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Assessment of the Toxicity to Algae of Colored Substances

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An assessment of the toxicity of substances to aquatic organisms is needed for European Classification and Risk Assessment purposes (EEC 1993a, 1993b). This involves measuring the acute toxicity of the substance to fish, *Daphnia magna* and algae. The assessment uses the acute data obtained to extrapolate to chronic effects, and so should not take into account gross physical effects. In testing colored substances, reduction of light is such a physical effect and is present when using the current OECD and EC guidelines (OECD 1984; EEC 1992) for measuring their toxicity to algae.

A recent study sponsored by Ecological and Toxicological Association of Dyes and Organic Pigments Manufacturers (ETAD) has clearly demonstrated that it is possible to show the effect of light reduction on the growth of algal cells, independently of any toxicity due to the coloured substance (*personal communication, Memmert, 1993*). This study compared the effect on the alga of having the coloured substance present in the test vessel and between the light source and a test vessel with no test substance present. In all but one test the measured "toxic" effect was reduced and found to be mainly due to light reduction. The estimation of the actual toxicity, independent of the light reduction effect is, however, not measured using this approach.

The work presented in this paper proposes modifications to the current OECD method, thus allowing the measurement of the chemical toxicity of colored substances. The proposed modifications involve increasing the light intensity, lowering the initial algal cell density and reducing the volume of solution (and hence the effective light path) within the test vessels. It involves a minimum of extra effort and should provide an end-point that may be used for Classification and Risk Assessment of coloured substances.

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The basis of the approach is that algal growth rate increases with increasing light intensity, up to a plateau region (light saturation). Beyond this plateau, the effect of further increasing light leads to a decline in the algal growth rate, as the light becomes too intense and physically inhibits the algal cells.

The current protocols set the incident light intensity at 6000 - 10000 lux, in the region where changes in the light intensity alter the algal growth rate; hence the effect noted in the ETAD study. By decreasing the depth (light path) of the test solution and increasing the light intensity towards the plateau region, the effect of light absorbance by the coloured substance on algal growth rate is minimised.

Some initial experiments showing the effect of these protocol changes on the toxicity of non-coloured substances are presented. These are seen as important in the general validation of the procedure.

MATERIALS AND METHODS

Two reference chemicals were tested, 3,5-dichlorophenol obtained from Aldrich Chemical Company, Gillingham, and potassium dichromate obtained from Fisons, Loughborough. For each chemical a standard and modified algal growth inhibition test were carried out.

The standard test was carried out according to OECD guideline 201 (OECD 1984) and is described below. The modified protocol differed only in that the light intensity was increased to approximately 16000 lux, the volume of the test solutions was reduced to 30 mL and the inoculum level of alga reduced to 0.3×10^4 cells mL^{-1}. The OECD method suggests the light intensity is 8000 ± 20% while the EEC method suggests 6000 - 10000 lux. In both cases the level of the inoculum is 1.0×10^4 cells mL^{-1}.

Since higher growth rates were expected for the modified test procedure solutions, a lower test start cell density was utilized to maintain exponential growth throughout the study duration.

In each test a solution was prepared in sterile culture medium (Miller et al. 1978) at the highest test concentration. All lower concentrations were prepared by dilution of the solution with sterile medium. The control consisted of sterile medium only.

The test vessels were borosilicate glass conical flasks of 250 cm^3 nominal capacity closed with polyurethane foam bungs. The cultures were incubated at nominal 24 °C under continuous "cool-white" illumination, with orbital

shaking at 160 rpm.

Six replicate cultures of the control and triplicate cultures at each concentration of the test substance were employed. The position of the vessels in the incubator were re-randomised, daily, by rows. One blank vessel (without algal inoculum) for each control and treatment was incubated concurrently.

The test organism used was the unicellular green alga *Selenastrum capricornutum* Printz (Strain ATCC 22662) from laboratory cultures maintained under axenic conditions. A culture of the alga in the exponential growth phase was used as inoculum for the test. The culture was grown in the medium and under the environmental conditions described for the standard OECD test.

Each standard method vessel was inoculated with the inoculum culture to give a nominal cell density of 1.0×10^4 cells mL^{-1}. Modified test procedure flasks were also inoculated with the same culture, but to give a nominal cell density of 0.3×10^4 cells mL^{-1}.

The algal cell densities of the inoculum and test cultures were determined by electronic particle counting, using a Coulter counter model ZB, counting at a lower threshold equivalent spherical diameter of approximately 2.3 μm.

After 24, 48 and 72 hr (1, 2 and 3 d), samples were removed from each test and blank vessel. The appropriate blank particle count was subtracted from that of the test culture to obtain the cell density.

The pH of each test solution was measured at the start of the test, using the excess remaining after filling the test vessels. At the end of the test the pH of two of the replicate test solutions (containing algae) from each control and test concentration was determined. The temperature of the incubator was measured daily and was continuously monitored, with hourly recording of values, using an automatic recording system linked to a thermistor. Light intensities were measured once during the study, in a range of thirteen sample test positions.

RESULTS AND DISCUSSION

At the start of the test the pH of the excess test solutions was 7.3 ± 0.1. At the end of the test by the standard test method, the pH ranged from 7.3 to 10.1. These values represent a shift of 2.8 pH units during the course of the test procedure. This is due to the high algal cell densities allied to fast growth rates. For example in the two standard tests the mean control value increased by over 280 times the original cell density.

A high orbital shaking rate of 160 rpm was employed to minimize this pH shift.

The pH for the modified test procedure solutions at the end of the test ranged from 7.3 to 7.9. This much lower shift may be due to the difference in the inoculum level and/or the greater gaseous exchange possibly due to a higher surface area to volume ratio of the solutions in the modified procedure.

The light intensities measured in a representative sample of flask positions, were 8000 ± 1000 and 16000 ± 3000 lux (by cosine receptor), for the standard and modified procedures respectively. The higher variation of the light in the modified procedure possibly reflects design limitations in the present experimental set-up. The system will need to be optimized to reduce this potential source of variability.

Independent sets of statistical analyses were performed for the standard and modified test procedures. The area under the growth curve (days 0 to 3), equivalent to the biomass, was calculated for each replicate culture, according to the formula given in the OECD protocol (OECD 1983). These areas were examined by one-way analysis of variance and Dunnett's procedure (Dunnett 1964) used to identify significant differences ($P \leq 0.05$) from the control. The biomass expressed as percentages of that of the control and the significant differences identified, are given in Table 1 (3,5-dichlorophenol) and Table 2 (potassium dichromate).

The average (days 0 to 3) growth rate of each replicate culture was calculated according to the formula:

$$\text{Growth rate} = \frac{\text{Log}_n(N_2/N_1)}{t}$$

where N_1 = Cell density at start
N_2 = Cell density at end
t = Time interval (3 d)

These data were analyzed as described for the area method. The growth rates expressed as percentages of the control and the significant differences identified are given in Tables 1 and 2. For both biomass and growth rate, the percent control data were transformed to probability scale and analyzed by linear regression against log concentration, to estimate the median effective concentration (E_bC50 for biomass, E_rC50 for growth rate), defined as the calculated concentration causing a 50% decrease in the growth parameter (see Table 3).

Table 1. 3,5-dichlorophenol, biomass (areas under the growth curve) and growth rates as percentage of the control indicating statistically significant differences.

Nominal concn of 3,5 dichlorophenol (mg L^{-1})	Percentage of Control			
	Standard test procedure		Modified test procedure	
	Biomass	Growth rate	Biomass	Growth rate
0.095	99	99	108	102
0.19	98	99	97	99
0.38	92	98	92	98
0.75	77*	95	79*	96
1.5	15*	63*	12*	61*
3.0	1*	4*	1*	10*
6.0	1*	10*	1*	11*
12	1*	7*	1*	9*

Table 2. Potassium dichromate, biomass (areas under the growth curve) and growth rates as percentage of the control indicating statistically significant differences.

Nominal concn of potassium dichromate (mg L^{-1})	Percentage of Control			
	Standard test procedure		Modified test procedure	
	Biomass	Growth rate	Biomass	Growth rate
0.032	93	98	102	100
0.056	93	99	99	100
0.10	91	98	100	100
0.18	89*	98	84	96
0.32	69*	92*	42*	83*
0.56	26*	72*	6*	42*
1.0	3*	28*	2*	24*
1.8	2*	16*	1*	14*

The results show that the changes made to the OECD protocol do not significantly alter the measured toxicity

of these non-coloured substances. The absorption of the potassium dichromate solutions is minimal at the concentrations tested. A 1 mg L^{-1} solution has a light absorption of approximately 0.45 at 360 nm, equivalent to a three-fold light reduction. This validation step is important and will probably need to be extended further if the method were to be adopted by the regulatory authorities.

Table 3. EC50's derived for 3,5-dichlorophenol and potassium dichromate using standard and modified conditions.

	Standard Protocol	Modified Protocol
Conditions		
Light Intensity (approx)	8000 lux	16000 lux
Volume of test soln.	100 mL	30 mL
Inoculum cell density	1×10^4	0.3×10^4
3,5-dichlorophenol (results in mg L^{-1})		
E_rC50	1.8	2.2
NOEC	0.75	0.75
E_bC50	0.89	1.0
NOEC	0.38	0.38
Potassium dichromate (results in mg L^{-1})		
E_rC50	0.87	0.64
NOEC	0.18	0.18
E_bC50	0.28	0.31
NOEC	0.10	0.18

Using these modified conditions, a study was performed on a coloured substance with an absorbance at 640 nm of 1.2 for a 10 mg L^{-1} solution in a cell with a 4-cm path length.

The observed E_rC50 changed from 80 to over 200 mg L^{-1} and the E_bC50 increased from 15 to 90 mg L^{-1} when the modified test protocol was used. These results confirm the reduction of the light absorbance effect; however, whether the proposed modifications eliminate light reduction entirely from the measured toxic effect is not yet confirmed.

Although further validation is required to ensure there is no impact on the results obtained for a wide range of non-coloured substances, the suggested protocol should be the basis of a method, suitable for the accurate measurement of the chemical toxicity of coloured substances in the alga test. In this way the test will be capable of being used for classification and risk assessment purposes for such substances.

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