GENOTOXICITY TESTING OF THIRTEEN POLYCYCLIC AROMATIC COMPOUNDS USING THE MUTATOX® ASSAY

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NWRI Contribution No. 98-060

Genotoxicity Testing of Thirteen Polycyclic Aromatic Compounds Using the Mutatox® Assay

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Management Perspective

Several polycyclic aromatic compounds (PACs) are carcinogenic or mutagenic and can enter aquatic environments through production and use of fossil fuels. One example on the production side is self-contained, below ground systems (the "wet landscape" option for site reclamation) that have been proposed for fine tailings management by the oil sands industry in northeastern Alberta. As part of a PERD-funded project (No. 57219) on PACs in the base/neutral fraction of oil sands fine tailings we have isolated and identified several PACs from mature fine tailings porewater. For ecotoxicological assessment of these PACs and base/neutral extracts, our emphasis has been on potential chronic toxicity expressed as mutagenicity.

For a screening test for mutagenicity, we chose the Mutatox® test for its simplicity relative to the longer and better established Ames test. Results are presented for testing of 13 PACs consisting of polycyclic aromatic hydrocarbons and dibenzothiophene, and alkylated and oxygenated derivatives of them. This report also describes results of experiments on pre-dilution of test compounds in the organic solvent used to deliver them to the test system versus the standard procedure wherein the dilutions are carried out in the aqueous medium. Most PACs have low water solubility, and because of this the mode of delivery was tested to see how it affected the outcome.

Sommaire à l'intention de la direction

Plusieurs composés aromatiques polycycliques (CAP) provenant des secteurs de la production et de l'utilisation de combustibles fossiles sont cancérogènes ou mutagènes et peuvent pénétrer dans les milieux aquatiques. L'un des exemples mentionnés pour le secteur de la production est celui des systèmes autonomes souterrains (option d'assainissement des sites en milieu humide) qui ont été proposés pour la gestion des stériles fins de l'industrie des sables bitumineux du nord-est de l'Alberta. Dans le cadre d'un projet financé par le GRDE (n° 57 219) et portant sur les CAP dans la fraction basique/neutre des stériles fins des sables bitumineux, nous avons isolé et identifié plusieurs CAP dans les eaux interstitielles de stériles fins à l'équilibre. Notre évaluation écotoxicologique de ces CAP et des extraits de la fraction basique/neutre se concentrait sur la toxicité chronique possible exprimée en mutagénicité.

Nous avons choisi le test Mutatox® comme essai de dépistage de mutagénicité à cause de sa simplicité par rapport au test Ames plus ancien et plus reconnu. On présente les résultats des tests de 13 CAP (des hydrocarbures aromatiques polycycliques et le dibenzothiophène, ainsi que certains de leurs dérivés alkylés et oxygénés). Ce rapport décrit aussi les résultats d'expériences portant sur la prédilution de composés à l'essai dans le solvant organique utilisé pour les transférer dans le système expérimental, par rapport à la procédure normalisée qui prévoit des dilutions en milieu aqueux. La plupart des CAP sont peu solubles dans l'eau et pour cette raison, le mode de transfert a fait l'objet d'essais visant à déterminer ses effets sur les résultats.

Abstract

One of the tests for mutagenicity we have been using for oil sands polycyclic aromatic compounds (PACs) is the Mutatox® assay. A recent publication outlined some complications with using benzo[a]pyrene (B[a]P) as a positive control (with metabolic activation by S9) since nominal concentrations in the test solutions are considerably higher than measured dissolved concentrations (Klamer *et al.*, 1997). To determine if water solubility was operationally affecting the Mutatox® assay, we ran a series of assays with the test compounds (B[a]P and phenanthrene) diluted in aqueous medium - the standard protocol, and a series pre-diluted in methanol, the organic solvent we have used for delivery of the test compound. There were no detectable differences between the two methods and we have concluded that the standard procedure is operationally acceptable. We used the Mutatox® assay (with and without S9 activation) to assess the genotoxic potential of several unsubstituted PACs: fluorene, fluoranthene, phenanthrene, pyrene, dibenzothiophene and benzo[b]naphtho[1,2-d]thiophene; three alkylated PACs: 1-methylphenanthrene, 3,6-dimethylphenanthrene and 4-methyldibenzothiophene; and a number of oxygenated PACs: dibenzothiophene sulfone, 9-phenanthrol, 1-hydroxypyrene and 6-hydroxychrysene. In general, non-oxygenated PACs are inactive or weakly active with S9 activation and are strongly active without S9 activation. Oxygenated derivatives tended to be inactive both with and without S9 activation, except for 1-hydroxypyrene which was weakly active with S9 activation and active without S9 activation.

Résumé

Le test Mutatox® est l'un des tests de mutagénicité que nous avons utilisés pour les composés aromatiques polycycliques (CAP) des sables bitumineux. Une publication récente soulignait certaines complications dues à l'utilisation du benzo[a]pyrène (B[a]P) comme produit témoin positif (avec activation métabolique au S9), étant donné que les concentrations nominales des solutions d'essai sont beaucoup plus fortes que celles mesurées dans le milieu (Klamer *et al.*, 1997). Afin de déterminer si la solubilité dans l'eau avait des effets sur les résultats du test Mutatox® en conditions opérationnelles, nous avons réalisé une série de tests avec les composés à l'essai (le B[a]P et le phénanthrène) dilués en milieu aqueux (selon le protocole normalisé), et une autre série avec des prédilutions dans le méthanol, le solvant organique utilisé pour transférer le composé à l'essai. Il n'y avait pas de différences visibles entre les deux méthodes et nous avons conclu que le protocole normalisé est acceptable en conditions opérationnelles.

Nous avons utilisé le test Mutatox® (avec et sans activation au S9) pour évaluer le potentiel génotoxique de plusieurs CAP non substitués, soit le fluorène, le fluoranthène, le phénanthrène, le pyrène, le dibenzothiophène et le benzo[b]naphtho[1,2-d]thiophène; trois CAP alkylés, soit le 1-méthylphénanthrène, le 3,6-diméthylphénanthrène et le 4-méthyldibenzothiophène et enfin, un certain nombre de CAP oxygénés, soit la dibenzothiophènesulfone, le 9-phénanthrol, le 1-hydroxypyrène et le 6-hydroxychrysène. En général, les CAP non oxygénés étaient inactifs ou faiblement actifs après activation au S9 et ils étaient fortement actifs sans activation au S9. Par contre, les dérivés oxygénés avaient tendance à être inactifs avec et sans activation au S9, sauf le 1-hydroxypyrène qui était faiblement actif après activation au S9, et actif sans activation au S9.

Introduction

The detection of mutagens (DNA-damaging compounds) in environmental samples has been carried out by a number of assays. The Ames test, developed by Maron and Ames (1983), is one of the most widely used bacterial assays to screen for chemicals that may have genotoxic potential. The Mutatox®[1] test, was developed by Azur Environmental (Carlsbad, CA) as a short-term, simpler procedure for screening for mutagenicity in environmental samples. In the Mutatox test, a special dark mutant of luminescent bacteria (*Vibrio fischeri*, strain M 169) is used to detect the presence of genotoxic agents (Azur, 1995). These bacteria exhibit increased light production when grown in the presence of genotoxic agents. The Mutatox assay has been evaluated by a number of researchers including Johnson (1992a), Sun and Stahr (1993) and Legault *et al.* (1994) and correlates favorably with the Ames test. The Mutatox test has also been used to measure the relative genotoxicity of complex mixtures and sediments (Ho and Quinn, 1993; Johnson, 1992b; Kwan *et al.*, 1990).

A number of polycyclic aromatic compounds (PACs) are known carcinogens and many carcinogens also exhibit mutagenicity. We used the Mutatox test to determine the genotoxic potential of individual PACs by using pure compounds dissolved in methanol. A number of the compounds tested have been found in oil sands fine tailings and the results will be used to examine the mutagenic potential of PACs released from the tailings.

A recent publication outlined some complications with using benzo[a]pyrene (B[a]P) as the positive control for the Mutatox S9 assay (metabolic activation with S9) since nominal concentrations in the test are considerably higher than measured dissolved concentrations (Klamer *et al.*, 1997). We ran a series of assays with the test compounds diluted in aqueous medium (standard protocol) and pre-diluted in the organic solvent (methanol) to determine if water solubility was operationally affecting the Mutatox test.

Method

Chemicals: phenanthrene, fluorene, fluoranthene, pyrene, and benzo[a]pyrene were obtained from Supelco Canada, Mississauga, ON; 3,6-dimethylphenanthrene, dibenzothiophene sulfone, benzo[b]naphtho[2,1-d]thiophene and 9-phenanthrol were obtained from Aldrich Chemical, Milwaukee, WI; 1-hydroxypyrene and 6-hydroxychrysene were obtained from Accustandard, New Haven, CT; dibenzothiophene and 1-methylphenanthrene were acquired from Chem Service, West Chester, PA. We received 4-methyldibenzothiophene as a gift from Dr. J. Andersson, University of Ulm, Germany.

Solvent: methanol (distilled in glass) was obtained from Caledon Laboratories Inc., Georgetown, Ontario.

[1] Mutatox® is a Registered Trademark of Azur Environmental.

Mutatox reagents: growth medium with and without S9 (rat liver microsomes) was purchased in lyophilized form from Azur Environmental, Carlsbad, CA and stored at -30°C until used. The bacterial culture, a dark mutant M169 strain of *V. fischeri*, was also purchased in lyophilized form from Azur and stored at -30°C prior to use.

Assay Protocol

Stock solutions of each compound were made in methanol at a concentration of 1 mg/mL, except 4-methyldibenzothiophene (1.17 mg/mL) and benzo[b]naphtho[2,1-d]thiophene (0.5 mg/mL). For all tests, positive and negative controls were used with each vial of bacteria. Benzo[a]pyrene (0.5 mg/mL) was used as the positive control for the S9 assay and phenol (10 mg/mL in reconstitution solution) was the positive control in the direct assay. For both tests, methanol was the negative control.

The Mutatox test was carried out according to the protocol described by Azur Environmental. Each lyophilized vial of medium was reconstituted with 15 mL of reconstitution solution and kept cold prior to use. The test chemical (10µL stock solution) was added to the cuvette containing 500 µL reconstituted medium and 1:2 serial dilutions were made by transferring 250 µL from cuvette to cuvette (each containing 250 µL medium) with mixing after each transfer; 250 µL was discarded from the last cuvette. Ten dilutions were made:

Cuvette No.	1	2	3	4	5	6	7	8	9	10
µg/mL	20	10	5	2.5	1.25	0.63	0.31	0.16	0.08	0.04
µg/cuvette	5	2.5	1.25	0.63	0.31	0.16	0.08	0.04	0.02	0.01

Sample concentrations are nominal because in serial dilutions one assumes complete solubility of test chemical; and dilution error is cumulative. To each cuvette, 10µL rehydrated bacteria (1.1 mL of reconstitution solution per vial of microbial reagent) were added. Test solutions with S9 activation were incubated at 35°C for 45 minutes then both assays (with and without S9) were incubated at 27°C. Bioluminescence determinations were made with an Azur Environmental M500 Toxicity Analyzer set in the Mutatox mode. Light readings were taken at 14, 18 and 22 hours and the readings were recorded as arbitrary units using the Mutatox Data Capture Software.

Evaluation of Organic Solvent

Most PACs have limited water solubility; therefore a suitable organic solvent is required for sample delivery. The Mutatox test is compatible with dimethylsulfoxide, methanol, ethanol and acetone up to a 4% final concentration; however, some effects may be seen at this concentration (Azur, 1995). Because of the limited water solubility of PACs, we decided to determine if the dilution series in medium (water-based) was adequate to

deliver the test compound to the bacteria. To test both the solvent effect and sample delivery, we made the sample dilutions in methanol (serial 1:2) and then added 5 µL or 10 µL of each dilution directly to the cuvettes containing 250 µL medium (in triplicate). This represented a 2% and a 4% final concentration of methanol in the test solutions. Benzo[a]pyrene was used in the dilution series for medium with S9 activation and phenanthrene in the dilution series for medium without S9. The positive controls (benzo[a]pyrene and phenol) were added as per Azur protocols. Solvent controls were 5 or 10 µL methanol added directly to each cuvette (5 replicates).

Results and Discussion

Solvent Effect

There is no apparent effect on the bacteria with 2% methanol in medium with or without S9 activation. Results show that the dilution series in methanol and the Azur protocols give similar results suggesting that the compound is delivered to the bacteria in an operationally equivalent way. In the direct assay, peak light level for phenanthrene was at 18 h and maximum light production occurred at 5 µg/mL of phenanthrene in all three modes: standard method (dilution in medium), pre-dilution in methanol at 2% concentration in test solution, and pre-dilution in methanol at 4% concentration in test solution (Figure 1). In the S9 assay, peak light levels for benzo[a]pyrene was at 14 h and maximum light production occurred at 5, 10, and 2.5 µg/mL for the standard, 2% methanol and 4% methanol series, respectively (Figure 2); however, there is an apparent acute toxic effect in the first two dilutions of the 4% methanol series. Both assays were performed in triplicate and the results for pre-dilution of sample in methanol are quite reproducible. Figures 3 and 4 show the replicate results for 2% methanol in the test solution.

The relatively similar pattern between the three modes indicate that the standard protocol and pre-dilution in methanol will give similar enough results that the pre-dilution in methanol (a time consuming step) is not necessary and that the standard protocol is an operationally acceptable method of preparing serial dilutions of even relatively water insoluble compounds such as benzo[a]pyrene.

PAC Mutagenicity

In the Mutatox test, a positive genotoxic response occurs when at least two consecutive dilution tubes show a light increase two times the average media control value (Azur, 1995). For all positive results we plotted response against tested concentrations to show minimum and maximum active concentrations. We used the arbitrary light reading of 10-100 as weakly positive, 100-1000 as moderately positive and >1000 as strongly positive for mutagenic activity.

No mutagenic activity was found for 3,6-dimethylphenanthrene, dibenzothiophene sulfone, benzo[b]naphtho[2,1-d]thiophene and 6-hydroxychrysene with either direct assay or with S-9 activation. 1-Methylphenanthrene was weakly positive in the S9 assay at the 14 hour reading only, with no activity at later readings. In addition, dibenzothiophene and 9-phenanthrol showed no activity with S9 activation.

In the direct assay, 9-phenanthrol gave positive light readings in the sixth and seventh dilution only, and was consistent at 14, 18 and 22 hours. Figure 5 shows the concentration-response results for the tested PACs by the direct assay. Phenol concentrations (positive control) are shown at 1/10 of actual concentration. Concentration-response results for compounds with positive results in the S9 activated assay are show in Figure 6. Phenanthrene, fluoranthene and fluorene are strongly positive in the direct assay; however, with S9 activation the activity, as measured by light output, for the same compounds is much lower. Dibenzothiophene and 4-methyldibenzothiophene are moderately active in the Mutatox direct assay but are inactive or slightly active, respectively, with S9. Table 1 summarizes the results of the 18-hour light readings for the Mutatox assay, with and without S9, for the compounds tested and are the results of a single test for each compound.

Table 1. Mutatox results for tested chemicals after 18 hours incubation. Number indicates the dilution which gave maximum response and + indicates the potency of response (arbitrary light units). + = 10 - 100; ++ = 100 - 1000; +++ = >1000; - = no activity.

	Maximum Light (dilution #)	
Compound	**+ S9**	**- S9**
Fluorene	+ (2)	+++ (1)
Phenanthrene	+ (1)	+++ (1)
Fluoranthene	+ (1)	+++ (1)
Pyrene	+ (2)	+ (2)
1-Methylphenanthrene	-	-
3,6-Dimethylphenanthrene	-	-
Dibenzothiophene	-	+++ (2)
4-Methyldibenzothiophene	+ (1)	+++ (1)
Dibenzothiophene sulfone	-	-
9-Phenanthrol	-	+ (6)
1-Hydroxypyrene	+ (2)	+++ (5)
6-Hydroxychrysene	-	-
Benzo[b]naphtho[2,1-d]thiophene	-	-

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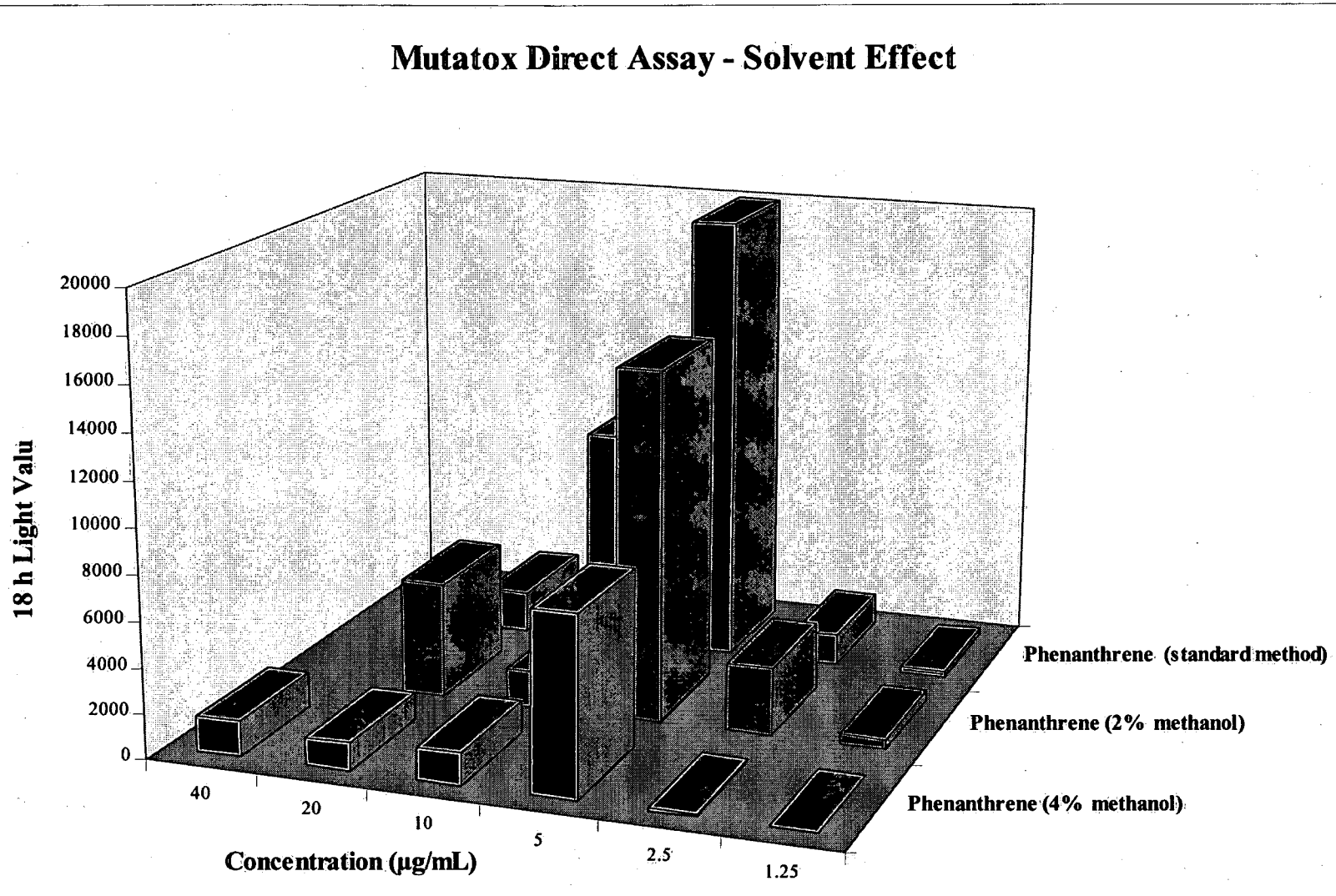


Figure 1. Effects with 2% and 4% methanol in Mutatox test solutions.

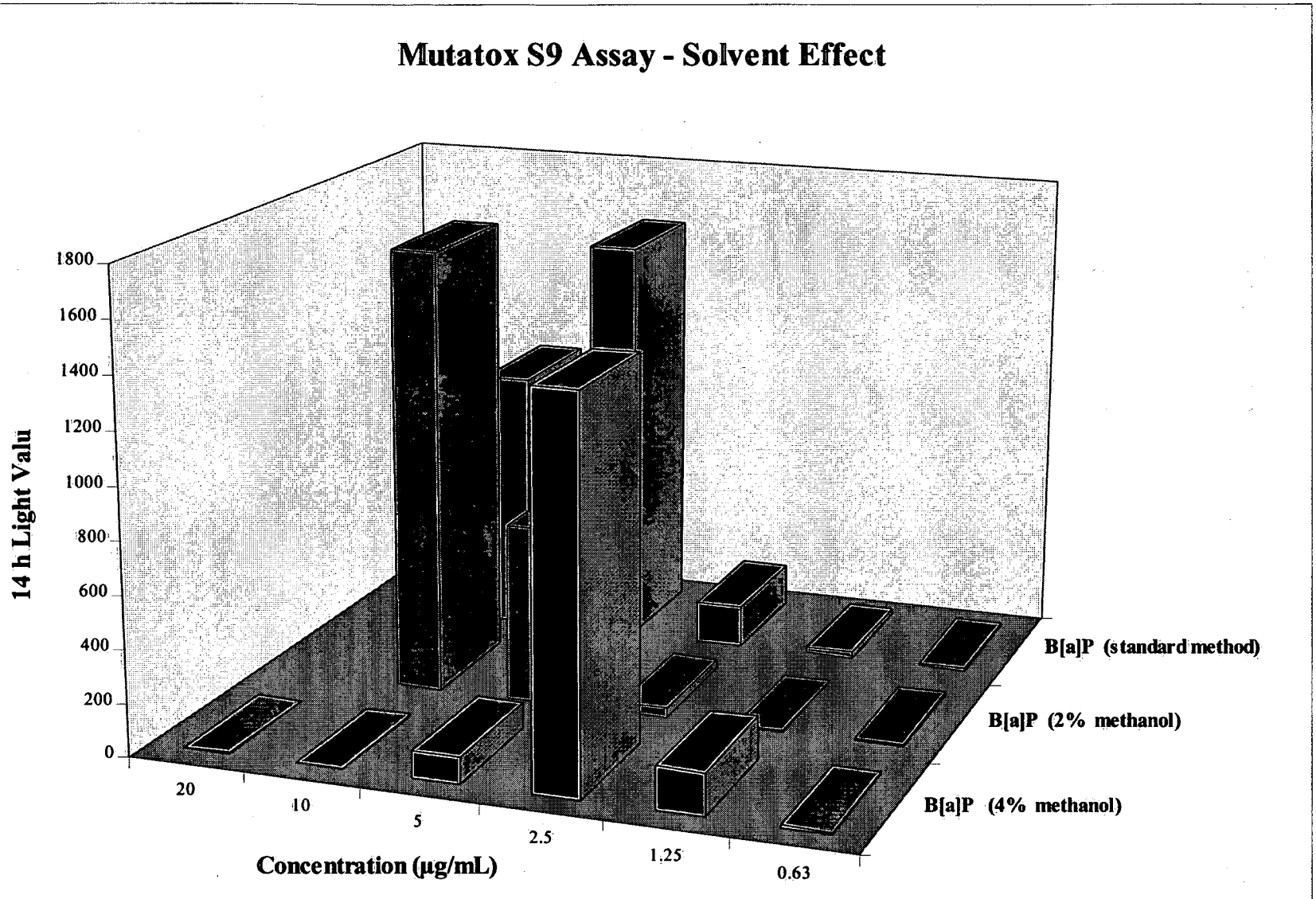


Figure 2. Effects with 2% and 4% methanol in Mutatox test solutions.

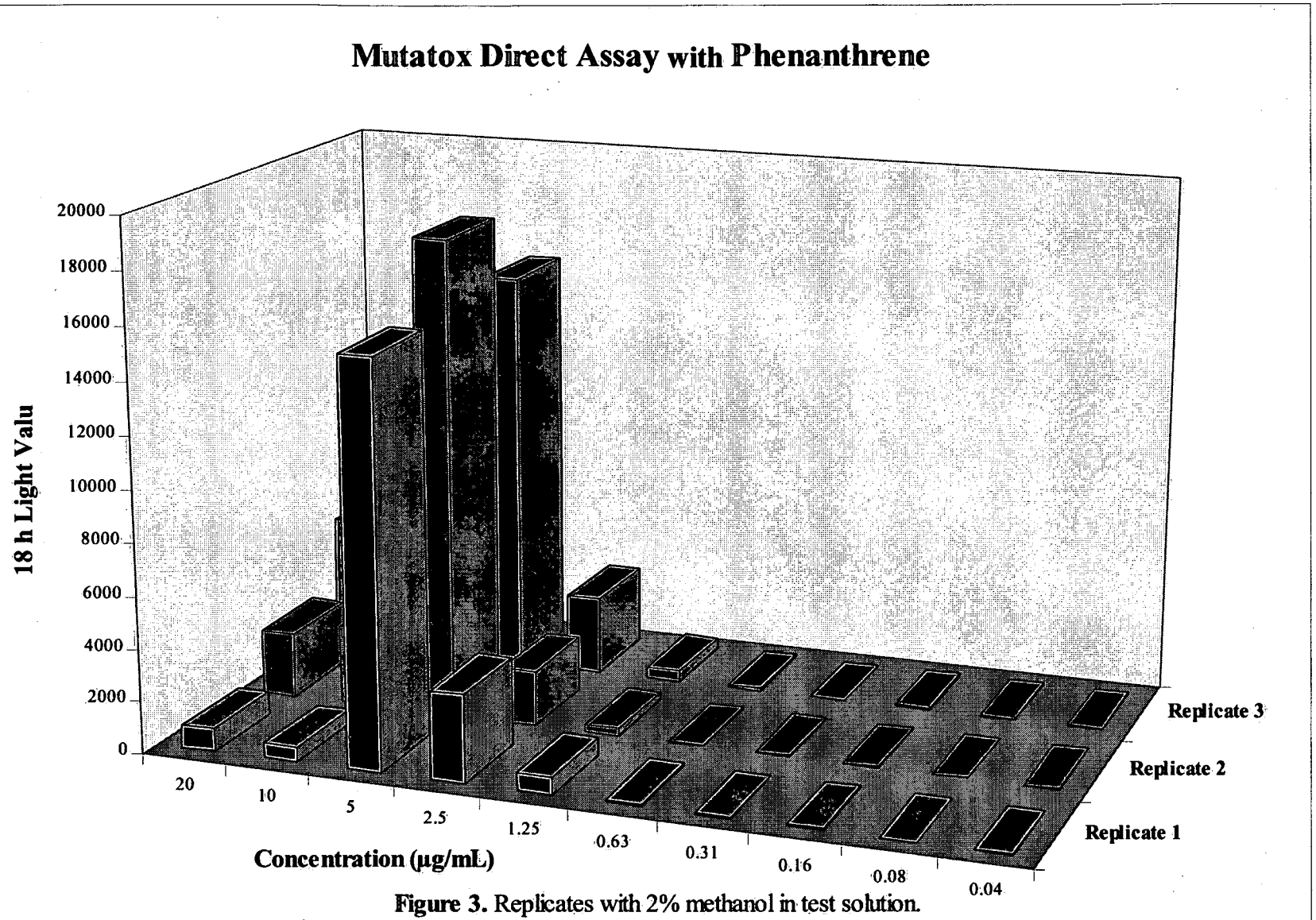


Figure 3. Replicates with 2% methanol in test solution.

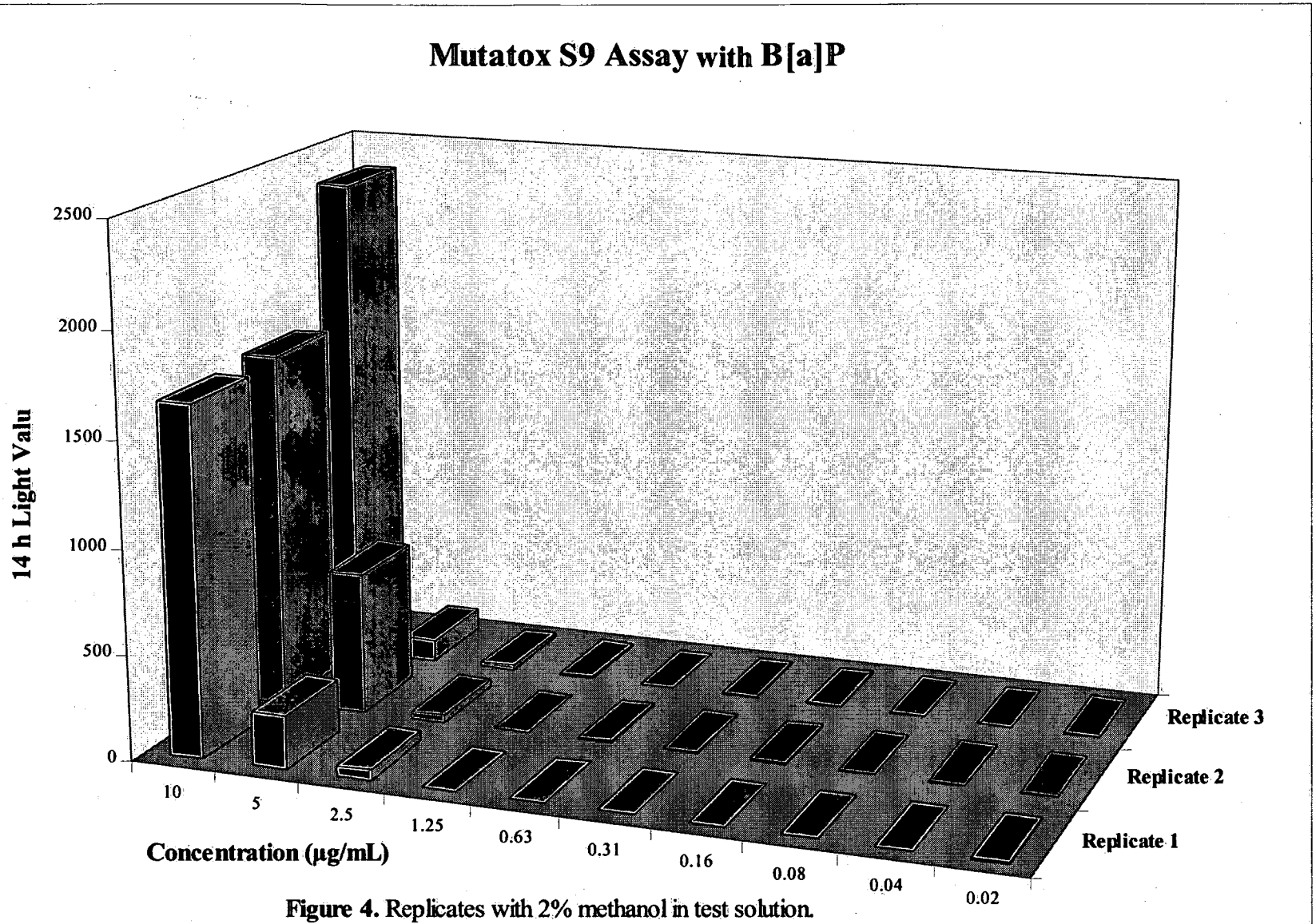


Figure 4. Replicates with 2% methanol in test solution.

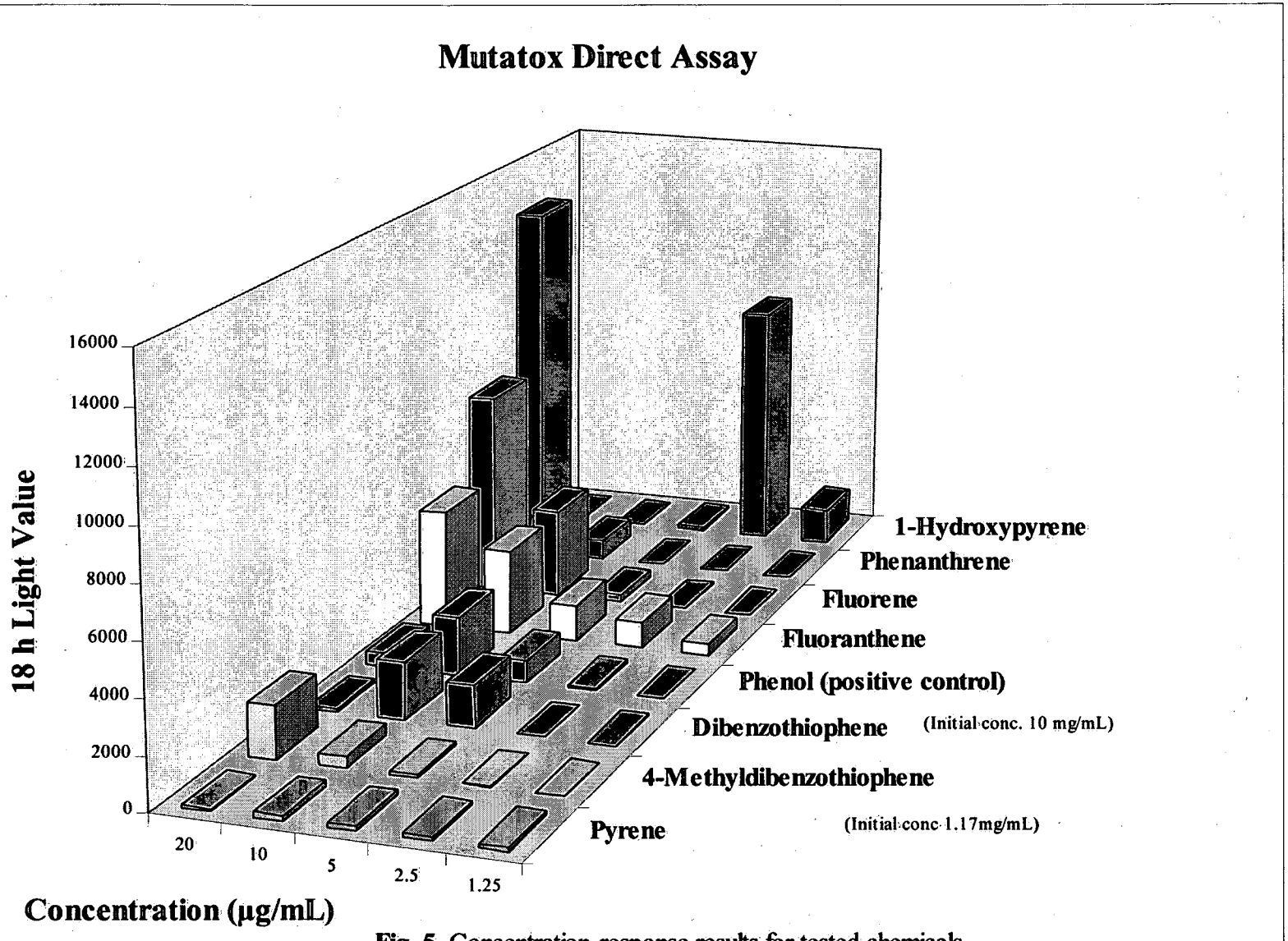


Fig. 5. Concentration-response results for tested chemicals.

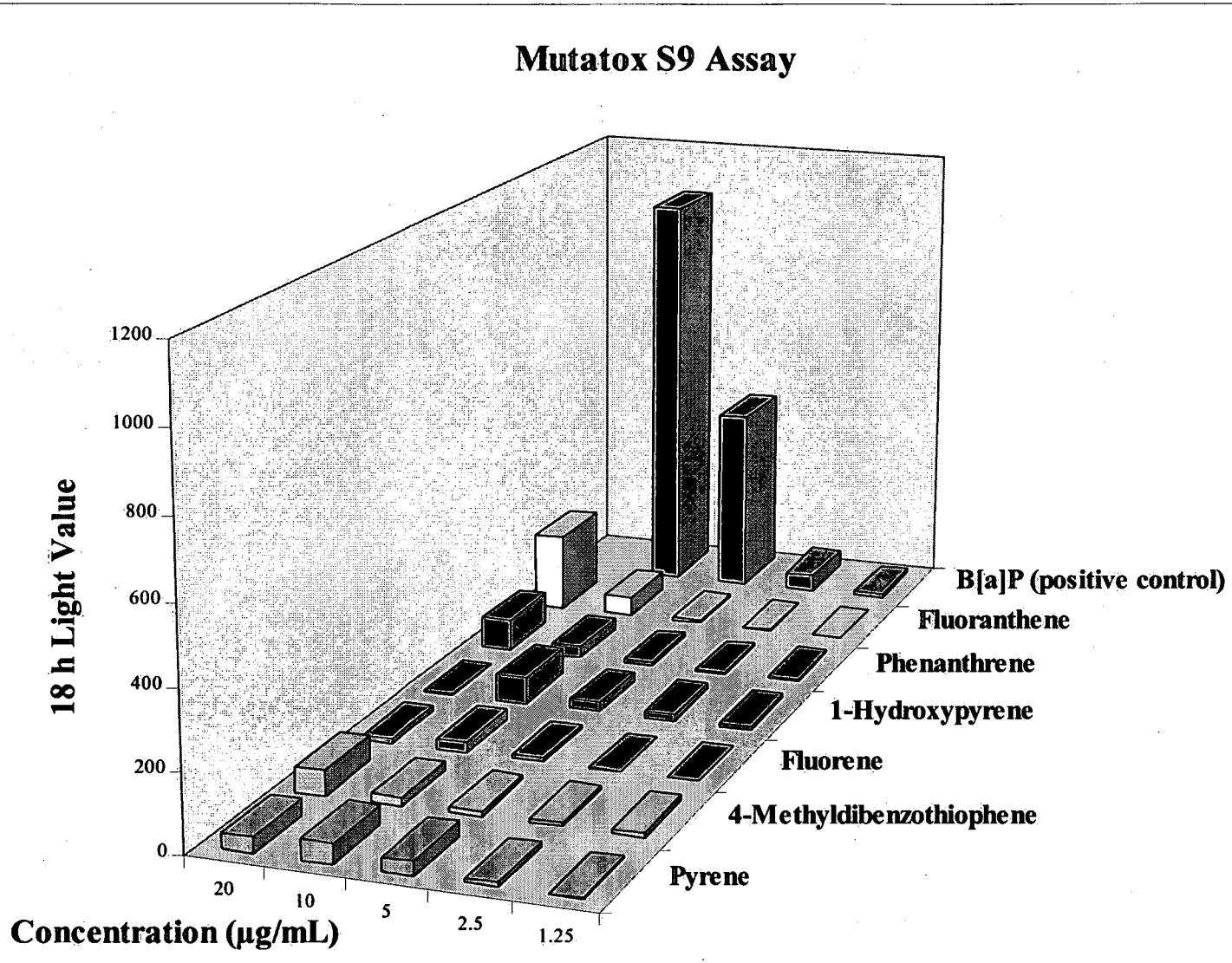
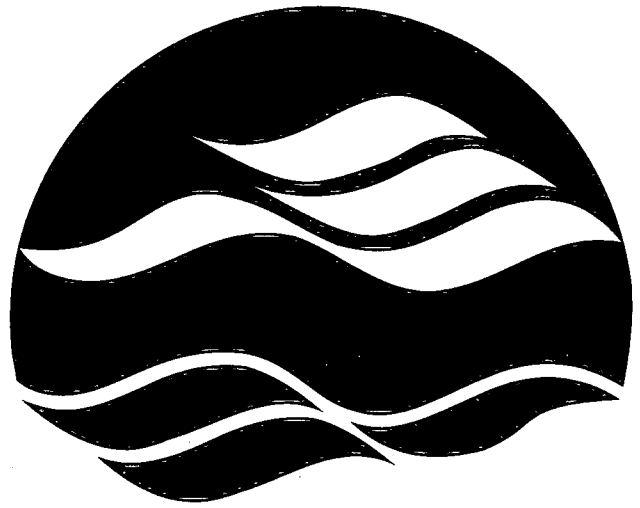


Fig. 6. Concentration-response results for tested chemicals.



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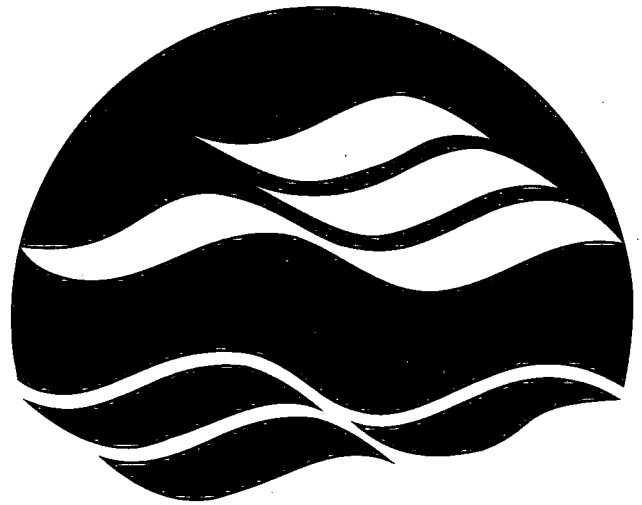

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