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Abstract

Textile and tannery wastewaters are complex mixtures of toxic pollutants and only a battery of ecotoxicity tests can assess their potential environmental impact and the actual effectiveness of alternative treatments. In this work the toxicity of four simulated textile and tannery wastewaters was evaluated by means of a battery of seven bioassays, using organisms that belong to different trophic levels. Moreover, since the outputs of the bioassay battery were quite difficult to compare, a novel synthetic index for environmental risk assessment was applied to the outputs of the test battery. All four simulated wastewaters were very toxic but they showed no mutagenic effect. The alga *Pseudokirchneriella subcapitata* was the most sensitive organism. In addition, the use of two mathematical models pointed out the interaction effect between dyes and salts, which resulted in a synergistic effect of wastewater toxicity.

Keywords

Textile wastewaters; Dyes; Salts; Ecotoxicity; Synthetic index; Risk assessment

1. Introduction

Water pollution is becoming a very worrisome phenomenon. In particular, textile and tannery industries contribute enormously to water deterioration by discharging in the environment large volumes of wastewaters and are regarded as one of the most polluting among all industrial sectors (Savin and Butnaru, 2008 and Soupilas et al., 2008). These wastewaters are complex mixtures of toxic pollutants, often in high concentrations, such as dyes and pigments, salts, heavy metals, biocides, carriers, surfactants and various other organic and inorganic components that can rise complexation phenomena (Eremektar et al., 2007, Sharma et al., 2007 and Verma, 2008).

Their toxicity is one of the major causes of the failure of biological treatment plants, resulting in non-compliance with discharge permit limits (Vijayaraghavan and Ramanujam, 1999, Aguayo et

al., 2004 and Alinsafi et al., 2006). Thus, textile and tannery wastewaters can lead to very serious environmental consequences, especially to aquatic ecosystems. The research on wastewaters toxicity carried out so far shows how the action of toxic pollutants occurs at different levels of the food chain, from producers (i.e. algae and plants) to secondary consumers (i.e. crustaceans and fishes) (Sharma et al., 2007 and Gómez et al., 2008).

Moreover, the discharge of textile and tannery effluents in small streams, from which water is taken for irrigation, causes deleterious effects on soil, such as deflocculation of soil particles, an increase in the N, P, K and Na levels and in the pH too. Salinisation and alkalisation of ground water due to these effluents are also reported (Chhonkar et al., 2000).

In many countries, scientists and legislators have underlined the importance to evaluate wastewaters ecotoxicity. Actually, chemical procedures alone cannot provide sufficient information about potential harmful effects of pollutants on the environment and are unable to predict the effect on organisms in the ecosystem (Daniel et al., 2004, Latif and Licek, 2004 and Sponza, 2006).

So far, the attention has been focused primarily on dyes toxicity, since colour is the most noticeable aspect of textile and tannery wastewaters. Single dye molecules have been tested, often resulting very toxic or genotoxic (Moawad et al., 2003, Birhanli and Ozmen, 2005, Dogan et al., 2005, Chang et al., 2007, Işik and Sponza, 2007, Vajnhandl and Le Marechal, 2007 and Abd El-Rahim et al., 2008).

However, the wastewaters toxicity and its impact on the receiving environment cannot be reliably predicted from the toxicity of single constituents; actually, this approach does not detect the combined effects of all chemical species and their potential synergistic effects. Thereby, the risk related to two or more species simultaneously present in wastewaters is not always equal to the sum of their respective toxicities (Daniel et al., 2004). The total impact of pollutants can be only detected by testing samples of industrial wastewaters as a whole (EC Directive 2008/1 IPPC).

Ecotoxicity analyses of wastewaters before their introduction into the biodegradation process can be useful to predict their impact on the activated sludge and the necessity for additional treatments. Moreover, the continuous monitoring of the potential toxic properties of effluents, prior to their discharge in the environment, is fundamental to assess the effectiveness of wastewater treatment plants (Cordova Rosa et al., 2001 and Soupilas et al., 2008).

A critical aspect of bioassays is the unrealistic representation of an ecosystem by means of a single organism. In fact, it is difficult to predict the impact on other species because they have different sensitivities to the same pollutant. Therefore, the application of a battery of tests with organisms belonging to different trophic levels is recommended. However, the obtained data may be difficult to compare, since different toxicological principles and endpoints are often used and results can be discordant (Latif and Licek, 2004, Novotný et al., 2006, Sponza, 2006, Soupilas et al., 2008 and Grinevicius et al., 2009). Thus, there is a clear need to find indices that summarise multiple toxicity measurements into a single numerical value. Some simplified composite indices have already been proposed; however, none of the classification methods have found general acceptance at the international level, so they are still under development or undergoing revision (Costan et al., 1993, Persoone et al., 2003 and Canna-Michaelidou and Christodoulidou, 2008).

This work aims to evaluate the toxicity of four simulated textile and tannery wastewaters by means of a battery of ecotoxicity tests, in order to select the most sensitive organisms. Moreover, a novel synthetic index for the environmental risk assessment, developed by UNICHIM Water Quality

Commission (UNICHIM, 2008), was applied to the outputs of the battery of tests. Finally, we determined the toxicity due to dyes and salts in wastewaters and their interaction.

2. Materials and methods

2.1. Dyes and preparation of simulated wastewaters

Four wastewater models (W1–W4), designed to simulate effluents produced during cotton, wool and leather dyeing processes were developed by the industrial partners of the EC FP6 Project SOPHIED (NMP2-CT-2004-505899). They were prepared using mixtures of industrial dyes purchased from Town End (Leeds, UK) plc. These wastewater models simulate the industrial ones also for the presence of different salts, often in high concentrations, and for the pH values. Their composition is reported in Table 1. Details of their preparation were previously reported by Prigione et al. (2008).

Table 1.

Wastewaters composition and pH.

Wastewater	Dyes and salts	Concentration (mg l^{-1})	pH
Acid bath for wool (W1)	Abu62	100	5
	AY49	100	
	AR266	100	
	Na_2SO_4	2000	
Acid bath for leather (W2)	ABk210	100	5
	ABk194	100	
	AY194	100	
Reactive bath for cotton (W3)	Rbu222	1250	10
	RR195	1250	
	RY145	1250	
	Rbk5	1250	
	Na_2SO_4	70000	
Direct bath for cotton (W4)	DrBu71	1000	9
	DrR80	1000	
	DrY	1000	
	NaCl	5000	

In order to assess the toxicity due to dyes and salts in W3, this wastewater was prepared with salts concentration from 0 to 70 g l^{-1} and without dyes too.

2.2. Bacteria test

Luminescent bacteria tests were performed according to the standard UNI EN ISO 11348-3, using the Microtox® toxicity system (Microtox Model 500; Microbics Corp., USA) with an automatic record of the luminescence. Freeze-dried marine luminescent bacteria (*Vibrio fischeri* strain NRRL B-11177) were bought at Ramcon A/S (Birkeroed, Denmark). All dose–response curves consisted of eight dilutions, each in duplicate and with four controls. The luminescence intensity in all cuvettes was measured before the addition of the wastewaters and after 5, 15 and 30 min. Automatic colour correction was performed when necessary. The inhibitory effect was calculated according to the principle described in the standard, with the computer program for Microtox Acute Toxicity Test (Azur Environmental Ltd., UK).

2.3. Algae test

The algae tests were performed according to the standard UNI EN ISO 8692:2005 using a monospecies culture of *Pseudokirchneriella subcapitata* (Korshikov) Hindak (ex *Selenastrum capricornutum* Prinz.) originating from Agenzia Regionale per la Protezione dell'Ambiente (ARPA Piemonte, Grugliasco, TO, Italy). Each dose–response curve consisted of 12 dilutions in triplicate and the control was performed with six repetitions.

After 48 h of incubation, the cells concentration was measured with a Coulter Counter (Beckman Coulter Z2) calibrated for 3–5 μm size cells. The inhibition percentage was plotted on dose-effect charts and, when possible, the EC50, its confidence limits (p=0.05) and toxic units ($100/EC_{50}$) were estimated using standard procedures.

2.4. Lemna test

The tests with the aquatic plant *Lemna minor* L. was performed according to the standard ISO SO/WD 20079. The test was performed in triplicate, in 250 ml glass beakers, with a working volume of 150 ml and with a sample dilution of 1:10. Distilled water was used as control in the test.

Ten fronds of *L. minor* (two or three fronds per colony) of similar size were used as inoculum. The test was carried out in a climatic exposure test cabinet, calibrated at 24±2 °C, with fluorescent tubes on the top, which provided continuous lighting (light intensity 100 $\mu E\ s^{-1}\ m^{-2}$) for seven days. At the end of the experiment, fronds number and plant dry weight were used to calculate the growth inhibition, using standard procedures.

2.5. Phytotoxicity tests

Two dicotyledonous plants, *Cucumis sativus* L. and *Lepidium sativum* L., were used for phytotoxicity tests, according to the standard method UNICHIM N. 1651 (2003). The seeds (90% germination warranty) were purchased from Ingegnoli S.p.A. (Milano).

All dose–response curves consisted of eight dilutions, each in four replicates. For both species, ten seeds were placed in 9 cm Petri dishes, containing 5 ml of sample and a paper filter (Whatman No.1). The control was performed in four replicates, using distilled water. The seeds were incubated for 72 h in the dark at 25 °C. At the end of the test, the germinated seeds were counted and radical extension was measured using standard procedures. The results were plotted on a dose-effect chart

and, when possible, the EC_{50}, its confidence limits (p=0.05) and toxic units were estimated using standard procedures.

2.6. *Daphnia magna* test

The test was performed according to the UNI EN ISO 6341:99. The used strain of *Daphnia magna* Straus (Cladocera, Crustacea) has been cultured at ARPA Piemonte as specified by the standard method. To test the *Daphnia* sensitivity, acute toxicity tests with $K_2Cr_2O_7$ were performed at regular intervals.

All dose–response curves consisted of at least six dilutions, each in four replicates of five animals. The test volume was 5 ml and 6-well plates were used. Control was performed with six repetitions. Immobile animals were counted after 24 h and the response was given as percent mobile animals with respect to the control and the results were plotted on a logarithmic-probability chart. When possible, the EC_{50}, its confidence limits and toxic units (p=0.05) were estimated using standard procedures (Litchfield and Wilcoxon simplified method).

2.7. Ames test

The strains TA98 and TA100 of the bacterium *Salmonella typhimurium* were used to detect frameshift and base-pair substitution mutations. The strains were obtained from IMTECH (Chandigarh) and they were tested to confirm their genetic features according to Maron and Ames (1983). The test was performed using standard pre-incubation procedure (ISO 16240/2005) with and without the S9 mix metabolic activation, in order to observe whether the parent molecule's metabolites formed in the hepatic system are positive or not. For each plate, 0.5 ml of 10% S9 mix and 0.1 ml of sample were used. After 48 h incubation at 37 °C the count of bacterial colonies was performed.

2.8. Statistical analysis

Statistically significant differences among inhibition effects were analysed using ANOVA (p<0.05). Statistically significant differences between dose-effect regression lines were analysed using T test (p<0.05 for line slope, p<0.001 for translation), for all the possible pairs.

2.9. Synthetic index and ecotoxicological risk assessment

The synthetic index was developed by UNICHIM (2008) as a modification of the model proposed by Hartwell (1997). The method allows the comparison of the outputs of batteries, in which the same tests are performed, by calculating the toxicity score of the battery (BTS) as the mean of the relative toxicity of each test (RTendpoint). These last values are expressed as a percentage, as follows:

$$RT_{endpoint} = 100 - 100\frac{[\log(CEC_x)RS]_{max} - [\log(CEC_x)RS]_{endpoint}}{[\log(CEC_x)RS]_{max}}$$

where C is a statistical corrective (C=2 if the EC_x is higher than 100%; C=1 if the EC_x and its 95% confidence limits are lower than 100%); S is a score depending on the considered endpoint (mortality=8; bioluminescence=7; development=6; reproduction=5; growth=4; genotoxicity=3; mutagenicity=2, behaviour=1); R is the rank of toxic concentrations and it is assigned from the lowest concentration to the highest one.

Moreover, the risk score of the battery (BRS), expressed as a percentage, has been calculated according the following formula:

$$BRS = \frac{\text{mean of the } RT_{endpoint}[(\sum RT_{enpoint} + \text{consistence})/\sqrt{N}]}{(\sum RT_{enpoint})/\sqrt{N}}$$

where *N* is the number of total endpoints; consistence is the half of total endpoints to which non-significant endpoints are subtracted. The consistency indicates the agreement rate among different endpoints: it is high (positive value) if all tests give results in agreement with each others; on the contrary, it is low (negative value) if tests are discordant. The role of the consistence is to increase or decrease the risk score, according to the number of significant endpoints.

Moreover, the relevance of the battery has been calculated according the following formula:

$$\text{Total relevance } \% = 100(S_{mean})/8$$

The toxicity score of the battery is converted in a scale based on the expert judgment as follows: BTS≤5%=negligible toxicity; 5%<BTS≤20%, consistence ≤0=moderate toxicity; 5%<BTS≤20%, consistence >0=high toxicity; BTS>20%=very high toxicity; BTS>50%=extremely high toxicity. As well, the risk score is converted in the following scale: BRS≤5%=negligible risk; 5%<BRS<10%=moderate risk; 5%<BRS<20%=high risk; BRS>20%=very high risk; BRS>50%=extremely high risk.

Eventually, this method allows the calculation of the EC_x and its confidence limits for the battery as follows:

$$\text{Battery } EC_x = 10^{(\text{mean}(\log EC_x))}$$

$$\text{Battery } L_{low} = 10^{(\text{mean}(\log \text{of the lower confidence limit}))}$$

$$\text{Battery } L_{up} = 10^{(\text{mean (log of the upper confidence limit)})}$$

Only the tests showing a significant toxicity (i.e. calculable EC_x) contribute to the estimation of the battery EC_x.

2.10. Toxicity interaction between dyes and salt

The toxicity interaction between reactive dyes and salt (Na_2SO_4) in W3 was analysed by means of two models for the toxicity prediction of mixtures: the concentration addition (CA) and the independent action (IA) models. The percent deviation of the predicted effect from the measured effect was calculated. A positive deviation indicates synergism, whereas a negative deviation indicates antagonism. Negligible deviation indicates additive behaviour without interactions. The classification based on the magnitude of percent deviation proposed by Parvez et al. (2009) was applied. This approach allows not only to characterise mixtures as synergistic/antagonistic, but also to classify them on the basis of their degree of synergism/antagonism.

3. Results

3.1. Ecotoxicity and mutagenicity tests

Results of ecotoxicity tests are reported in Table 2. When possible, wastewaters toxicity was expressed as effective concentration (EC_{50}) and toxic unit (TU). *D. magna* was the most sensitive organism towards W1, whereas *P. subcapitata* was the most sensitive organism towards W2, W3 and W4. While the algal test allowed calculating the EC_{50} for each wastewater, the *D. magna* test was not sensible to W2, since no crustacean was immobilised at the 100% dose.

Table 2. Wastewater models toxicity: EC_{50} (% v/v), 95% confidence limits and toxic units (TU) resulted from the different ecotoxicity tests.

	W1				**W2**				**W3**				**W4**			
	EC_{50}	**L_{low}**	**L_{up}**	**TU**	**EC_{50}**	**L_{low}**	**L_{up}**	**TU**	**EC_{50}**	**L_{low}**	**L_{up}**	**TU**	**EC_{50}**	**L_{low}**	**L_{up}**	**TU**
Microtox bioluminescence																
5’	46.2	38.5	55.6	2.2	>100	–	–	<1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
15’	38.8	29.4	50.0	2.6												
30’	36.4	29.4	45.5	2.8												
P. subcapitata growth	18.8	16.6	21.1	5.3	17.7	10.8	24.6	5.6	2.2	1.9	2.5	45.5	31.7	23.6	39.7	3.2
L. minor fronds development	>100	–	–	<1	>100	–	–	<1	>100	–	–	<1	>100	–	–	<1
L. minor biomass development	>100	–	–	<1	>100	–	–	<1	>100	–	–	<1	>100	–	–	<1
L. sativum root development	17.2	–	–	5.8	>100	–	–	<1	2.8	–	–	35.7	>100	–	–	<1
C. sativus root development	37.3	–	–	2.7	>100	–	–	<1	4.4	–	–	22.7	>100	–	–	<1
D. magna immobilisation	12.6	11.1	14.4	7.9	>100	–	–	<1	7.2	5.9	8.8	13.9	74.4	71.5	77.4	1.3

n.d. = EC_{50} not determined because of colour interference.

Microtox® assessed the EC_{50} only for W1, which ranged between 36% and 46%, according to the exposure time. The EC_{50} for W2 was always higher than 100%, but EC_{30} was 32%, 18% and 25% for 5, 15 and 30 min exposition, respectively. The bacterial test was not able to give nor the EC_{50}

nor the EC_{30} for both W3 and W4, because of their high colour intensity that interferes with light emission measurements.

According to the phytotoxicity tests with *L. sativum* and *C. sativus*, the EC_{50} of W1 were 17% and 37%, respectively, while the EC_{50} of W3 were 3% and 4%, respectively. These tests did not allow to calculate the EC_{50} towards W4, since at the 100% dose the inhibition effect was 37% and 47% for *L. sativum* and *C. sativus*, respectively. On the contrary, W2 caused biostimulation of the root growth for both the plants (at the 100% dose, the corresponding inhibition effects were −11% and −2%, respectively).

L. minor resulted in the less sensitive organism and the toxic effects (up to 49% fronds number and up to 42% for the biomass development) never reached the EC_{50} value.

The Ames test did not find any linear relationship between toxicants concentration and mutagenic effect; actually there was no increase of the revertants number compared to the negative control.

3.2. Synthetic index and ecotoxicological risk assessment

Since the adopted mathematical model is based on the assumption that batteries are comparable only if they are the same for each wastewater, the *V. fischeri* test was not considered for the calculation of the synthetic index and the risk assessment, because of the missing results about W3 and W4.

The relative toxicity of W1 ranged from 0% (according to the *L. minor* and Ames tests) to 100% (according to the *L. sativum* test). The relative toxicity of W2 was 0% according to all tests, except for the *P. subcapitata*, whose relative toxicity was 100%. The relative toxicity of W3 ranged from 0%, according to the *L. minor* and Ames tests, and 100%, according to the *P. subcapitata* test. The relative toxicity of W4 was 35% according to the *P. subcapitata* test and 30% according to the *D. magna* test; the other tests showed a null toxicity (Table 3).

Table 3. Toxicity of wastewater models resulting from the outputs of the synthetic index applied to the battery: EC50 (% v/v), 95% and confidence limits of the battery.

	W1	**W2**	**W3**	**W4**
Battery EC_{50}	19.7%	17.7%	3.7%	30.6%
L_{low}	13.6%	10.8%	3.4%	28.2%
L_{up}	17.5%	24.6%	4.7%	33.4%

Both the toxicity score (BTS) and the risk score (BRS) of the battery were calculated (Fig. 1). The highest toxicity score was achieved by W3 (38.8%), followed by W1 (31.0%), W2 (14.3%) and W4 (9.3%). The corresponding scale attributes toxicity value from high to very high to all wastewater models. The highest total relevance of the battery was obtained by W3 (65.7%), whereas that of the other wastewaters was 40%.

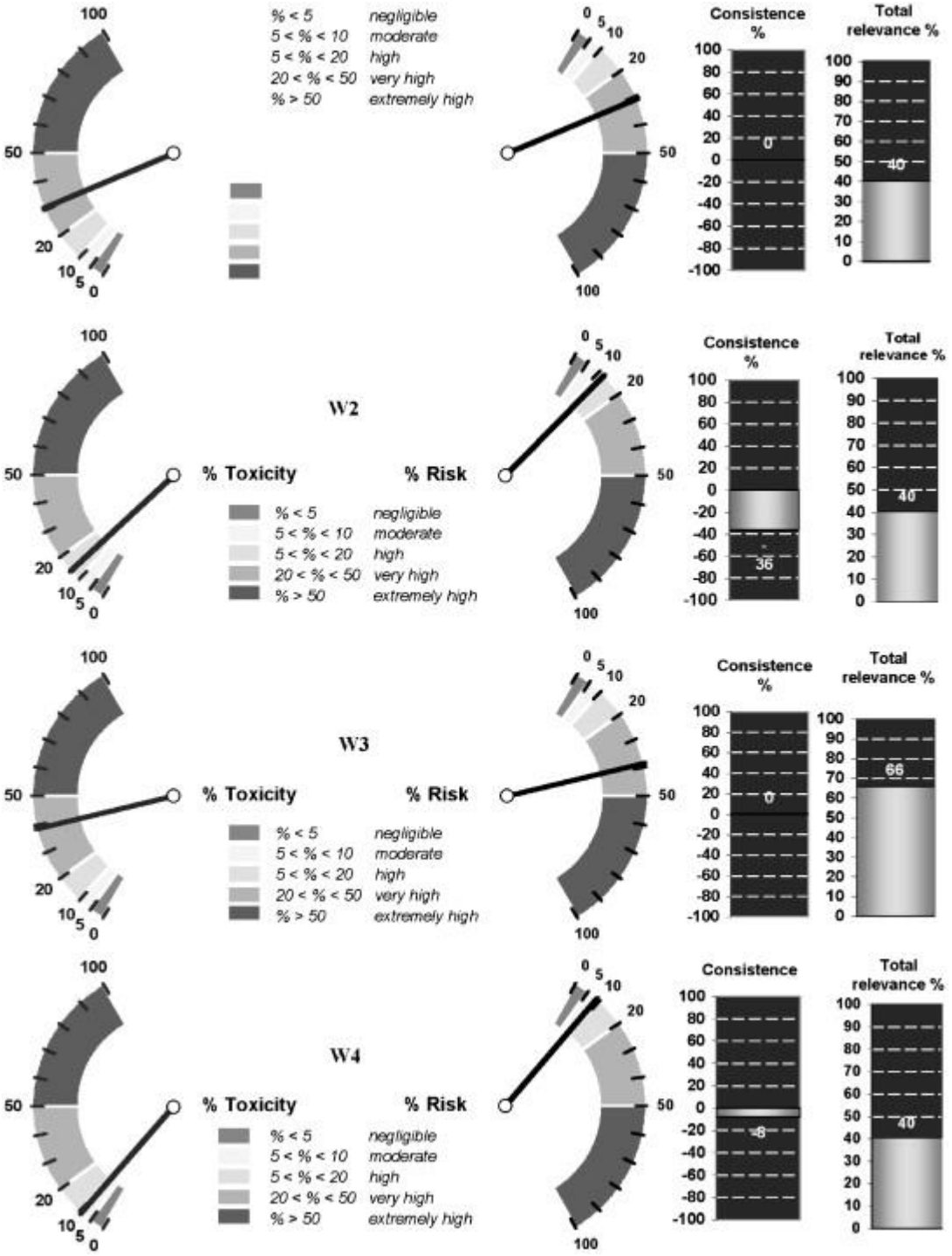

Fig. 1.

Toxicity and risk scores, consistence and total relevance of the battey for W1, W2, W3 and W4, according to the synthetic index. BTS=toxicity score of the battery and BRS=risk score of the battery.

The highest consistency of the battery was obtained by W1 and W3 (0.3%), followed by W4 (−7.9%) and W2 (−36.4%). Consequently, the highest risk score was achieved by W3 (38.8%), followed by W1 (14.3%), W2 (12.1%) and W4 (8.8%). The corresponding scale attributes a high to very high risk to all wastewaters.

Finally, the EC_{50} of the battery for each wastewater are reported in Table 3. W3 showed the lowest EC_{50} (3.7%), whereas W4 showed the highest EC_{50} (30.6%).

3.3. Salt contribution to toxicity and combined effect with dyes

P. subcapitata was selected as the most sensitive organism for this experiment focused on the evaluation of the salt and dyes contribution to the toxicity of W3.

The algal growth inhibition percentages caused by W3 without dyes or with different salt concentrations are reported in Fig. 2. The sample without dyes showed the lowest toxicity (EC_{50} 12.5%), followed by the sample without salt (EC_{50} 4.1%). The other samples showed EC_{50} ranging from 2.8% to 1.8%.

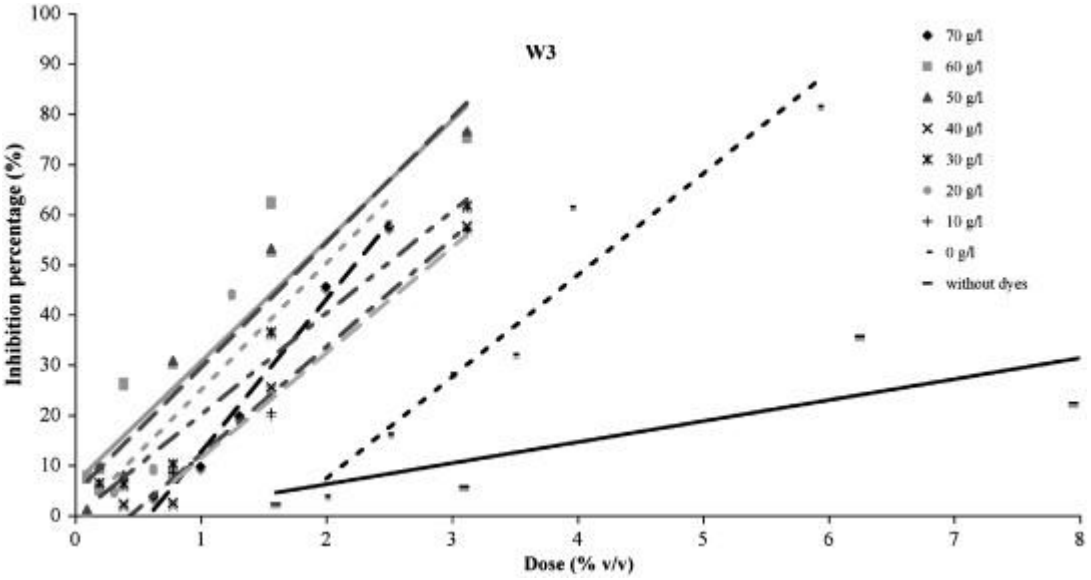

Fig. 2.

Dose-effect chart according to the *P. subcapitata* toxicity test (inhibition of algal growth): regression lines of reactive bath W3 containing different salt concentrations or without dyes.

Only the linear regressions of W3 without dyes and W3 without salt are clearly distinguished. The other regression lines were quite overlapped. This reflects the results of the *T* test statistical analysis: only these two regression lines were always significantly different, whereas the others showed similarity with at least another regression line (Table 4).

Table 4. *P. subcapitata* toxicity test (inhibition of algal growth) towards reactive bath W3: significant differences among the dose-effect regression lines according to T test.

	0 mg l^{-1} salt	**10 mg l^{-1} salt**	**20 mg l^{-1} salt**	**30 mg l^{-1} salt**	**40 mg l^{-1} salt**	**50 mg l^{-1} salt**	**60 mg l^{-1} salt**	**70 mg l^{-1} salt**	**Without dyes**
0 mg l^{-1} salt		≠	≠	≠	≠	≠	≠	≠	≠
10 mg l^{-1} salt	≠		=	=	=	≠	=	≠	≠
20 mg l^{-1} salt	≠	=		=	=	=	=	≠	≠

	0 mg l^{-1} salt	10 mg l^{-1} salt	20 mg l^{-1} salt	30 mg l^{-1} salt	40 mg l^{-1} salt	50 mg l^{-1} salt	60 mg l^{-1} salt	70 mg l^{-1} salt	Without dyes
30 mg l^{-1} salt	≠	=	=		=	≠	≠	≠	≠
40 mg l^{-1} salt	≠	=	=	=		≠	=	≠	≠
50 mg l^{-1} salt	≠	≠	=	≠	≠		=	≠	≠
60 mg l^{-1} salt	≠	=	=	≠	=	=		=	≠
70 mg l^{-1} salt	≠	≠	≠	≠	≠	≠	=		≠
without dyes	≠	≠	≠	≠	≠	≠	≠	≠	

≠ indicates significant differences for both slope (p<0.05 *T* test) and translation (p<0.001 *T* test) rates.

= indicates absence of significant differences for slope (p<0.05 *T* test) and/or translation (p=0.001) rates.

The percent deviation of the mixture predicted effect with respect to the measured effect by CA and IA were −29% and +135%, respectively. Thus, a moderate antagonistic effect between salt and dyes was pointed out according to CA models, whereas very high synergistic effect was pointed out according to IA model.

4. Discussion

4.1. Sensitivity of different organisms

The seven species belonging to different trophic levels showed different sensitivity to the four wastewaters. *P. subcapitata* appears to be the most sensitive one towards three (W2, W3 and W4) of the four tested wastewaters. This alga has already been reported as a good test organism for dyed wastewater (Novotný et al., 2006), even if some authors underlined the incapacity of this kind of organisms to distinguish between chemical toxicity and growth inhibition due to physical shading, when coloured substances like dyes are evaluated (Cleuvers and Ratte, 2002 and Bilinova, 2004). Nevertheless, in our opinion it is very important to consider also this last inhibiting factor on photosynthetic organisms, in order to better evaluate the effect of pollutants on the ecosystem. Actually, colour is a parameter regulated by the Italian legislation, independently from the chemical toxicity of wastewaters.

D. magna was the most sensitive organism towards W1, which simulates the effluent generated by wool dyeing with acid dyes. It is noteworthy that W1 is a foaming wastewater, because of surfactants contained in the dye powders, and bubbles could seriously damage the daphnids. Thus, *D. magna* test is probably useful for evaluating the effect of the “physical toxicity” due to surfactants, not detectable by other organisms.

On the contrary, the three angiosperms, L. minor, *C. sativus* and *L. sativum*, were less sensitive and in some cases they showed even biostimulation when put in contact with the wastewaters. However,

some caution must be expressed in this regard because these bioassays were short-term tests (acute toxicity) and hence they could not provide information on possible long-term effects (chronic toxicity). Moreover, the biostimulation effects should be seriously taken into account since they could predict eutrophication potential of wastewaters and this phenomenon should become progressively more significant within toxicological evaluation and risk assessment (Calabrese, 2008).

In addition to these considerations, it is noteworthy that dyes were partially and selectively adsorbed by filter paper during phytotoxicity tests. In the light of this, the use of inert supports (i.e. glass beads) for experiments on coloured samples can be preferable. Actually, with respect to previous measurements performed towards the same wastewaters using inert supports (Anastasi et al., 2010), filter paper caused an underestimation of the wastewaters toxicity in phytotoxicity tests.

The *V. fischeri* test was the only one that pointed out some limits in the toxicity assessment of the dark-coloured baths W3 and W4, because of the interference with the measure of the light emission by the bacterium. Other authors have already underlined this problem, nevertheless when the colour intensity of samples allowed the employment of this test, the luminescent bacteria showed high sensitivity to the toxicity of the tested wastewaters (Wang et al., 2002).

4.2. Wastewaters toxicity

All the four simulated wastewaters were toxic for at least one organism. The reactive bath W3, which has the highest concentration of dyes and salts, was the most toxic one according to all species. The toxicity order of the other wastewaters was W1>W4>W2 according to *L. sativum*, *C. sativus* and *D. magna*, whereas exclusively the algae test measured a higher toxicity in W2 than in W1 and W4.

Coloured wastewaters may represent a carcinogenic risk. Actually, they usually contain chemicals, including dyes, that are toxic, carcinogenic, mutagenic, or teratogenic to various organisms; moreover, many of their derivates, especially azo- and nitro-compounds, can be reduced to potentially carcinogenic amines in sediments of aquatic bodies that spread in the ecosystem (Møller and Wallin, 2000, Moawad et al., 2003, Umbuzeiro et al., 2005 and Novotný et al., 2006). In addition, the mutagenicity of this kind of dyes and their derivates is not eliminated by drinking water treatment plants (Alves de Lima et al., 2007 and Oliveira et al., 2007).

In our case, no wastewater exhibited mutagenic effects towards *Salmonella typhimurium*, both in the presence and in the absence of S9 metabolic activation. Nevertheless, it must be considered that sulphonic groups in dye molecules can decrease the amines mutagenic effect (Møller and Wallin, 2000). Moreover, concern about the presence of these molecules in wastewaters still remains, as bacteria are not a perfect model for eukaryotic cells and could not predict carcinogenic potency for higher organisms, including humans (Stravric, 1994).

According to the Italian law (DM 152/06), the toxic effect of a wastewater must be lower than 50% of inhibition of the tested organism. In the light of this, none of the simulated wastewater could be discharged because of the exceeding of this legal threshold value for at least one test. Since organisms responded differently to wastewaters exposure, the use of a single ecotoxicity test for the toxicity assessment results in an improper procedure. However, batteries are rarely carried out during the discharging phase of a wastewater, due to the absence of a specific legislation about their compulsoriness. Moreover, the fact that several analyses are time consuming and require specific expertise dissuades industries from performing more than one test.

4.3. Synthetic index and risk assessment

The need to apply a synthetic index appears with all its evidence when the outputs of the single ecotoxicity tests are contrasting, as in our case. While a tests battery can give the information about the most sensitive organism to pollutants, the application of a synthetic index for toxicity and risk score could be useful to elaborate a realistic projection of the environmental impact of wastewaters.

The model we used allows to compare the outputs of the single tests by giving different weights to the considered endpoints, which contribute differently to the assignment of the toxicity score of the battery, according to the weight of their endpoints. On the basis of this elaboration, the impact of the phytotoxicity tests with *L. sativum* and *C. sativus* increased, especially in the case of W1, because the root development is the endpoint with the heaviest weight.

Considering the data elaborated with this method, high or very high toxicity and risk were associated to all wastewaters, indicating that this kind of wastewater represents a serious danger for the environment. This datum confirms the study of Costan et al. (1993), who employed the Potential Ecotoxic Effects Probe index to compare eight wastewaters from different industrial sectors and found that the textile wastewater was the second most toxic effluent after pulp and paper sector effluent.

The order of the wastewaters toxicity and risk were W3>W1>W2>W4. It is important to note that dyes concentration was not the preponderant toxicity factor; actually, W4 (3000 mg l^{-1}) was less toxic than W1 and W2 (both 300 mg l^{-1}). A possible explanation is that these last wastewaters contain dye molecules bound to fluorine, chloride and heavy metals, such as cobalt and chromium that could contribute to increase the toxicity of this kind of dyes.

4.4. Contribution of dyes and salt to toxicity

The W3 was always the most toxic wastewater, both according to the single tests and the synthetic index, thus further experiments were performed in order to assess the contribution of reactive dyes and Na_2SO_4 to toxicity. Actually, in toxicity assessment of some textile wastewaters, dyes were found less toxic than other components, such as surfactants, heavy metals, salts, alkali and acids (Galassi and Benfenati, 2000 and Sharma et al., 2007).

In our case, the algal test pointed out that the dyes alone determine a higher toxicity than the salt alone. Their combined toxicity did not result in a simple additive combined effect when these pollutants were simultaneously present: according to the CA model they acted with a moderate antagonistic effect on the target organism, since the mixture toxicity was lower (−29%) than the addition of the single pollutants toxicity. On the contrary, the IA model pointed out a contrasting result showing a very high synergistic effect between dyes and salt, since the mixture toxicity was 135% higher than the predicted effect.

Literature reports that these models can often present dramatically different results depending on the slope of the dose–response curve of a single substance, thus the choice of the most appropriate model is of fundamental importance. In this case, the IA model seem more mathematically well-founded than the CA one, since the physiological mechanisms in which dyes and salt explicate their toxic effect are probably different (salt toxicity is basically due to the extreme osmotic pressure that leads to plasmolysis of algae cells, while the mechanism in which dyes act is not known). Nevertheless, some authors pointed out that this predictive model can underestimate the combined effect of substances and consider the CA model preferable because of a generally higher biological plausibility (Goldoni and Johansson, 2007).

No significant difference among dose–response regression lines was found when the salt is present together with dyes in the W3 mix. This could probably indicate that the interaction between salt and dyes achieves the highest effect already at the lowest tested salt concentration (10 g l^{-1}). Actually, the osmotic pressure can lead to the plasmolysis of the fresh water alga cells even at this salinity value (Vijayaraghavan and Ramanujam, 1999).

5. Conclusions

The results obtained in these experiments confirmed that toxicity test battery, followed by the data elaboration with synthetic index, is the most correct method for the evaluation of wastewaters toxicity; actually, the output of a single test cannot be exhaustive of wastewaters toxicity and, when more tests are performed, there is the need for a clear and concise way to properly sum up the results.

Another general conclusion of this research is that, in the presence of strongly coloured wastewaters, great care must be taken in choosing of target organisms and test procedures, since deep colour can interfere with the instrumentation (i.e. spectrophotometric reading of Microtox) and dyes can interact with supports (i.e. filter paper in phytotoxicity tests).

All the tested effluents are toxic and represent a risk for the environment. In addition to dyes, salts contained in textile wastewaters are dangerous for the environment too and a synergistic effect between dyes and salts occurs already at low salt concentration. Thus, this kind of wastewater should be treated with an adequate method, unaffected by their high toxicity, in order to completely remove all pollutants in wastewater.

Finally, the algae *P. subcapitata* was the most sensitive organism towards almost all the simulated wastewaters; consequently it is suggested for the assessment of textile and tannery wastewaters toxicity and to assess the effectiveness of remediation methods.

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