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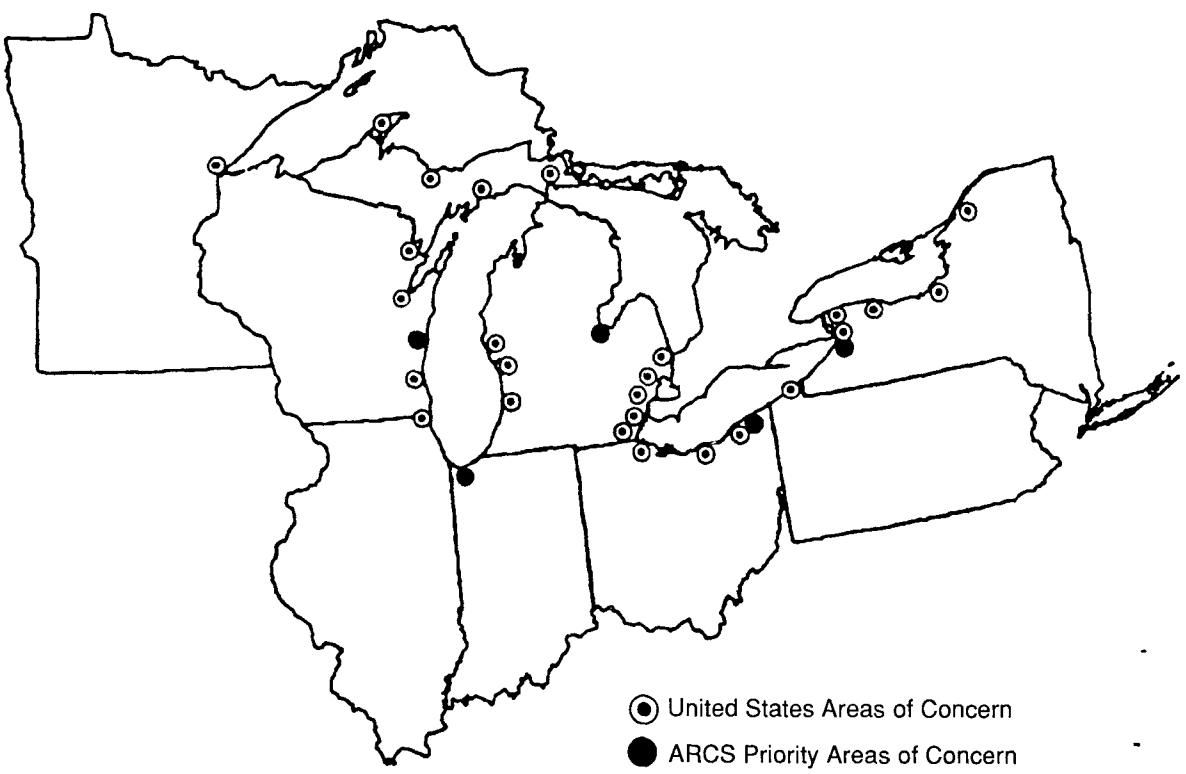
Detection of Genotoxins in Contaminated Sediments: An Evaluation of a New Test for Complex Environmental Mixtures



DETECTION OF GENOTOXINS IN CONTAMINATED SEDIMENTS: AN EVALUATION OF A NEW TEST FOR COMPLEX ENVIRONMENTAL MIXTURES

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DETECTION OF GENOTOXINS IN CONTAMINATED SEDIMENTS: AN EVALUATION OF A NEW TEST FOR COMPLEX ENVIRONMENTAL MIXTURES.

OVERVIEW

This document reviews a new approach to detect genotoxins in contaminated freshwater sediments and summarizes the lessons learned from this investigation of the Great Lakes Basin (Johnson, 1992a, 1992b, 1993a, 1993b). This study was conducted as part of the U.S. Environmental Protection Agency, Great Lakes National Program Office, Assessment and Remediation of Contaminated Sediment (ARCS) Program in cooperation with the National Biological Survey Contaminant Research Center (Johnson, 1993a). The objective was to detect genotoxic chemical contamination in Great Lakes Basin sediments (Bro et al., 1987).

BACKGROUND

In the last few decades, genetic toxicology -- a new discipline -- has emerged with the generally accepted view that some chemicals (genotoxins) can induce DNA damage in cells that may result in lethality, mutagenesis, carcinogenesis, and potential eco-genotoxicological expressions (Wurgler and Kramers, 1992). To ascertain potential environmental hazards, numerous short-term qualitative tests (Epler, 1980; Brockman and DeMarini, 1988; DeMarini et al., 1989) have been developed to detect genotoxic agents. These bioassays, in most instances, were poorly suited for extensive environmental surveys of complex mixtures in sediments because they were not well-adapted to field applications, were costly, and required sophisticated technical expertise. In addition, these bioassays encountered vexing cytotoxicity problems in sample analysis that frequently negated their effective use in complex environmental samples such as sediments. New tests were needed to evaluate environmental mixtures.

One of NBS primary tasks was to develop and explore innovative techniques to detect environmental genotoxins in complex mixtures associated with sediments. A primary objective of this investigation was to search for a new genotoxicity bioassay that was suited for field studies of large geographic areas, that was short-term and cost effective, and that was simple to use. An assay developed by Microbics Corporation[2]

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(Carlsbad, CA), under the trade name Mutatox™, that is currently undergoing trials in laboratories throughout this country (Sun and Stahr, 1993; Ho et al., 1994) and Canada (Khan et al. 1990; Legault and Blaise, 1994) satisfied these criteria. The developmental goals in this project with the Mutatox™ assay were fourfold:

(1) to validate the relative sensitivity and selectivity of the assay using model genotoxins and nongenotoxins in simple, binary and model complex mixtures and to validate its use for biohazard assessment of complex pollutants in freshwater sediments.

(2) to develop a protocol to detect and estimate chemical genotoxins in complex environmental samples;

(3) to use this assay to determine the potential genotoxicity of contaminated sediments (environmental samples) from selected areas of concern (AOC) sites in the Great Lakes Basin; and

(4) to compare Mutatox™ performance for sensitivity, utility, and cost with the well-validated *Salmonella* -- microsome mutagenicity test, frequently referred to as the Ames test (Ames et al., 1973; Maron and Ames, 1983).

This report relates a series of unique events -- protocol development, validation, and field application --in the exploration of a genotoxicity assay for use in complex mixtures. No effort was made to test any other short-term assays to detect genotoxins. The Ames test served as a benchmark for comparisons. This report focused only on a new bioluminescent procaryotic bioassay --- Mutatox™ with its specific application to detect genotoxins in complex environmental mixtures. These were the highlights of the findings.

MUTATOX™ ASSAY: THEORY

The Mutatox™ assay detects genotoxins with a dark mutant strain of the luminescent bacterium *Photobacterium phosphoreum*. DNA-damaging substances are recognized by measuring the ability of a test sample to restore the luminescent state in the bacterial cells (Johnson, 1992a). Light produced by luminescent bacteria makes an easy quantitative endpoint in the genotoxicity assay. The amount of light increase indicates the genotoxicity of the sample. Various genotoxins, base-substitution or frame-shift, DNA synthesis inhibitors, and DNA-intercalating agents, have been detected with the Mutatox™ assay (Johnson, 1992a; A. A. Bulich, Microbics Corp., personal communication).

MUTATOX™ ASSAY: PROTOCOL

Mutatox™ protocol is very simple, requiring minimal expertise. The assay may be initiated and completed in less than 24 hours. Prepackaged dehydrated media, freeze-dried bacteria, and standard disposable reaction tubes require only a few hydration, mixing, and dilution steps and eliminate the rigors, tedium and cost of sterile

technique. Bacteria are nonpathogenic, clonal, and require no reisolation or reculturing; in addition, they are completely disposable without causing any environmental harm. Incubation time is short which reduces the possibility of cross-contamination with bacterial contaminants. Quality assurance and quality control are simply maintained because the protocol develops a clear paper trail and the archival tester strains and test media are easily stored for inspection or audit. The volume of the reactants is low; as a result, the quantity of toxic wastes and the cost of their disposal is significantly reduced. The assay is conducted with exogenous metabolic activation, traditionally a rat hepatic microsomal mixture. Results and confirmation of a suspected genotoxic substance are obtained in <24 hours. These operational procedures require minimal technical or microbiological training (Johnson, 1992a, 1993b). Most importantly, Mutatox™ is unlike most genotoxicity assays because the assay is available on demand and requires no preculture of test organisms.

Unlike traditional toxicity tests where lethal concentration or lethal dose is the endpoint, metabolic activation systems are required in many genotoxicity tests. Most environmental genotoxins are found in an inactive state (progenotoxin) and must be metabolically activated to become DNA-damaging substances. The incorporation of a mammalian (rodent) hepatic metabolic activation system -- the postmitochondrial supernatant fraction (commonly referred to as the S9 fraction) -- into a genotoxicity test (Ames et al. 1973) significantly improved the assay's sensitivity to a broader spectrum of genotoxins. The use of fish S9 (Johnson, 1993c) has increased the ecological relevancy of these tests in aquatic ecosystems. This addition of a hepatic metabolic activation system has become an important milestone in the development of environmental genotoxicity testing (Brusick, 1990).

MUTATOX™ ASSAY: VALIDATION

Validation experiments for Mutatox™ were performed with selected EPA priority pollutants (Callahan et al. 1979; Richards, D.J. and W.K. Shieh. 1986.). The assay detected the priority pollutants that are known to be found in organic sediment extracts from complex environmental samples (Jacobs, 1993). For example, 2-aminoanthracene (2-AA) and benzo(a)pyrene (BaP) were dose-responsive with a maximum detected concentration (MDC) of 5 μg and 2.5 μg, respectively; a lowest detected concentration (LDC) of 0.07μg and 0.07μg respectively; and dose-response numbers of 7 and 6 respectively (Figs.1 and 2). A chemical was identified as genotoxic when there were three or more responses in each dilution series (dose-response number ≥3). In general, the sensitivity of the Mutatox™ assay to these priority pollutants was ≤1 μg/cuvette (Table I). A partial list of chemicals evaluated with Mutatox™ and Ames is compared in Table II.

Mutatox™ validation experiments delineated the assay's relative spectrum of detectability, focused on pollutants of interest -- PAH types, and confirmed the ability of Mutatox™ to detect expected genotoxins that could be encountered in contaminated

sediments. Selection of specific models was based on sediment residue work of Jacobs et al. 1993 and the EPA pollutant priority series (Callahan et al. 1979; Richards, D.J. and W.K. Shieh. 1986). Limited experiments were performed to simulate the interactions of complex mixtures and their potential influence on genotoxin detection. The reader is cautioned that it is axiomatic in toxicology that there is always toxicity in dosage. The term cytotoxicity is used in the traditional sense: lethality to the test cell. Cytotoxicity is described here in relationship to sample doses and observed genotoxicity responses. As expected, *Photobacteria* do show cytotoxicity to environmental pollutants; however, they seem less effected than other bacterial tester strains. It must be remembered that some environmental samples contain several cytotoxic substances, which may or may not also be genotoxic, that may potentially interfere with an assay's sensitivity.

MODEL COMPLEX MIXTURES

Genotoxins

Binary mixtures of four pollutants (2-AA + 2-aminofluorene (2-AF), 2-AA + BaP, 2-AA + pyrene (PY), 2-AF +BaP, 2-AF + PY, and BaP + PY) at concentrations of 10 to 0.6 μg/cuvette showed no evidence of inhibitory interactions (Table III) (Johnson, 1992b).

Non-genotoxins

The model complex mixture of carbofuran, di-2-ethylhexyl phthalate, malathion, simazine, permethrin, and Aroclor 1254, representing six classes of potential aquatic contaminants (both pesticides and industrial sources), showed no genotoxic response or cytotoxicity at test doses of ≤10 μg/cuvette, nor did the mixture interfere with the genotoxic expression of known progenotoxins (Table III) (Johnson, 1992b).

ENVIRONMENTAL SAMPLES: ORGANIC SEDIMENT SAMPLES

Thirty-eight sediment samples were collected from 28 sites in three Great Lakes priority AOCs (Grand Calumet River in Indiana, Buffalo River in New York, and Saginaw River in Michigan), extracted with the solvent dichloromethane, and evaluated for genotoxicity with the Mutatox™ assay (Johnson, 1992b). For example, 210 genotoxic measurements were made from seven sites along the Grand Calumet River. All grab samples collected in August 1989 were genotoxic, with an average 5.5 (0.8) dose-response number/site (Fig. 3). The MDC detected ranged from 50 to 12 mg eq. sediment/mL and the LDC ranged from 0.7 to 0.09 mg eq. sediment/mL -- a single data set of site six in shown in Figure 4. Similar samplings were taken from Buffalo River in New York and the Saginaw River in Michigan (Johnson, 1992b). The Saginaw extracts (Fig. 5) demonstrated various genotoxic responses with two sites designated "Negative," one "Suspect," and four "Genotoxic." The Mutatox™ assay clearly

demonstrated the ability to detect environmental genotoxins in complex environmental mixtures. Cytotoxicity was not observed in either spot-plate or tube turbidity tests; positive controls (2-AA, 2-AF, BaP and PY) were within acceptable sensitivity limits. All findings are reported as mean • standard deviations. These data show that large geographic areas can be sampled for Mutatox™ determination of environmental hazards.

QUALITATIVE TOXICITY TESTING

Mutatox™ is a qualitative toxicity test. The assay produces a yes-no answer to the question: Is the sample genotoxic? Two arbitrary values are used to determine if a test substance is genotoxic. First, a light emission value of or greater than 100 indicates that the single-dilution sample is genotoxic. Second, three or more dilution series responses of or greater than 100 indicate that the sample is genotoxic. The relative light responses are irrelevant because a simple yes or no designation -- a qualitative designation (Figs. 1, 2, and 3) -- is the assay's endpoint. The rationale for qualitative toxicity evaluation is straightforward -- there are no partially genotoxic substances (although there frequently are suspects, samples that may require additional testing.).

Other toxicity tests measure multiple organisms over time in a series of chemical concentrations to determine the lethality of an aquatic community. The resulting dead or immobile organisms are easily quantified, usually in the form of a 50% effect: e.g., the EC50 (Effective Concentration) or LC50 (Lethal Concentration). This number is then used to estimate the acute toxicity of the test chemical. These assays provide a quantitative answer.

MUTATOX™ AND AMES TEST COMPARISON

The Mutatox™ assay compared favorably with the Ames *Salmonella* Mutagenicity Test (Tables II and IV)(Johnson, 1992a). Parallel Mutatox™ and Ames bioassays with EPA priority pollutants and other model genotoxins showed comparable spectra of sensitivity (Table II). In complex environmental mixtures, Mutatox™ assays and Ames tests compared favorably with 96% (27/28) site agreement in detecting evidence of genotoxic substances in all three priority areas: Grand Calumet River, Buffalo River, and Saginaw River (Johnson, 1992b).

Importantly, the Mutatox™ system showed low cytotoxicity in testing complex environmental mixtures (Johnson, 1992b). Toxic chemicals did not induce bacterial cytotoxicity in the Mutatox™ assay at the test dosage. The Mutatox™ tester strain *Photobacterium* is not a nutritional auxotroph; therefore, it does not require a tedious and time-consuming confirmation test. In contrast the Ames test needs to demonstrate the reversion from auxotroph to prototroph of the histidine-deficient tester strain *Salmonella.*

Unlike the Ames test, which requires a 12 to 18-hours preincubation period of the tester strain, Mutatox™ is available on demand. The use of prepackaged test materials makes conducting Mutatox™ simpler. Mutatox™ results were obtained in <24 hours whereas the Ames test required ≥68 hours. The liquid wastes from the Mutatox™ assay were only 2% of the volume from the Ames test. The Mutatox™, however, was easier and more rapid to perform with environmental samples and, as a result, more affordable than the Ames test. The three most common indexes of performance -- sensitivity, selectivity, and predictability (Purchase 1982) -- demonstrated that the Mutatox™ assay is a valuable monitoring tool for detection of complex environmental genotoxins, and that it should be considered for routine assessment of contaminant toxicity.

HAZARD EVALUATION: POTENTIAL VERSUS BIOAVAILABILITY OF ENVIRONMENTAL GENOTOXINS

Most toxicological bioassays, procaryotic or eucaryotic, single cell or multicellular, lack an important ingredient: the element of *in situ* exposure to the real world. The best test can only simulate. Most genotoxins recovered from chemically contaminated sediments are mobilized with strong organic solvents, concentrated from large soil samples, and dissolved in assay-compatible solvents. Therefore, genotoxicity findings must be prefaced with the word potential, i.e., existing in possibility, not in actuality.

The bioavailability of genotoxins in freshwater sediments -- how they move in pore water, how they sorb onto sediment components, and how they move through the food-chain -- is still poorly understood and worthy of further investigation. The widespread occurrence of anthropogenic polyaromatic hydrocarbons (PAHs) in the environment and the high sensitivity of Mutatox™ to detect these substances undoubtedly will create difficulties both in terms of scientific and political conclusions for governmental regulators and resource managers in their efforts to eliminate environmental hazards from chemical contaminants. The quantitative and qualitative toxicity testing of organic extracts from contaminated sediment offers only estimates of the true environmental hazards influencing the freshwater ecosystem.

CONCLUSIONS AND RECOMMENDATIONS

Validation experiments showed that Mutatox™ is a useful genotoxicity test with important field applications.

- It is sensitive: environmental genotoxins are detected in the low μg (<10 μg/cuvette) range.
- It is selective: non-genotoxins are differentiated; no false positives were observed with models in simple, complex or environmental mixtures.
- It is predictable: relative sensitivity and selectivity over time remained a

laboratory constant.

- It is rapid: the assay is available on demand; the incubation time is short; results are obtained in <24 hours.
- It is simple; minimal technical or microbiological training is required.
- It is economical; cost of large environmental samplings is minimal.
- It is cost effective; the volume of the reagents is low, reducing the quantity of toxic wastes and the cost of their disposal.
- It is quality assurance and quality control friendly; archival tester strains and test media are easily stored for inspection or audit.

These results suggest that Mutatox™, used with traditional organic extraction methods, shows potential as a tier I genotoxicity screening tool for hazard assessment of large freshwater bodies. The Mutatox™ Test System needs additional field sampling and laboratory testing to confirm its value as an effective and practical screening tool to detect environmental genotoxins.

ACKNOWLEDGMENT

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List of tables and figures.

Table I

List of selected EPA priority pollutants detected with Mutatox™[a] (*Photobacterium*-rat hepatic S9).

acenaphthene
acenaphthylene
2-aminoanthracene
2-aminofluorene
anthracene
benz(a)anthracene
benzo(a)pyrene
chrysene
fluoranthrene
fluorene
naphthalene
phenanthrene
pyrene

a. Sensitivity ≤ 1μg/cuvette

Table II. Partial list of chemicals evaluated with Mutatox and Ames Test for genotoxicity.

Compound	Mutatox	Ames
Aflatoxin B1	Positive	Positive
2-Aminoanthracene	Positive	Positive
2-Aminoflurorene	Positive	Positive
9-Aminoacridine	Positive	Positive
Benzene*	Positive	Negative
Benzidine	Positive	Positive
Benzoin*	Negative	Negative
Benzo(a)pyrene	Positive	Positive
Captan	Positive	Positive
2-Chloroethanol*	Positive	Positive
Cyclophosphamide	Positive	Positive
1,2-Dichloropropane	Positive	Positive
1,3-Dichloropropene	Negative	Positive
Dioxane	Negative	Negative
Ethylene glycol	Negative	Negative
8-Hydroxyquinoline*	Positive	Positive
Lindane	Negative	Negative
Monuron*	Postive	Negative
3-methylcholanthrene	Postive	Positive
Nalidixic acid	Positive	Negative
Pyrene	Postive	Negative

* Designated National Toxicology Program Chemical

Table III

Assay sensitivity and selectivity: Genotoxicity of progenotoxic chemicals[a] in model complex mixtures determined with and without activation in Mutatox™ (*Photobacterium*-rat hepatic S9).

CHEMICAL	TREATMENT		
	HEATED[b] S9	WITHOUT S9	WITH S9
PROGENOTOXINS	ND[c]	ND	GENOTOXIC
NON-GENOTOXINS[d]	ND	ND	ND
PROGENOTOXINS + NON-GENOTOXINS	ND	ND	GENOTOXIC
CONTROL[e]	ND	ND	ND
CONTROL[f]	ND	ND	ND

[a]Progenotoxins: As single and binary mixtures: (2-aminoanthracene (2-AA) + 2-aminofluorene (2-AF), 2-AA + benzo(a)pyrene (BaP), 2-AA + pyrene (PY), 2-AF + BaP, 2-AF + PY, and BaP + PY).

[b]Boiling water for 15 seconds.

[c]ND = not detected (genotoxic)

[d]Non-genotoxins: complex mixture of carbofuran (carbamate insecticide), di-2-ethylhexyl phthalate (plasticizer), malathion (organophosphate insecticide), simazine (triazine herbicide), permethrin (synthetic pyrethroid insecticide) and Aroclor 1254 (PCB product).

[e]Control sediment = methylene chloride sediment extract (Florissant, MO)

[f]Control solvent = dimethylsulfoxide

Table IV
Comparison: Mutatox™ assay and Ames test[a].

	MUTATOX™	AMES
TEST ORGANISM	*Photobacterium*	*Salmonella*
BACTERIAL REQUIREMENT	One isolate	Usually one to four isolates
ENDPOINT	Light emission	Colony formation
EXOGENOUS ACTIVATION	Optional	Optional
TEST DURATION	16-24 h	48-72 h
TEST TEMPERATURE	23 ± 2°C	37°C
RELATIVE SENSITIVITY[b]	≤1.0 μg/tube	≤1.0 μg/plate
STERILITY	Optional	Essential
PROCEDURE	Simple	Complex
INSTRUMENTATION	Luminometer	Particle counter[c]
COST Labor Materials Disposal	Low	High
SCIENTIFIC DEVELOPMENT	Validation	In common use[d]

a. *Photobacterium*-activation and *Salmonella*-activation genotoxicity assays.

b. Rat S9 activation with 2-acetamidofluorene, aflatoxin B1 , 2-amino-anthracene, 2-aminofluorene, 2-aminonapthalene, benzo(a)pyrene, 3-methylcholanthrene, and pyrene.

c. Particle counter is essential for enumeration of large samples.

d. Extensive literature and validation.

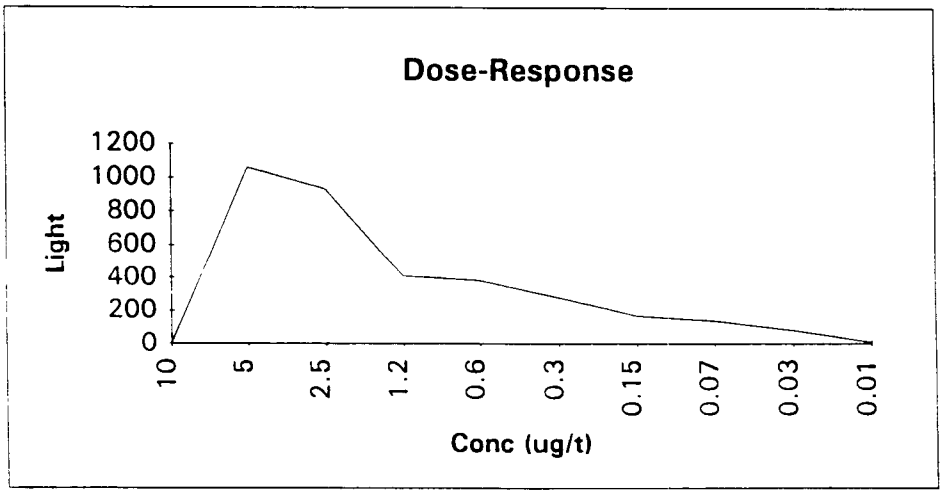
Fig. 1. Genotoxicity of 2-aminoanthracene (2-AA) determined with Mutatox.

[Raw Data Set: Light Emission Values Recorded]

2-Aminoanthracene

Ten-tube dilution series (microgram per cuvette)

Concentration	10	5	2.5	1.2	0.6	0.3	0.15	0.07	0.03
2-AA	0	1060	930	410	380	280	170	140	80
Control	0	2	2	3	3	3	5	6	6



Summary:

Maximum detected concentration = 5 micrograms per cuvette

Lowest detected concentration = 0.07 microgram per cuvette

Dose-response number (DRN) = 7

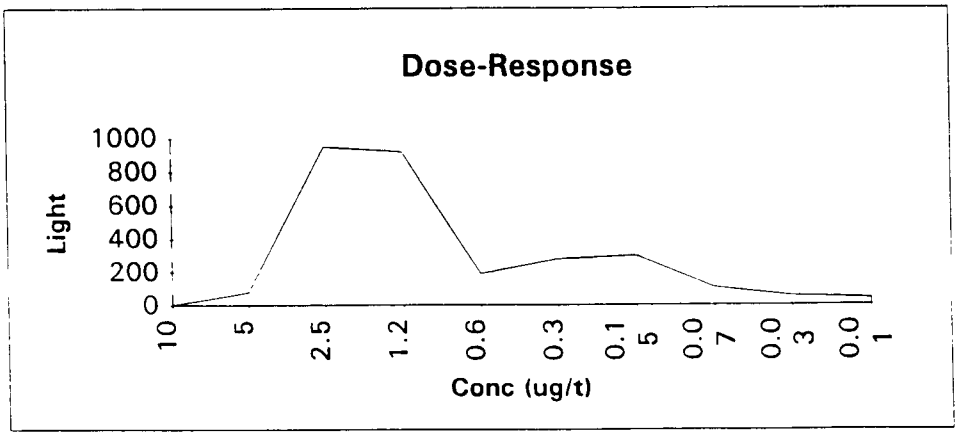
Conclusion: 2-Aminoanthracene is genotoxic.

Fig. 2. Genotoxicity of benzo(a)pyrene (B(a)P) determined with Mutatox.

[Raw Data Set: Light Emission Values Recorded]
Benzo(a)pyrene

Ten-tube dilution series (microgram per cuvette)

Concentration	10	5	2.5	1.2	0.6	0.3	0.15	0.07	0.03
B(a)P	0	80	950	920	190	280	300	106	56
Control	0	2	2	3	3	3	5	6	6



Summary:

Maximum detected concentration =2.5 micrograms per cuvette

Lowest detected concentration = 0.07 microgram per cuvette

Dose-response number (DRN) = 6

Conclusion: Benzo(a)pyrene is genotoxic.

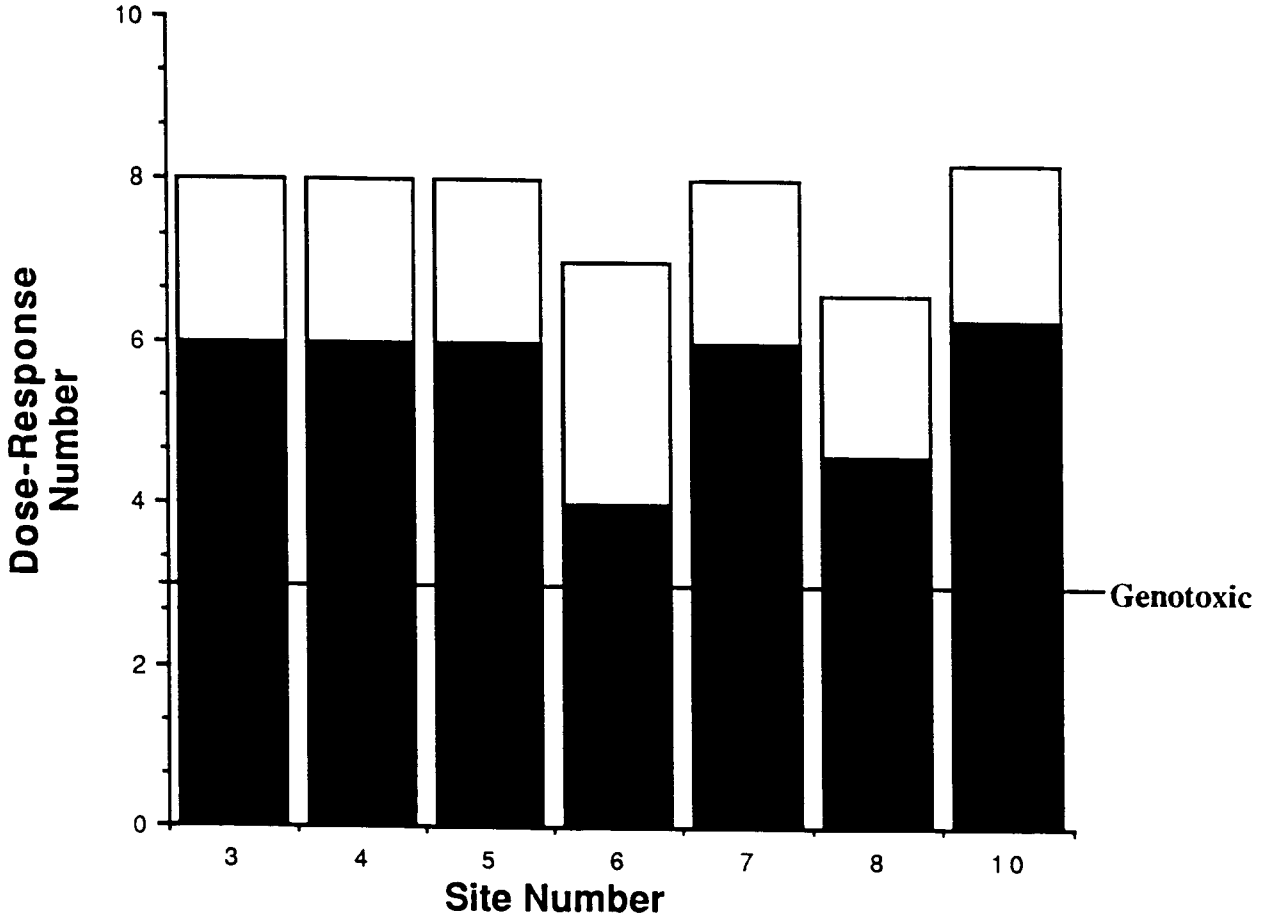


Fig. 3. Genotoxicity of sediment extracts from Grand Calumet River in Indiana determined with Mutatox (sensitivity ≤ 1 μg/tube.). Dose-response number = the mean (dark bar) of three replicates of a ten-tube dilution series with standard deviation (white bar).

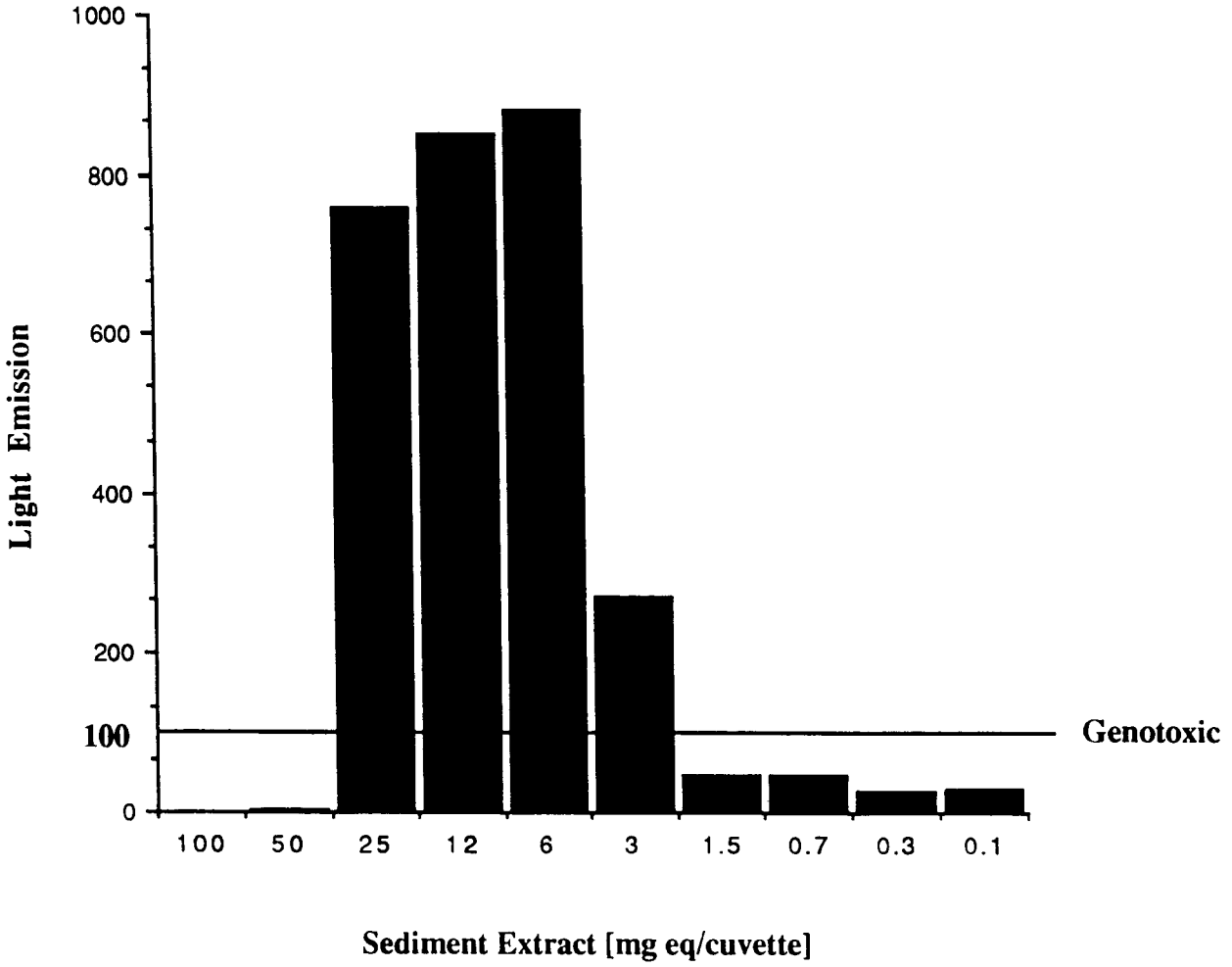


Fig. 4. Single genotoxicity data set of sediment extracts from site 6 of Grand Calumet River in Indiana determined with Mutatox.

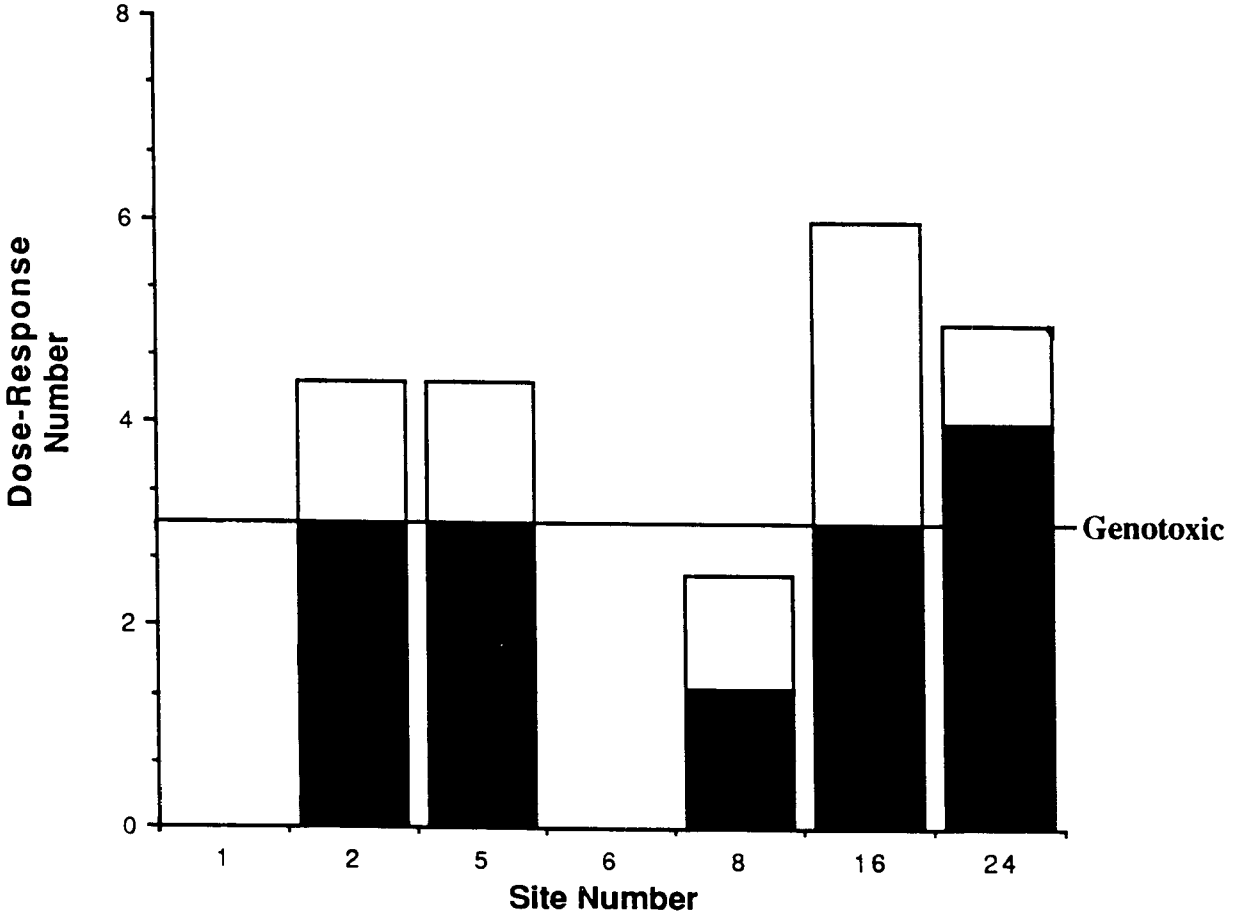


Fig. 5. Genotoxicity of sediment extracts from Saginaw River in Michigan determined with Mutatox (sensitivity ≤ 1 μg/tube.). Dose-response number = the mean (dark bar) of three replicates of a ten-tube dilution series with standard deviation (white bar).