86	0.020
Hydroxy Clean	soap	0.67	5.24
Dispersan PNG	dispersent	0.15	0.011
Peristal BFL	wetting agent	0.045	0.069

It is obvious that one bioassay cannot provide the equivalent results of another bioassay. The EC_{50} data for 8 auxiliaries covered over almost 4 orders of magnitude. The most toxic auxiliary tested to *Vibrio fischeri* was Peristal BFL, a wetting agent, with the 15-min EC_{50} of 0.045 gL^{-1}, whereas Setaglesin A could be listed as the auxiliary with the lowest toxic capacity with a 15-min EC_{50} of 22.91gL^{-1}. The observed acute toxicity ranking to luminescent bacteria *Vibrio fischeri* was: Peristal BFL > Dispersan PNG > Hydroxy Clean > Levelgal FTS > Breviol A69 > Perizym Red > Mollan 129 > Setaglesin A. The NR_{50} data for 8 auxiliaries covered over 3 orders of magnitude. The most toxic chemical tested to rat hepatoma cell was Dispersan PNG with NR_{50} of 0.011 gL^{-1}; and the lowest toxic one was Hydroxy Clean, a soap with NR_{50} of 5.24 gL^{-1}. The observed acute cytotoxicity ranking to rat heptoma cell was: Dispersan PNG > Levelgal FTS > Peristal BFL > Breviol A69 > Setaglesin A > Mollan 129 > Perizym Red > Hydroxy Clean, which was different from the ranking obtained from bioluminescence test.

NR_{50} and EC_{50} in the tests were used to express the toxicity data in a manner that allows for the ranking of the test agents according to their potencies. A comparison between acute toxicity results obtained by luminescent bacteria and Neutral Red test indicated that a difference in the sensitivity of the organism and cell to toxic substances. Neutral red test was more sensitive than bioluminescence test. The NR_{50} from Neutral red test using rat hepatoma cell were obviously different from those obtained with bioluminescent test using *Vibrio fischeri*. This is not surprisig since different species may be expected to respond quite differnetly to the same chemicals as suggested by Ahlf et al. (1989) that the comparability of bioassays is limited by the different test sample sensitivities of test organisms. This is the reason that a battery of biotests is necessary to assess the hazard effcets of chemicals and environmental samples.

It must be emphasized that toxicity is species specific and chemical specific. Variations in the sensitivity of organisms to toxicants can be quite large. No single species can be proven unquestionanly superior to all others. The small battery of bioassays is too restricteed to fully cover the potential toxic impact of chemicals on all types of biota. Using several species simultaneously in multispecies bioassays, the likelihood of making an erroneous conclusion may be reduced.

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