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TESTING OF COLOURED SAMPLES FOR TOXICITY BY THE ALGAL-ATP BIOASSAY MICROPLATE TECHNIQUE

by

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ABSTRACT

Algal-ATP bioassay is a relatively simple, inexpensive, reliable and fast testing procedure for toxicity assessment. This algal growth inhibition bioassay technique using Selenastrum capricornutum was used to assess the toxicity levels in pulp and paper mill and thermomechanical mill effluent samples. Pulp and paper mill and thermomechanical mill effluent samples contain a wide range of colours due to certain physico-chemical properties of the test samples. The optical densities of the samples ranged from 0.27 to >2.0. The colour variations in the test samples may interfere with ATP measurements. This paper presents data on the nature of intereferences due to certain physico-chemical properties of the test sample and the usefulness of developed controls to overcome the problems for a more realistic toxicity assessment of coloured waste samples.

RÉSUMÉ

Le bio-essai algue-ATP est une méthode d'essai pour l'évaluation de la toxicité, qui est relativement simple, peu coûteuse, fiable et rapide. Ce test d'inhibition de la croissance des algues au moyen de Selenastrum capricornutum a été employé pour évaluer le degré de toxicité des échantillons prélevés dans les effluents des usines de pâtes et papiers et des usines thermomécaniques. Ces échantillons contiennent une grande diversité de couleurs à cause de certains de leurs propriétés physico-chimiques. La densité optique des échantillons variait de 0,27 à > 2,0. Les variations de couleurs dans les échantillons mis à l'essai peuvent interférer avec les mesures de ATP. Ce rapport présente des données sur la nature des interférences dues à certaines propriétés physico-chimiques des échantillons mis à l'essai ainsi que sur l'utilité d'établir des contrôles pour résoudre le problèmes afin d'en arriver à une évaluation plus réaliste de la toxicité des échantillons de déchets colorés.

MANAGEMENT PERSPECTIVE

Toxicant screening procedures are becoming basic tools in environmental studies and with the increased use of these procedures there is a growing awareness of problems with these tests. Interferences due to various physico-chemical properties of the samples are surfacing and methods to alleviate some of these problems are being evaluated. This report describes a procedure that eliminates the problem caused by coloured samples. We have used the Selenastrum capricornutum (algal) growth inhibition test to illustrate the problem and the solution. ATP concentrations were used as the indicating system in this study. By following the described procedure for modifying problems due to coloured samples, the sensitivity of toxicant screening tests is enhanced and results can now be obtained from these samples by toxicant screening tests usually affected by coloured or turbid samples.

PERSPECTIVE DE GESTION

Les méthodes de dépistage des toxiques sont en train de devenir des outils de base dans les études de l'environnement et, étant donné qu'on utilise davantage ces procédures, on prend de plus en plus conscience des problèmes que soulèvent ces tests. On se rend compte des interférences qui se produisent à cause de diverses propriétés physico-chimiques des échantillons et l'on est en train d'évaluer des méthodes pour remédier à ces problèmes. Ce rapport décrit une méthode qui élimine le problème causé par les échantillons colorés. Nous avons employé le test d'inhibition de la croissance des algues en utilisant le Selenastrum capricornutum pour illustrer le problème et sa solution. Dans cette étude, nous avons utilisé les concentrations de ATP comme le système indicateur. En suivant la procédure décrite pour remédier aux problèmes dus aux échantillons colorés, on améliore la sensibilité des tests de dépistage des toxiques; on peut maintenant obtenir des résultats à partir de ces échantillons au moyen des tests de dépistage qui d'ordinaire étaient faussés par la coloration ou la turbidité des échantillons.

INTRODUCTION

The increased concern about the effects of potential environmental hazards entering our streams and lakes through domestic and industrial discharges and agricultural runoff has generated researchers to develop simple, sensitive, low cost, reliable and relatively fast toxicity screening bioassays. The Adenosine triphosphate (ATP) assay has been used extensively for biomass estimations in fresh water (Burglis, 1974; Deming et al., 1977; Dutka and Kwan, 1984), marine water (Hamilton and Holm, 1967; Holm, 1978) and sediments (Tobin et al., 1977). Also, the potential use of ATP to assess toxicant presence in waters and sediments has been frequently demonstrated by Patterson et al., 1970; Mahlom, 1979; Blaise et al., 1984; and Hao and Dutka, 1987. Recent research (Parker, 1983 and Blaise et al., 1984) has demonstrated that the ATP bioassay is simple, inexpensive, reliable, sensitive and fast.

However, there are certain physico-chemical properties within the test samples which may introduce interference to the measurement of ATP content during the ATP bioassay. During the course of our toxicity assessment studies of thermomechanical mill and kraft pulp and paper mill effluents using Algal-ATP bioassay, it became apparent that certain coloured substances in the effluents clearly posed technical problems which prevented a realistic assessment of the toxic nature of the samples.

This study describes the usefulness of certain necessary controls to overcome some of the technical problems when the ATP-Algal bioassay is applied to coloured samples to estimate toxicity levels.

MATERIALS AND METHOD

Sample and Sample Treatment

Composited pulp and paper mill and semi-chemical effluents were collected from kraft pulp and paper mills and thermomechanical mills respectively. Colour of the samples ranged from chalky to dark brown. Samples used in the study were sterilized by heating between 80°C-90°C in a water bath for five minutes since autoclaving (Miller et al., 1978) and membrane filtration technique (Tobin and Dutka, 1977) were found to alter the nature of the toxicity properties of the samples.

Microplate Test Procedure

Selenastrum capricornutum algal suspension was prepared by inoculating a fresh culture (Carolina Biological Supply Company) into 1.5 L of Bristol's medium (Stein, 1973) in a two litre flask and placed on a shaker under Vita light at 25°C for 7 days. At this temperature, 7 days produces maximum growth. Forty mL of the 7 day old Selenastrum capricornutum culture was then centrifuged for

30 minutes at 2500 rpm in a refrigerated centrifuge. After centrifugation, the supernatant was decanted. The algal pellet was mixed with 25 ml of sterile 15X concentration Bristol's medium on a vortex mixer for 30-40 seconds. After mixing, 20 µl of the mixture was transferred onto a haemocytometer using a sterilized Eppendorf pipette tip. Algal counts between 20,000 to 25,000 cells per mL were preferred and if the counts were too high, more 15X Bristol's medium was added to dilute to the appropriate cell concentration. After the desirable cell concentration was obtained, the mixture was vortexed for 30 seconds prior to transferring it into a sterile reservoir (Flow Laboratories, Inc.) (Figure 1) or a sterile petri dish. Immediately, 20 µl of the mixture was pipetted, using a multi-channel pipetter, into the 96 wells of the microplate which was previously labelled (Figure 2). Sterile Milli-Q reagent grade water (200 µl) was then transferred using a multiple channel pipetter into the 12 wells in the first row of the microplate and filling the rest of the wells with 200 µl of appropriate sample or sample dilutions. All samples were tested at 100% concentration and blanks and samples were run in replicates of six. Each microplate was sealed inside a transparent ziplock plastic bag to prevent evaporation and incubated for four days at 25°C, 16 inches below a bank of eight Vita lights (growth lights). An identical microplate without the algal culture served as a control for sterility, colour and physico-chemical properties.

After incubation, the microplates were examined visually for growth responses using a plate reading mirror (Figure 3). The six

wells from each sample and the blank were aspirated and mixed 3 to 5 times with a multi-channel pipetter before 200 µl sample was taken out of each well and transferred into a sterile reservoir. The sample in the reservoir was then mixed 3 to 5 times before three - 100 µl aliquots of the sample were transferred to three sterile polypropylene tubes (Turner Designs, California) with a multichannel pipetter. The remainder of the sample in the reservoir was discarded. Control samples were treated identically except six 100 µl aliquots of each sample were transferred into six sterile polypropylene tubes. Three tubes were used for sterility checks and the other three were used for color and physico-chemical checks.

Algal-ATP Test

Algal-ATP was measured using the Turner Model 20e Luminometer. ATP measurement was recorded in Relative Light Units (RLU) which were proportional to the amount of ATP present in the sample (100 µl volume).

To determine the toxic effects of the samples, 100 µl of Releasing Agent (Turner Designs) was added to the polypropylene tubes containing 100 µl control and test samples. Then 100 µl Hepes Buffer solution (Turner Designs) was added after a thirty minute contact time between the cells and solution to ensure complete lysis. Each tube was then placed into the Luminometer and 100 µl of luciferin-luciferase (Turner Designs) was injected into the tube and the Relative Light Units (RLU) were recorded.

The sterility of the samples was checked by adding 100 µl of Releasing Agent and 100 µl of Hepes Buffer solution to the tubes containing the blank (Milli-Q reagent grade water) and samples. Then each tube was reacted with 100 µl of luciferase-luciferin in the Luminometer and the Relative Light Unit was measured.

Calculation

The following formulae were used to calculate the final percentage inhibition of toxicant(s) in the samples:

$$R = \frac{B\ (algae) - S\ (algae)}{B\ (algae)}$$

$$C = \frac{B\ (ATP) - S\ (ATP)}{B\ (ATP)}$$

$$F = R - C$$

where,

R = Relative inhibition due to toxicants, colour and physico-chemical effects
B (algae) = Blank with algae
S (algae) = Sample with algae
C = Colour and physico-chemical corrections
B(ATP) = Blank with ATP Standard
S(ATP) = Sample with ATP Standard
F = Final inhibition due to toxicant(s) alone

Colour Reduction

Sample A, B and C having optical densities of 0.53, 0.27 and >2.0 were used to evaluate the relationship of colour and sample

concentrations. 20 µl of ATP standard which yielded 400-500 RLU was added into each sample and dilutions (100%, 50%, 25% and 12.5%). ATP measurements were performed as previously described. A colour reduction graph was plotted on semi-logarithmic paper (Figure 4).

RESULTS AND DISCUSSION

Table 1 presents the ATP values for the kraft pulp and paper mill and thermomechanical mill effluent samples as percentage of light inhibition. The total light inhibition is shown in column 1. Column 2 presents the RLU data obtained from samples due to colour and other physico-chemical properties. In column 3 the true RLU values are presented, representing the effects of growth inhibition due to toxicants. If the percentage of light inhibition in column 1 is greater than 100% as seen in samples H, J, and K, this is considered to be a stimulatory effect which promotes the algal growth and subsequently increases the ATP concentration in the samples. If the percentage of light inhibition in column 1 is less than those in column 2 as seen in samples E, F and I, this is considered to be colour or physico-chemical interference effects or light increases due to contaminants in the samples or both. Light increases due to contaminants can easily be rectified from the sterility check microplate. If the RLU in any tested sample is greater than the RLU in the control (blank) this indicates the presence of contaminants. Therefore, the sample should be repeated with proper sterilization.

Since, all samples were sterilized by heating at a temperature between 80°C-90°C, spore forming bacteria may survive and present false negative effects (increase in ATP content). Hence, sterility checks of each sample and its dilution are important factors in the Algal-ATP test.

If the light inhibition in column 2 is less than in column 1 this is considered to be an inhibitory response due to colour, physico-chemical and toxicant effects. Light inhibition caused solely by toxicants as shown in column 3, is shown by the difference of light inhibitions in columns 1 and 2. In Table 1, it can be seen that 10 of the 13 samples tested (column 1) produced a toxic effect while in column 3 only 7 of 13 samples produced a toxic effect after colour and physico-chemical effects were removed. Similarly, samples E, F and I would have been considered toxic if colour and physico-chemical effects were not accounted for. In summary, of the 13 samples tested, 7 were toxic, 3 produced stimulatory effects and 3 were negative for both toxic and stimulatory effects.

Figure 3 presents the colour reduction graphs of samples A, B and C. It can be seen that the colour reduction is not a function of concentration. Thus, in a dose-response study of more than one concentration it is important to be aware of the nature and extent of interferences due to the inherent complexity of some environmental samples. Therefore, it is necessary to use proper controls in order to realistically assess the toxicity of the sample.

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FIGURES

Table 1. ATP values expressed as percentage of light inhibition.

Sample	Total Light inhibition (%)	Light Inhibition Due to colour and physico-chemical (%)	Final Light Inhibition due to toxicants (%)
A	94	33	61
B	63	4	59
C	99	27	72
D	97	40	57
E	99	100	-1
F	99	100	-1
G	96	51	45
H	>100	14	S*
I	75	85	-10
J	>100	30	S
K	>100	18	S
L	87	38	49
M	82	10	72

*Stimulatory effect

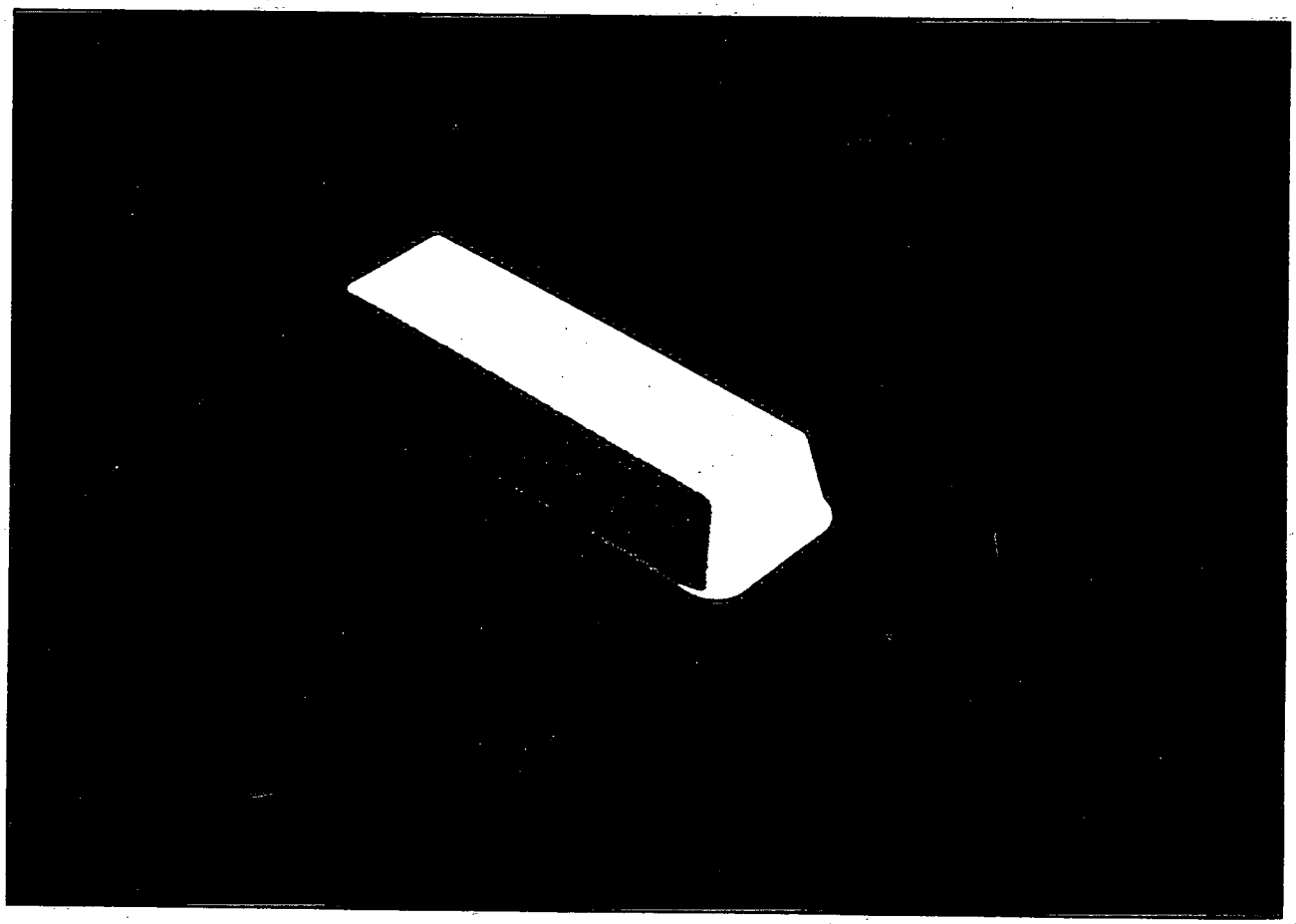

Figure 1. Reservoir

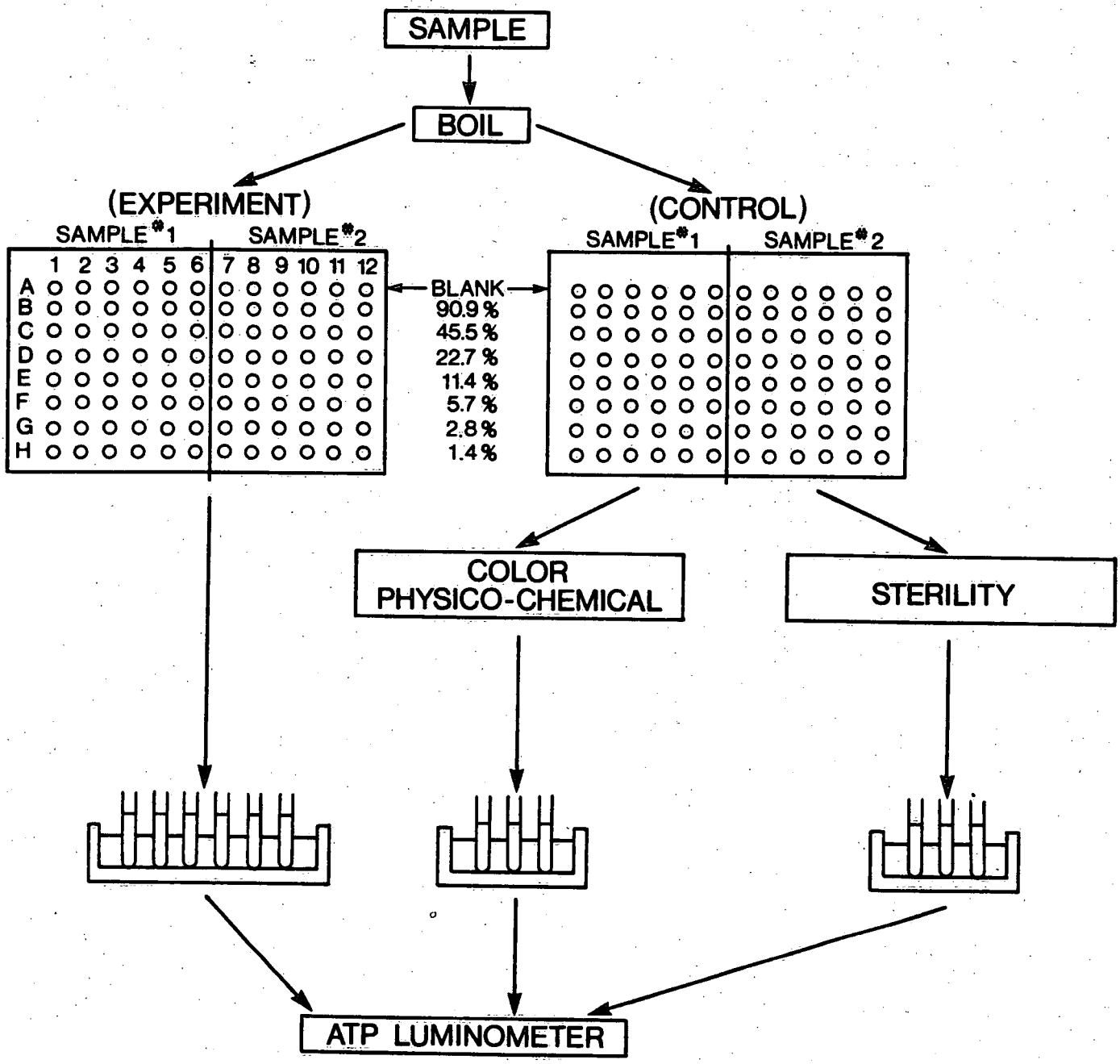

Figure 2. Algal-ATP microplate bioassay technique

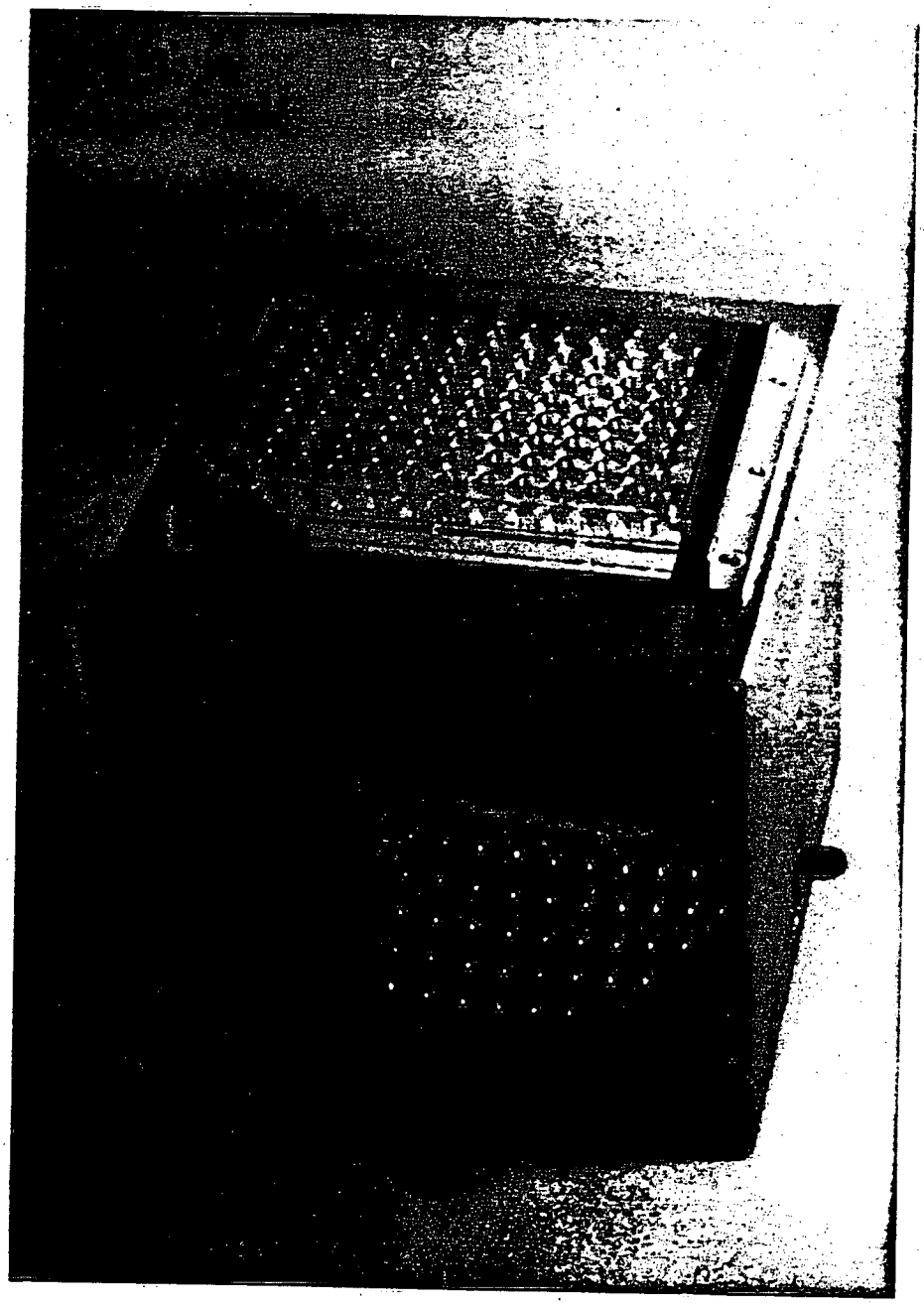

Figure 3. Plate reading mirror

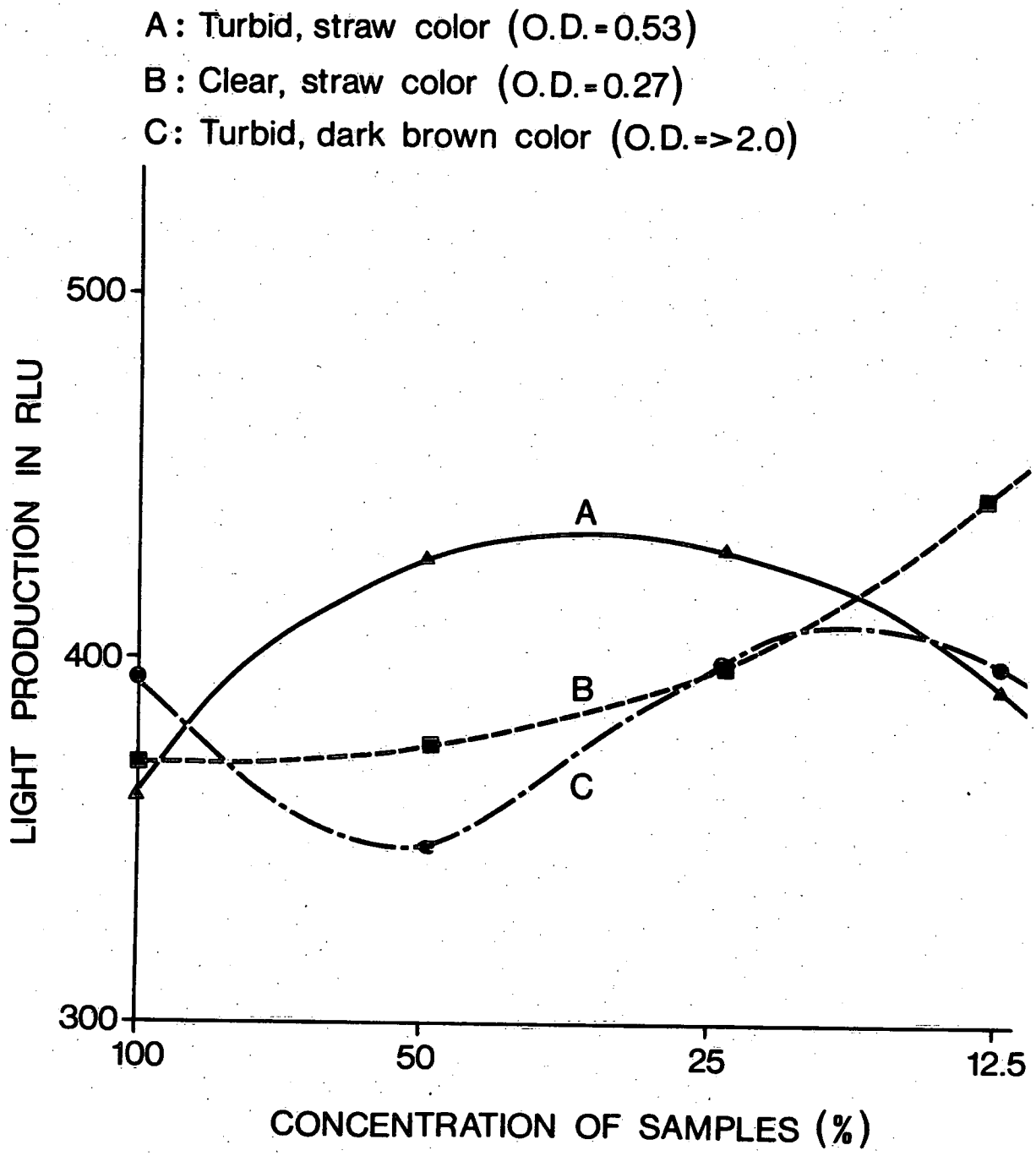

Figure 4. Color reduction graphs