DOE/ER/61193-1

ON-LINE MONITORING OF AEROBIC BIOREMEDIATION WITH BIOLUMINESCENT REPORTER MICROBES

Final Report
July 1991 - December 1994

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March 1995

PREPARED FOR THE U.S. DEPARTMENT OF ENERGY
UNDER GRANT NUMBER DE-FG05-91ER61193

DISCLAIMER

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Introduction

A critical issue in the biological characterization of contaminated sites and in the evaluation of relative bioremediation treatment efficiencies is the development of appropriate monitoring methods for the assessment of pollutant bioavailability and microbial *in situ* activity potential. In nature, pollutants are found dispersed among the solid, liquid and gaseous phases of the complex environments rendering the analytical estimation of their bioavailability and degradation more difficult and irrelevant. *Ex situ* and extractive analytical techniques have only been misrepresentative of the natural conditions and often resulted in inaccurate estimates of pollutants mass transfer.

Bacterial degradation of polyaromatic hydrocarbons such as naphthalene and phenanthrene have been dependent on the dissolved fraction of the compounds present in the environmental matrix (Stucki and Alexander, 1987; Wodzinski and Bertolini, 1972; Wodzinski and Coyle, 1974). The physical adsorption of naphthalene to soil particles is an added hindrance making it not readily bioavailable for bacterial degradation (Al-Bashir et al., 1990; Mihelcic and Luthy, 1991). Therefore, development of efficient, sensitive and specific quantitative methods to determine the bioavailability and degradation of specific pollutants has been a major issue in the field of bioremediation.

During the past decade, few novel bioanalytical and molecular diagnostic methods have been developed for environmental systems (Levin et al., 1992). Many of these techniques could be adopted for the biological characterization of contaminated sites and for the assessment of bioremediation efficiency (Heitzer and Sayler, 1993). The presence of specific pollutants and their degradation has been shown using specific gene probe

techniques. Gene probes have been used in the determination of the relative gene frequencies and distribution in the environment and they have also provided direct evidence on the catabolic gene expression levels (Bowman et al., 1993, Holben et al., 1992, Sayler and Layton, 1990). However, these techniques are not suitable and cannot be extended to provide any information on the precise bioavailability of a specific compound and on the physiological status of the microbial community involved in the degradation of the specific compound.

Since many of the genes involved in the degradation of organic pollutants are specific and inducible, the expression of such catabolic genes can be a measure of the presence, availability, and biodegradation of a particular pollutant or a group of pollutants. Measurement of gene expression can be done directly or indirectly. By the indirect method, instead of measuring the expression of the actual catabolic gene, the expression of a reporter gene fused to the actual catabolic gene is measured. Usually, the reporter genes are selected such that their gene product could be detected with readily and easily. The use of bioluminescent genes as reporters has been shown to be advantageous than many other genes. This gene produces light that can be measured with rapid, uncomplicated, inexpensive, sensitive and reliable devices.

Reporter gene technology has recently been a primary cause in the development of bioluminescent reporter bacteria, capable of sensing pollutants such as naphthalene, toluene and heavy metal like mercury (King et al., 1990; Selifonova et al., 1993). These bacteria produce light with a wave length of 490nm, when exposed to the respective catabolic substrate. The bioluminescence genes (*lux*) were originally cloned out of the

marine bacteria *Vibrio fischeri*. Promoterless *lux* gene cassette (*luxCDABE*) was then employed in creating gene fusions with desired catabolic gene promoters.

The bioluminescence reaction is catalyzed by a heterodimeric luciferase enzyme and a fatty acid reductase complex encoded by the *luxAB* and *luxCDE* genes, respectively.

$$RCHO + FMNH_2 + O_2 \text{ ---------- } RCOOH + FMN + H_2O + LIGHT\ (490nm)$$

$$RCOOH + NADPH + ATP \text{ ---------- } RCHO + NADP + AMP + PPi$$

The *lux* reaction requires oxygen, reduced flavomononucleotide, and fatty aldehyde substrate. The reductase complex is responsible for the production of the aldehyde and recycling of the corresponding fatty acid in the lux reaction. The reductase complex consists of a reductase, a transferase and a synthetase, encoded by *luxC*, *luxD* and *luxE* respectively, and is dependent on ATP and NADPH.

Engineered bacteria containing the *lux* gene cassette in critical gene operons of metabolic processes involved in bioremediation of organic pollutants can serve as "reporters", monitoring these critical processes as the parameters of the subsurface systems change or when manipulated. Recently, *Pseudomonas fluorescens* HK44 was constructed as a bioluminescent bacteria, capable of monitoring the availability and catabolism of naphthalene. Exposure of this bacteria to both naphthalene and its degradation intermediate, salicylate, resulted in increased gene expression and consequently, increased bioluminescence. A positive correlation between naphthalene concentration and the amount of bioluminescence has been revealed under continuos

cultivation conditions (King et al., 1990). Subsequently, linear relationship between naphthalene or salicylate concentrations and overall bioluminescence was demonstarted and resulted in the development of a quantitative and specific bioassay for the bioavailability of these two compounds (Heitzer et al., 1992). However, these assays could be done only in discontinuous fashion and *ex situ*. Therefore, the objective of this project was to develop a biosensor device that would allow continuos on-line monitoring of pollutant bioavailability and specific microbial activity potential.

A biosensor can be defined as an apparatus consisting of a biological sensing component, connected or integrated to a physico-chemical transducer. Numerous biosensors have be proposed; while most of them include macromolecules like protein or or nucleic acid, whole cell biosensors have also been reported. The transducer can be any electrical or chemical element that produces amperometric, potentiometric, conductimetric outputs or optical changes like light emission, reflection, absorption and fluorescence.

In this project, the bioluminescent bioreporter bacterium *P.fluorescens* HK44 was integrated to an optical device, capable of conducting emitted light, and used as an on-line biosensor of naphthalene and salicylate. The physiological requirements of the bacteria and the physical limitations of the biosensor were also determined.

OBJECTIVES

1) Development of a whole cell on-line biosensor for the monitoring of naphthalene and salicylate presence and bioavailability with *Pseudomonas fluorecens* HK44

2) Evaluation of the quantitative response of the biosensor to the bioluminescence signal stability, reproducibility and specificity

3) Demonstration of the environmental application of the biosensor

4) Establishment of the bioluminescence induction pattern and robustness of HK44 for an extended period of time, when subjected to stress.

5) Construction of bioluminescent reporter strain with *tod-lux* fusions, and apply it to detect toluene and co-metabolism of TCE

Materials and Methods

(I) *Pseudomonas fluorescens* HK44 as an on-line whole cell biosensor

Organism: *Pseudomonas fluorescens* HK44, a bioluminescent catabolic reporter bacterium was used in all the experiments conducted (King et al. 1990). This strain is able to degrade both salicylate and naphthalene and carries a *nah-lux* reporter plasmid , pUTK21, that allows naphthalene and salicylate catabolism to be monitored. The plasmid contains a transcriptional gene fusion between a *luxCDABE* gene cassette from *Vibrio fischeri and* the *nahG* gene of the salicylate operon.

Immobilization procedure: *Pseudomonas fluorescens* HK44, were harvested from an exponentially growing batch culture in a yeast extract-peptone-glucose medium at an optical density of 0.8 at 546 nm. The reporter was washed once in 0.9% NaCl and 12.5 ml of this culture was mixed with 25 ml of a sterile, low viscosity alginate solution 3.5% (w/w) in 0.9% NaCl and with 7.5 ml sterile glycerol. After mixing aliquots of 1.5 ml were

kept frozen at -70°C for further use. Probe tips were prepared by injecting the cell-alginate mixture into the ferrule on the liquid light guide and immersion in a stirred 0.1 mM $SrCl_2$ solution for 60 min to harden the strontium-alginate matrix.

Growth conditions and experimental set up: The probe tip was inserted into the biosensor device. A maintenance medium of the following composition was used: $SrCl_2.6H_20$, 666 mg/l; NH_4NO_3, 100 mg/l; glucose, 1 0 mg/l; peptone, 20 mg/l; Na_2S0_4, 7.2 mg/l; and tetracycline, 28 mg/l. The medium was buffered at pH 7.0 with 100 ml/l of a 50 mM potassium phosphate solution.

Waste stream solutions: Salicylate solutions, 10 mg/l, were prepared in sterile water. Saturated naphthalene solutions, 31 mg/l, were prepared by adding naphthalene crystals to either stirred, sterile water or sterile maintenance medium, the latter for experiments with changing naphthalene concentrations. All other test solutions were prepared in sterile water.

Manufactured gas plant (MGP) soil leachates were obtained from MGP soil from as site in the northeastern United States (Sanseverino, 1994). A sandy loam soil composed of 43.4% silt-clay was sieved, and a fraction containing particles that were <3.5 mm in diameter was used. The naphthalene concentration in this soil was 320 +/- 10 mg/kg (dry weigt)

Biosensor set-up: Figure 1 provides a scheme of the biosensor set up. The liquid volume of the biosensor cell was 1.5 ml. Both maintenance medium in combination with either a waste stream solutions or water for reconditioning of the biosensor were continuously added to the biosensor at flow rates of 2.5 ml/min each, resulting in a total

flow of 5 ml/min. Experiments were conducted at a constant, controlled temperature of 25°C. Viable plate counts were conducted after serial dilutions of samples and plating on yeast extract peptone glucose agar with or without 14 mg/l tetracycline.

Analytical and monitoring procedures:

Bioluminescence was monitored on line using a liquid light guide in combination with an photomultiplier (model 77340, Oriel, Stratford, CT) and a digital display (model 7070, Oriel, Stratford, CT). Data were stored automatically on a IBM PC using a software program written by Rod Bunn (University of Tennessee).

Salicylate was determined spectrophotometrically at 296 nm.

Naphthalene was measured using HPLC (model 5560,Varian, Palo Alto, CA) in combination with UV detection at 254 nm. Separation was achieved using a 25 cm C_{18} reverse phase column (VYDAC 201TPB5, Separations Group, Hesparia, CA) and a continuous aqueous acetonitrile gradient from 0 to 65% between minute 1 and 2. At the end of the 12.5 min program the column was equilibrated for 0.5 min with H_2O. A flow rate of 1.5 ml/min was used with sample injection occurring at 0.4 min (manual injection) or at 1 min (on-line automatic injection).

Glucose was determined colorimetrically using an enzyme assay kit (sigma Chemical Co., St. Louis, MO.).

Dissolved oxygen was determined in a 53 Biological Oxygen Monitor respirometer (Yellow Springs Instrument Co., Yellow Springs, OH.) by collecting effluent samples from effluent line and transferring into the measurement cell of the respirometer by a directly connected syringe.

Colony hybridizations were conducted as described by Sayler et al. 1985 with a PCR generated, single stranded, ^{32}P-labeled 1717 base fragment of the *luxAB* genes was used as a probe.

(II) Environmental application of *P.fluorescens* HK44 biosensor

Organism and growth media: Same as described in previous section.

Bioavailability assays: The bioavailability assays were conducted as described earlier (10). An overnight culture from a frozen stock of *P. fluorescens* HK44 was prepared in 300 ml Erlenmeyer flasks containing 100 ml YEPG medium. A subculture was then prepared and the optical density at 546 nm (OD_{546}) was monitored in 30 min intervals. At a predefined OD_{546} Of 0.35, 2 ml aliquots of the exponentially growing culture were added to 25 ml mineralization vials (Pierce, Rockford, IL) with teflon lined screw caps containing 2 ml of the test sample. Experiments were conducted at 25°C except for the soil extract samples from the petroleum contaminated samples from Kwajalein island which were conducted at 27° C. Bioluminescence was monitored after a defined incubation time of 1 hour if not otherwise indicated. Carbon starved, resting cultures were prepared as described earlier (Heitzer et al., 1992).

Contaminated soils: The soil samples consisted of coral sand and were from a core taken at a site on Kwajalein island, mostly contaminated with petroleum hydrocarbons (Walker & Walker, 1994). Sample A was a presumably uncontaminated control soil from the top of the core while sample B was heavily contaminated with petroleum hydrocarbons, exhibiting a characteristic odour. Soil portions of 10 g and 10 ml distilled water were added to 25 ml Corex glass centrifuge tubes (Corning Inc., Corning, NY) with

teflon lined screw caps. The samples were incubated at 27°C for 1 hour on a shaker in order to extract the contaminants. The soil phase was separated by centrifugation at 7311 x g for 10 min at 27°C and 2 ml aliquots of the aqueous phases were transferred to the test vials for bioavailability assays.

Solvent solutions: Saturated solutions of either JP-4 jet fuel, toluene or n-alkanes were prepared by adding 1 ml solvent to 20 ml sterile water in a 25 ml mineralization vial (Pierce, Rockford, IL) with a teflon lined screw cap and mixing for several hours on a rotary-shaker. After phase separation samples of the aqueous phase were transferred into test vials. A 1 % (v/v) acetone solution was prepared in sterile water. The saturated naphthalene solution was obtained by adding naphthalene crystals to sterile water and equilibration under stirring over night. Different naphthalene concentrations for a standard curve were obtained by preparing appropriate dilutions. Heavy metal and cyanide solutions of the following concentrations in milligrams per liter were prepared in water: $CdCl_2$, 366; $HgCl_2$, 542; KCN, 172.

Analytical procedures:

Bioluminescence measurements: The test vials were inserted into a light tight measurement cell and the light output was measured using a liquid light cable connected to a photomultiplier (model 77340, Oriel, Stratford, CT) and a digital display (model 7070 Oriel, Stratford, CT). The effect of n-decanal was investigated by adding 160 µl of a freshly prepared, vigorously shaken 1% (v/v) aqueous solution of n-decanal to the test sample prior to the light reading. Larger volumes of n-decanal solution did not result in a

further bioluminescence increase in samples induced with salicylate. For comparisons, the peak bioluminescence value was used.

Naphthalene estimation: HPLC analyzed as described in the previous section.

Membrane fatty acids extraction: These were extracted and analysed as described by Guckert et al. (1991) and the same nomenclature was used. For the extraction 3 ml of a culture with an OD_{546} Of 0.32 was centrifuged at 7311 x g for 5 min at 2°C and washed once. The pellets were kept frozen at -70°C for further processing.

mRNA-extraction and probing: The 4 ml culture contents of the test vials were frozen immediately by transferring them into precooled centrifuge tubes on dry ice and ethanol. The samples were kept frozen at -70°C for further processing. The RNA extraction was then conducted as described by Oelmuller et al. (1990). For slot hybridization RNA in concentrations of approx. 10, 2.5, 0.5 and 0.25 µg were loaded on nylon membranes and processed as described in Sambrook et al. (1989). In order to prove that only RNA and not DNA was processed, a separate filter membrane containing from each sample a fraction that was treated with DNase free RNase (Boehringer Mannheim Corp., Indianapolis, IN) was prepared as control. All the filter membranes were subsequently prehybridized and hybridized according manfacturer's instructions.
In order to detect the *lux*-mRNA a PCR generated, digoxigenin-labeled, single stranded antisense DNA probe was used for hybridization. The 1717 base *luxAB* fragment extends from base 4254 in the *luxA* gene to base 5971 in the *luxB* gene (Baldwin et al., 1989). The sequence of the primer used for the PCR reaction was GTCATCATGAGACCCTACTGC. The reaction mixture contained 10 µl of previously

generated double stranded *luxAB* fragment as template, 2.5 µl primer (5ng/ml), 20 µl dNTP labeling mixture (Genius, Boehringer Mannheim Corp., Indianapolis, IN), 10 µl reaction mix (10x) (Gene^{amp}, Perkin Elmer), 1 µl Taq polymerase and 66.5 µl H_20. Thermocycler conditions for the 38 cycles were: 1 minute at 94°C for strand melting, 2 minutes at 55°C for primer annealing and 3 minutes at 72°C for strand extension. The filters were washed and the color was developed according to the manufacturers protocol (Genius, Boehringer Mannheim Corp., Indianapolis, IN). Photographs were taken from the developed filters using a Polaroid type 665 positive/negative film. The hybridization bands on the negatives were scanned and quantified using a BioImage (Millipore Corp., Bedford, Mass) imaging system and a Whole Band analysis software package (Millipore Corp., Bedford, Mass). The image of the negative was converted into a positive picture using the image processor prior to analysis. All the values obtained were divided by the exact amounts of RNA loaded onto the filter membranes in order to obtain specific *lux*-mRNA amounts that can be compared to each other. The data reported were obtained from 3 independently prepared filter membranes and RNA isolations and represent averages.

(III) Survival and bioluminescence induction of HK44 when incubated in groundwater

Organism and culture conditions: To investigate the effects of groundwater possessing different pH values, HK44 was grown to exponential stage in YEPG medium and transferred to groundwater as alginate-encapsulated beads for long-term assay. The groundwater was prepared in the lab and contained, in mg per liter of distilled water, the following: $CaCl_2$, 166; $MgCl_2.6H_2O$, 85; $BaCl_2.2H_2O$, 1.8; $SrCl_2.6H_2O$, 0.6;

$FeSO_4.7H_2O$, 25; and KNO_3, 17. Double strength groundwater was prepared and diluted with respective buffer solutions to yield single strength groundwater containing pH values 3, 4, 5, 6 and 7. The following stocks of buffer solutions were used to adjust to the respective pH: 0.2 M Potassium hydrogen phthalate with 0.2 M HCl for pH 3 and 4 or with 0.2 M NaOH for pH 5 and 6; and 0.2 M Tris with 0.2 M HCl for pH 7. The groundwater was thoroughly mixed, passed through Whatman filter paper and sterilized by autoclaving.

Long-term incubation and induction of HK44: The set up consisted of alginate beads, encapsulating HK44, incubated in groundwater and induced at intervals for bioluminescence response. The cells were encapsulated as described in the first section of materials and methods. Five hundred milligrams of beads were weighed into 3 ml of groundwater in sterile 25 ml mineralization vials. For every type of groundwater a set of triplicates were set up and each set were in multiples of atleast 7. Because, a set of three was sacrificed everytime, when induced for light, and there were 6 separate inductions on day 1, 7, 14, 21, 28 and 35. The extra vials were for back ups, if required. The induction and light measurements were performed as described in the previous section. Control included vials consisting of beads incubated in 0.1 strength YEPG (a rich medium control). From each set of groundwater controls consisting no inducer were used to measure background light production. Upon induction, light outputs were measured every 30 minutes from time zero onwards to 5 hours.

Inducer solutions. Simple induction solutions provided a final concentration of 19 mg l^{-1} of sodium salicylate, complex induction solution provided a final concentration of

19 mg l^{-1} of sodium salicylate from a stock prepared with distilled water and containing YEP.

Analytical procedures:

Bioluminescence measurements, salicylate concentrations and viable colony counts were performed as described in section I, and section II of materials and methods.

(IV) *tod-lux* bioluminescent reporter for TCE co-metabolism

Creation of *tod-lux* reporter *Pseudomonas putida* B2: The strains and plasmids used in this study are *E.coli* DH5α, *E.coli* HB101, *E. Coli* DF1020 and *Pseudomonas putida* F1 and pDTG514, pUCD615 and pRK2013, respectively. *Eschericia coli* was grown in Luria broth (LB) and on LB agar plates at 37°C. *Pseudomonas putida* Fl was grown on a yeast extract-peptone-glucose (YEPG) medium. *E. coil* transformants were screened on LB plates containing ampicillin (50 μg/ml). The reporter plasmid pUTK30 was generated by cloning the *tod* promoter fragment in front of the *lux* genes of pUCD615. This was accomplished by directionally cloning a 2.75 kb EcoRI, XbaI fragment from pDTG514. One liter cultures of *E. coli* DH5α harboring the appropriate plasmid were harvested and plasmid DNA was isolated using a modified alkaline lysis procedure (Maniatis et al., 1982). The plasmid DNA was then subjected to CsCl density gradient purification, followed by butanol extraction and ethanol precipitation. Plasmid DNA was resuspended in TE buffer (10 mM Tris, 1 mM EDTA) and stored at 4°C until use. Restriction enzymes and T4 ligase were obtained from GIBCO BRL (Gaithersburg, MD U.S.A.) and used according to manufacturers protocols. Cloning techniques were performed as outlined in Manniatis et al (1982). Transformations were performed using

subcloning efficiency competent cells purchased from GIBCO BRL according to manufacturers protocol. Plasmid minipreps of transformants were performed as described by Holmes and Quigley (1981) and the DNA was cleaved with BamHI to confirm the location of the *tod* fragment The resulting *tod-lux* reporter plasmid was designated pUTK30.

Triparental matings were carried out using a modified version of the filter technique. Pure cultures of donor (JBF-7), Helper (2013), and Recipient (F-1) were grown to an A_{546} of approximately 1.0 in LB broth with selective pressure. Cells were harvested by centrifugation at 7311 x g for 10 min. The pellets were resuspended and washed three times in 100 ml 50mM KH_2PO_4, after which they were resuspended in 50 ml KH_2PO_4.

A PC membrane (47mm X 1.0 μm) was placed on a non-selective (LB) agar plate and the three strains were mixed using a ratio of 2:1:1 (Donor:Helper:Recipient). The mixtures were incubated overnight and the filter was then removed and washed in 1.5 ml of 50 mM KH_2PO_4. A serial dilution was carried out and the resultant reporter plasmid pUTK30 was mated into *P. putida* F1. Exconjugants were selected for light production by incubation for 48 hr on *pseudomanas* isolation agar plates containing kanamycin (50 mg/ml) and toluene vapor. Five of the exconjugants were selected for further study while strain B2 was selected for use in the TCE DVR experiments.

Reactor application of pUTK30 in monitoring TCE cometabolism: Alginate encapsulated strain B2 was used in a plug flow differential volume reactor (DVR) to correlate toluene induced light emmission with toluene degradation and TCE co-

metabolism. Strain B2 was grown in 1 L LB broth to an A_{600} of 1.0. The cells were then centrifuged at 6000 x g for ten minutes and washed in 0.9%(w/v) NaCl. The final wash was resuspended in 120 ml of an alginic acid solution. Aliquots of the cell-alginate suspension (app. 60 mg cells total) was placed in a 60 ml syringe, forced through a 25 gauge needle, and allowed to drop into a 0.5 M $CaCl_2$ solution. The alginate was cross linked by the Ca^{+2} ions, thus encapsulating the cells. The cells were then placed in a fresh solution of $CaCl_2$ and allowed to sit for 30 minutes prior to use.

Differential volume reactor set-up: For the study an 80 ml volume of encapsulated B2 strain was used to prepare the packed bed for the, DVR (figure 2). The bed dimensions were 1.75 in diameter by 2.0 in height. The flow rate across the bed was 0.4 ml/min and using an estimated void fraction of 0.45 the retention time was calculated at 1.5 hr. The system was equipped with three Millipore stainless steel substrate containers rated to 690 kPa. The feed from the substrate vessels to the reactor inlet was controlled by two Gilson 301 HPLC pumps. The substrate vessels were pressurized with either oxygen or air to provide the system with an electron acceptor. All medium vessels contained trace mineral medium with the addition of 3 mg/1 pyruvic acid and a 0.1 M solution of trizma base kept at a pH of 7.0. In addition to medium, the different vessels contained either TCE or toluene. The reactor effluent entered a 12.7 cm stripping column that was packed with 3mm glass beads to provide adequate surface area for the volatile separation process. The volatiles were stripped with helium, the GC carrier gas, in a concurrent fashion through the stripper. The outlet of the stripping column was attached directly to a gas chromatograph (Packard 5890 Series H) with an electron capture

detector (ECD) through a heated sample line maintained at 75°C. Automatic injections (25 µl sample loop) were made on the GC by a computerized control process (Hewlett Packard computer, HP Chem Station software). The GC was equipped with a cross-linked methyl silicone capillary column (internal diameter of 0.2 mm, 0.33 µm film thickness) while the oven temperature was operated isothermally at 60°C. Other operating parameters include an injection temperature of 150°C, the detector temperature of 200°C, and a split ratio was set at 10:1. This system was equipped with a bypass line around the reactor in order to calibrate the stripping column. Gas sampling ports for measuring inlet and effluent toluene concentrations were available. Liquid phase toluene samples were quantified by head space analysis using a Schimadzu GC-9A gas chromatograph equipped with a Poropak N packed column (2.44 m long and 3.2 mm diameter) and a flame ionization detector. The isothermal temperature of the oven was 210°C, and both the detector and injector temperatures were 220°C. An Oriel model 770 photomultiplier, with a liquid light pipe and culminating beam probe and computer were used to collect the bioluminescence data.

Results

(I) On-line biosensor

Biosensor response to a step change in salicylate concentration: In figure 3 the bioluminescence response of the biosensor to a step change from 0 to 10 mg/l salicylate (5 mg/l in the measurement cell) in the waste stream is shown. The bioluminescence signal

increased approximately 15 to 20 min after exposure to salicylate from a baseline level of 0.6 namp to an average level of 13+/-3 namp during the following 8 hours after the step change. Similar results were also obtained for the priority pollutant naphthalene.

Biosensor response to gradual changes in naphthalene concentration: The event of a passing pollution plume in a waste stream was simulated using gradual step changes in the naphthalene feed concentration from 0 to 3.1, 10.33, 15.5 and 31 mg/l in 2 hour increments (Fig. 3). The corresponding biosensor cell naphthalene concentrations were 0, 1.55, 5.16, 7.75 and 15.5 mg/l. The naphthalene solution was prepared in maintenance medium to provide a constant carbon substrate background since concentration was varied using different flow ratios between the maintenance medium pump and the naphthalene source pump. The total flow was kept constant at 5 ml/min. The bioluminescence response followed closely the naphthalene concentration pattern with a time delay. For the naphthalene degradation pathway inducer salicylate, similar results were obtained.

Biosensor response to repetitive naphthalene and salicylate perturbations: The reproducibility of the biosensor response was investigated using repetitive defined perturbations of 1 hour duration with either 10 mg/l salicylate or 31 mg/l naphthalene (5 mg/l and 15.5 mg/l biosensor cell concentrations, respectively). In figure 4 the bioluminescence response to salicylate perturbations is shown. Good reproducibility of the peak levels at 5 namp was observed. The recovery time necessary from the maximum bioluminescence level to the initial baseline bioluminescence of 0.6 namp was approx. 5 hours. For naphthalene, generally similar results were obtained with significantly higher

peak bioluminescence levels at 110 to 120 namp. Response times of the biosensor were 15 min for naphthalene and 15 to 20 min for salicylate.

Comparison of non-inducing compounds with naphthalene: The response of the biosensor to a model waste stream containing a high, easily degradable carbon load was investigated. In figure 5A the carbon load was increased at 1 hour from 0 to 1 g/l glucose (0-5 g/l in biosensor cell). Figure 5B shows the response to a complex medium containing 1 g/l peptone, 0.5 g/l glucose and 100 mg/l yeast extract. In both cases a minor increase in the bioluminescence response was observed 1 hour after the step change. However, as compared to the response to 31 mg/l naphthalene (Fig. 5C) the response time was much longer and the absolute magnitude of the bioluminescence signal was minor. In figure 5D the response to a 25% saturated toluene solution (aqueous phase of a biphasic solution containing 100 ml toluene in 1 l water) is shown. After exposure to toluene, the overall bioluminescence decreased slightly. After 2 hours exposure, a good recovery of the system was observed (results not shown).

Response to complex environmentally relevant and real pollutant mixtures: In order to evaluate the practical utility of the biosensor system experiments were conducted using relevant pollutant mixtures. In figure 6A the bioluminescence response to an aqueous solution, saturated with JP-4 jet fuel (biphasic 100 ml jet fuel and 1 l water) is shown. JP-4 jet fuel contains 0.5% (w/w) naphthalene (Riser & Roberts 1992). Naphthalene was detectable at concentrations of 0.55 mg/l in the effluent of the biosensor. The response time of the bioluminescence signal was similar to aqueous naphthalene solutions at lower concentrations. Figure 6B illustrates the bioluminescence response to

the aqueous leachate from a manufactured gas plant (MGP) soil. A soil column packed with 135 g (dry weight) of MGP soil was continuously flushed with water at a flow rate of 2.5 ml. The aqueous volume in the column was 32 ml. The naphthalene concentration in the effluent of the soil column was 0.6 mg/l (0.3 mg/l biosensor effluent) during the experiment. The bioluminescence response increased from a baseline level at 0.5 namp to a maximum of approx. 10 namp two hours after exposure to the leachate.

General characterization and performance of the biosensor: The biosensor probe tip contains approximately 2×10^7 viable cells of the reporter bacterium *P. fluorescens* HK44 at the beginning of an experiment. During the course of an experiment reporter bacteria are released from the biosensor cell. Four days after the beginning of an experiment the effluent cell concentration was approx. 3×10^4 cells/ml. All colonies probed positive during colony hybridizations using a *lux* gene probe. Experiments were conducted continuously for 14 days with the same probe tip. The formation of a biofilm in the biosensor cell was observed during the course of a long term experiment. After 9 days of operation the bioluminescence produced by this biofilm corresponded to approximately 10% of the total light readout.

Oxygen concentration: An important operating parameter is the oxygen partial pressure in the biosensor system. Measurement in the effluent of the biosensor under non induced conditions provided values of 80 to 95 % saturation. Comparative studies conducted under induced conditions with a 10 mg/l salicylate solution which was either degassed with helium or aerated, revealed that the bioluminescence signal was not affected

even at effluent saturation levels of 49% saturation which corresponds to a situation of an anaerobic waste stream since the maintenance medium is completely saturated.

(II) Physiological affects of HK44 when exposed to environmental contaminants

Bioluminescence response of HK44 when exposed to environmental contaminants: The response due to naphthalene presence and bioavailability in aqueous extracts of soils contaminated with petroleum hydrocarbons are summarized in Table 1. For each experimental set which involved two different soils, uncontaminated soil A and a heavily contaminated soil B, a series of naphthalene concentrations were included as well, in order to prepare a bioluminescence versus naphthalene standard curve. For all the samples investigated, the final biomass concentrations were identical and remained unaffected by the contaminants. The regression lines obtained were for set a: $y = 12.8436x + 1.418$ with $r2 = 0.992$ for 1 1 concentration samples over a range from 0 to 0.725 mg/l naphthalene and for set b: $y = 11.5327x + 1.8063$ with $r2 = 0.983$ for 13 concentration samples over a range from 0 to 0.711 mg/l naphthalene. Based on these curves and on the average bioluminescence response obtained from triplicate test samples, the amount of bioavailable naphthalene was estimated. The concentration ranges for the estimated naphthalene concentrations were calculated using the intersection points of the upper and lower standard deviation limits of the measured bioluminescence values in the test samples and the lower and upper 95% confidence curves around the standard regression lines, respectively. From Table 1 it can be seen that for soil A the estimated naphthalene concentrations were close to zero while for soil B the estimated concentrations were about 90 μg/l. This naphthalene concentration pattern was qualitatively reflected in the

analytically determined naphthalene concentrations of these samples, but a quantitative comparison of estimated and analytically determined naphthalene concentrations for soil B revealed a reproducible and significant discrepancy.

In figure 7 the bioluminescence response of *P. fluorescens* HK44 to different amounts of JP-4 jet fuel is shown. The dilution factors used referred to an aqueous solution, saturated with JP-4. A good linearity was found for the dilution investigated which included 12 samples with a range from 0.03 to 0.5 and a control with water. The regression line was described by the equation: $y = 104.83x + 1.2656$ with $r2 = 0.967$. For all the samples investigated, the final biomass concentrations were identical and remained unaffected by the amount of JP-4. The effect of different aliphatic and aromatic hydrocarbons present in diesel fuel, gasoline or jet fuel, on the bioluminescent response in *P. fluorescens* HK44 was investigated. Table 2 summarizes the results for a number of relevant compounds: It was interesting to note that 64 mg/l toluene and 22 mg/l p-xylene as well as 980 mg/l acetone, an industrially relevant pollutant, caused a significant bioluminescence increase as compared to a control with water. In addition, for mixtures of the same concentrations of either acetone and toluene or acetone and p-xylene the responses were exactly additive but not for a mixture of toluene and p-xylene. In contrast to the bioluminescence results obtained without n-decanal, the bioluminescence values after addition of n-decanal were not significantly different from each other for all these combinations. For 50% saturated aqueous solutions of the alkanes n-decane, n-undecane and n-dodecane only minor increases in the bioluminescence responses were found and the

presence of either 183 mg/l $CdCl_2$, 271 mg/l $HgCl_2$ or 86 mg/l KCN resulted in significantly decreased bioluminescence levels compared to the control.

The effect of the physiological growth state of *P. fluorescens* HK44 on the bioluminescence response after exposure to 64 mg/l toluene and 3.9 mg/l naphthalene, either individually or as a mixture, is presented in figure 8. For comparative reasons bioluminescence values were presented in nanoampere per optical density units, since the final biomass levels were different for growing and resting cultures. Figures 8A and 8B illustrate the bioluminescence levels without the addition of n-decanal for growing and carbon-starved, resting cultures, respectively. In figures 8C and 8D the bioluminescence response after the addition of n-decanal for growing and carbon-starved, resting cultures are presented, respectively. The exposure of a growing culture (Fig. 8A) to either toluene or naphthalene resulted in a significantly increased bioluminescence response compared to the control. A mixture of naphthalene and toluene results in a bioluminescence level which is higher than the sum of the individual responses to both toluene and naphthalene. In contrast, the addition of aldehyde to growing cultures under identical conditions results in a different response pattern (Fig. 8C). The bioluminescence levels of both control and toluene were identical and significantly smaller than the level for naphthalene and naphthalene plus toluene which were also identical. For the carbon-starved, resting culture exposure to toluene provides no bioluminescence, identical to the control, while for both naphthalene and naphthalene plus toluene a significant response was obvious in the absence of n-decanal (Fig. 8B). The addition of n-decanal to carbon-starved, resting cultures resulted in an analogous bioluminescence response pattern as demonstrated in

Figure 8D with the only difference that for the control and for the toluene sample a significant bioluminescence was found. In general, the bioluminescence responses found for the growing cultures were about an order of magnitude higher than for the carbon-starved, resting cultures. Further, the bioluminescence levels did increase by about one order of magnitude after the addition of n-decanal for both physiological culture states.

In figure 9 a comparative analysis of the response of the growing *P. fluorescens* HK44 culture after exposure to water (control), 64 mg/l toluene, 3.9 mg/l naphthalene and an 8x dilution of an aqueous solution saturated with JP-4 jet fuel is shown with respect to the bioluminescence patterns without n-decanal (Fig. 9A) or in the presence of n-decanal (Fig. 9C), and with respect to the membrane fatty acid composition ratios (Fig. 9B) and the *nah-lux*-mRNA levels as presented in figure 9D. The overall response patterns were qualitatively and quantitatively similar. For all the three solvent treatment conditions significantly higher bioluminescence levels were obvious than found for the control. In contrast, the addition of n-decanal after 60 minutes (Fig. 9C) resulted in a different response pattern with almost identical bioluminescence values for the control and toluene and a slightly increased level for JP-4 jet fuel and a significantly higher response for naphthalene. A very similar relative response pattern was found for the *nah-lux* -mRNA levels under the four different treatment conditions after 30 minutes exposure as shown in figure 9D. Here, the slightly increased *nah-lux* mRNA level after exposure to JP-4 was more obvious than in figure 9C.

In Figure 9B the membrane fatty acid ratios for the unsaturated C_{16} trains to is (I), the unsaturated C_{18} trains to is (II) and the C_{17} cyclopropyl to unsaturated C_{16} cis fatty

acids (III) are shown. While exposure to toluene resulted in a pronounced increase in all three fatty acid ratios, naphthalene caused a still significant but smaller increase for the ratios I and II. The response observed after exposure to JP-4 jet fuel indicated only a small increase for all three ratios.

(III) Incubation of HK44 in groundwater and its robustness

Induction of bioluminescence response: Alginate encapsulated HK44 were inducible throughout the 35 day incubation in groundwater. Figures 10A and 10B, are representations of induction response of HK44 when induced with the complex and simple inducer solutions respectively. Every time, the induction was found specific to the inducer. The bioluminescence response showed discriminate patterns in their inducibility based on the type of incubation pH of the groundwater and the inducer solution. At pHs 3, 4 and 5 there was no induction with either of the inducer solutions.

The light production was found to be consistent at pH 6, within an order of magnitude, when induced with either of the inducer solutions. Interestingly, at pH 6 the response on day 1 was very low when induced with salicylate alone. This may be due to slow uptake of the inducer and any induction after 5 hours may have been unrecorded as the no light measurements were made more than that time period. However, as the light production became consistently higher during subsequent induction days, the bacteria appear adapted at pH 6 groundwater and responded with rapid uptake of the inducer. The percent uptake of salicylate at pH 6 was found to be higher than at pH 7 or when incubated in a rich medium, 0.1x YEPG (Matrubutham et al.) At pH 7, the bioluminescence response reached an overall minimum on day 21 when induced with

simple inducer and began to show gradual increase on days 28 and 35. The reason for such a trend could not be speculated at this time because of insufficient data. A moderately consistent response was displayed throughout the observation period of 35 days with complex inducer. The induction response of HK44 incubated in 0.1x YEPG, decreased gradually overtime (Fig. 10A) or was highly inconsistent (Fig. 10B).

Survival of encapsulated HK44: The robustness of HK44 was indicated by its ability to survive in groundwater without any organic carbon substrate. The population counts in the encapsulated beads were found to remain constant at pHs 6 and 7 (Fig. 11). Though some colonies formed from beads incubated in lower pHs, they did not form during every sampling period of the experiment. The colonies formed may be an anomaly of incubation conditions, where most of the encapsulated cells but for those buried in the central core of the bead, were killed due to the low pH conditions.

(IV) Toluene bioreporter for TCE co-metabolism

Bioluminescene response and TCE co-metabolism: During DVR operation, toluene at 10 mg l^{-1} was fed to the reactor in 20 hr cycle, square wave inputs. Bioluminescent response was virtually instantaneous to toluene exposure. Figure 12 shows both the light response of the reporter strain in the reactor and the step changes made with the inlet concentration of the inducer, indicating a direct response of bioluminescence of B2 with respect to toluene present in the system. During the cycle, light emission increased by 16.3 +/- 1.2 nA/h. The toluene effluent concentration approached zero after the toluene feed was stopped, and the light response in the reactor decreased at a rate of 3.4 +/- 0.8 nA/hr. When the system was induced with toluene, light

was emitted and TCE was degraded (Fig. 12). A direct relationship between light emission and TCE degradation was observed. The maximum light response was 43.4 +/- 6.8 nAmps. The system degraded approximately 20% of TCE and 50% of toluene. This gave a steady-state TCE effluent concentration, when toluene was being introduced into the system, of 16.5 +/- 0.2 mg/l, and an effluent toluene concentration of 5.8 +/- 0.1 mg/l, which represents a ratio of 1.7 μmol toluene degraded/μmol TCE degraded.

Discussion and Conclusion

A whole cell biosensor based on the bioluminescent reporter/catabolic bacterium *P.fluorescens* HK44 was developed and designed around a commercially available light detection device for rapid on-line monitoring of the presence and bioavailability of pollutants in waste streams. Exposure of the biosensor to either naphthalene or its degradation product salicylate resulted in a rapid increase in the bioluminescence response. Under defined conditions, the response was shown to be highly reproducible, stable and specific to the inducers. The observed response was rapid the response time varied with the concentration of the inducing substrate.

The *tod-lux* bioreporter *Pseudomonas putida* B2, was successfully constructed and tested as a potential biosensor of toluene availability and TCE degradation. When used in a differential volume reactor, strain B2 displayed specific and proportional bioluminescence response to the concentration of toluene feed. Since toluene was the inducer of the *lux* system and the co-metabolic degradation of TCE as well, the

bioluminescence response of B2 correlated with the degradation of TCE. Approximately only 20% of TCE was found to be degraded with a simultaneous 50% removal of toluene. However, future studies will provide better understanding on the stability and performance efficiency of B2.

With *Pseudomonas fluorescens* HK44, the bioluminescence signal was found to be reproducible both for naphthalene and salicylate upon repetitive-pertubations. The biosensor responded to real pollutants such as JP-4 jet fuel and MGP soil leachates. The response demonstrated that the biosensor can be used under realistic environmental waste conditions. The magnitude of bioluminescence correlatd with the presence of naphthalene or salicylate in the pollutant mixtures. With the JP-4 jet fuel, an inverse relationship between bioluminescence and the inherent concentration of naphthalene was observed. This anomaly should be attributed as an effect of toxic materials present in the jet-fuel that were inhibiting the bioluminescence reaction. MGP-soil leachates did not display any toxic effect. However, number of solvents have been shown to reduce the bioluminescence response of *Phosphobacterium phosphoreum* in the Microtox assay (Bulich, 1986). Future studies on the inhibition of bioluminescence under induced catabolic systems will elucidate more on the toxicity effects.

When HK44 was subjected to substrate replacement (non-inducers) the bioluminescence signal decayed. The half-life of signal decay varied based on the inducing compound that was replaced. Replacement of naphthalene resulted in a shorter half-life of signal decay compared to salicylate replacement. The causes for signal decay may include i) reduced *de novo* synthesis rate of the luciferase enzyme, ii) limitation of reaction co-

factors like O_2, aldehyde, reduced flavin mononucleotide, NADPH, and ATP, iii) intracellular dilution by cellular growth, and iv) intrinsic stability of the enzyme. The temperature at which the biosensor was set-up could be responsible for the rate of decay. However, luciferase enzyme has been reported to have an half-life of only 1 minute at 38°C (Ruby & Hastings, 1980) and all experiments in the project was conducted at 25°C, the enzyme could have had longer half-life and resulted in a slower signal decay rate (24-30 minutes).

As a biosensor, HK44 displayed strong specificity for naphthalene and salicylate. When tested with solvents and other non-inducing substrates like glucose and peptone, there was insignificant bioluminescene response. The insignificant light production could easily be discriminated from the specific bioluminescence response on the basis of magnitude and response time. However, specificity becomes a major issue when dealing with complex environmental organic mixtures. Some solvents have been reported to cause significant changes in the fatty acid composition of bacterial membranes by affecting their synthesis (Ingram, 1977 &1982). Consequently, such changes can affect the fatty acid -aldehyde cycle of the bioluminescence reaction. This type of solvent mediated changes results in what is called the "membrane-solvent effect". Control bioreporter bacteria should then be the solution to distinguish the specific bioluminescence response from solvent mediated response.

Pseudomonas fluorescens HK44 survived environmental stress conditions when incubated in groundwater for 35 days and was inducible for bioluminescence. The ability to survive an extended incubation in groundwater is a proof of its robustness. The

inducibility of bioluminescence is an important attribute, if the bacterium is desired to be employed for long term biosensing purpose. The groundwater incubation study, provided preliminary insight on the possibility of a real time biosensor that could be installed in the water table under ground. Besides, the consistency in the magnitude of bioluminescence observed in the study assures the realiability of bioluminescent technology for biosensors even when the contaminant is the only carbon and energy source. Besides, in the presence of limited oxygen and reduced co-factors, the bioluminescence induction that was seen in the groundwater study enhances the application possibility of HK44 under ground. The luciferase reaction could be directly responsible for as much as 10-20% of the total oxygen consumed in cultured cells (Dunlap 1984). With this if the amount of energy required to synthesize the luciferase was also added, then the cellular energy for the luxurious light emission may become considerably high. According to past studies by Nealson and Karl (1980), the estimated quantum efficiency (quanta emitted per oxygen molecule) of the luciferase reaction in vivo may possibly be as high as 1.0. Based on the literature and the results from the long-term study, it is obvious more must be performed to establish steady-state bioluminescence by HK44, when subjected to physiologically stressful conditions.

Potential applicability of bioluminescent biosensors in monitoring naphthalene and TCE degradation was realized by this project. Such biosensors are non-invasive and can be developed as a routine monitoring and analytical device in the assessment of biodegradation and site characterization. As the bioreporter organisms act as "surrogates" of the native biodegradative community, any biosensor constructed with them and installed at the contaminated site will provide sufficient information on the physiological status of a

related microbial community and enable approriate manipulation of processes to efficiently bioremediate the site. Creation of similar or related biosensors capable of sensing other environmental contaminants may help bioremediation processes. As significant accomplishments of the project, the following could be expected when biosensors are properly employed in environmental systems along with other analytical tools:

1) Specific assessment and quantification of contaminant bioavailability and degradation based on cellular and molecular activities.

2) Real-time monitoring of physiological events to facilitate constant and appropriate fabrication of processes leading to effective biodegradation.

3) Generation of case specific and comprehensive data bases enabling futuristic integrated approaches.

4) Dynamics with respect to natural and experimental processes.

References

1. Al-Bashir, B., T.Cseh, R. Leduc, and R. Samson. 1990. Effect of soil/contaminant interactions on the biodegradation of naphthalene in flooded soil under denitrifying conditions. Appl. Microbiol. Biotechnol. 34:414-419.

2. Bowman, J. P., L. Jimenez, I. Rosario, T. C. Hazen, and G. S. Sayler. 1993. Characterization of the methanotrophic bacterial community present in a trichloroethylene-contaminated subsurface groundwater site. Appl. Environ. Microbiol. 59:2380-2387.

3. Bulich, A. A. 1986. Bioluminescence assays, p 57-74. In G. Bitton and B. J. Dutka (ed.), Toxicity testing using microorganisms, vol. 1. CRC Press, Inc. Boca Raton, FL.

4. Dunlap, P. 1984. Biological Bulletin 167:410.

5. Holben, W.E., B.M. Schroeter, V.G.M. Calabrese, R.H. Olsen, J.K. Kukor, V.O. Biederbeck, A.E. Smith, and J.M. Tiedje. 1992. Gene probe analysis of soil microbial populations selected by amendmend with 2, 4-dichlorophenoxyacetic acid. Appl. Environ. Microbiol. 58:3941-3948.

6. Guckert, J. B., D. B. Ringleberg, D. C. White, R. S. Hanson, and B. J. Bratina. 1991. Membrane fatty acids as phenotypic markers in the polyphasic taxonomy of methylotrophs within the proteobacteria. J. Gen. Microbiol. 137:2631-2641.

7. Heitzer, A., and G. S. Sayler. 1993. Monitoring the efficacy of bioremediation. Trends Biotechnol. 11:334-343.

8. Heitzer A, Webb OF, Thonnard JE and Sayler GS. 1992. Specific quantitative assessment of naphthalene and salicylate bioavailability using a bioluminescent catabolic reporter bacterium. Appl. Environ. Microbiol. 58:1839-1846.

9. Holmes, D. S and M. Quigley. 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. 114:193-197.

10. Ingram, L. O. 1977. Changes in lipid composition of *Escherichia coli* resulting from growth with organic solvents and with food additives. Appl. Environ. Microbiol. 33:1233-1236.

11. Ingram, L. O. 1982. Regulation of fatty acid composition in *Escherichia coli*: A proposed coomon mechanism for changes induced by ethanol, chaotropic agents, and a reduction of growth temperature. J. Bacteriol. 149:166-172.

12. Karl, D. M., and Nealson, K. H. 1980. Journal of General Microbiology. 117:357.

13. Levin, M. A., R. J. Seidler, and M. Rogul. 1992. Microbial ecology: Principles, methods and applications. McGraw-Hill, New York, p. 945.

14. Maniatis R., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold spring harbor, N.Y.

15. Matrubutham, U., J. Thonnard, and G. S. Sayler. 1995. Bioluminescence induction response and survival of *Pseudomonas fluorescens* HK44 under stress. In preparation.

16. Mihelcic, J. R., and R. G. Luthy. 1991. Sorption and microbial degradation of naphthalene in soil-water suspensions under denitrification conditions. Environ. Sci. Technol. 25:169-177.

17. Oelmuller, U., N. Kruger, A. Steinbuchel and C. G. Friedrich. 1990. Isolation of prokaryotic RNA and detection of specific mRNA with biotinylated probes. J. Microbiol. Methods 11:73-84.

18. Riser-Roberts, E. 1992. Bioremediation of petroleum contaminated sites, C. K. Smoley p. 471.

19. Ruby, E. G., and J. W. Hastings. 1980. Formation of hybrid luciferases from subunits of different species of *Photobacterium*. Biochemistry 19:4989-4993.

20. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual

21. Sanseverino, J., Werner, C., Fleming, J.T., Applegate, B.M., King, J.M.H. and Sayler, G.S. 1993. Molecular diagnostics of polycyclic armoatic hydrocarbon biodegradation in manufactured gas plant soils. Biodegradation. 4:303-321.

22. Sayler, G. S., and A.C. Layton. 1990. Enviornmental application of nucleic acid hybridization. Annu. Rev. Microbiol. 44:625-648.

23. Selifonova O, Burlage R and Barkay T. 1993. Bioluminescent sensors for the detection of bioavailable Hg(II) in the environment. Appl. Environ. Microbiol. 59:3083-3090.

24. Stucki, G., and M. Alexander. 1987. Role of dissolution rate and solubility in biodegradation of aromatic compounds. Appl. Environ. Microbiol. 53:292-297.

25. Walker, J. R., and A. B. Walker. 1994. The Kwajalein bioremediation demonstration: Final technical report for U. S. DOE., Grant DE-AC05-840R21400.

26. Wodzinski, R. S., and D. Bertolini. 1972. Physical state in which naphthalene and biphenyl are utilized by bacteria. Appl. Microbiol. 23:1077-1081.

27. Wodzinski, R. S., and J. E. Coyle. 1974. Physical state of phenanthrene for utilization by bacteria. Appl. Microbiol. 27:1081-1084.

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Figure 1. A) Probe tip. A, strontium-alginate matrix containing bioluminescent reporter; E, epoxy resin; F, stainless steel ferrule; L, liquid guide; M, stainless steel mesh; N, hex nut for probe fixation; P, light guide probe; S, screw for ferrule fixation; W, wire for mesh fixation; Z, stainless-steel cylinder. All dimensions are in millimeters. B) Biosensor set-up. B, biosensor; C, computer; E, effluent waste tank; H, dH_2O tank; M, maintenance medium tank; P, pollutant tank; PR, photomultiplier and recorder; P1, P2, HPLC pumps; S1, S2, S3, sampling ports; T, thermostat; V2, 2-way valve; V3, 3-way valve.

Figure 2. Schematic representation of the on-line system used to monitor the co-metabolism of TCE.

Figure 3. Biosensor response to a step change in salicylate concentration from 0 to 5 mg/l. A) Salicylate concentration (dotted line), bioluminescence response (squares). B) Naphthalene concentration (dotted line), bioluminescence (squares).

Figure 4. Reproducibility of biosensor signal: Response to repetitive perturbations. A) Salicylate, B) Naphthalene. Pollutant concentration profile (dotted line), bioluminescence response (squares).

Figure 5. Specificity of biosensor response: A) Step change from 0 to 0.5 g/l glucose; B) step change from 0 to 25% YEPG medium; C) step change from 0 to 25% saturated aqueous toluene solution. Pollutant concentration profile (dotted line), bioluminescence response (squares).

Figure 6. Biosensor response to complex environmentally relevant pollutant mixtures containing naphthalene: A) JP-4 jet fuel 50% saturated aqueous solution. B) Manufactured gas plant soil leachate 50% (Insert: response over large time scale). Pollutant concentration profile (dotted line), bioluminescence response (squares).

Figure 7. Relationship between the amount of JP-4 jet fuel and the bioluminescence response of growing cultures of *P.fluorescens* HK44 after 1 hour incubation. The amount of JP-4 is expressed as a factor of an aqueous solution, saturated with JP-4. $Y = 104.83x + 1.2656$; $r^2 = 0.967$.

Figure 8. Effect of the physiological growth state of the *P.fluorescens* HK44 reporter culture on the bioluminescence response after 1 hour exposure to toluene and naphthalene: A) Growing culture without n-decanal addition; B) Starved culture without n-decanal addition; C) Growing culture with n-decanal addition; D) Starved culture with n-decanal addition. The control was water and the concentration of toluene and naphthalene used were 64 mg/l and 3.9 mg/l respectively.

Figure 9. Comparative analysis of the response of the growing *P.fluorescens* HK44 reporter culture after exposure to water (control), toluene, naphthalene and JP-4 jet fuel: A) Bioluminescence response without n-decanal addition after 30 min (hatched bars) and 60 min (black bars) exposure; B) Fatty acid ratios for 16:1ω7t/c (back bars), 18:1ω7t/c (gray bars) and cy17:0/16:1ω7c (open bars) after 60 min exposure; C) Bioluminescence response with n-decanal addition after 60 min exposure; D) Gene expression measured as nah-lux mRNA after 30 min exposure. The toluene and naphthalene concentrations were 64 mg/l and 3.9 mg/l, respectively, and the JP-4 jet fuel solution was an eightfold dilution of a saturated aqueous solution. For comparative reasons all values were normalized to the controls and are expressed as relative numbers.

Figure 10. Bioluminescence induction response of encapsulated HK44. A) The induction response at different pHs when induced with complex inducer (YEPSS), B) The induction response at different pHs when induced with simple inducer (salicylate).

Figure 11. Robustness of HK44. Survival of encapsulated HK44 incubated in groundwater with different pHs. Cultural units refer to viable plate counts.

Figure 12. Bioluminescence response and co-metabolism of TCE by *P.putida* B2. Response based on square wave perturbations with 10 mg/l toluene in 20 hour cycles.

Table 1: Analysis of aqueous extracts from soils contaminated with petroleum hydrocarbons: Estimation of naphthalene content based on the bioluminescence response of the reporter *P. fluorescens* HK44.

Sample	Experimental set [a]	Bioluminescence [nAmp ± SD]	Estimated naphthalene [b] (conc. range) [c] [mg/l]	Analytically determined naphthalene [mg/l ± SD]
Soil A	a	1.75 ± 0.12	0.026 (-0.003 ≤ x ≤ 0.052)	0
	b	1.79 ± 0.08	-0.001 (-0.015 ≤ x ≤ 0.028)	0
Soil B	a	2.75 ± 0.08	0.104 (0.081 ≤ x ≤ 0.126)	0.015 ± 0.009
	b	2.77 ± 0.15	0.083 (0.048 ≤ x ≤ 0.116)	0.016 ± 0.001

[a] Each experimental set was conducted in triplicate samples (n=3).

[b] For each experimental set a standard curve for the relationship between naphthalene concentration and bioluminescence was prepared by linear regression: Set a: $y = 12.8436x + 1.418$; $r^2 = 0.9920$; $n = 11$; naphthalene concentration range 0 - 0.725 mg/l. Set b: $y = 11.5327x + 1.8063$; $r^2 = 0.9830$; $n = 13$; naphthalene concentration range: 0 - 0.711 mg/l. The estimated concentrations were determined based on these equations.

[c] The concentration ranges were numerically calculated based on the 95 % confidence limit-curves of the regression line and the intersection with the lines described by the upper (nAmp + SD) and lower (nAmp - SD) bioluminescence limits of the triplicate samples.

Table 2: Effects of different organic and inorganic pollutants on the bioluminescence response of the reporter bacterium *P. fluorescens* HK44.

	relative Bioluminescence [-] x ± SD	
Compound [a]	no n-decanal	with n-decanal
H_2O (control)	1 ± 0.03 [b]	1 ± 0.00 [c]
acetone	4.20 ± 0.17 [b]	1.24 ± 0.36 [c]
toluene	10.41 ±0.17 [b]	0.71 ± 0.20 [c]
p-xylene	4.17 ± 1.09 [b]	0.98 ± 0.16 [c]
acetone + Toluene	13.96 ±0.67 [b]	0.87 ± 0.01 [c]
acetone + p-xylene	8.25 ± 0.26 [b]	1.13 ± 0.26 [c]
toluene + p-xylene	7.61 ± 0.81 [b]	0.78 ± 0.02 [c]
n-decane	2.01 ± 0.07 [c]	n.d.
n-undecane	1.56 ± 0.00 [c]	n.d.
n-dodecane	1.29 ± 0.36 [c]	n.d.
$CdCl_2$	0.23 ± 0.03 [c]	n.d.
$HgCl_2$	0.006 ± 0.000 [c]	n.d.
KCN	0.012 ± 0.003 [c]	n.d.

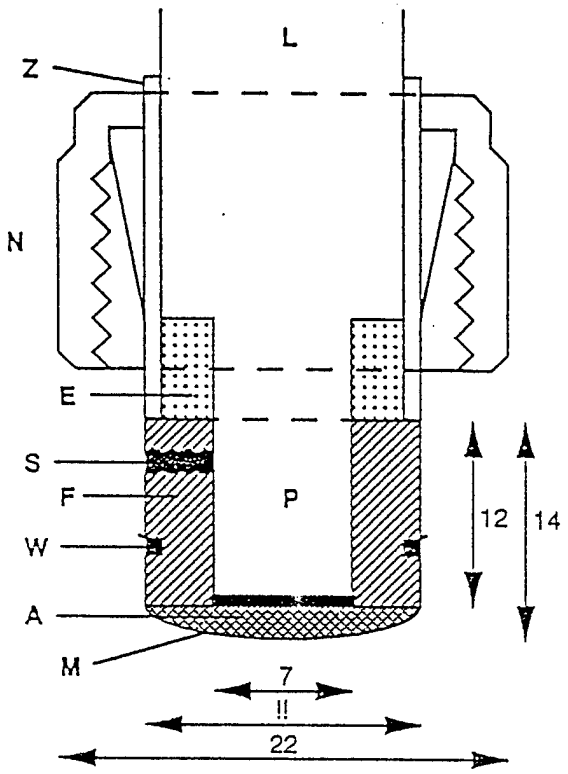
[a] The initial concentrations were: Acetone 980 mg/l; toluene 64 mg/l; p-xylene 22 mg/l; n-decane, n-undecane and n-dodecane 50% saturated aqueous solutions; $CdCl_2$ 183 mg/l; $HgCl_2$ 271 mg/l; KCN 86 mg/l.

[b] n = 4.

[c] n = 2.

figure 1

A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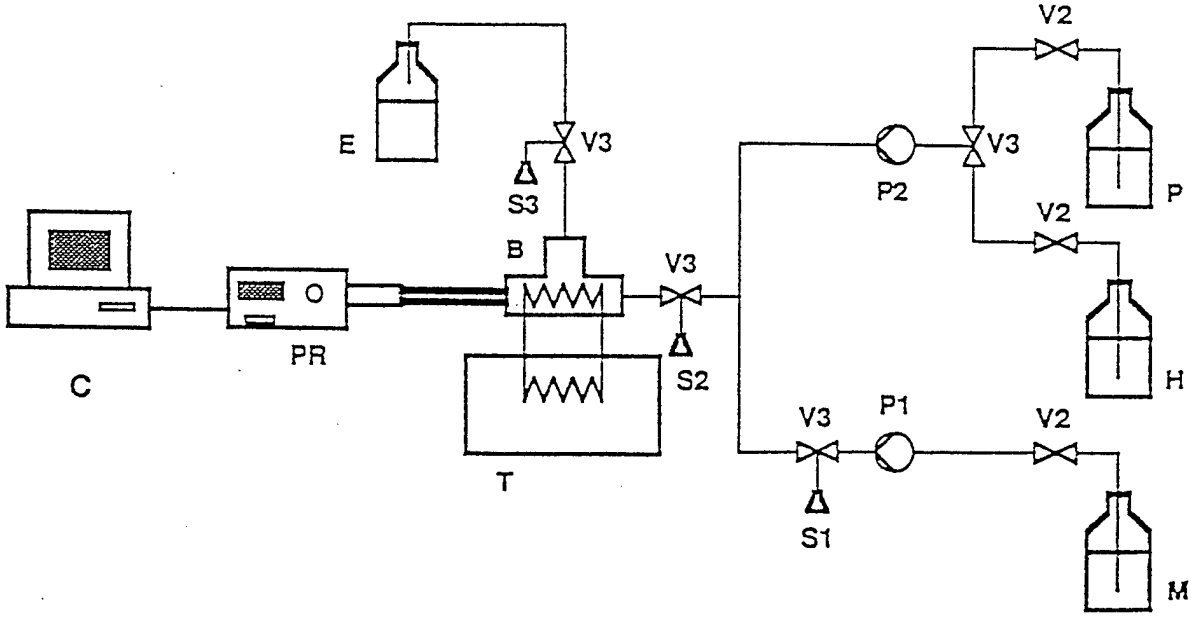
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figure 2

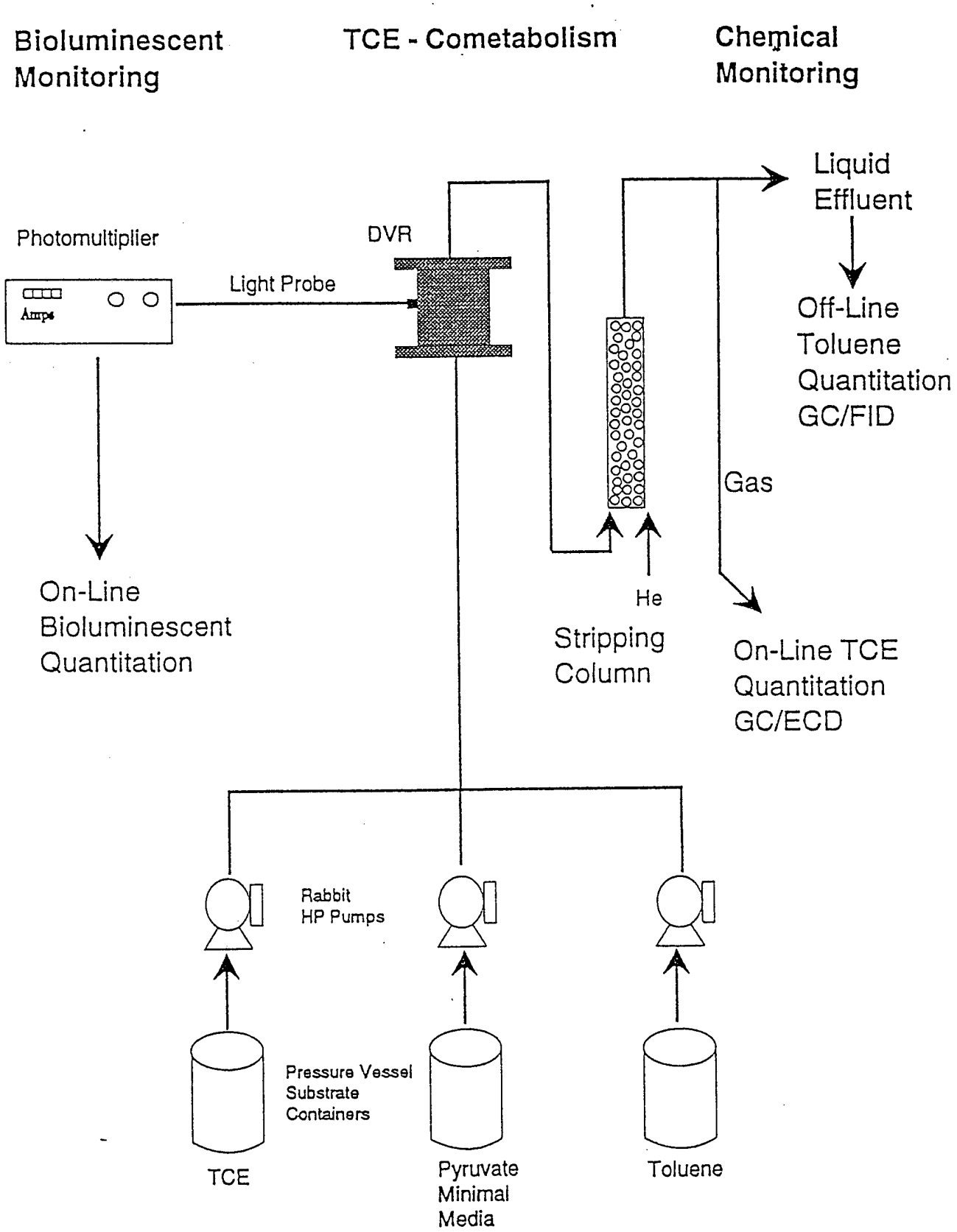
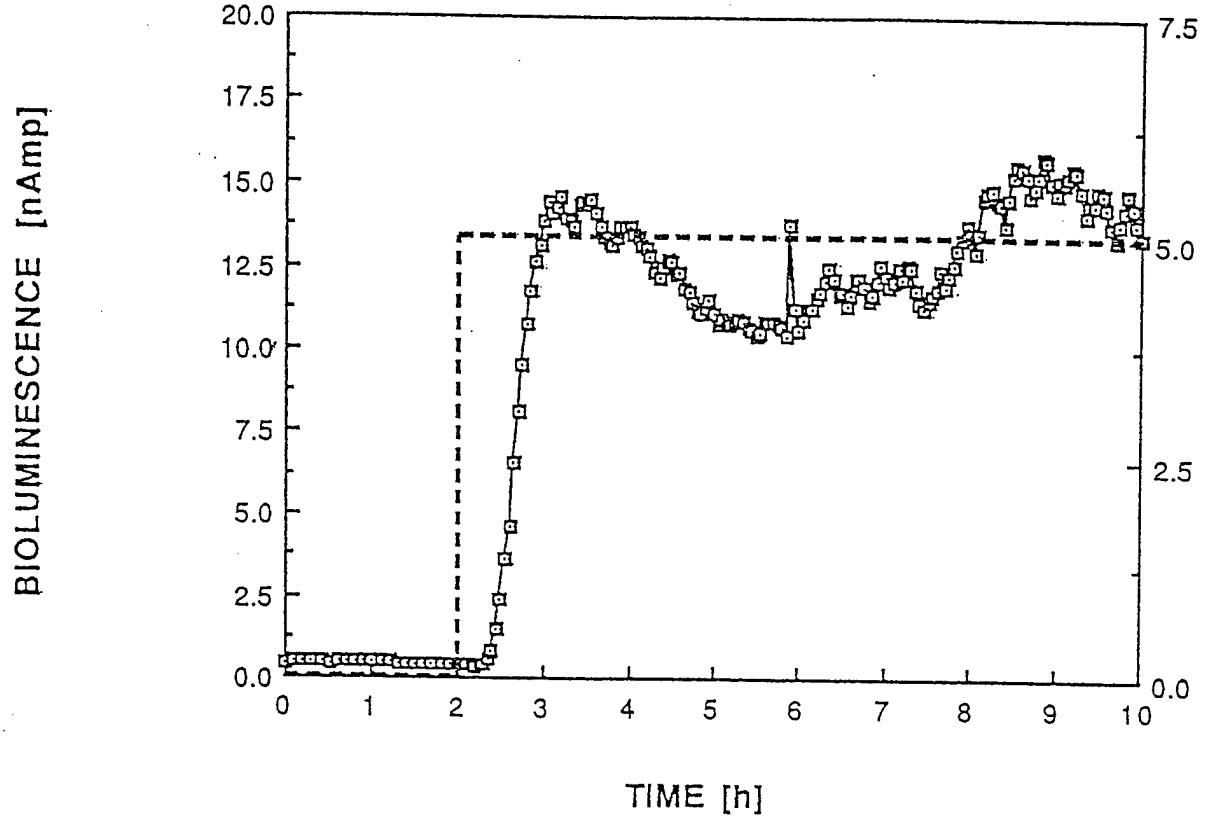

Bioluminescent Monitoring
TCE - Cometabolism
Chemical Monitoring
Photomultiplier
Amps
Light Probe
DVR
Liquid Effluent
Off-Line Toluene Quantitation GC/FID
Gas
On-Line Bioluminescent Quantitation
He
Stripping Column
On-Line TCE Quantitation GC/ECD
Rabbit HP Pumps
Pressure Vessel Substrate Containers
TCE
Pyruvate Minimal Media
Toluene

Figure 3


BIOLUMINESCENCE [nAmp]
SALICYLATE [mg/l]
TIME [h]
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
7.5
5.0
2.5
0.0
0
1
2
3
4
5
6
7
8
9
10

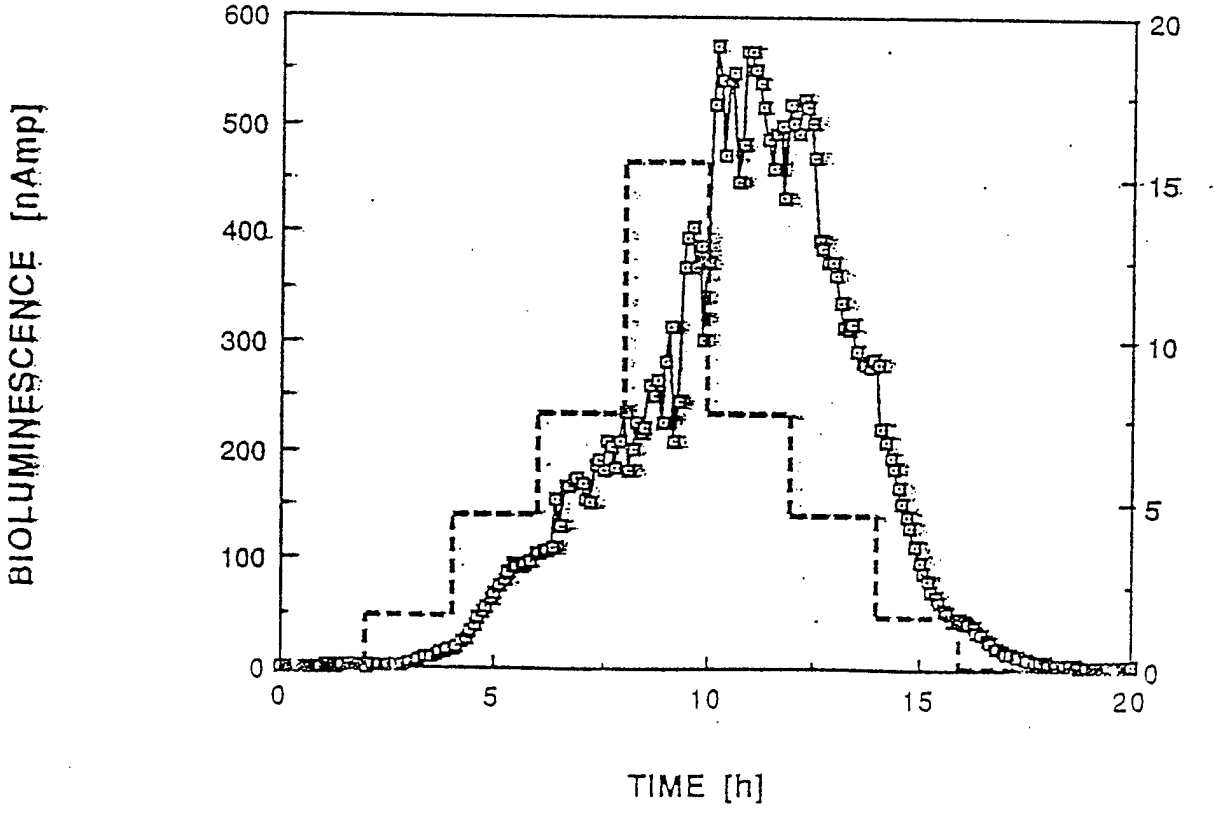
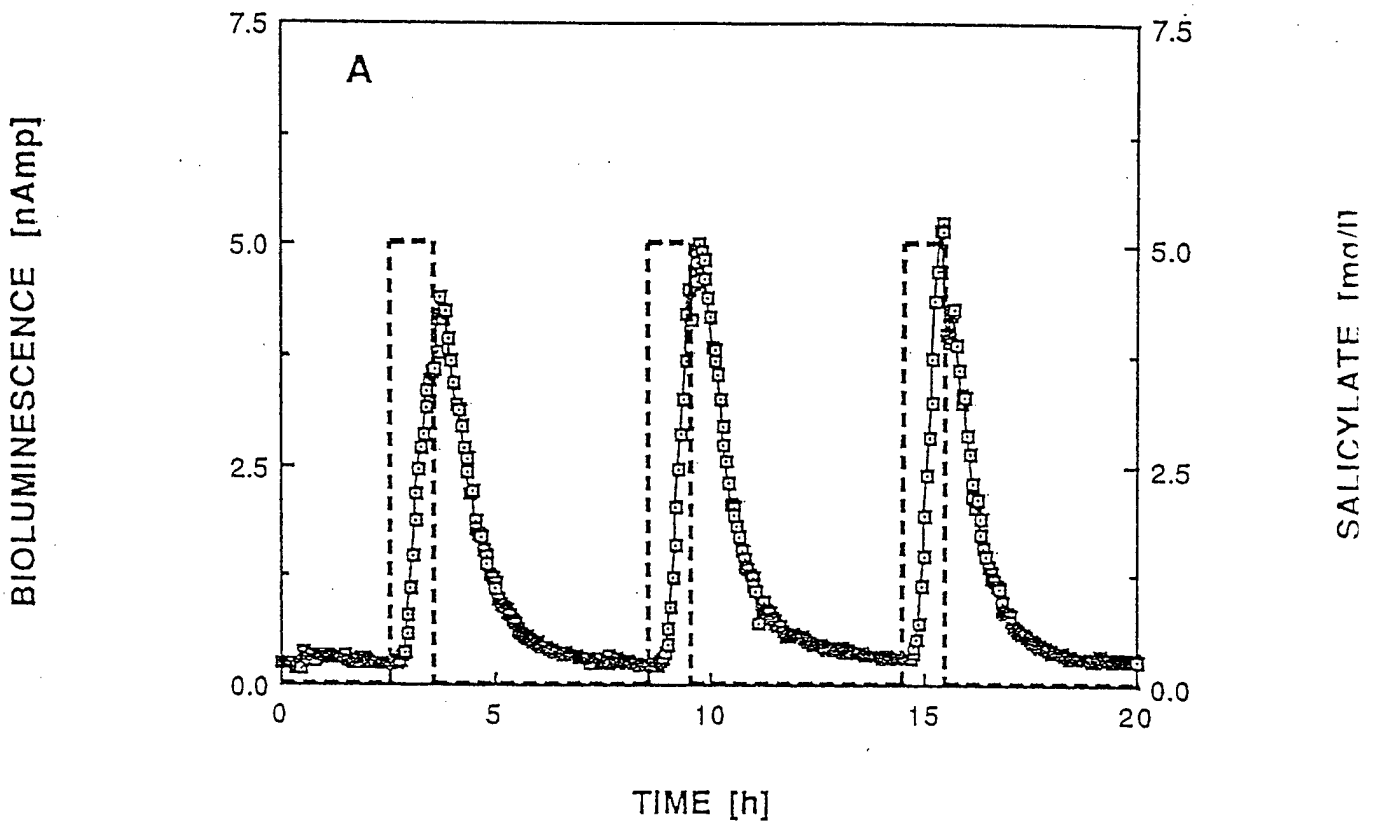

BIOLUMINESCENCE [nAmp]
NAPHTHALENE [mg/l]
TIME [h]
600
500
400
300
200
100
0
20
15
10
5
0
0
5
10
15
20

figure 4


A
BIOLUMINESCENCE [nAmp]
SALICYLATE [mg/l]
7.5
5.0
2.5
0.0
0
5
10
15
20
TIME [h]

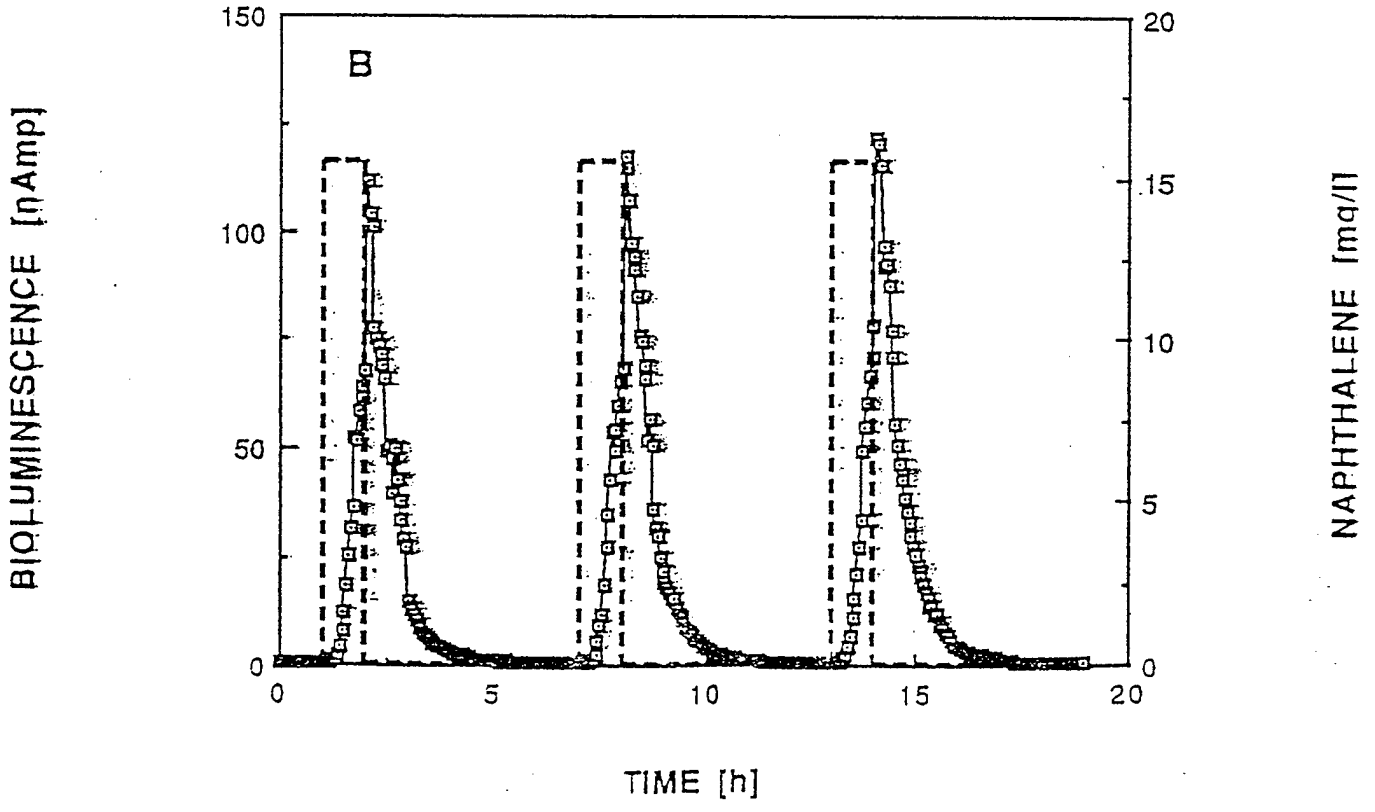

B
BIOLUMINESCENCE [nAmp]
NAPHTHALENE [mg/l]
150
100
50
0
20
15
10
5
0
0
5
10
15
20
TIME [h]

Figure 5

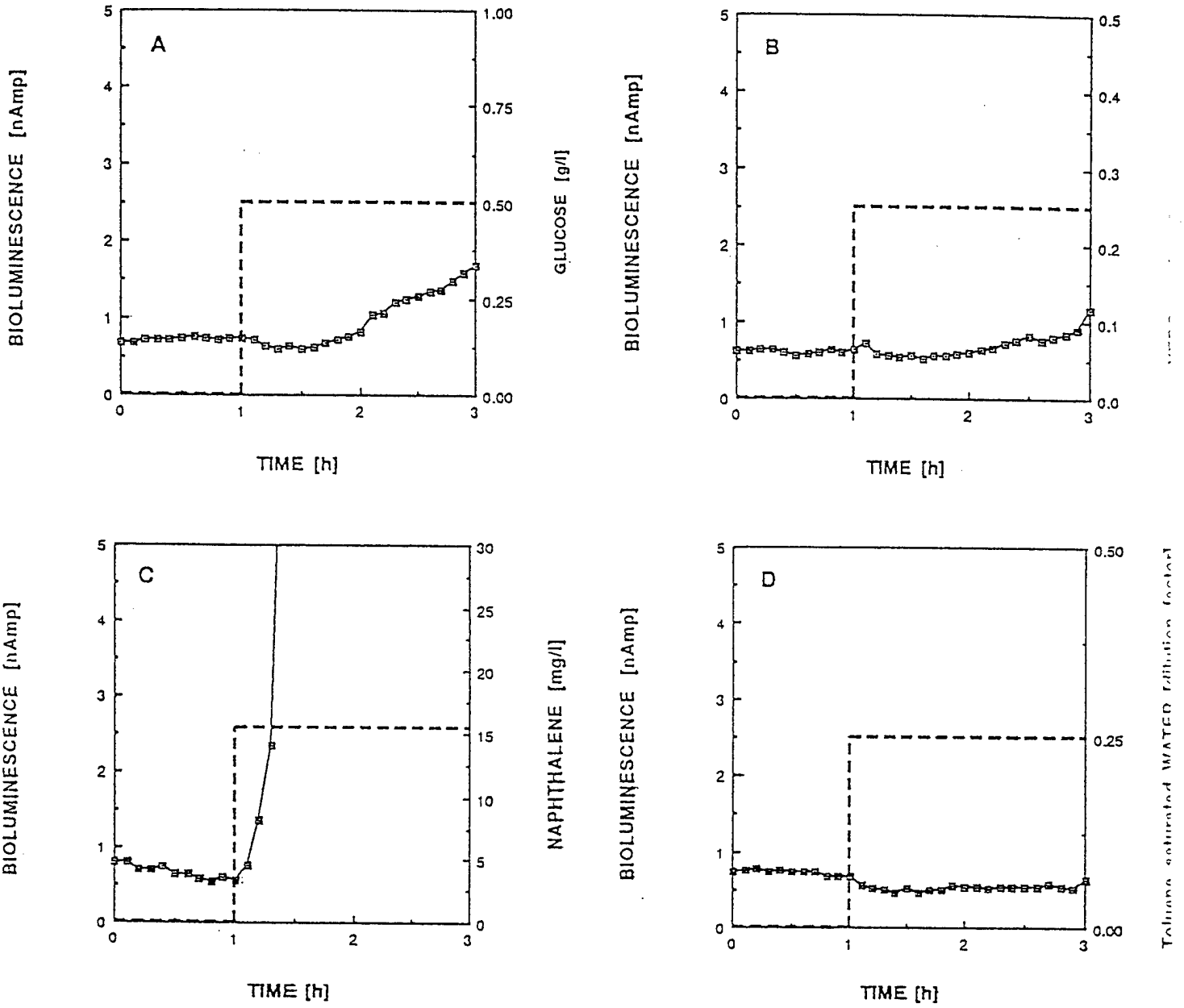

A
B
C
D
BIOLUMINESCENCE [nAmp]
GLUCOSE [g/l]
NAPHTHALENE [mg/l]
TIME [h]
0
1
2
3
0.00
0.25
0.50
0.75
1.00
0.0
0.1
0.2
0.3
0.4
0.5
5
10
15
20
25
30

figure 6

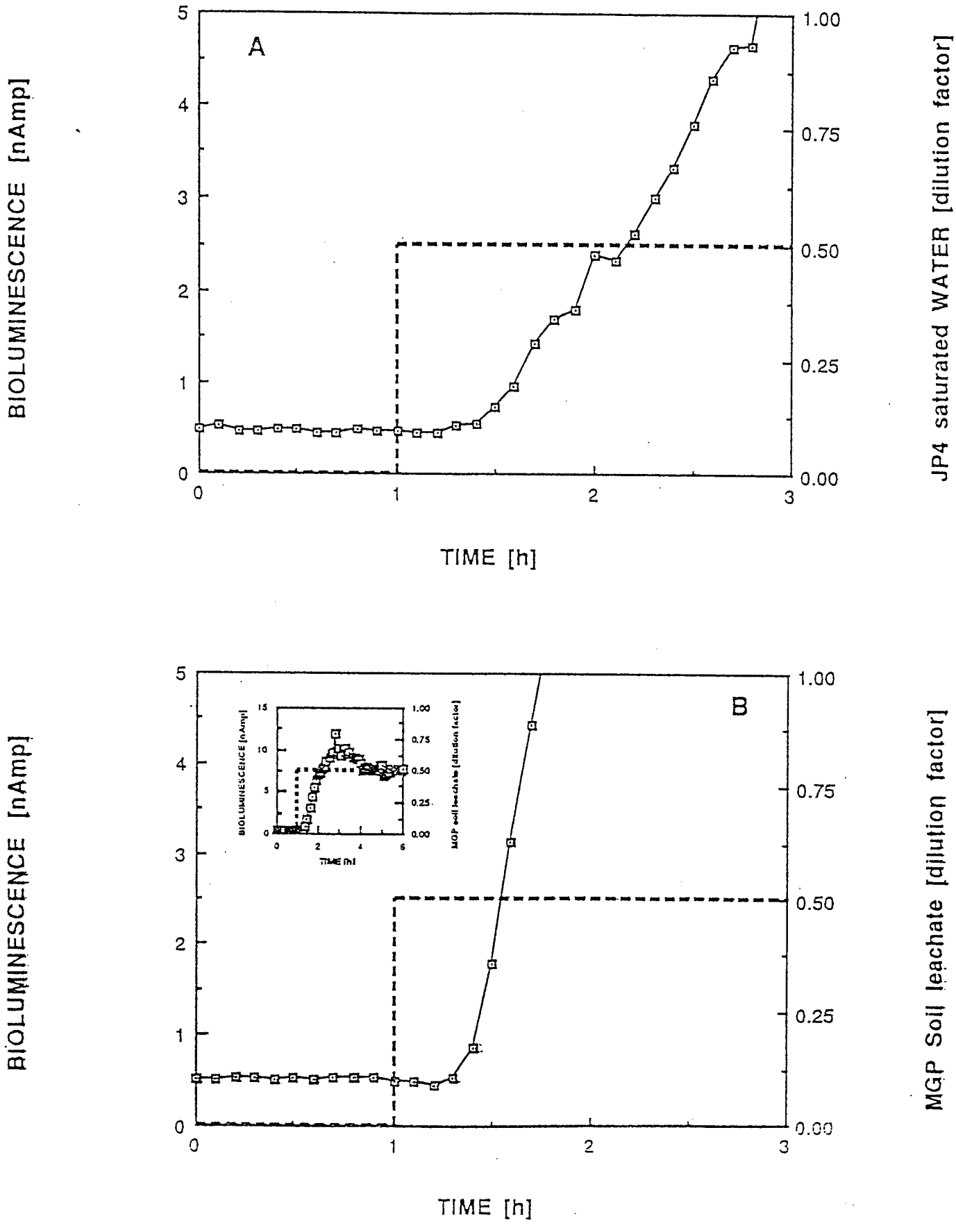

A
B
BIOLUMINESCENCE [nAmp]
JP4 saturated WATER [dilution factor]
MGP Soil leachate [dilution factor]
TIME [h]
0
1
2
3
4
5
0.00
0.25
0.50
0.75
1.00

Figure 7

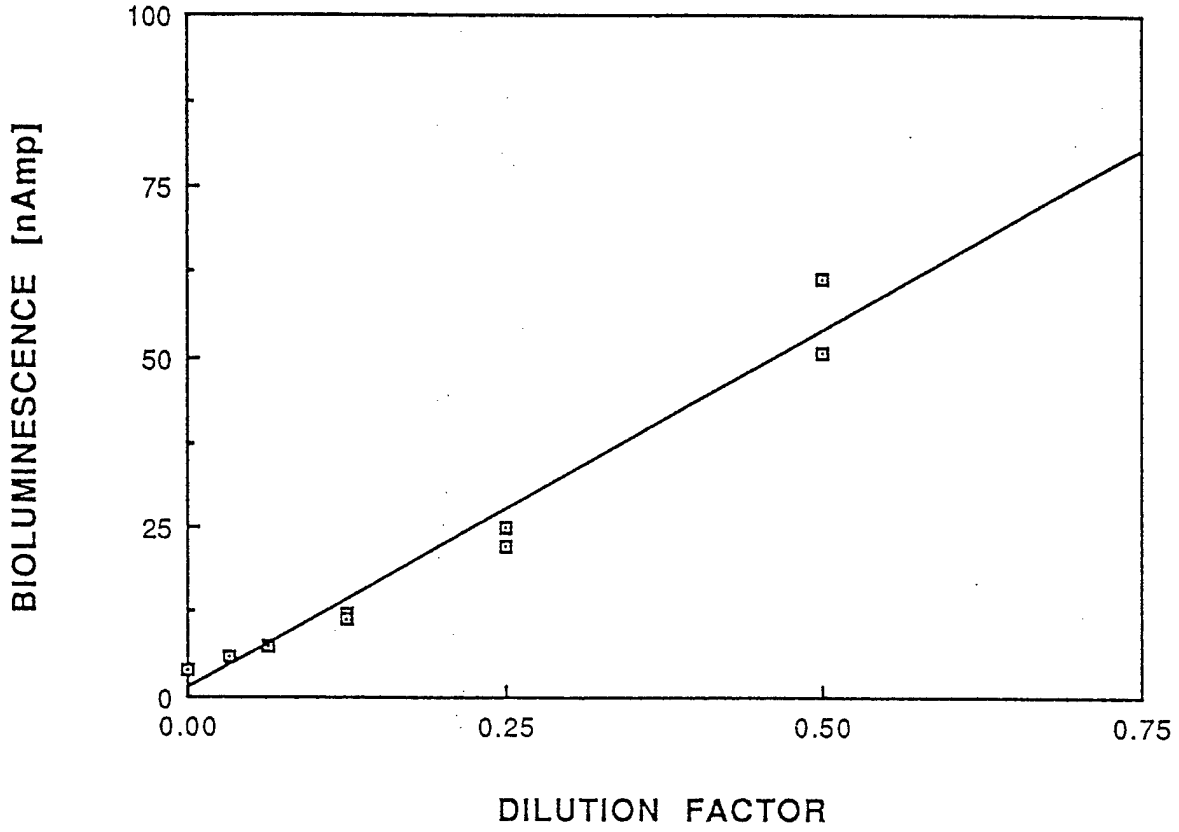
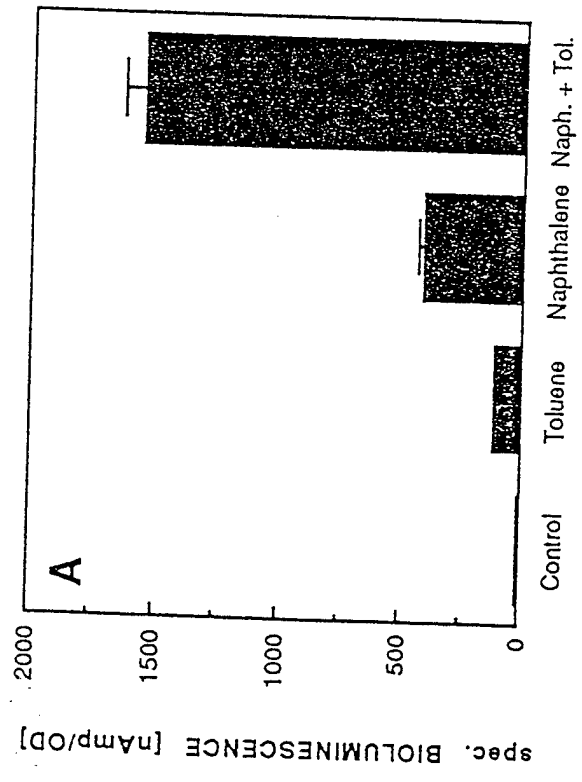
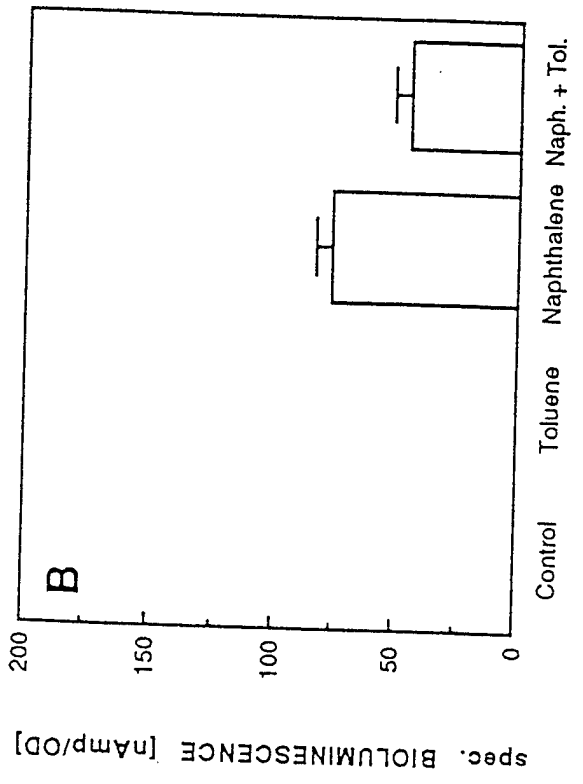
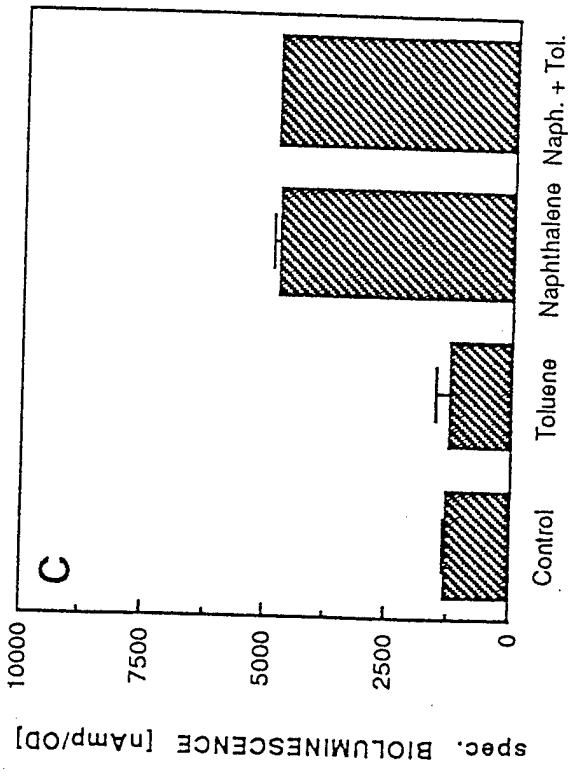
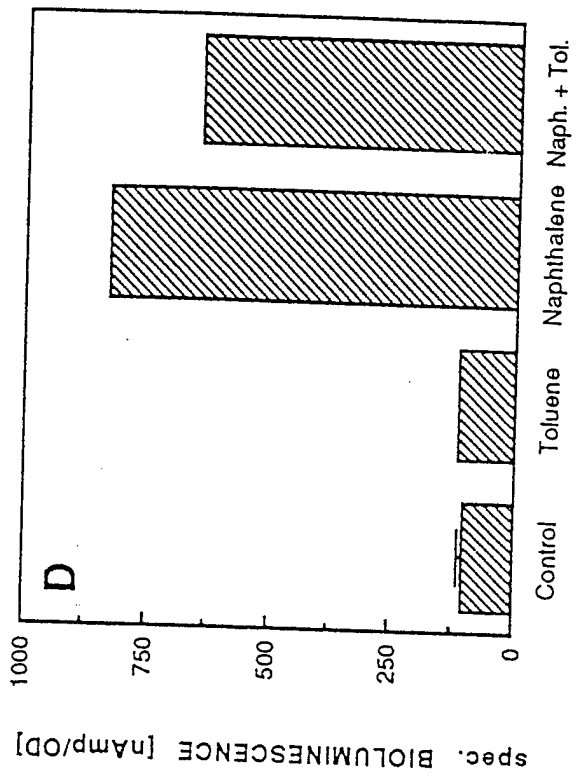

BIOLUMINESCENCE [nAmp]
100
75
50
25
0
0.00
0.25
0.50
0.75
DILUTION FACTOR

figure 8


A
2000
1500
1000
500
0
spec. BIOLUMINESCENCE [nAmp/OD]
Control
Toluene
Naphthalene
Naph. + Tol.


B
200
150
100
50
0
spec. BIOLUMINESCENCE [nAmp/OD]
Control
Toluene
Naphthalene
Naph. + Tol.


C
10000
7500
5000
2500
0
spec. BIOLUMINESCENCE [nAmp/OD]
Control
Toluene
Naphthalene
Naph. + Tol.


D
1000
750
500
250
0
spec. BIOLUMINESCENCE [nAmp/OD]
Control
Toluene
Naphthalene
Naph. + Tol.

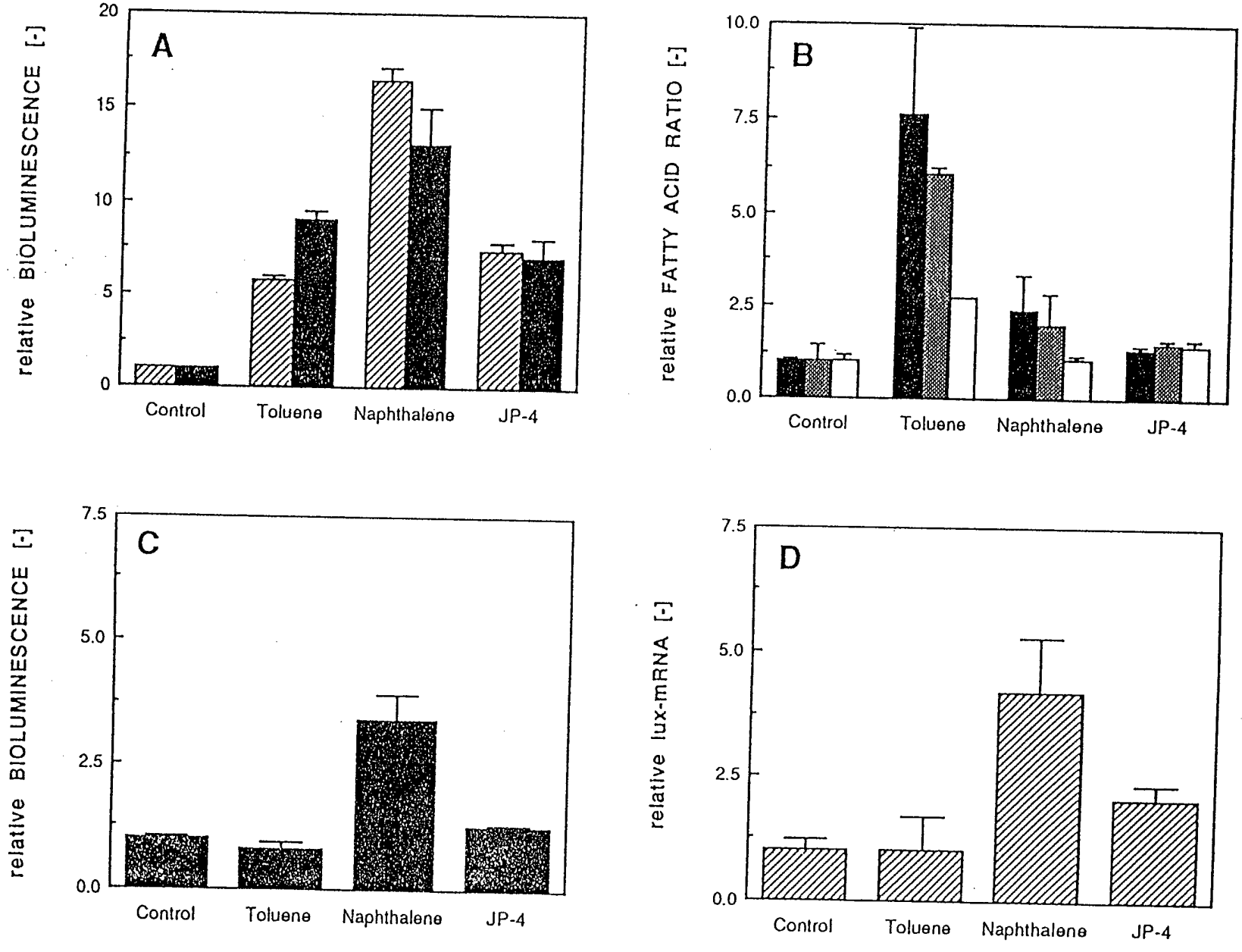
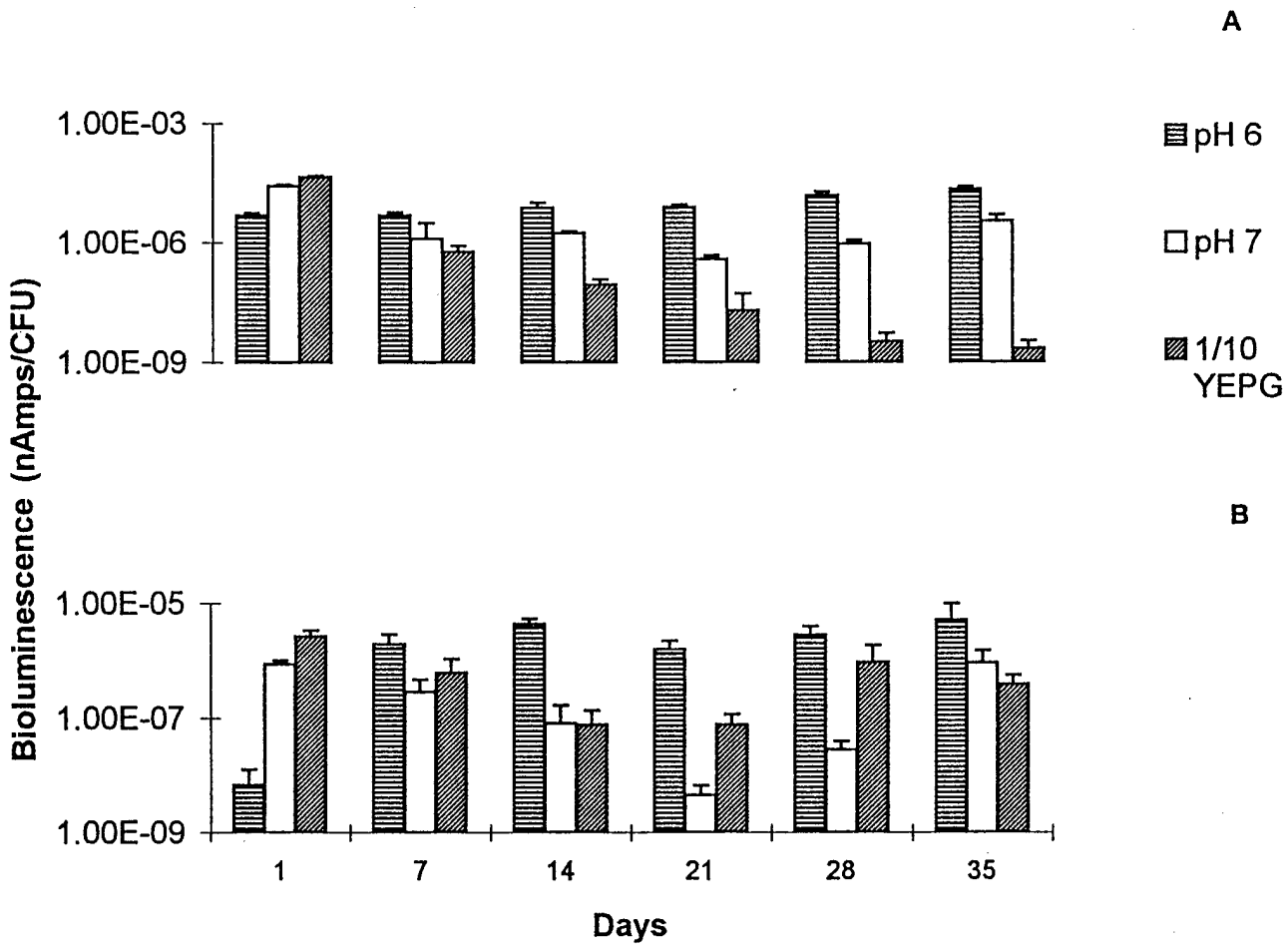
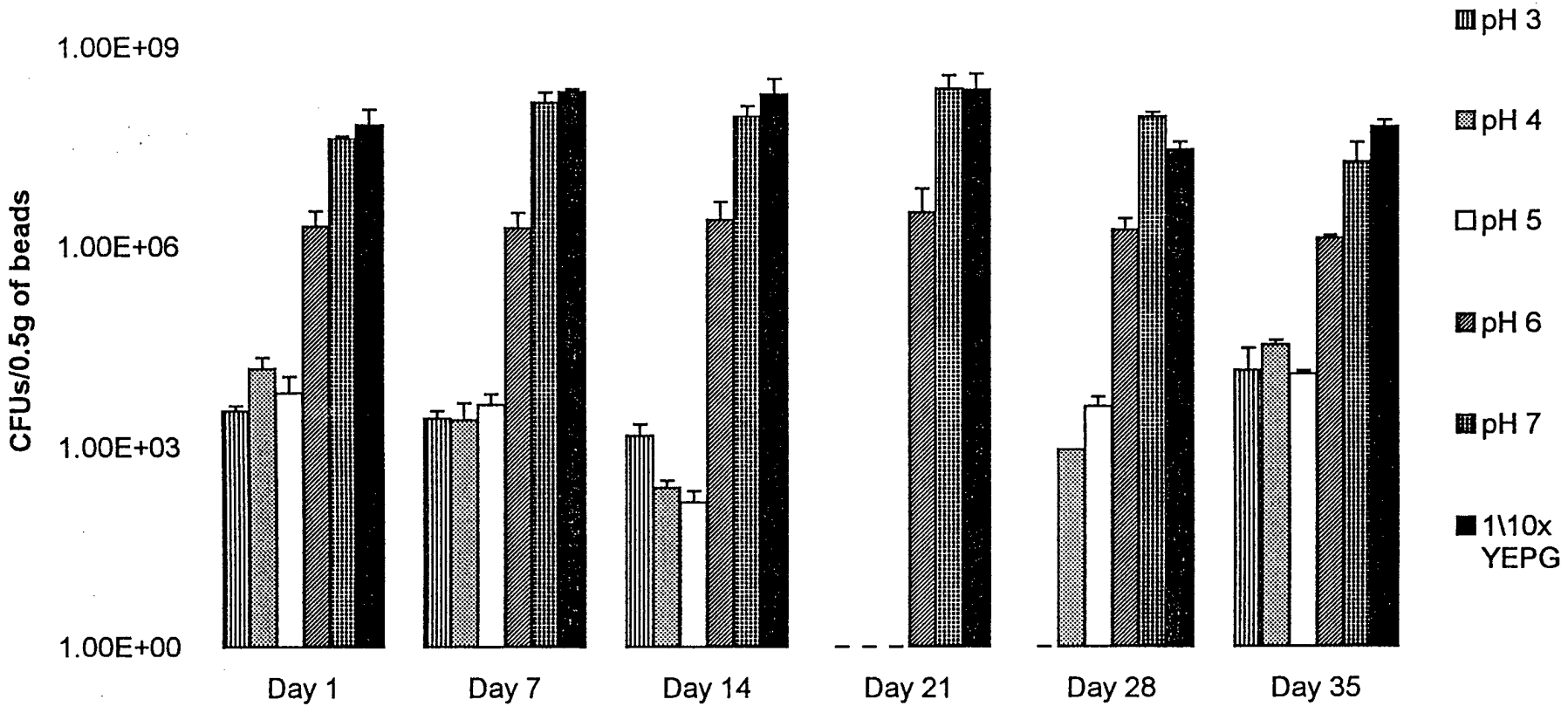
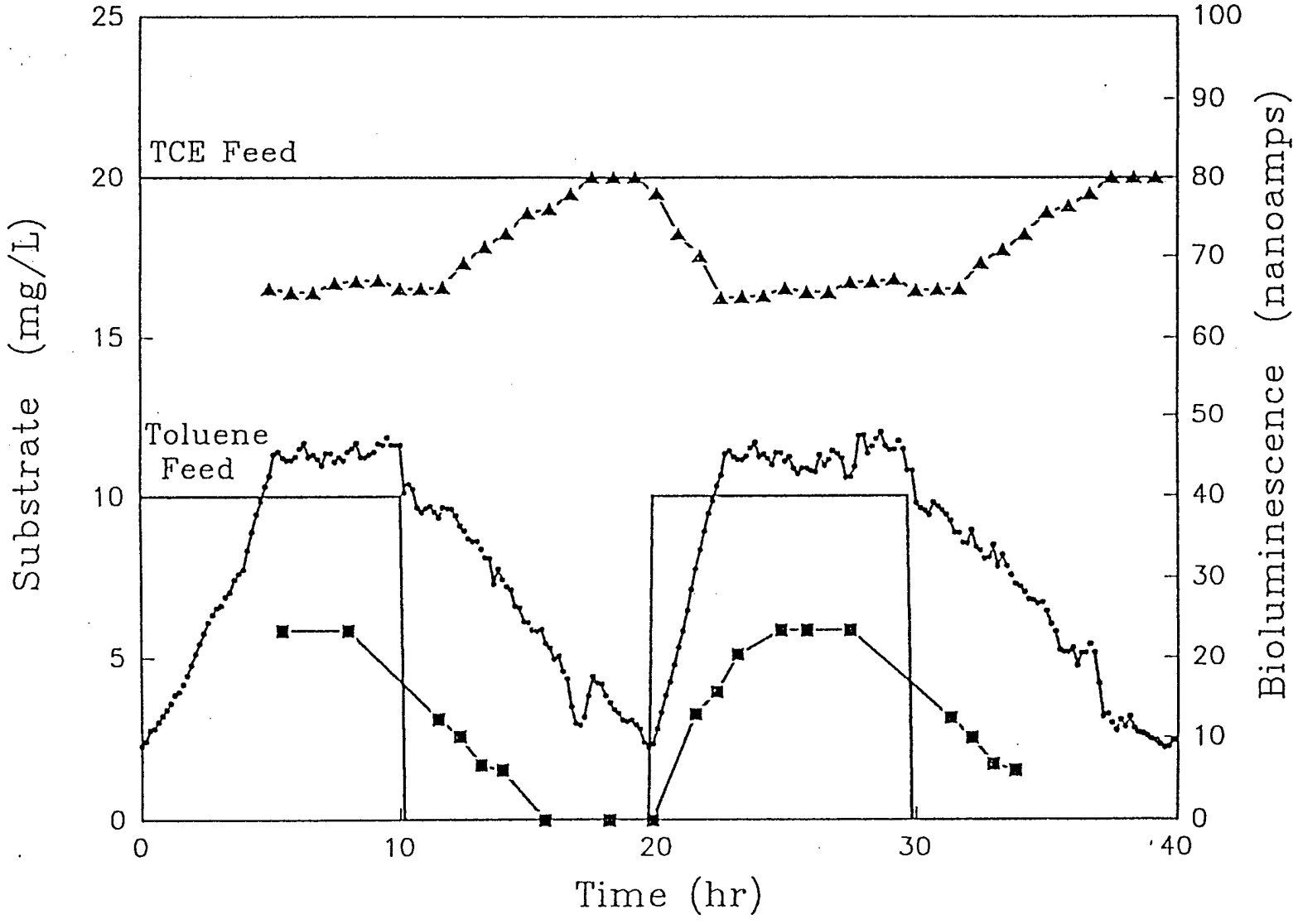

A
relative BIOLUMINESCENCE [-]
20
15
10
5
0
Control
Toluene
Naphthalene
JP-4
B
relative FATTY ACID RATIO [-]
10.0
7.5
5.0
2.5
0.0
Control
Toluene
Naphthalene
JP-4
C
relative BIOLUMINESCENCE [-]
7.5
5.0
2.5
0.0
Control
Toluene
Naphthalene
JP-4
D
relative lux-mRNA [-]
7.5
5.0
2.5
0.0
Control
Toluene
Naphthalene
JP-4

Figure 6


A
1.00E-03
1.00E-06
1.00E-09
pH 6
pH 7
1/10 YEPG
B
1.00E-05
1.00E-07
1.00E-09
Bioluminescence (nAmps/CFU)
1
7
14
21
28
35
Days


CFUs/0.5g of beads
1.00E+09
1.00E+06
1.00E+03
1.00E+00
Day 1
Day 7
Day 14
Day 21
Day 28
Day 35
pH 3
pH 4
pH 5
pH 6
pH 7
1\10x YEPG


TCE Feed
Toluene
Feed
Substrate (mg/L)
Bioluminescence (nanoamps)
Time (hr)
0
5
10
15
20
25
0
10
20
30
40
0
10
20
30
40
50
60
70
80
90
100

Report Number (14) DOE/ER/61193--1

Publ. Date (11) 199503

Sponsor Code (18) DOE/ER, XF

UC Category (19) UC-400, DOE/ER

DOE