(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
21 October 2004 (21.10.2004)

PCT

(10) International Publication Number
WO 2004/090156 A2

(51) International Patent Classification[7]: C12Q 1/00

(21) International Application Number:
PCT/GB2004/001578

(22) International Filing Date: 8 April 2004 (08.04.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0308546.1 11 April 2003 (11.04.2003) GB

(71) Applicant *(for all designated States except US)*: **CROWN VISION SYSTEMS LIMITED** [GB/GB]; 14 Bleriot Crescent, Whiteley, Fareham, Hants PO15 7JD (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **LEHARNE, Steven** [GB/GB]; Crown Vision Systems Limited, 14 Bleriot Crescent, Whiteley, Fareham, Hants PO15 7JD (GB). **NAGLER, Martin** [GB/GB]; Crown Vision Systems Limited, 14 Bleriot Crescent, Whiteley, Fareham, Hants PO15 7JD (GB). **NEAL, Gordon** [GB/GB]; Crown Vision Systems Limited, 14 Bleriot Crescent, Whiteley, Fareham, Hants PO15 7JD (GB). **EZZI, Mufaddal** [GB/GB]; Crown Vision Systems Limited, 14 Bleriot Crescent, Whiteley, Fareham, Hants PO15 7JD (GB). **COKER, Raymond** [GB/GB]; Crown Vision Systems Limited, 14 Bleriot Crescent, Whiteley, Fareham, Hants PO15 7JD (GB). **BELL, Edward** [GB/GB]; Crown Vision Systems Limited, 14 Bleriot Crescent, Whiteley, Fareham, Hants PO15 7JD (GB). **ROTHER, Joachim** [GB/GB]; Crown Vision Systems Limited, 14 Bleriot Crescent, Whiteley, Fareham, Hants PO15 7JD (GB). **PHILLIPS, Pamela** [GB/GB]; Crown Vision Systems Limited, 14 Bleriot Crescent, Whiteley, Fareham, Hants PO15 7JD (GB). **DAVIES, Lyn** [GB/GB]; Crown Vision Systems Limited, 14 Bleriot Crescent, Whiteley, Fareham, Hants PO15 7JD (GB). **ANDREOU, Michael** [GB/GB]; Crown Vision Systems Limited, 14 Bleriot Crescent, Whiteley, Fareham, Hants PO15 7JD (GB). **SOARED, Fernando** [GB/GB]; Crown Vision Systems Limited, 14 Bleriot Crescent, Whiteley, Fareham, Hants PO15 7JD (GB).

(74) Agent: **BROWN, David, Leslie**; Haseltine Lake, Imperial House, 15-19 Kingsway, London WC2B 6UD (GB).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2004/090156 A2

(54) Title: TEST SYSTEM

(57) Abstract: An assay is provided for assaying a sample of soil, sand, sediment or other particulate material for the presence of contaminants such as polycyclic aromatic hydrocarbons (PAHs), organic pesticides, petroleum hydrocarbons, or polychlorinated biphenyls (PCBs), and preferably comprises: extracting an assayable amount of the contaminant from the particulate material into a water-miscible solvent, such as an organic alcohol, capable of dissolving the contaminant, the solvent optionally containing a surfactant; mixing the resultant solvent solution of the contaminant with water and optionally a surfactant, for example to a dilution factor between about 10 and about 25, whereby a mixture is obtained containing water, solvent, surfactant and any extracted contaminant; and exposing the bioluminescent organism *Vibrio fischeri* to the mixture under conditions in which the inhibition, by the contaminant, of light emitted by the organism can be related to the presence of the contaminant in the mixture. The method is sufficiently quantitative that it can be determined whether the contaminant is present in the particulate material at a concentration above or below a certain - e.g. a legally specified - level. A test kit is provided, whereby the method can be performed in the field and the result showing whether the contaminant is present at a legally acceptable or unacceptable level can be displayed.

TEST SYSTEM

Field of the Invention

The present invention relates to a test system, and more particularly to an assay method for testing samples of soil and the like for the presence of toxic contaminants. The invention also relates to test kits, particularly portable field kits and consumables, for carrying out the novel assay.

Background of the Invention

Modern environmental legislation and awareness have created a need for ever more sophisticated assays for testing samples of soil, sand, sediments and other particulates quantitatively or qualitatively for the presence of toxic contaminants.

Bioassays are generally known, in which a chemical to be assayed is exposed to an organism which can generate a detectable signal which is inhibited in the presence of the chemical. In a particularly preferred example, the signal is emitted light in the form of bioluminescence.

In *Wat. Res.*, Vol. 14, pp. 3448-3456 (2001), the disclosure of which is incorporated herein by reference, Jennings *et al.* evaluated three commercial bioassay systems based on the inhibition of bioluminescence by water-soluble chemical contaminants, the bioluminescent organism being the marine bacterium *Vibrio fischeri*. However, the study deliberately excluded chemicals which were "virtually insoluble in water and those with a solubility considerably less than the predicted EC50" (p. 3449, column 1, final paragraph). The authors admitted that "solubility in water was the limiting factor in determining the selection of chemicals" (p. 3450, column 1, "Results", paragraph 1).

Environmental particulates such as soil, sand and sediments can include a range of substantially water-insoluble organic contaminants, especially polycyclic aromatic

hydrocarbons (PAHs), organic pesticides and polychlorinated biphenyls (PCBs), and there is a need for such contaminants to be assayable in a sufficiently reliable and preferably quantitative manner, particularly using a method which can be used in the field as well as the laboratory.

Traditional assay methods for such substantially water-insoluble contaminants have used the Soxhlet extraction procedure to obtain an assayable sample from the particulate material in high yield. In this extraction procedure, a suitable organic solvent is contacted with the particulate material over a period of about 6 to 16 hours in a Soxhlet apparatus. As is well known in the art, the Soxhlet apparatus is a complex piece of laboratory equipment, in effect a refluxing system which is arranged to pass heated solvent vapour from a solvent reservoir to a reflux condenser at the top of the apparatus, to drip pure solvent from the reflux condenser onto the sample held in a receptacle, and to periodically return contaminant-containing solvent from the receptacle into the solvent reservoir. The result is a gradually increasing concentration of the contaminant in the solvent reservoir, which tends to a maximum as the extraction nears completion.

In *Chemosphere*, Vol. 37, Nos. 14-15, pp. 2895-2909 (1998), the disclosure of which is incorporated herein by reference, Guzzella describes the use of two of the commercial bioluminescent assay systems (Microtox and LUMIStox) evaluated by Jennings *et al.*, in assays of contaminated sediment from the banks of the River Po in Italy. With the aim of accessing the organic contaminants, Guzzella transported sediment material to the laboratory at 4°C, lyophilised the material, sieved it, Soxhlet extracted for 8 hours with 1:1 acetone:hexane, controlled solvent exchanged with dimethyl sulphoxide (DMSO), and subsequently diluted the DMSO solution with Microtox diluent (a 2% aqueous sodium chloride solution) to reduce the DMSO concentration to the sub-lethal level of 0.5% for exposure to the bioluminescent organism. The procedure used is slow, complex and expensive, and does not offer a realistic basis for a simple field extraction technique that would make bioluminescent assays readily available for assaying organic contaminants in particulate materials.

In *Environmental Toxicology and Chemistry* 12, pp. 615-625 (1993), the disclosure of which is incorporated herein by reference, Ho *et al.* describe the use of methanol, acetone and acetonitrile as possible solvents for extracting toxins from soil, using the Microtox bioassay as an end-point. The authors claimed to find that acetone provided the most toxic extracts and that acetonitrile and methanol gave extracts that had similar degrees of toxicity to each other. However, there is insufficient data to identify the cause of the observed toxicity.

We have now found, surprisingly, that a bioassay can be used for testing soil samples for contaminants when a particular extraction system is used prior to exposure of the extracted contaminant to the organism.

Brief Description of the Invention

In a first aspect, the present invention provides a bioassay method for testing a sample of soil, sand, sediment or other particulate material for the presence of a contaminant, comprising: extracting an assayable amount of the contaminant from the particulate material into a water-miscible solvent capable of dissolving the contaminant, the solvent optionally containing a surfactant; mixing the resultant solvent solution of the contaminant with water and optionally a surfactant, whereby a mixture is obtained containing water, solvent, surfactant and any extracted contaminant; and exposing an organism to the mixture under conditions in which the inhibition, by the contaminant, of a signal generated by the organism can be related to the presence of the contaminant in the mixture.

The method is preferably sufficiently quantitative to yield a result which shows whether the particulate material contains a contaminant loading above or below a certain – e.g. a legally specified - concentration.

The assay method does not preclude a different order of steps in the procedure, or adding additional steps to the recited procedure.

Preferably, the signal is the emission of light by a bioluminescent organism, most preferably *Vibrio fischeri*.

Preferably, the contaminant is one or more substantially water-insoluble organic contaminant or a combination of one or more substantially water-insoluble organic contaminant and one or more relatively water-soluble contaminant.

The mixing of the solvent solution, containing the contaminant and optional surfactant, with the water and optional surfactant preferably results in dilution of the solvent solution of the contaminant. The amount of water mixed with the solution of the contaminant will be selected so as to provide in the mixture to be contacted with the organism an assayable concentration of the contaminant. The amount of water used will preferably be up to about 25 times the volume of the solvent solution of the contaminant being diluted, more preferably between about 10 and about 20 times, and most preferably between about 12 and about 15 times.

The surfactant may be introduced into the extraction system and/or the bioassayable (diluted) mixture at any suitable and effective time in the overall procedure and in any suitable and effective manner and amount. The options will be readily apparent to one of ordinary skill in this art.

The conditions in which the inhibition, by the contaminant, of a signal generated by the organism can be related to the presence of the contaminant in the mixture will preferably include measurement of a corresponding signal generated by a similar organism in a reference mixture, the reference mixture preferably corresponding to the mixture under assay but omitting the contaminant. The difference in the measured inhibition between the mixture under assay and the reference mixture can then be related to the presence or, within the accuracy of the system, concentration of contaminant in the particulate material, by means of calibration data relating the measured signal to the concentration of contaminant

in the mixture under assay and data concerning the amount of particulate material and the volume of the mixture used.

In a second aspect of the present invention, there is provided an apparatus (e.g. a kit) for performing the assay of the first aspect of the invention, the apparatus comprising: a first container containing the water-miscible solvent capable of extracting an assayable amount of a contaminant from a sample of soil, sand, sediment or other particulate material when contacted therewith; a second container containing the surfactant, optionally in aqueous solution; a third container containing the organism; means for detecting the signal generated by the organism; means for relating the signal to the presence of the contaminant in the mixture; and instructions, and optionally guidance indicia on at least one of the containers, for performing the assay according to the first aspect of the invention.

The apparatus preferably further comprises means for determining and displaying whether the contaminant is present above or below a certain – e.g. legally specified – concentration in the particulate material.

The water-miscible solvent is preferably non-aqueous. A relatively small amount of water – i.e. an amount which does not substantially adversely influence the extraction – may, however, be present and may indeed arise naturally from water present in the particulate material. The solvent may be a single compound or a mixture. It is preferred that the method is performed at normal temperature and pressure, e.g. between about 5°C and about 30°C and atmospheric pressure.

The water-miscible solvent and the surfactant should each be substantially less toxic to the organism at the concentration obtained, after dilution, in the mixture to be presented to the organism, than is the organic contaminant at the concentration in which it is present in the same mixture. The water-miscible solvent and the surfactant are preferably each substantially non-toxic to the organism at the concentration obtained, after dilution, in the mixture presented to the organism.

Without wishing to be bound by theory, it is believed that the surfactant serves to prevent the contaminant coming out of solution, e.g. as a precipitate, emulsion, suspension or in any other way, when the extraction solution is diluted for presentation to the organism. This effect and its efficiency and reliability is surprising and is believed to be a primary reason for the relative accuracy and reproducibility observed in the assay of the present invention.

The present invention provides a very robust and simple assay technique for field or laboratory use, whereby a range of contaminants in particulate materials can be measured. The assay is not capable of identifying contaminants in terms of their chemical composition, but that can be done separately using the obtained extract and conventional analytical techniques. Where the assay is used in the laboratory, for example as part of controlled tests, the identity of the contaminants may anyway already be known.

Detailed Description of the Invention

The Water-Miscible Solvent

Suitable solvents will typically have an LD_{50} on oral administration to rats of at least about 1500mg/kg, preferably greater than about 2000mg/kg, and be officially classed as non-hazardous and non-toxic.

Examples of suitable water-miscible solvents are organic alcohols, more preferably those containing two to eight carbon atoms. Alkyl mono-ols containing three to six carbon atoms are generally preferred. Iso-propanol is particularly mentioned.

Dimethyl sulphoxide (DMSO) may be present in admixture with the organic alcohol, for example in a volume ratio alcohol:DMSO between about 25:75 and about 75:25, more preferably between about 50:50 and about 70:30, and most

preferably about 60:40. Such a mixture is found to improve the extraction efficiency in comparison with the organic alcohol alone.

Furthermore, and surprisingly, the presence of DMSO reduces the toxicity of the solvent system in comparison to what would have been expected having regard to the individual toxicities of the organic alcohol and the DMSO, and may also increase the sensitivity of the organism to the contaminant.

The Surfactant

Any suitable surfactant may be used, provided it has the ability to prevent the contaminant coming out of solution on dilution, and the required low biotoxicity relative to the contaminant at the concentrations to which the organism is exposed, as explained above.

Suitable surfactants will typically have an LD_{50} on oral administration to rats of at least about 2000mg/kg, preferably greater than about 3000mg/kg, and be officially classed as non-hazardous and non-toxic.

The surfactant will preferably be substantially biodegradable, by which is meant at least about 80% primary biodegradability under the Strum test, OECD 301B.

Examples of suitable surfactants include, for example, fatty alcohol ethoxylates, alkylamine ethoxylates, mixtures of fatty alcohol ethoxylates and alkylamine ethoxylates, polyoxyalkylene polymers (e.g. block copolymers of more than one alkylene oxide) and any combination thereof. Poloxamer surfactants are particularly suitable. These comprise polyoxyethylene-polyoxypropylene block copolymers (typically OE_x-OP_y-OE_x) in a wide range of oxyethylene:oxy-propylene (OE:OP) weight ratios (deriving from the selected x and y block lengths), e.g. between about 90:10 and about 10:90, between about 80:20 and about 20:80 or between about 70:30 and about 30:70, w/w OE:OP. The surfactants Biononex (a mixture of fatty alcohol ethoxylates and alkylamine ethoxylates),

Lutrol F68 (INCI name "Poloxamer 188", available from BASF, an approximately 80:20 w/w OE:OP polyoxyethylene-polyoxypropylene block copolymer) and Lutrol F127 (INCI name "Poloxamer 407", available from BASF, an approximately 70:30 w/w OE:OP polyoxyethylene-polyoxypropylene block copolymer) are especially mentioned.

The surfactant may be used at an amount of less than about 5% by weight relative to the water, more preferably less than about 2% by weight, e.g. about 1% by weight of the water.

Possible Additional Components

The diluted mixture containing the contaminant in preparation for the bioassay may if desired include one or more additional components, typically solutes. Such additional components may be added individually or together to the mixture, to components thereof, or during the mixing. For example, the water used to dilute the solvent solution of the contaminant may suitably include salts (e.g. about 2% sodium chloride) to prevent osmotic stress on the bioluminescent organism.

The Extraction Method

Solvent Extraction

The water-miscible solvent is preferably contacted with the particulate material at a relative amount in the range between about 10 and about 30 ml per 10g of particulate, more preferably about 20ml per 10g. The contacting preferably takes place in a closed container such as a glass or plastic bottle, suitably under vigorous shaking for a period of up to about 5 minutes, e.g. about 2 minutes, until the extraction is substantially complete.

It is preferred that substantially (e.g. at least about 80%) complete extraction of the contaminant should be achieved under normal temperature and pressure by simple contact between particulate and solvent in a hand-held container for a period of a few minutes, e.g. between about 1 and about 5 minutes, if necessary with vigorous shaking of the container.

The resultant contaminant solution may optionally be separated from the particulate material before further handling. This will avoid turbidity, which would interfere with the assay.

Separation of the contaminant solution from the particulate material may suitably be achieved by allowing the particulate material to settle in the solution, e.g. for a period of up to about 5 minutes, and removing a sample of the supernatant solution to a fresh container, e.g. using a pipette or other conventional separation method or device.

Dilution

The diluent (i.e. the water and any optional surfactant) is preferably used in a considerable excess with respect to the amount of the extract (i.e. the solvent solution of the contaminant), e.g. a volume ratio of diluent to extract in excess of 10:1, e.g. in excess of 12:1, suitably up to about 30:1 or about 25:1, and preferably in the range of about 12: 1 to about 20:1, e.g. about 14:1 or 15:1. This degree of dilution has been found to be satisfactory, to reduce the concentration of the solvent and the surfactant to sub-toxic levels while retaining the concentration of the contaminant at assayable levels.

It is to be noted in particular, that the use of DMSO in the solvent has been found to be effective at reducing the extent of dilution required. Without DMSO, a relatively greater degree of dilution - e.g. around 25-fold instead of around 15-fold – may typically be required.

The exact level of dilution may need to be selected according to the contaminant, its concentration, and the particular bioluminescent organism and its conditions. However, such selection will be well within the capacity of one of ordinary skill in this art.

As a preferred resultant mixture containing the contaminant, we provide in the present invention an aqueous medium comprising, and preferably consisting essentially of, about 3% to about 5%, e.g. about 4%, by volume iso-propanol, about 2% to about 4%, e.g. about 3%, by volume DMSO, about 0.5% to about 1.5%, e.g. about 1%, by volume surfactant, and the balance water and the contaminant and optionally a salt (preferably sodium chloride, and preferably at a concentration of between about 1% and about 3%, e.g. about 2%, by weight). This composition of matter is believed to be novel as such, and constitutes a further feature of the present invention.

The same composition of matter, but without the contaminant in any effective amount, will be used as a reference mixture in the assay, as described below. Such a composition is also novel as such, and constitutes a further aspect of the present invention.

After dilution of the extract with the diluent, the mixture is preferably thoroughly mixed, e.g. by vigorous shaking for a short period of time (e.g. up to about 30 seconds).

This vigorous mixing is likely to froth the mixture, due to the presence of the surfactant, and it may be necessary to allow the froth to subside. When this has happened, typically within two minutes, a suitable amount of the dilute aqueous assayable medium may be withdrawn and contacted with the organism in a suitable container.

The Bioluminescent Organism

A number of bioluminescent organisms are known, which can provide the basis for a bioluminescent assay. Examples of bioluminescent bacteria include *Photobacterium* species such as *P. splendidum, P. mandapamensis, P. phosphoreum*, *Vibrio* species such as *V. fischeri, Lucibacterium* species such as *L. harveyi* and *Achromobacter*. Some of this nomenclature may be interchangeable as a result of taxonomic revision. In addition to the various bacteria, other types of microorganisms are known to exhibit luminosity, such as, for example, certain marine dinoflagellates such as *Noctiluca* and *Gonyaulax*. Certain varieties of fungi (*Basidiomycetes*) also exhibit luminescence, including for example *Armilleria mellea, Panus stipticus, Mycena polygramma* and *Omphalia flavida.* Luminous bacteria and fungi in particular are widely commercially available.

The Gram-negative bacterium currently named as *Vibrio fischeri* is the preferred bioluminescent organism for use in the present invention. This organism bioluminesces at temperatures of between about 15°C and about 25°C, which is suitable for present purposes.

The *V. fischeri* bacteria are preferably supplied and stored as freeze-dried or lyophilized cells. The freeze-dried or lyophilized material is reconstituted for use as recommended by the supplier, typically by addition of an aqueous sodium chloride reconstitution solution, e.g. phosphate-buffered saline, at a temperature within the range stated above.

The Contaminant

The contaminant to be assayed is preferably one or more PAH, one or more organic pesticide, one or more petroleum hydrocarbon, one or more PCB, or any combination thereof. The contaminant may contain additional, relatively water-soluble, components, such as heavy metal compounds.

In the case of a contaminant extracted from a natural particulate material, the identity of individual contaminants cannot usually be determined from the assay of the invention alone; in that case, individual contaminants can, if desired, be identified in an extract by means of conventional chemical analytical techniques, as will be well understood by those skilled in this art. In the case of a contaminant extracted from a deliberately spiked or seeded sample of a particulate material - for example as part of a controlled trial or experiment - the identity of the contaminant assayed by means of the present invention may be known already, in which case subsequent analysis may not be necessary.

Examples of individual PAHs assayable using the present invention include: acenaphthene, anthracene, fluoranthene, chrysene, benzo[a]anthracene, benzo[a]pyrene, dibenzo[a,h]anthracene, naphthalene, 2-chloronaphthalene, 2-bromonaphthalene, carbazole, dibenzofuran, acenaphthylene, fluorene, phenanthrene, pyrene, benzo[b]fluoranthrene, benzo[k]fluoranthrene, benzi[ghi]perylene, and indeno[1,2,3-cd]pyrene.

Examples of individual organic pesticides assayable using the present invention include: gamma-BHC, dieldrin, alpha-HCH, beta-HCH, lindane, quintozene, chlorothalonil, heptachlor, aldrin, triadimefon, pendimethalin, heptachlor epoxide, o,p'-DDE, endosulphan I, endosulphan II, p,p'-DDE, p,p'-TDE (DDD), endrin, o,p'-DDT, p,p'-DDT, endosulfan sulphate, o,p'-methoxychlor, p,p'-methoxychlor, the pyrethroids (including permethrin, flumethrin, cypermethrin, alpha-cypermethrin, and deltamethrin), the carbamates, and the organophosphorus pesticides (including dimethoate, propetamphos, diazinon, etrimphos, chlorpyrifos-methyl, ethyl-parathion, pirimphos-methyl, fenitrothion, malathion, fenthion, chlorpyrifos, chlorfenviphos, ethion, triazophos, carbophenothion, phosalone, azinphos-ethyl, and azinphos-methyl).

Examples of individual petroleum hydrocarbons assayable using the present invention include: straight-chain aliphatic hydrocarbons having carbon numbers in the range of about C_8 to about C_{40}, branched aliphatic hydrocarbons having carbon

numbers in the range of about C_8 to about C_{40}, diesel range organics (DROs), particularly C_{10} to C_{30} and C_{30+}, petroleum range organics (PROs), mineral oils, BTEX (benzene toluene ethylbenzene xylene) hydrocarbons, semi-volatile organic compounds (SVOCs), and phenols.

Examples of individual PCBs assayable using the present invention include: 2,4,4'-trichlorobiphenyl, 2,2',5,5'-tetrachlorobiphenyl, 2,3',4,4'-tetrachlorobiphenyl, 2,4,4',5-tetrachlorobiphenyl, 3,4,4',5-tetrachlorobiphenyl, 2,2',4,4',5-pentachlorobiphenyl, 2,2',4,5,5'-pentachlorobiphenyl, 2,3,3',4,4'-pentachlorobiphenyl, 2,3',4,4',5-pentachlorobiphenyl, 3,3',4,4',5-pentachlorobiphenyl, 2,2',3,3',4,4'-hexachlorobiphenyl, 2,2',3,4,4',5'-hexachlorobiphenyl, 2,2',3,4',5,-5'-hexachlorobiphenyl, 2,2',4,4',5,5'-hexachlorobiphenyl, 2,3,3',4,4',5-hexachlorobiphenyl, 2,3,3',4,4',5'-hexachlorobiphenyl, 2,3',4,4',5,5'-hexachlorobiphenyl, 3,3',4,4',5,5'-hexachlorobiphenyl, 2,2',3,3',4,4',5-heptachlorobiphenyl, 2,2',3,3',4,5,5'-heptachlorobiphenyl, 2,2',3,3',4,5',6'-heptachlorobiphenyl, 2,2',-3',5,5',6-heptachlorobiphenyl, 2,2',3,4,4',5,5'-heptachlorobiphenyl, 2,2',3,4,4',-5'6-heptachlorobiphenyl, and 2,2',3,4',5,5'6-heptachlorobiphenyl.

The Bioluminescent Assay Method

The commercially available bioluminescent assay systems described in the prior art acknowledged herein, particularly the Jennings and Guzzella publications, may conveniently be used for assaying the contaminants extracted from the particulate material using the extraction procedure provided by the present invention. Trivial modifications to such commercially available assay systems, in ways which will be well within the capacity of those of ordinary skill in this art.

In order that the bioluminescence of the organism can be related to the concentration of the contaminant in the particulate material, it is necessary for a reference assay to be performed, preferably simultaneously with the assay of the extracted contaminant. This is suitably done in the same apparatus, using a control sample of the mixture of the diluted extraction system, i.e. a mixture

corresponding to the assay sample but without any contaminant. The difference between the bioluminescent assay performed on the diluted extract and the reference assay provides the data to relate the measured inhibition of the bioluminescence to the contaminant.

Calibration data may then applied to express the measured inhibition of the bioluminescence as the concentration or amount of the contaminant in the mixture or the particulate material. Such calibration data are suitably pre-obtained and the measurements and calculations operated by conventional microprocessors operating under suitable computer software. This enables the assay results to be read off or printed out by the user immediately, or saved or relayed back to a base memory or database for storage and printing out as desired.

The Apparatus

The apparatus according to the second aspect of the present invention may typically comprise: the first, second and third containers, the third container containing a bioluminescent organism; at least one additional container; as part of the means for relating the signal to the contaminant, a luminometer and ports for receiving two of the containers for differential simultaneous bioluminescence measurements on a reference mixture and on a contaminant-containing mixture; optionally a sample tray or other support surface; a plurality of small volume (preferably about 0.1 to about 3 ml) measuring devices for liquids (e.g. pipettes and/or syringes), at least some of which are optionally marked to show 0.5ml volume; optionally at least one glove (e.g. of rubber); a measuring device for the particulate sample; associated microprocessors and software for the luminometer, or data connection means to permit connection of the luminometer to a remote computer; and means as necessary, for establishing connection to ancillary equipment.

The preferred embodiment of the test kit according to the invention will contain: at least about seven containers; a luminometer (preferably having a timer device)

and ports for receiving two of the containers for differential simultaneous bioluminescence measurements on a reference mixture and on the contaminant-containing mixture; a sample tray or other support surface; at least about four of the small volume measuring devices for liquids, at least some (e.g. at least three) of which are marked to show 0.5ml volume; at least one glove (e,g, of rubber); a measuring device (e.g. a measuring spoon, suitably of plastic) for the particulate sample; associated microprocessors and software for the luminometer, or data connection means to permit connection of the luminometer to a remote computer; and means as necessary, for establishing connection to ancillary equipment such as printers, electrical batteries, etc.

The seven containers will suitably be as follows:

A – containing the water-miscible solvent (component A');

B – containing a measured quantity of aqueous diluent containing the surfactant and preferably also sodium chloride (component B');

C - containing a reconstitution solution for the bacteria (component C');

D – containing the *V. fischeri* bacteria, preferably in freeze-dried or lyophilized form ;

E – empty to start with;

F – empty to start with; and

G – containing a reference mixture consisting of components A' and B' in the same amounts as they are present in the diluted extract obtained from a soil sample according to the method of the invention, but not containing any contaminant (component G').

Container A is closable and has a mark (e.g. a red line) showing the height of the solvent A' when the correct amount of soil has been added.

The containers A, B, C, D, G may suitably be tubes, e.g. test tubes or the like.

The containers E and F may suitably be constructed, shaped and dimensioned for use in the luminometer, e.g. cuvettes or the like.

Brief Description of the Drawings

Examples of the present invention will now be described, without limitation and purely by way of illustration, with reference to the accompanying drawings.

In the drawings:

Figure 1 shows the extraction efficiency of PAHs from various particulate materials, using as the water-miscible solvent (a) iso-propanol alone and (b) 60:40 v/v iso-propanol/DMSO ("QuikX"), PAH measured by HPLC;

Figure 2 shows the effect of shaking time, as between (a) 5 and (b) 2 minutes on the percent extraction of PAHs from soil using as the water-miscible solvent 60:40 v/v iso-propanol/DMSO, PAH measured by HPLC;

Figure 3 shows a comparison in the extraction efficiency of PAHs as between fresh and dry soils using as the water-miscible solvent 60:40 v/v iso-propanol/DMSO PAH measured by HPLC;

Figure 4 shows the efficiency of retention of PAHs on 15-fold dilution as between (a) 1% aqueous Lutrol F127 surfactant solution, (b) water, (c) 1% Biononex surfactant solution, (d) 5% Biononex surfactant solution with vortexing, (e) 5% Biononex surfactant solution with shaking, and (f) 10% Biononex surfactant solution with shaking, PAH measured by HPLC;

Figure 5 shows the levels of six key PAHs in spiked fresh Devon, UK soil, (a) nominal levels as spiked, (b) as extracted using as the water-miscible solvent 60:40 v/v iso-propanol/DMSO, (c) as extracted using as the water-miscible solvent 60:40 v/v iso-propanol/DMSO followed by dilution with F127 and salt

solution and gentle shaking, (d) as extracted using as the water-miscible solvent 60:40 v/v iso-propanol/DMSO followed by dilution with F127 and water and gentle shaking, (e) as extracted using as the water-miscible solvent 60:40 v/v iso-propanol/DMSO followed by dilution with F127 and water and hard shaking, and then addition of salt, (f) as extracted using as the water-miscible solvent 60:40 v/v iso-propanol/DMSO followed by dilution with F127 and water and vortexing, and then addition of salt, and (g) as extracted using as the water-miscible solvent 60:40 v/v iso-propanol/DMSO followed by dilution with F127 and water and vortexing, PAH measured by HPLC;

Figure 6 shows the results of tests to determine optimum shaking time on recovery of PAHs, and whether prior addition of salt before shaking had an effect on recovery, PAH measured by HPLC;

Figure 7 shows the extraction and dilution results for a certified reference material as described below, PAH measured by HPLC;

Figure 8 shows a dose response curve relating to the inhibition of the bioluminescence of *V. fischeri* exposed to PAHs;

Figure 9 shows the results of a bioluminescent assay on clean soil, using the assay method of the present invention, to show that clean soil does not produce an extract toxic to *V. fischeri*;

Figure 10 shows the results of monitoring the bioluminescence of *V. fischeri*, with addition of a PAH contaminant during the procedure (the PAH was dissolved initially in 60:40 v/v iso-propanol/DMSO and subsequently diluted using water, F127 surfactant and salt using the preferred protocol of the invention, prior to contact with the bacteria), versus a blank (uncontaminated) water and salt control;

Figure 11 shows the results of monitoring the bioluminescence of *V. fischeri*, with addition of a cocktail of PAH contaminants during the procedure (the PAHs were

dissolved initially in 60:40 v/v iso-propanol/DMSO and subsequently diluted using water, F127 surfactant and salt using the preferred protocol of the invention, prior to contact with the bacteria), versus a blank (uncontaminated) control of 60:40 v/v iso-propanol/DMSO diluted using water, F127 surfactant and salt using the preferred protocol of the invention; and

Figure 12 shows the results of a bioluminescent assay using the method of the present invention, performed on a certified reference soil LGC6140, versus a blank (uncontaminated) control.

Examples and Detailed Description of the Drawings

Example 1

PAH Extraction Studies

Extraction studies were carried out with soil types including sandy, clay, and garden samples spiked at the 1 and 60 ppm levels with a cocktail of six key PAHs, namely: acenaphthene, anthracene, fluoranthene, chrysene, benzo[a]pyrene, and dibenz[a,h]anthracene. The spiked soils were left for at least 12 hours before extraction experiments were conducted.

Using a reference BSI method of extraction, which required a 6 hour Soxhlet extraction with dichloromenthane, followed by quantification using a reference HPLC method that enabled the simultaneous quantification of 15 NIST PAH standards, it was shown that extremely high recoveries of PAH were possible. When a range of soil types was spiked at the 1 ppm level with the six key PAHs, mean recoveries ranging from 94% to 102% were found. When similarly spiked soils were extracted with dichloromethane by simply shaking 10 g of spiked soil with 20 ml of solvent for 5 minutes, high recoveries in the order of 90% were obtained. Further, when iso-propanol was used instead of dichloromethane and the simple shaking procedure for 5 minutes was used, recoveries were as high, if

not higher. The results for such a study conducted using a sandy soil are given in Table 1 below.

Table 1. Example of Recovery of PAH from a sandy soil spiked at 1 ppm with 6 key PAHs when extracted by Soxhlet with dichloromethane (DCM) for 6 hours, shaken with DCM for 5 minutes, and shaken with iso-propanol (IP) for 5 minutes

PAH	Recovery (%) of Spiked PAH		
	SoxDCM	ShakeDCM	ShakeIP
Acenaphthene	77.5	*64.5**	*96.4*
Anthracene	81.5	*84.1*	*109.2*
Fluoranthene	111.4	*101.1*	*111.0*
Chrysene	95.9	*101.8*	*112.8*
Benzo[a]pyrene	84.6	*91.6*	*95.5*
Dibenz[a,h]anthracene	114.9	*92.0*	*91.0*
Mean Recovery	94.3	*89.2*	*103*

* Not protected by toluene during concentration step

It was therefore demonstrated that simply shaking 10 g of soil with a suitable solvent could achieve excellent recoveries of PAH. This 'field method' procedure was then validated for 4 soil types: sandy (Soil 83), clay (Soil 84), clay (Soil 85), and garden (Soil 87) spiked at the 10 ppm level with each of the key PAHs. A comparison was made between the extraction efficiency of iso-propanol with that of iso-propanol/ DMSO mixture in the ratio of 60:40 v/v and termed QuikX. The results are shown in Figure 1 of the drawings.

QuikX was found to give excellent recovery of spiked PAH at the 10 ppm level, and this was in the order of 10% greater than that achieved using iso-propanol alone.

Further experiments were conducted to determine if the nature of the bottle used for extraction by shaking was important. Naturally contaminated garden soil from Glasgow, UK was used and triplicate extractions in acid-washed glass and polystyrene bottles was performed. It was found that extraction using glass bottles resulted in a total of 12.8 ppm PAHs, whereas the plastic bottles appeared

to give a 10% lower recovery, at 11.5 ppm. Hence the nature of the bottle does appear to have a bearing, but the cost, weight and safety advantages of polystyrene may outweigh the slight decrease in extraction efficiency.

Reducing the shaking time from 5 minutes to 2 minutes was also studied and found to result in only a 4% loss in recovery of PAHs; see Figure 2 of the drawings. For field extraction, therefore, it may be sufficient to shake soil and solvent together for only 2 minutes.

The above procedure had been developed using dried soils, so performance using natural fresh, wet soils were also performed. It was found that both clays and sandy soils dispersed readily in QuikX and recovery from soils spiked with 6 key PAHs each at a level of 10 ppm, giving a total of 60 ppm, was at least 80% and was comparable to that achieved using dried soils. Results for triplicate tests are shown in Figure 3 of the drawings.

Extraction studies were also carried out on certified reference material soils, such as CRM 104 with a certified total PAH content of 78 ppm. QuikX extraction, using a 5 minute shaking period resulted in 83% extraction, which was consistent with the 80%+ extraction efficiencies found using spiked soils. This CRM result is included in Figure 7 of the drawings, discussed in more detail below, where recoveries on dilution are also presented.

Example 2

Toxicity Studies

Toxicity studies with luminescent bacteria have established that *Vibrio fischeri* bacteria can be tolerant to organic solvents, if they are diluted sufficiently.

Firstly, methanol and iso-propanol toxicity was tested on *V. fischeri* and compared. The bacteria were reconstituted from freeze-dried by soaking for 5

minutes with an osmotic regulator before addition of the toxicant. The results are given in Tables 2 and 3 below.

Table 2. The percentage inhibition caused by the presence of methanol at different concentrations.

		% inhibition compared to a control with diluent solution only		
Concentration of methanol (%v/v)		1.25	2.5	5
Time elapsed from adding toxicant (mins)	15	20	56	71
	30	20	58	85

These results were very variable, and there were differences between the response of freshly diluted and stored-diluted methanol samples.

Table 3. The percentage inhibition caused by the presence of isopropanol at different concentrations.

		% inhibition compared to a control with diluent solution only					
Concentration of isopropanol (v/v)		0.5	1	2.5	5	10	15
Time elapsed from adding toxicant (mins)	10	6	18	36	75	98	100
	20	7	20	37	75	99	100

Good repetitions of the results were observed, with no variation between freshly diluted and stored-diluted isopropanol. Isopropanol caused less of a toxic response when compared to methanol at 2.5% concentration.

The toxicity of the surfactants Triton X100 (a polyethoxylated octyl phenol surfactant having an Oral Rat LD_{50} = 1800 mg/kg), Lutrol F68 (a

polyoxyethylene-polyoxypropylene block copolymer surfactant, available from BASF, having an Oral Rat LD_{50} greater than 15000 mg/kg), and Lutrol F127 (a polyoxyethylene-polyoxypropylene block copolymer surfactant, available from BASF, having an Oral Rat LD_{50} greater than 10000 mg/kg), was then tested with *V. fischeri*. The results are given in Table 4 below.

Table 4. Comparison of surfactants toxicity on V. fischeri

		% inhibition compared to a control with diluent solution only		
Concentration (%v/v)		Triton X100 (0.1)	F68 (0.25)	F127 (0.25)
Time elapsed from adding toxicant (mins)	10	97	10	0
	20	97	10	10

The toxicity of Triton X100 was significantly higher than the other two surfactants at a lower concentration, and was therefore not suitable.

The inhibitions caused by a mixture of isopropanol with and without the surfactants Lutrol F68 and Lutrol F127 were then assessed. The results are given in Table 5 below.

Table 5. Percentage inhibitions caused by isopropanol (0.5%v/v) and surfactant (0.5%v/v)

		% inhibition compared to a control with diluent solution only				
		Isopropanol (IP)	F68	F127	IP + F68	IP + F127
Time elapsed from adding toxicant (mins)	10	5	25	14	24	10
	20	4	25	15	20	10

Lutrol F127 gave the least toxic response and became the most promising surfactant of choice. Further tests were performed at differing concentrations of isopropanol in a 1% F127 solution. The results are given in Table 6 below.

Table 6. Percentage inhibitions with a range of concentrations of isopropanol in a 1% F127 solution

		% inhibition compared to a control with diluent solution only			
Concentration of isopropanol (%v/v)		2	4	7	10
Time elapsed from adding toxicant (mins)	10	5	10	40	67
	20	7	11	52	72

Comparing Table 6 with Table 3, it appears that Lutrol F127 actually moderated the toxic effect of isopropanol. DMSO toxicity in 1% F127 was assessed with *V. fischeri*. The results are given in Table 7 below.

Table 7. Percentage inhibitions caused by a range of concentrations of DMSO in a 1% Lutrol F127 solution.

		% inhibition compared to a control with diluent solution only						
Concentration of DMSO (%v/v)		2	4	6	8	10	15	20
Time elapsed from adding toxicant (mins)	10	33	35	50	66	70	82	90

Corresponding studies were performed, with 5% iso-propanol present in the solution. The results are shown in Table 8 below.

Table 8. Percentage inhibitions caused by 5% (v/v) isopropanol, 1% (v/v) F127 and a range of concentrations of DMSO.

		% inhibition compared to a control with diluent solution only			
Concentration of DMSO (%v/v)		2	2.5	3	4
Time elapsed from adding toxicant (mins)	10	22	28	31	40
	15	22	33	30	40

It was found that the maximum preferred concentrations of iso-propanol and dimethylsulphoxide were 4% and 3%, respectively, when diluted with 1% aqueous F127 surfactant.

Example 3

Dilution Studies

To achieve the concentration of 4% iso-propanol and 3% dimethylsulphoxide, a QuickX extract needs to be diluted 15-fold. Experiments were therefore conducted to determine the percentage of PAH retained on dilution of selected extracts with a range of possible diluents. In addition to F127 (1%) surfactant, water and a range of concentrations of Biononex (a mixed fatty alcohol ethoxylate (60-100%) and alkylamine ethoxylate (30-60%) surfactant; Rat Oral LD_{50} of fatty alcohol ethoxylates > 2000 mg/kg; Rat Oral LD_{50} of alkylamine ethoxylates > 5000 mg/kg) were tried. The results are shown in Figure 4 of the drawings.

It can be seen that negligible PAH is retained in solution when the QuikX extract is simply diluted with water. Surfactant F127, however, retained up to 80% of the PAH dibenz[a,h]anthracene in solution, and the mean retention for the six key PAHs was 60%. Biononex was far less efficient than F127: even at extremely

high concentrations of 10% it could only retain 27% PAH. Hence F127 was selected as the surfactant of choice, but Biononex was shown to have some utility.

The dilution experiment described above was performed using vortex mixing for 15 seconds to mix the extract with the diluent. For a field method, shaking would be more practical than vortex mixing, so a series of experiments was conducted to test the efficiency of shaking for a range of periods, both gently and vigorously with salt either being added before mixing or afterwards. A QuikX extract of fresh Devon, UK, soil, spiked at 10 ppm with the six key PAHs, was diluted with F127 (1%) surfactant and either shaken for 1 minute, or vortex mixed for 15 seconds. The results are shown in Figure 5 of the drawings.

It can be seen that gentle shaking results in very low retention of PAHs, but that vigorous shaking for 1 minute results in good retention, similar to that achieved using vortex mixing.

It would be desirable to reduce the shaking time, and also to add the salt (required for presentation to the bacteria) prior to shaking. Salt is needed to ensure the bacteria are at the optimum osmotic potential. If salt could be added prior to shaking, then it could be incorporated into the aqueous diluent, saving a step in the procedure. An experiment was therefore conducted to determine, in triplicate, the retention of PAH on 15-fold dilution of a QuikX extract, using vigorous shaking for periods of 60, 30, and 15 seconds. Salt was added after mixing for these three shaking times, but, in addition, it was added prior to shaking for 15 seconds. The high performance liquid chromatography (HPLC) results for this experiment are shown in chart form in Figure 6 of the drawings.

It is concluded that there was very little, if anything, to be gained by shaking for longer than 15 seconds, and that furthermore there was good evidence to suggest that salt could be added prior to mixing.

The preferred extraction and dilution protocol was therefore established as the following:

Step 1	Measure 10 g of soil sample into a plastic bottle and add 20 ml of QuikX solvent
Step 2	Cap bottle and shake vigorously for 2 minutes
Step 3	Let soil settle until adequate supernatant is visible
Step 4	Take 500 µl of supernatant and add to 7 ml of Diluent (F127 @ 1% plus sodium chloride to 2%)
Step 5	Shake vigorously for 15 seconds to form thick froth
Step 6	As soon as sufficient liquid re-forms (~1 minute) remove 0.5 ml and add to bacteria in sample measuring cuvette

This extraction and dilution protocol was validated using a certified reference material soil (CRM104 ex the Laboratory of the Government Chemist, UK). The results are summarised in Figure 7 of the drawings.

The results were consistent with our earlier studies, yielding 83% extraction and 65% retention of extracted PAH on dilution.

Example 4

Bioluminescence Tests

Luminescence-based toxicity studies were carried out on *V. fischeri* bacteria reconstituted from the freeze-dried commercially available stock by soaking in 2% salt water, using a range of commercial luminometers, as well as a specially designed and built test rig. The bulk of the work was performed using the bespoke kit which allowed real-time monitoring of both the test and control cuvettes. With simultaneous monitoring of the control cuvette sample with the test cuvette it allowed the reconstitution time of the bacteria to be shortened. In other protocols, 15 minutes is needed to allow the bacteria's luminescence to plateau, but this

work indicated the luminescence plateau was achieved within 5 minutes. The blank (reference sample) in these experiments consisted of reconstituted bacteria with QuikX diluted 15 times with 1% F127.

A number of dose response studies were conducted, and the results of one are shown in Figure 8 of the drawings. In this study, concentrations of total PAH in the range 1.7 to 66.7 μg/ml gave a classic bioluminescent response. These data demonstrate that semi-quantitative screening of PAH and other contaminants is possible using the method of the present invention.

Studies were conducted on soils containing negligible PAH contamination, to dispel the fear that all soil extracts may be toxic to the luminescent bacteria. The results of such an experiment are given in Figure 9 of the drawings.

Inhibition caused by the sample of uncontaminated soil did not exceed that of the solvent blank. Hence it was shown that 'clean' soil, uncontaminated with PAHs, did not produce an extract toxic to *Vibrio fischeri*.

The toxicity of individual PAHs, mixtures of PAHs, and extracts of spiked soils and certified reference materials, were then determined as outlined below.

One of the most toxic PAHs was shown to be acenaphthene, and this was tested using a variety of bacterial preparations. Its effect on a *Vibrio fischeri* preparation is given in Figure 10 of the drawings.

Here there is a dramatic difference in inhibition between the control (blank) in Rig 1 and the PAH dissolved in the QuikX solution at a concentration of 67μg/ml in Rig 2. It should also be noted that the toxic effect is immediate. This is a general finding for this invention, and allows for extremely rapid toxicity determination.

Other PAHs shown to give a large toxic response include: phenanthrene and fluoranthene, but some PAHs give lower responses using this protocol. A mixture

of the six key PAHs recited in Example 1, at a total PAH concentration 67μg/ml, has consistently given a significant toxic response to a variety of bacterial preparations. This PAH cocktail was, for example, tested using *Vibrio fischeri* bacteria grown and prepared by us. The results are shown in Figure 11 of the drawings.

Although the control has, on this occasion, caused a significant level of inhibition, it can be clearly seen that the sample has resulted in greater inhibition. The difference in inhibition between the control and the sample will be the parameter used to assess the toxicity of PAHs.

The PAH extraction and dilution protocols have also been applied to certified reference materials CRM104 and LGC6140 containing 78 and 490 ppm total PAH respectively. Both have given conclusive positive responses, and the latter is shown in Figure 12 of the drawings.

It is concluded that the novel extraction of PAHs from soil and the novel dilution to give a solution that can be presented to bioluminescent organisms for toxicity testing has been demonstrated. Further, the immediate toxic response to PAHs prepared in this way has been demonstrated and the advantage of dual monitoring of sample and control has been established.

Example 5

Apparatus

The preferred embodiment of the test kit according to the invention will contain seven containers, a luminometer having a timer device and ports for two cuvettes for differential simultaneous bioluminescence measurements on the control and sample, a sample tray, four small volume measuring devices for liquids selected from pipettes and/or syringes, at least three of which are marked to show 0.5ml volume, a pair of thin rubber gloves, a measuring spoon, e.g. of plastic, for the

particulate sample, associated microprocessors and software for the luminometer, or data connection means to permit connection of the luminometer to a remote computer, and means for establishing any necessary connection to ancillary equipment such as printers, electrical batteries, etc.

The seven containers will suitably be identified as follows:

A – the QuikX solvent;

B – 7.5ml of aqueous diluent containing F127 surfactant and sodium chloride

C - a reconstitution solution (aqueous sodium chloride) for the bacteria

D – the freeze-dried *V. fischeri* bacteria

E – empty to start with

F – empty to start with

G – a reference mixture consisting of the QuikX solvent and the diluent in the amount that is present in the test sample on introduction into the luminometer, but omitting any contaminant.

Container A is closable and has a mark (e.g. a red line) showing the height of the solvent when the correct amount of soil has been added.

The containers A, B, C, D, G may suitably be a test tubes or the like.

The containers E and F may suitably be cuvettes or the like, for use in the luminometer.

The above kit is suitable for testing a sample of soil weighing approximately 10g. A further approximately 500g of soil will typically be obtained at the same time as the sample, labelled and kept for chemical analysis, to provide a source for repeat test runs, or for storage as a future reference sample.

Method Using this Apparatus

Approximately six heaped measuring spoonfuls of soil are placed in a sample tray and inspected for stones, debris, plant or organic material. Such material is removed and clods of soil broken up manually.

The soil is added carefully to container A so that the liquid level moves up to the mark on the container. If necessary, the soil can be pushed under the surface of the solvent using the spoon.

The container A is then closed and shaken vigorously for two minutes, and then left to stand until the soil particles have settled.

The contents of container C are removed using a pipette or syringe and added to container D. Using the same pipette or syringe, the contents of container D are then mixed by carefully aspirating with the pipette or syringe. Then 0.5ml of the fluid is withdrawn from the container D using the same pipette or syringe, by reading the required volume from the pipette or syringe marking, and added to container E. A further 0.5ml of the fluid is withdrawn from the container D using the same pipette or syringe, and added to container F.

Containers E and F are then placed in their respective ports of the luminometer and the Start button pressed. A period of 5 minutes must pass, which is timed by the instrument. The containers are left in the instrument for this time.

Using a fresh pipette or syringe, 0.5ml of the supernatant of container A is extracted from just below the surface of the liquid, added to container B, and container B shaken vigorously for at least 15 seconds, until a froth has formed.

Immediately that the timer device of the instrument indicates the time has passed, using a fresh pipette or syringe, the entire contents of container G are added to container F in the instrument.

Using a further fresh pipette or syringe, 0.5ml of the liquid from container B are added into container E in the instrument, and the Start button is pressed. This must be done within a maximum of ten minutes after the bacteria were first placed in the instrument.

The microprocessor and the memory and software of the instrument will process and store the % inhibition and the green/red response of the bacteria, from which the contaminant concentration can be calculated by reference to calibration data.

The foregoing broadly describes the present invention, without limitation. Variations and modifications as will be readily apparent to those of ordinary skill in this art are intended to be within the scope of this application and any subsequent patent(s).

CLAIMS

1. A bioassay method for testing a sample of soil, sand, sediment or other particulate material for the presence of a contaminant, comprising: extracting an assayable amount of the contaminant from the particulate material into a water-miscible solvent capable of dissolving the contaminant; mixing the resultant solvent solution of the contaminant with water and optionally a surfactant, whereby a mixture is obtained containing water, solvent, surfactant (if present) and any extracted contaminant; and exposing an organism to the mixture under conditions in which the inhibition, by the contaminant, of a signal generated by the organism can be related to the presence of the contaminant in the mixture.

2. An assay according to claim 1, wherein the contaminant assayed comprises one or more polycyclic aromatic hydrocarbon (PAH), one or more organic pesticide, one or more petroleum hydrocarbon, or any combination thereof.

3. An assay according to claim 1 or claim 2, wherein the signal is the emission of light by a bioluminescent organism.

4. An assay according to any one of claims 1 to 3, wherein the signal is the emission of light from *Vibrio fischeri.*

5. An assay according to any one of the preceding claims, wherein the amount of water mixed with the solution of the contaminant is up to about 30 times the volume of the solvent solution of the contaminant being diluted.

6. An assay according to claim 5, wherein the amount of water mixed with the solution of the contaminant is between about 10 and about 25 times the volume of the solvent solution of the contaminant being diluted.

7. An assay according to claim 5, wherein the amount of water mixed with the solution of the contaminant is between about 12 and about 15 times the volume of the solvent solution of the contaminant being diluted.

8. An assay according to any one of the preceding claims, wherein the water and the surfactant are mixed with the solvent solution of the contaminant as an aqueous surfactant solution.

9. An assay according to any one of the preceding claims, wherein the water-miscible solvent and the surfactant are each substantially less toxic to the organism at the concentration obtained, after dilution, in the mixture to be presented to the organism, than is the contaminant at the concentration in which it is present in the same mixture.

10. An assay according to claim 9, wherein the water-miscible solvent and the surfactant are each substantially non-toxic to the organism at the concentration obtained, after dilution, in the mixture presented to the organism.

11. An assay according to any one of the preceding claims, wherein the water-miscible solvent comprises one or more organic alcohol.

12. An assay according to claim 11, wherein the organic alcohol, or at least one of the organic alcohols, contains two to eight carbon atoms.

13. An assay according to claim 11 or claim 12, wherein the organic alcohol, or at least one of the organic alcohols, is an alkyl mono-ol containing three to six carbon atoms.

14. An assay according to any one of the preceding claims, wherein the water-miscible solvent comprises iso-propanol.

15. An assay according to any one of the preceding claims, wherein the water-miscible solvent comprises dimethyl sulphoxide (DMSO).

16. An assay according to any one of the preceding claims, wherein the water-miscible solvent consists essentially of one or more organic alcohol, DMSO, or a mixture thereof.

17. An assay according to any one of the preceding claims, wherein the water-miscible solvent consists essentially of a mixture of an organic alcohol and DMSO in a volume ratio of alcohol:DMSO between about 25:75 and about 75:25.

18. An assay according to claim 17, wherein the volume ratio of alcohol:DMSO is about 60:40.

19. An assay according to any one of claims 16 to 18, wherein the organic alcohol is as defined in any one of claims 12 to 14.

20. An assay according to any one of the preceding claims, wherein the surfactant is selected from fatty alcohol ethoxylates, alkylamine ethoxylates, mixtures of fatty alcohol ethoxylates and alkylamine ethoxylates, polyoxyalkylene polymers, block copolymers of more than one polyoxyalkylene, and any combination thereof.

21. An assay according to any one of the preceding claims, wherein the surfactant comprises Biononex, Lutrol F68, Lutrol F127, or any combination thereof.

22. An assay according to any one of the preceding claims, wherein the surfactant is used at an amount of less than about 5% by weight relative to the water.

23. An assay according to any one of the preceding claims, wherein the surfactant is used in an amount of less than about 2% by weight relative to the water.

24. An assay according to any one of the preceding claims, wherein the diluted mixture containing the contaminant includes one or more additional components.

25. An assay according to claim 24, wherein the additional component, or at least one of the additional components, is a further solute.

26. An assay according to claim 25, wherein the further solute is sodium chloride.

27. An assay according to any one of the preceding claims, wherein the solvent solution of the contaminant, or at least that portion of the solvent solution of the contaminant that is to be used for the assay, is substantially separated from the particulate material before mixing with at least one of the water and the surfactant.

28. An assay according to any one of the preceding claims, wherein the extraction of the contaminant into the solvent, the mixing of the solvent solution with the water and the surfactant, or both, is carried out under vigorous agitation.

29. An assay according to any one of the preceding claims, wherein the conditions in which the inhibition, by the contaminant, of a signal generated by the organism can be related to the presence of the contaminant in the mixture comprise measurement of a corresponding signal generated by a similar organism in a reference mixture.

30. An assay according to claim 29, wherein the reference mixture corresponds to the mixture under assay, but omits the contaminant.

31. An assay according to claim 29 or claim 30, wherein the difference in the measured inhibition between the mixture under assay and the reference mixture is related to the concentration of contaminant in the mixture under assay or in the particulate material, by means of calibration data relating the measured signal to the concentration of contaminant in the mixture under assay and data concerning the amount of particulate material and the volume of the mixture.

32. An assay according to claim 31, wherein the measurement is sufficiently quantitative to enable the determination to be made as to whether the concentration of the contaminant in the particulate material is above or below a certain level.

33. An assay according to claim 32, wherein the certain level is a legally specified level.

34. Apparatus for performing an assay as defined in any one of the preceding claims, the apparatus comprising: a first container containing the water-miscible solvent capable of extracting an assayable amount of a contaminant from a sample of soil, sand, sediment or other particulate material when contacted therewith; a second container containing the surfactant, optionally in aqueous solution; a third container containing the organism; means for detecting the signal generated by the organism; means for relating the signal to the presence of the contaminant in the mixture; and instructions, and optionally guidance indicia on at least one of the containers, for performing the assay.

35. An apparatus according to claim 34, further comprising means for determining and displaying whether the contaminant is present above or below a certain concentration in the particulate material.

36. An apparatus according to claim 35, wherein the certain level is a legally specified level.

37. An apparatus according to claim 35 or 36, wherein the parts are present as a kit in a portable container therefor.

38. An apparatus according to any one of claims 34 to 37, comprising: the first, second and third containers, the third container containing a bioluminescent organism; at least one additional container; as part of the means for relating the signal to the contaminant, a luminometer and ports for receiving two of the containers for differential simultaneous bioluminescence measurements on a reference mixture and on a contaminant-containing mixture; optionally a sample tray or other support surface; a plurality of small volume (preferably about 0.1 to about 3 ml) measuring devices for liquids (e.g. pipettes and/or syringes), at least some of which are optionally marked to show 0.5ml volume; optionally at least one glove; a measuring device for the particulate sample; associated microprocessors and software for the luminometer, or data connection means to permit connection of the luminometer to a remote computer; and means as necessary, for establishing connection to ancillary equipment.

39. An apparatus according to claim 38, wherein in total at least seven containers are present.

40. A composition comprising about 3% to about 5% by weight of at least one organic alcohol, about 2% to about 4% by weight dimethyl sulphoxide (DMSO), about 0.5% to about 1.5% by weight of a surfactant, and the balance water and optionally a salt and optionally additional components.

41. A composition acording to claim 40, consisting essentially of the said amounts of the at least one organic alcohol, the DMSO, the surfactant, and the water and, if present, the salt, with less than about 10% by weight of any additional components.

42. A composition according to claim 40 or 41, wherein the organic alcohol is as defined in any one of claims 12 to 14.

43. A composition according to any one of claims 40 to 42, wherein the surfactant is as defined in any one of claims 20 to 23.

44. A composition according to any one of claims 40 to 43, wherein the salt, when present, comprises sodium chloride.

45. A composition according to any one of claims 40 to 44, wherein the salt, when present, is at a concentration of between about 1% and about 3% by weight.

46. A composition according to any one of claims 40 to 45, further comprising one or more polycyclic aromatic hydrocarbon (PAH), one or more organic pesticide, one or more petroleum hydrocarbon, or any combination thereof.

Figure 1

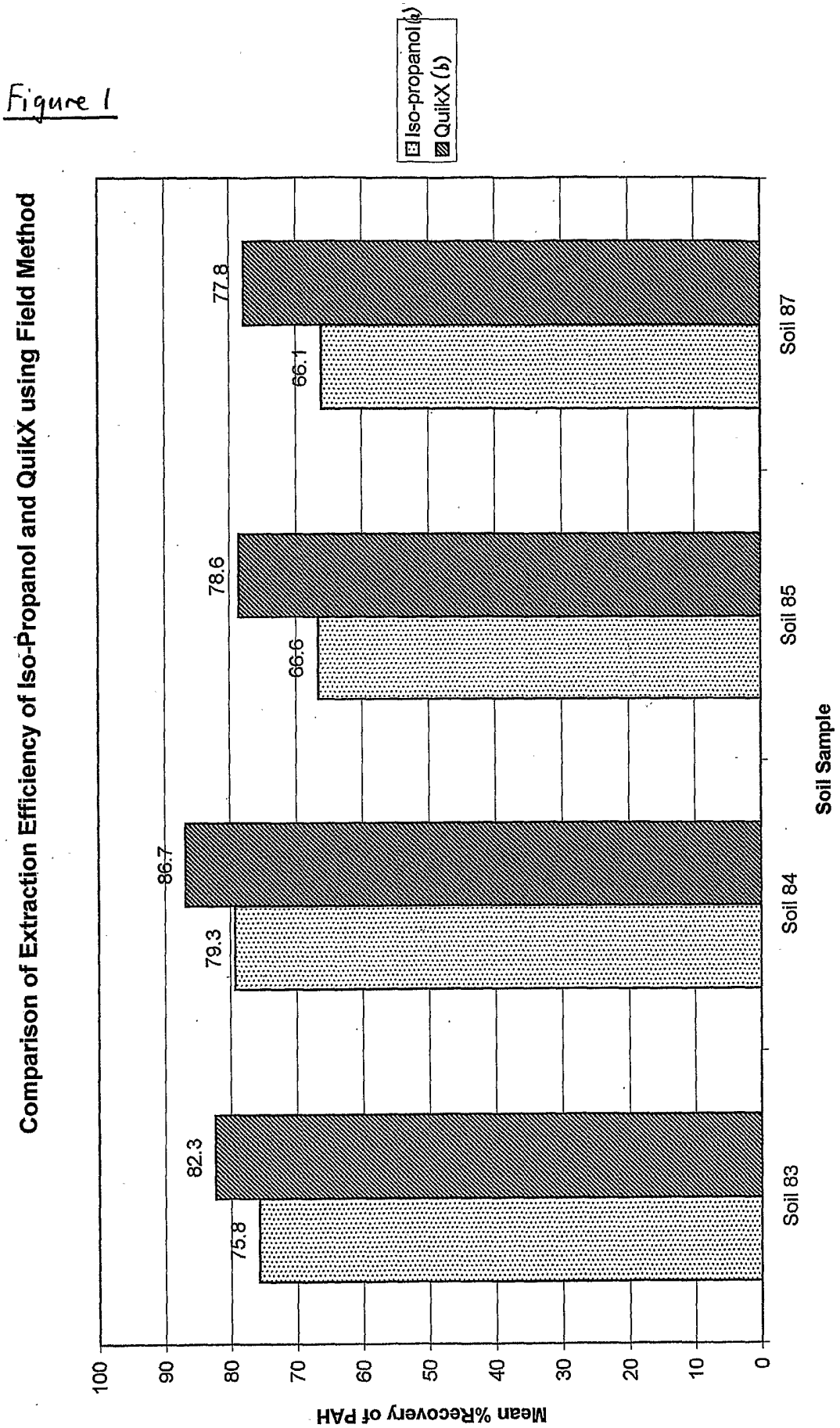

Comparison of Extraction Efficiency of Iso-Propanol and QuikX using Field Method
Iso-propanol (a)
QuikX (b)
77.8
66.1
78.6
66.6
86.7
79.3
82.3
75.8
Soil 87
Soil 85
Soil 84
Soil 83
Soil Sample
Mean %Recovery of PAH
100
90
80
70
60
50
40
30
20
10
0

Figure 2

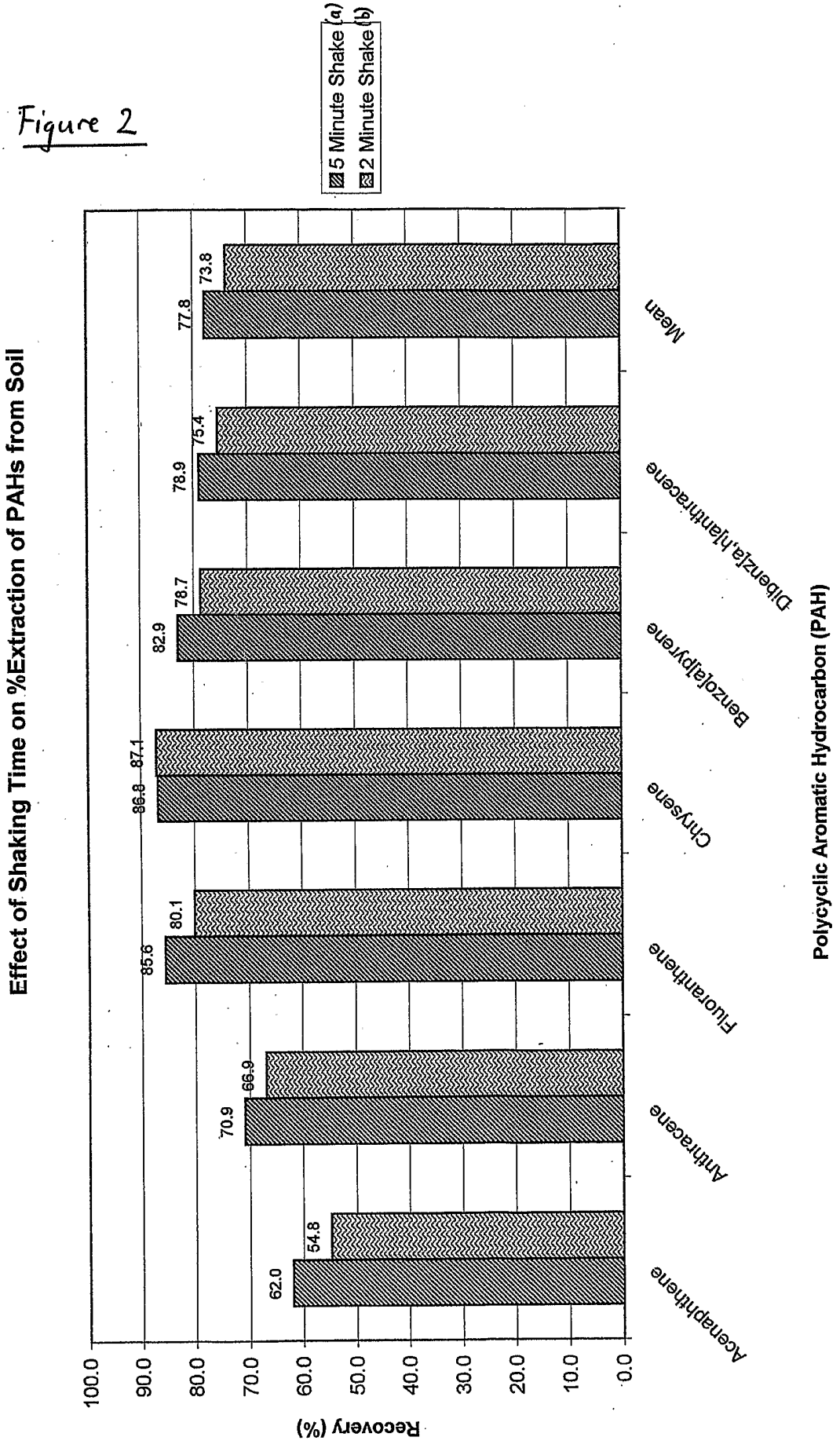

Effect of Shaking Time on %Extraction of PAHs from Soil
5 Minute Shake (a)
2 Minute Shake (b)
Recovery (%)
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Acenaphthene
62.0
54.8
Anthracene
70.9
66.9
Fluoranthene
85.6
80.1
Chrysene
86.8
87.1
Benzo[a]pyrene
82.9
78.7
Dibenz[a,h]anthracene
78.9
75.4
Mean
77.8
73.8
Polycyclic Aromatic Hydrocarbon (PAH)

Figure 3

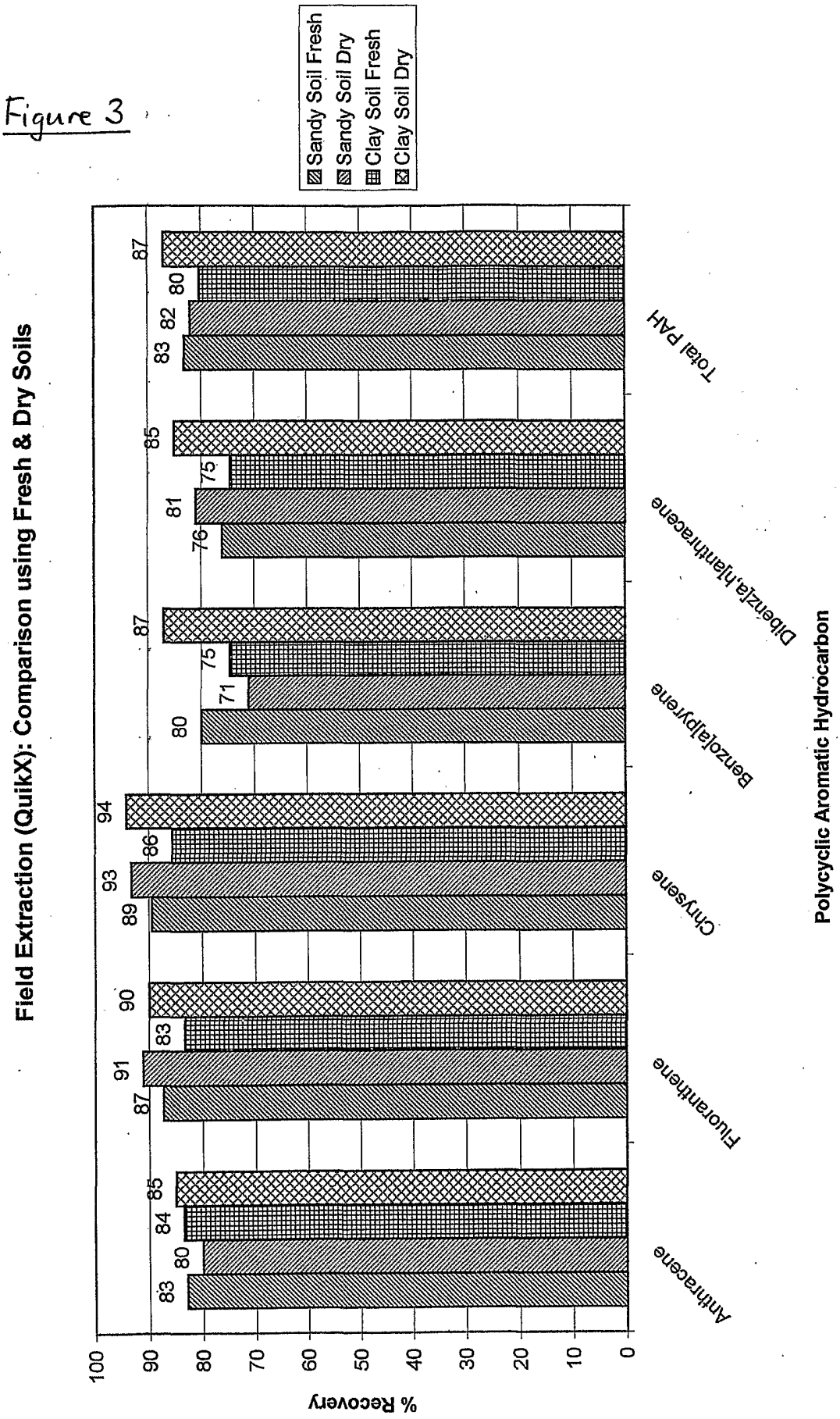

Field Extraction (QuikX): Comparison using Fresh & Dry Soils
Sandy Soil Fresh
Sandy Soil Dry
Clay Soil Fresh
Clay Soil Dry
% Recovery
100
90
80
70
60
50
40
30
20
10
0
Anthracene
83
80
84
85
Fluoranthene
87
91
Chrysene
89
93
86
94
Benzo[a]pyrene
80
71
75
87
Dibenz[a,h]anthracene
76
81
75
85
Total PAH
83
82
80
87
Polycyclic Aromatic Hydrocarbon

Figure 4

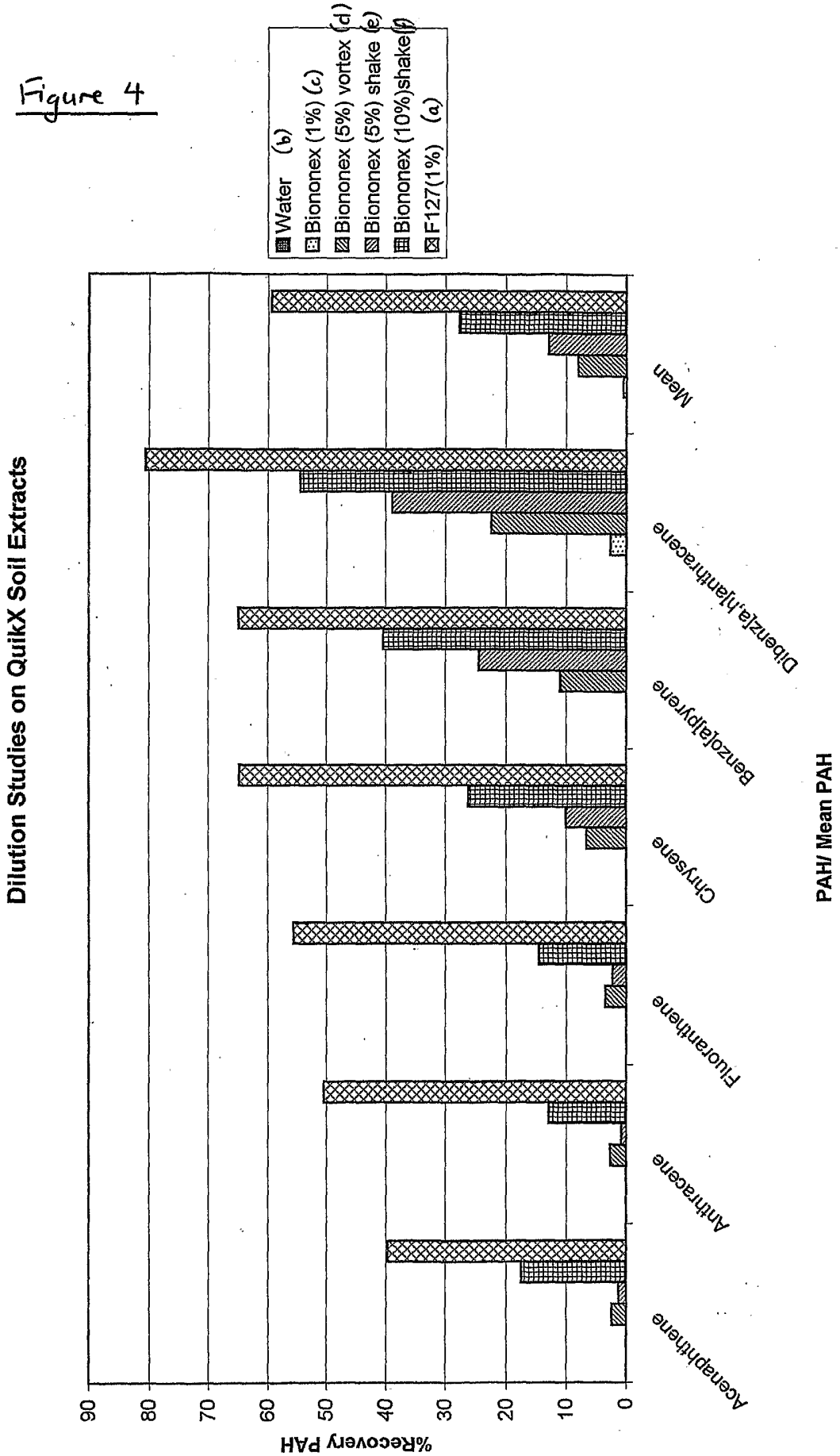

Dilution Studies on QuikX Soil Extracts
Water (b)
Biononex (1%) (c)
Biononex (5%) vortex (d)
Biononex (5%) shake (e)
Biononex (10%)shake (f)
F127(1%) (a)
%Recovery PAH
0
10
20
30
40
50
60
70
80
90
Acenaphthene
Anthracene
Fluoranthene
Chrysene
Benzo[a]pyrene
Dibenz[a,h]anthracene
Mean
PAH/ Mean PAH

Figure 5

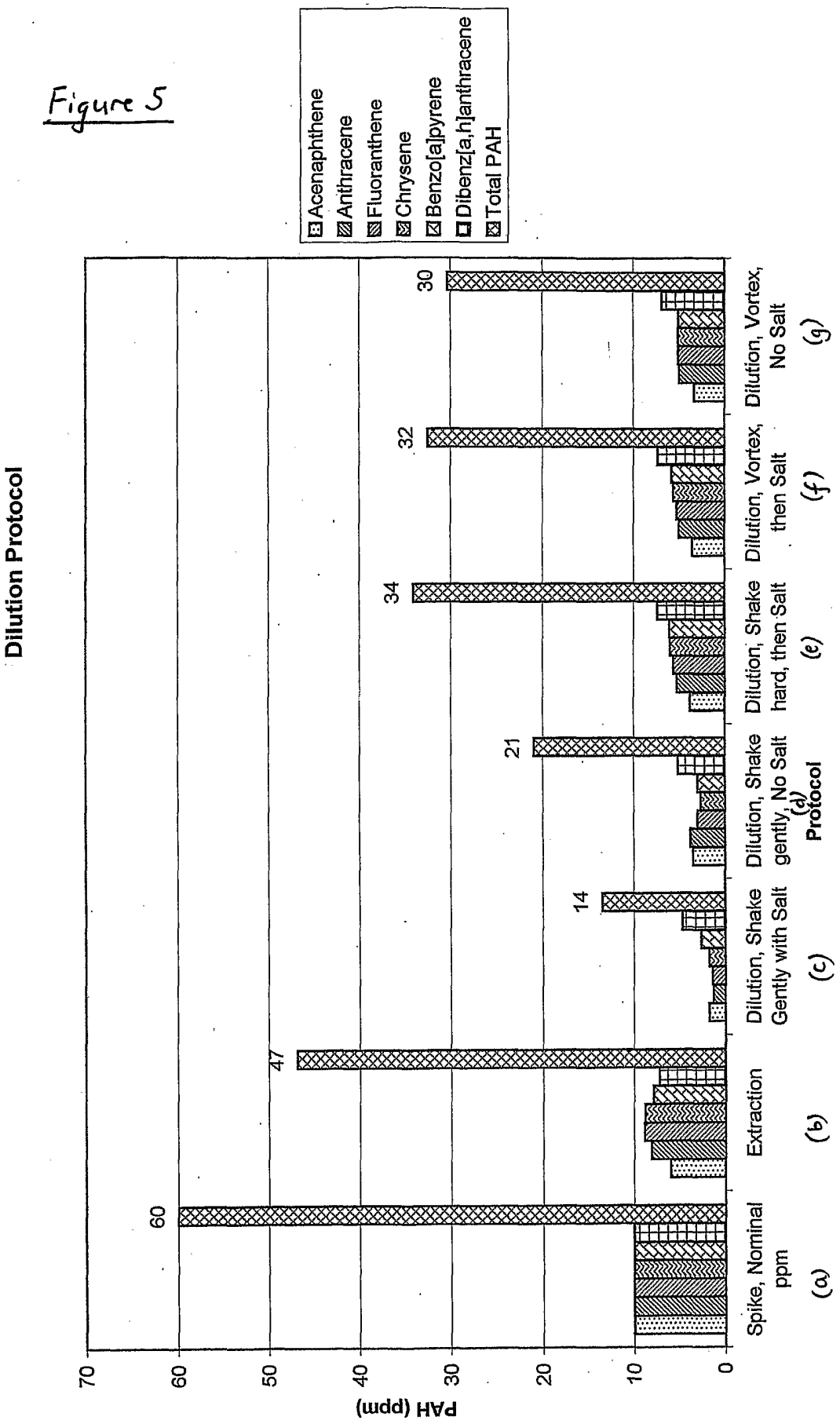

Dilution Protocol
Acenaphthene
Anthracene
Fluoranthene
Chrysene
Benzo[a]pyrene
Dibenz[a,h]anthracene
Total PAH
PAH (ppm)
0
10
20
30
40
50
60
70
60
47
14
21
34
32
30
Spike, Nominal ppm
(a)
Extraction
(b)
Dilution, Shake Gently with Salt
(c)
Dilution, Shake gently, No Salt
(d)
Dilution, Shake hard, then Salt
(e)
Dilution, Vortex, then Salt
(f)
Dilution, Vortex, No Salt
(g)
Protocol

Figure 6


Dilution Studies
60 Sec.
30 Sec.
15 Sec.
15Sec. with Salt
PAH (ppm)
120.0
100.0
80.0
60.0
40.0
20.0
0.0
PAH
Acenaphthene
Anthracene
Fluoranthene
Chrysene
Benzo[a]pyrene
Dibenz[a,h]anthracene
Total PAH
52.5
52.7
56.4
63.4
61.1
60.7
62.2
61.9
61.7
61.6
59.8
59.6
67.2
66.8
61.5
61.1
77.5
75.2
69.9
68.4
98.0
95.8
92.1
92.4
69.9
69.0
66.9
67.5

Figure 7

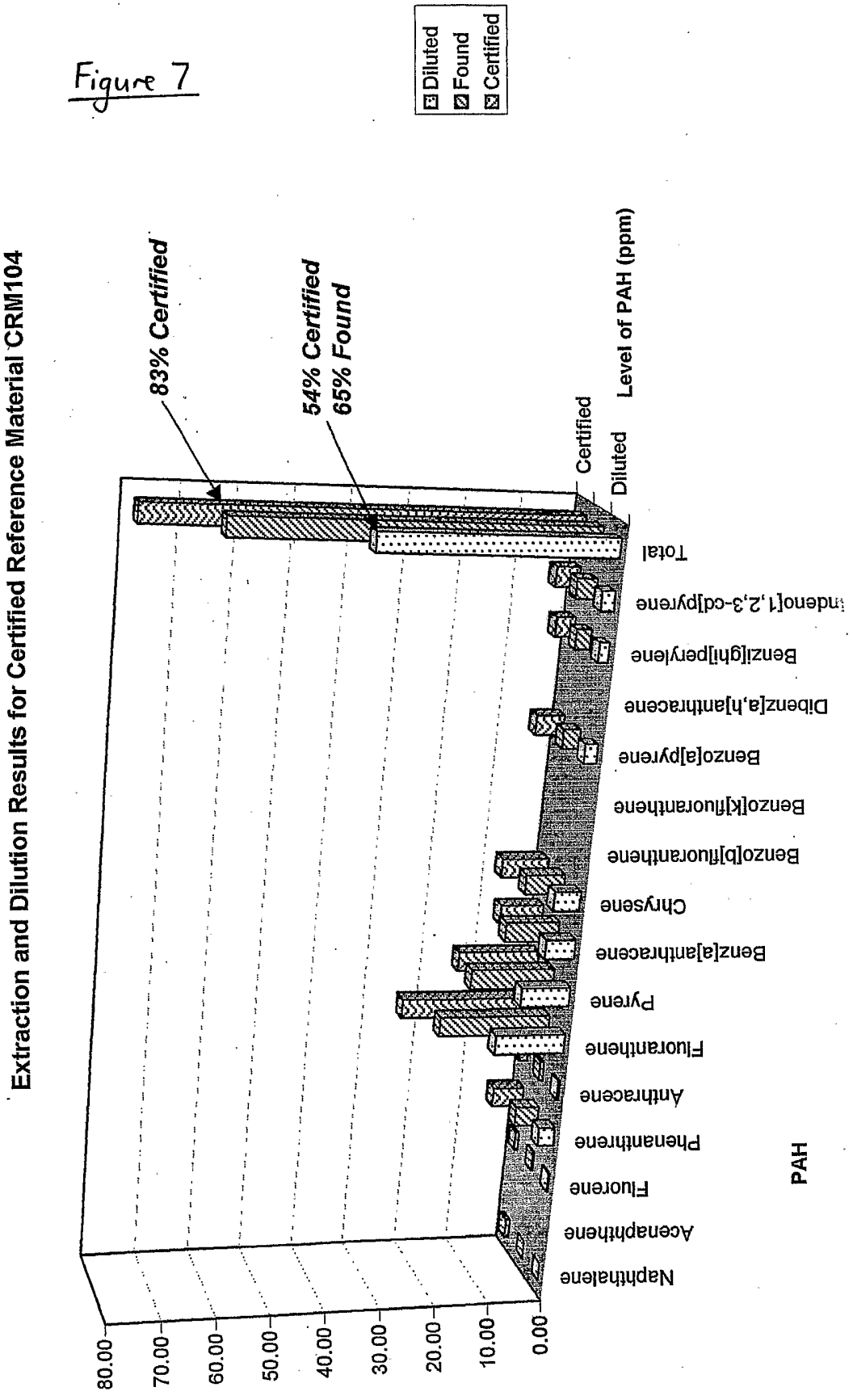

Extraction and Dilution Results for Certified Reference Material CRM104
Diluted
Found
Certified
83% Certified
54% Certified
65% Found
Level of PAH (ppm)
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Certified
Diluted
Total
Indeno[1,2,3-cd]pyrene
Benzi[ghi]perylene
Dibenz[a,h]anthracene
Benzo[a]pyrene
Benzo[k]fluoranthene
Benzo[b]fluoranthene
Chrysene
Benz[a]anthracene
Pyrene
Fluoranthene
Anthracene
Phenanthrene
Fluorene
Acenaphthene
Naphthalene
PAH

Figure 8

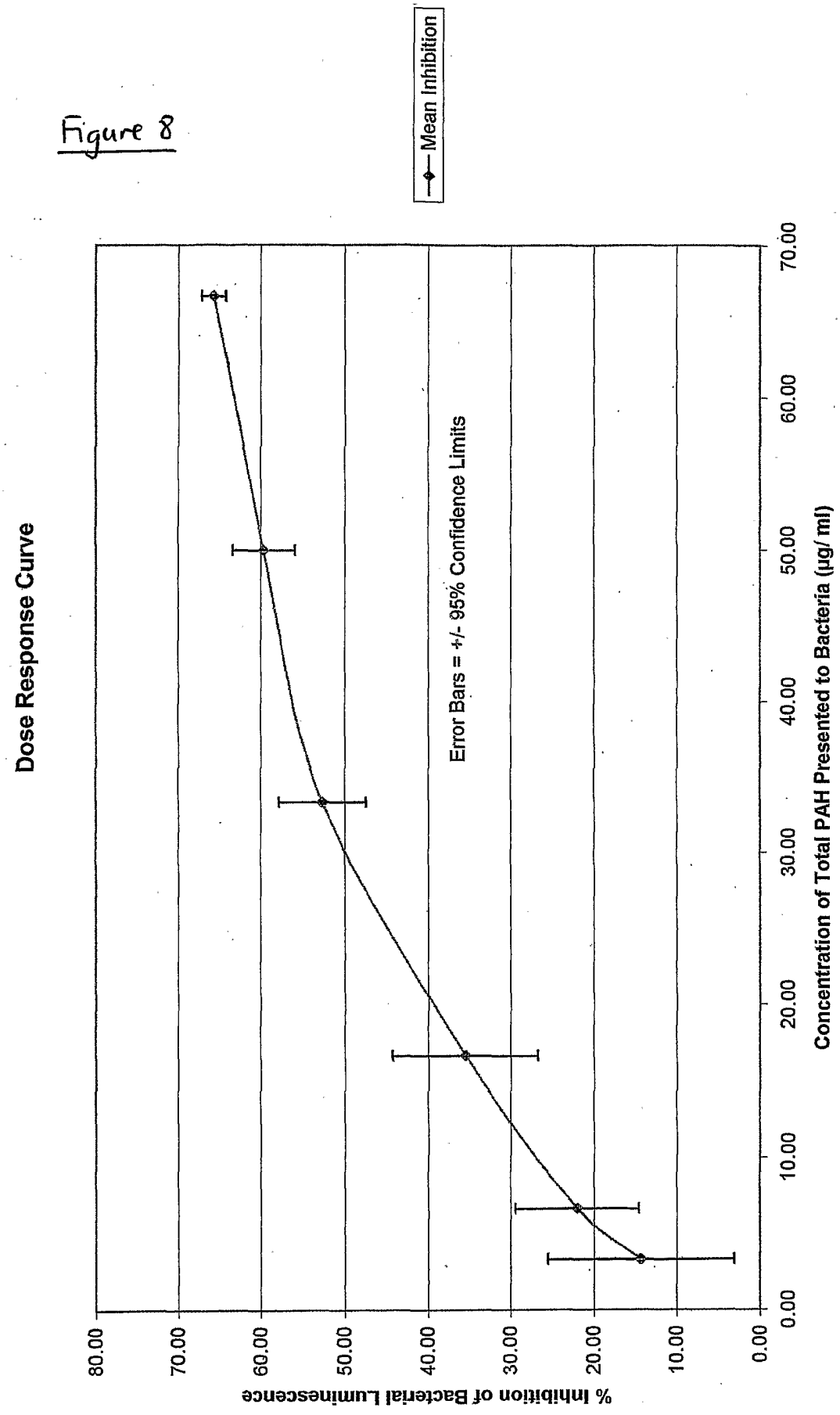

Dose Response Curve
Mean Inhibition
Error Bars = +/- 95% Confidence Limits
% Inhibition of Bacterial Luminescence
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
Concentration of Total PAH Presented to Bacteria (µg/ ml)

Figure 9

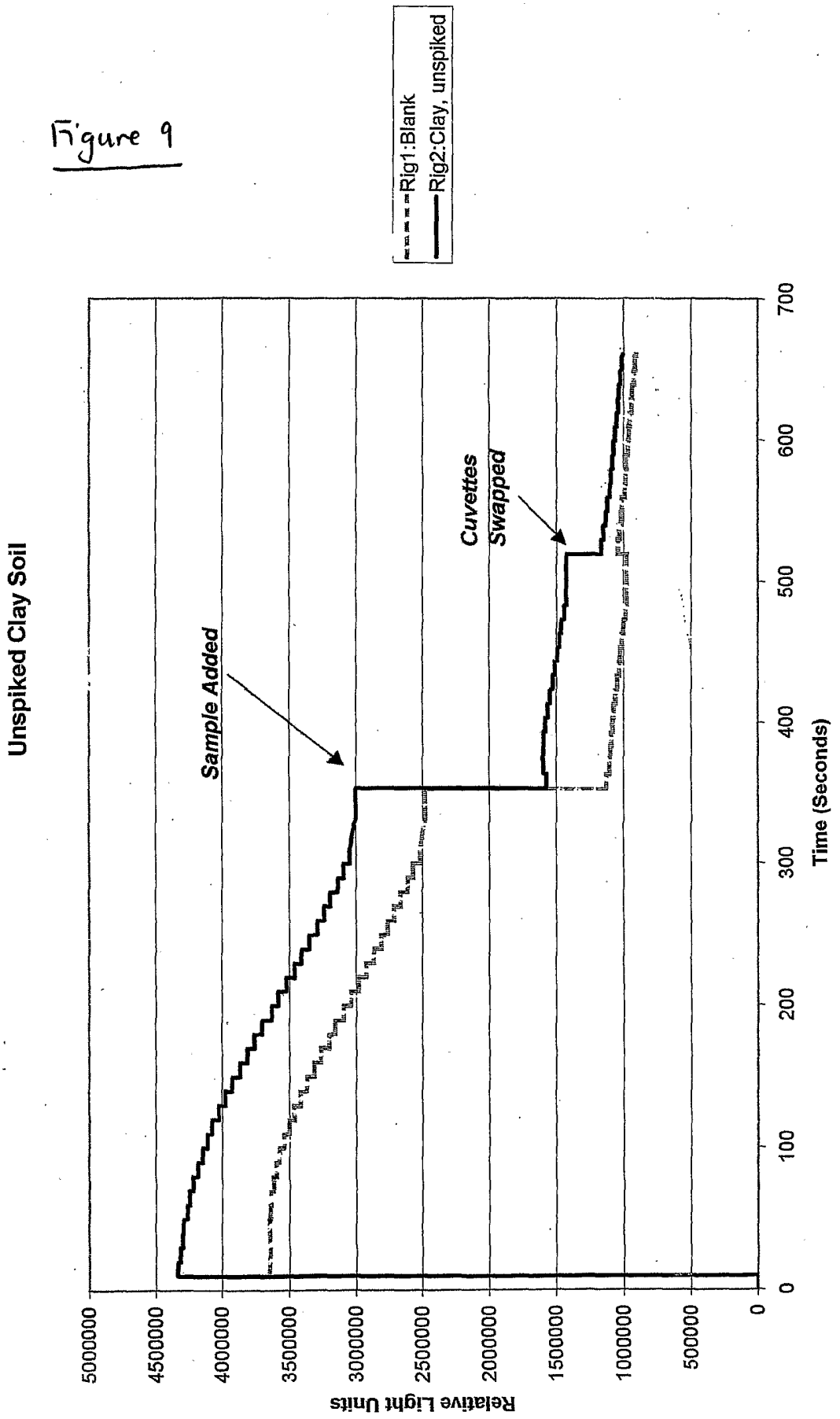

Unspiked Clay Soil
Rig1:Blank
Rig2:Clay, unspiked
Sample Added
Cuvettes Swapped
Relative Light Units
Time (Seconds)
5000000
4500000
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
0
100
200
300
400
500
600
700

Figure 10

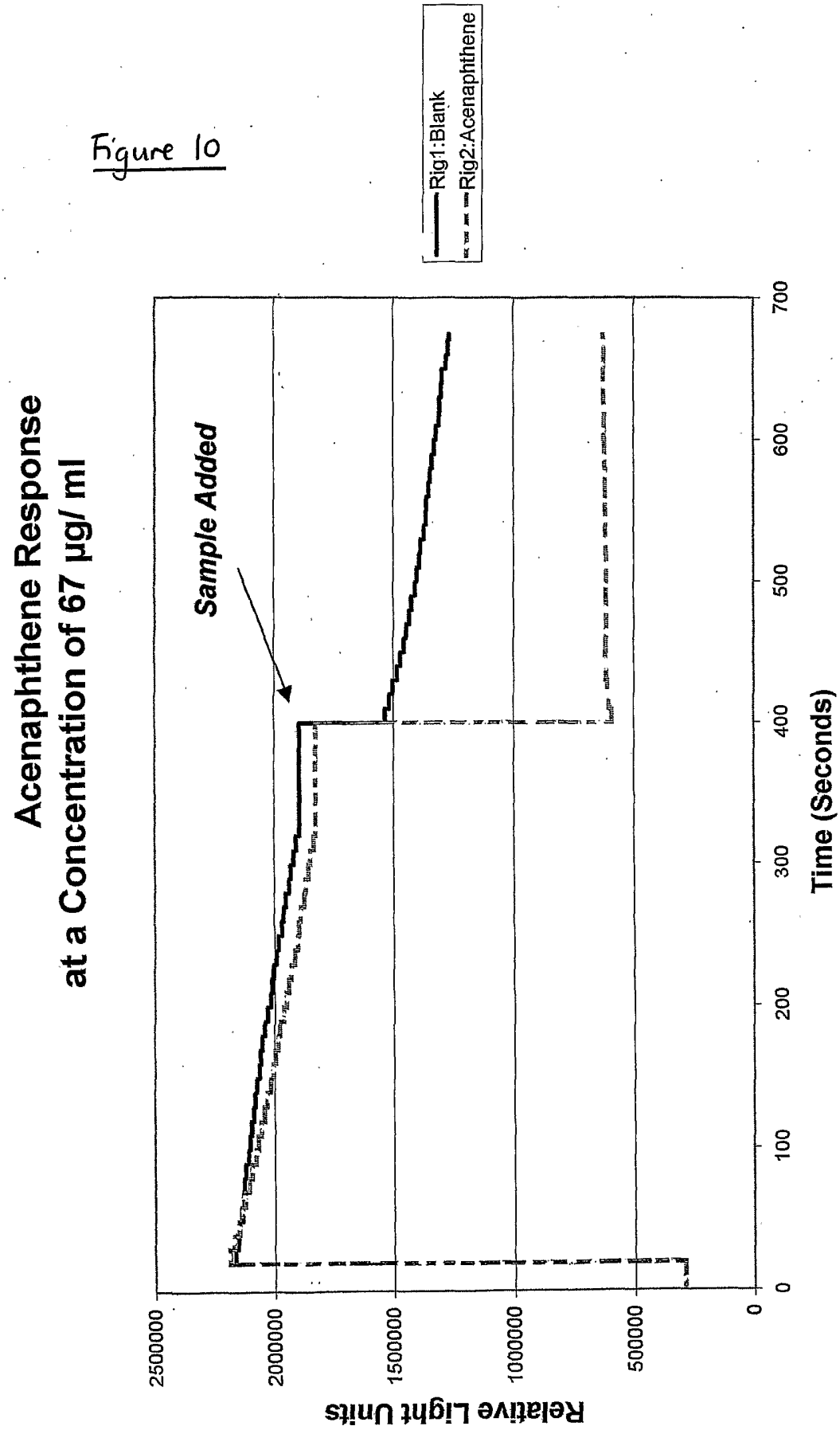

Acenaphthene Response
at a Concentration of 67 µg/ ml
Rig1:Blank
Rig2:Acenaphthene
Sample Added
Relative Light Units
Time (Seconds)
0
500000
1000000
1500000
2000000
2500000
0
100
200
300
400
500
600
700

Figure 11

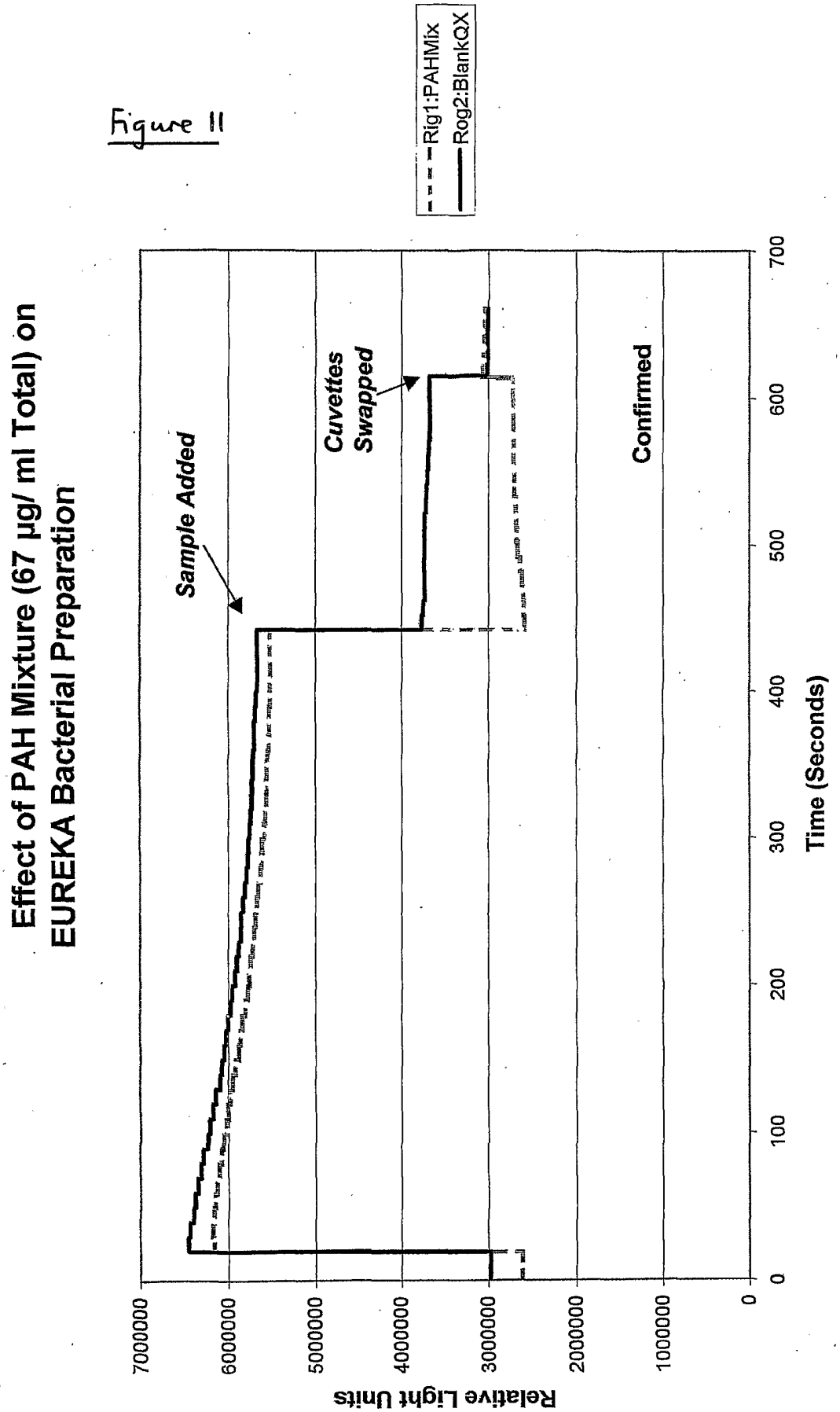
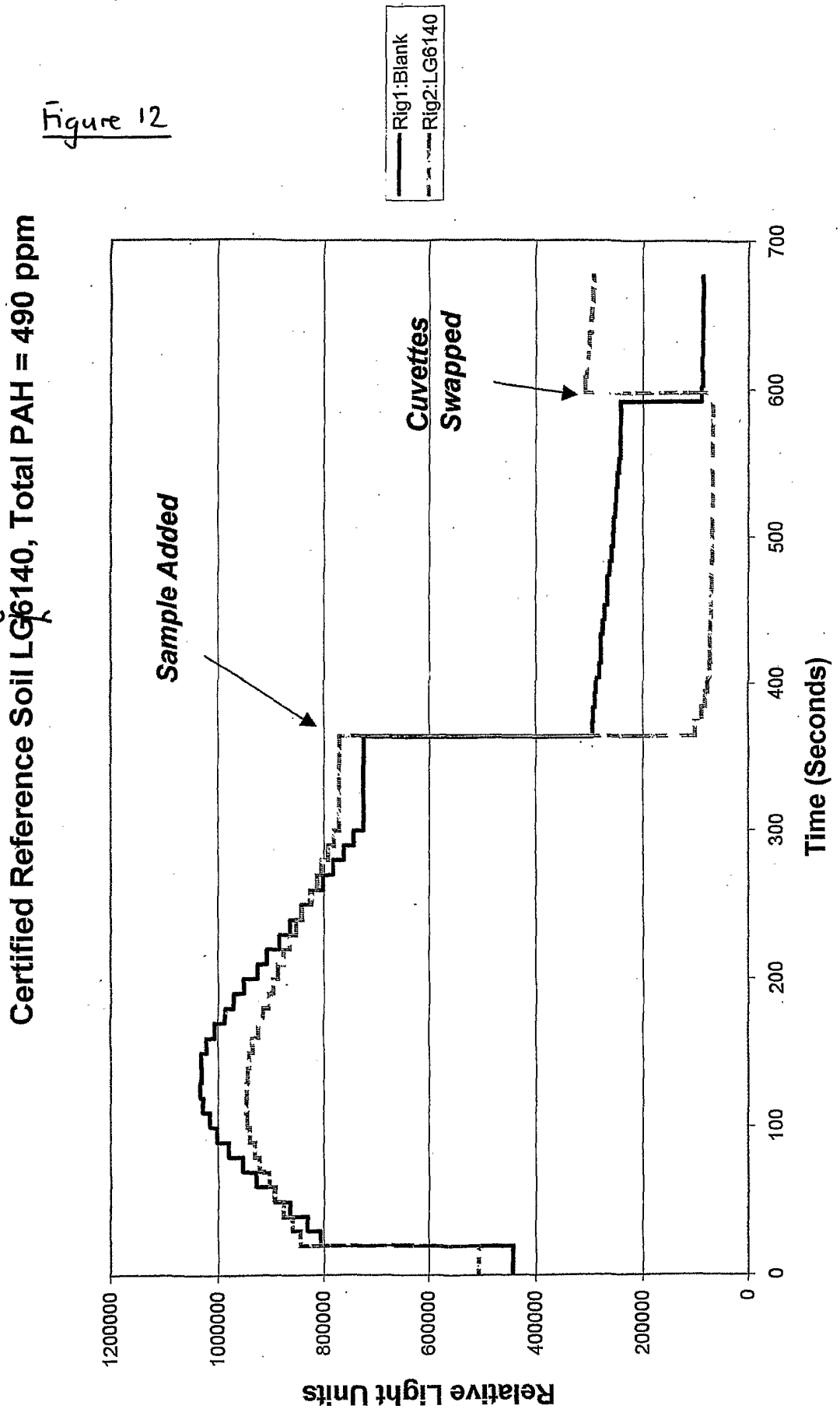

Effect of PAH Mixture (67 µg/ ml Total) on EUREKA Bacterial Preparation
Rig1:PAHMix
Rog2:BlankQX
Sample Added
Cuvettes Swapped
Confirmed
Relative Light Units
Time (Seconds)
0
1000000
2000000
3000000
4000000
5000000
6000000
7000000
0
100
200
300
400
500
600
700

Figure 12


Certified Reference Soil LG6140, Total PAH = 490 ppm
Rig1:Blank
Rig2:LG6140
Sample Added
Cuvettes Swapped
Relative Light Units
Time (Seconds)
0
200000
400000
600000
800000
1000000
1200000
0
100
200
300
400
500
600
700