APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1994, p. 1487–1494
0099-2240/94/$04.00+0

Vol. 60, No. 5

Optical Biosensor for Environmental On-Line Monitoring of Naphthalene and Salicylate Bioavailability with an Immobilized Bioluminescent Catabolic Reporter Bacterium

ARMIN HEITZER,[1,2]† KEN MALACHOWSKY,[1,2] JANEEN E. THONNARD,[2] PAUL R. BIENKOWSKI,[2,3] DAVID C. WHITE,[1,2,4] AND GARY S. SAYLER[1,2,4]*

Department of Microbiology,[1] Graduate Program in Ecology,[4] Department of Chemical Engineering,[3] and Center for Environmental Biotechnology,[2] The University of Tennessee, Knoxville Tennessee 37932

Received 3 November 1993/Accepted 23 February 1994

An optical whole-cell biosensor based on a genetically engineered bioluminescent catabolic reporter bacterium was developed for continuous on-line monitoring of naphthalene and salicylate bioavailability and microbial catabolic activity potential in waste streams. The bioluminescent reporter bacterium, *Pseudomonas fluorescens* HK44, carries a transcriptional *nahG-luxCDABE* fusion for naphthalene and salicylate catabolism. Exposure to either compound resulted in inducible bioluminescence. The reporter culture was immobilized onto the surface of an optical light guide by using strontium alginate. This biosensor probe was then inserted into a measurement cell which simultaneously received the waste stream solution and a maintenance medium. Exposure under defined conditions to both naphthalene and salicylate resulted in a rapid increase in bioluminescence. The magnitude of the response and the response time were concentration dependent. Good reproducibility of the response was observed during repetitive perturbations with either naphthalene or salicylate. Exposure to other compounds, such as glucose and complex nutrient medium or toluene, resulted in either minor bioluminescence increases after significantly longer response times compared with naphthalene or no response, respectively. The environmental utility of the biosensor was tested by using real pollutant mixtures. A specific bioluminescence response was obtained after exposure to either an aqueous solution saturated with JP-4 jet fuel or an aqueous leachate from a manufactured-gas plant soil, since naphthalene was present in both pollutant mixtures.

For comprehensive microbiological characterization of contaminated sites and monitoring of bioremediation processes, it is necessary to develop appropriate methodological approaches to the assessment of critical biological parameters. Relevant biological parameters include the spatial and temporal distribution and frequency of specific indigenous or introduced microbial catabolic genotypes, their specific catabolic activities, and the bioavailability of pollutants. Quantification of these parameters is also important for evaluation of the treatment efficacy of a particular bioremediation method.

A number of molecular diagnostic techniques for detection of specific catabolic genotypes and their activities have been reported (2, 32, 33). Many of these approaches, such as direct isolation of DNA (25) or mRNA (8) from environmental samples and subsequent analysis with specific gene probes, provide information on in situ conditions and do not require additional cultivation or activation of the indigenous bacterial population. Another novel approach for environmental monitoring of bacterial distribution and activities involves the application of genetically engineered bacteria with specific bioluminescent reporter functions that allow monitoring both in situ and on line (5).

Bacterial bioluminescence has been characterized physiologically and biochemically (10, 21), as well as genetically (22). The bioluminescence reaction is catalyzed by a heterodimeric luciferase enzyme encoded by the *luxA* and *luxB* genes. The light reaction itself is dependent on O_2, reduced flavin mononucleotide, and an aldehyde substrate. The synthesis of the aldehyde is catalyzed in an ATP- and NADPH-dependent manner by a multienzyme fatty acid reductase complex comprising a reductase, a transferase, and a synthetase encoded by the *luxC*, *luxD*, and *luxE* genes, respectively. Environmental applications of *lux* genes include their use as constitutively expressed markers for monitoring of the fate of bacteria introduced into the environment (7, 26, 37, 38) and construction of transcriptional fusions between *lux* gene cassettes and inducible catabolic and detoxification genes of environmental relevance (5, 6, 16, 36, 41). In addition, numerous other diagnostic and analytical applications of bioluminescent bacteria have been described (4, 40).

Recently, the construction of genetically engineered bioluminescent reporter bacteria for monitoring of naphthalene and salicylate bioavailability and catabolism was reported (6, 16). One of these bacteria, *Pseudomonas fluorescens* HK44, carries a reporter plasmid, pUTK21, which encodes naphthalene degradation and the bioluminescence reporter function (16). The reporter plasmid contains a transcriptional fusion between a *luxCDABE* gene cassette from *Vibrio fischeri* (39) and the *nahG* gene of the lower naphthalene (salicylate) degradation operon (43). Exposure of *P. fluorescens* HK44 to both naphthalene and its degradation intermediate, salicylate, resulted in increased gene expression and, consequently, increased bioluminescence. Studies with suspended reporter cultures under continuous cultivation conditions revealed a positive correlation between naphthalene catabolism and bioluminescence (16). It was subsequently demonstrated that a linear relation-

* Corresponding author. Mailing address: Center for Environmental Biotechnology, The University of Tennessee, 10515 Research Drive, Suite 100, Knoxville, TN 37932-2567. Phone: (615) 974 8080. Fax: (615) 974 8086.

† Present address: Department of Environmental Sciences, Swiss Federal Institute of Technology, ETH-Zentrum, VOD, CH-8092 Zurich, Switzerland.

ship exists between the naphthalene or salicylate concentration and overall bioluminescence, which resulted in the development of a quantitative and specific bioassay for the bioavailability of these pollutants (13). Since such assays could be conducted only in a discontinuous fashion, it was of interest to develop a biosensor device that allows continuous on-line monitoring of pollutant bioavailability and specific microbial catabolic activity potential.

The characteristics of a biosensor can be defined as follows: (i) specific recognition of the analyte by a biologically active compound (receptor), (ii) conversion of the physicochemical effect caused by the interaction between the analyte and the receptor into an electrical signal by a transducer, and (iii) signal processing and amplification (34). Numerous types of biosensors have been proposed; while most biosensor receptors involve macromolecules such as enzymes and antibodies (35) or nucleic acids (9), whole-cell systems have also been reported. These living-cell biosensors are commonly based on electrochemical transducers which include, for example, amperometric systems for oxygen monitoring to assess biological parameters such as the biological oxygen demand (15) or the presence of chlorinated aromatic pollutants (27). Other systems involve thermistors as transducers for the measurement of reaction enthalpies caused by the metabolism of aromatic compounds by immobilized cells (42).

The development of optical biosensors for detection of chemi- and bioluminescence reactions has been recently reviewed (1). While all of these sensors were based on enzymes, it was the goal of this work to develop and investigate the performance of an optical whole-cell bioluminescence biosensor for specific on-line monitoring of naphthalene and salicylate bioavailability and microbial catabolic activity potential with *P. fluorescens* HK44. It was demonstrated that the bioluminescent reporter bacterium *P. fluorescens* HK44 is fully functional as immobilized cultures in calcium alginate, which provided a translucent matrix that allowed bioluminescence to be easily monitored (11). Studies conducted with reporter cultures immobilized in calcium alginate beads revealed linear correlations between the salicylate or naphthalene concentration and maximum bioluminescence levels. The use of strontium alginate instead of calcium alginate was recently described, and it was stated that strontium alginate provides a stronger matrix than calcium alginate (17). On the basis of these findings, a biosensor was developed around commercially available light-sensing equipment while taking the physiological requirements of the whole-cell receptor into account.

MATERIALS AND METHODS

Organism. *P. fluorescens* HK44, a genetically engineered, bioluminescent catabolic reporter bacterium, was used in all of the experiments reported here (16). This strain carries a *nah-lux* reporter plasmid, pUTK21, which contains a transcriptional gene fusion between a *luxCDABE* gene cassette from *V. fischeri* and the *nahG* gene of the salicylate operon. *P. fluorescens* HK44 produces light after exposure to salicylate and naphthalene and is able to degrade both compounds.

Growth media. Before immobilization, *P. fluorescens* HK44 was grown in a yeast extract-peptone-glucose (YEPG) medium containing, in grams per liter, the following: yeast extract, 0.2; polypeptone, 2.0; glucose, 1.0; NH_4NO_3, 0.2. The medium was buffered at pH 7.0 with a 0.05 M K_2HPO_4-NaH_2PO_4 mixture, and 14 mg of tetracycline per liter was added for positive selection of the *lux* transposon. Plating was conducted by using the YEPG medium supplemented with 15 g of Bacto Agar per liter.

For biosensor studies, a maintenance medium with the following composition, in milligrams per liter, was used: $SrCl_2 \cdot 6H_2O$, 666; NH_4NO_3, 100; glucose, 10; peptone, 20; Na_2SO_4, 7.2; tetracycline, 28. Strontium chloride was provided to maintain the integrity of the alginate immobilization matrix. All of the components were added through sterile filtration with a 0.2-μm-pore-size membrane filter. The medium was buffered at pH 7.0 with 100 ml of a sterile 50 mM potassium phosphate solution per liter.

Waste stream solutions. Salicylate solutions, 10 mg/liter, were prepared in water by using sterile filtration. Saturated naphthalene solutions were prepared by adding naphthalene crystals to either stirred, sterile water or sterile maintenance medium, the latter for experiments with changing naphthalene concentrations. The solutions were stirred overnight before use. A saturated naphthalene solution contains approximately 31 mg of naphthalene per liter (19).

Sterile glucose and a complex medium solution were prepared in water containing (in grams per liter) either glucose (1.0) or peptone (1.0), yeast extract (0.1), and glucose (0.5), respectively. Saturated aqueous toluene and JP-4 jet fuel solutions were prepared by adding 100 ml of each solvent to 1 liter of sterile water. The solutions were stirred overnight in a closed glass container before use. An aqueous solution saturated with toluene contains approximately 500 mg of toluene per liter (18), and JP-4 jet fuel contains 0.5% (wt/wt) naphthalene (28).

For soil leachate studies, a manufactured-gas plant (MGP) soil from a site in the northeastern United States was used. 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 weight).

Immobilization procedure. A culture of *P. fluorescens* HK44 grown in YEPG medium in 300-ml Erlenmeyer flasks at 25°C was harvested in the exponential growth phase at an optical density at 546 nm of 0.8. The culture was washed once and resuspended in a sterile 0.9% NaCl solution. Of the washed bacterial suspension, 12.5 ml was mixed with 25 ml of a sterile, low-viscosity alginate solution (3.5% [wt/wt] in 0.9% NaCl) and 7.5 ml of sterile glycerol. The cell-alginate-glycerol mixture was kept in 1.5-ml aliquots frozen at −70°C for further use.

Biosensor probe tips were prepared by injecting the cell-alginate mixture into the ferrule cavity on the liquid light guide. Subsequently, the ferrule was immediately immersed in a stirred, sterile 0.1 M $SrCl_2$ solution for 60 min at room temperature to harden the strontium-alginate matrix.

Biosensor setup and bioluminescence monitoring. The biosensor probe tip, shown in Fig. 1A, consists of a stainless-steel ferrule mounted onto the measuring end of a liquid light guide (Oriel, Stratford, Conn.). A stainless-steel woven mesh with a 104-μm pore size (Spectra/Mesh; Spectrum Medical Industries, Inc., Houston, Tex.) formed into a spherical shape provided, together with the liquid light guide surface, a cavity with a volume of approximately 200 μl that contained the reporter culture immobilized in strontium-alginate. To monitor the bioluminescence emitted by the reporter culture, the biosensor probe tip was connected through the liquid light guide to a 77340 photomultiplier (Oriel) and a 7070 digital display (Oriel). Data were stored automatically on a personal computer. Figure 1B provides a scheme of the complete biosensor setup. The biosensor probe tip was inserted into a stainless-steel measurement cell which consisted of a modified stainless-steel union tee (Swagelok Co., Solon, Ohio). The liquid volume of this biosensor cell was 1.5 ml. Delivery of the maintenance medium and waste stream solutions or water was

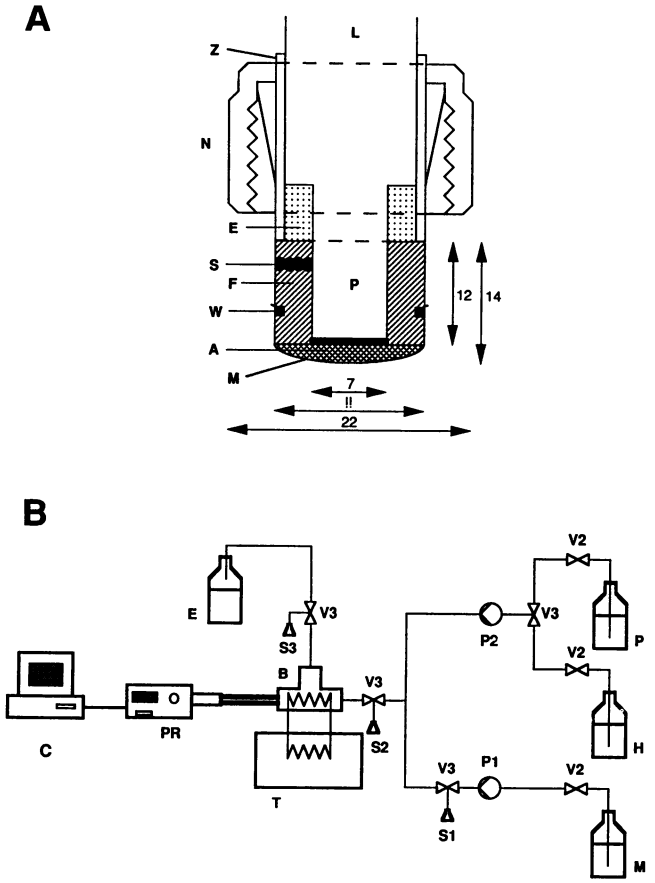


FIG. 1. (A) Biosensor probe. A, strontium-alginate matrix containing the bioluminescent reporter culture; E, epoxy resin; F, stainless-steel ferrule; L, liquid light guide; M, stainless-steel mesh; N, hex nut for probe fixation; P, light guide probe tip; S, set screw for ferrule fixation; W, wire for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

conducted with high-pressure liquid chromatography (HPLC) pumps (Rabbit-HP solvent delivery system; Rainin Instruments Co., Woburn, Mass.). All of the tubing (Alltech, Deerfield, Ill.) and valves (Whitey Co., Highland Heights, Ohio) used were made of stainless steel, and the latter contained Teflon inserts. The test solutions were stored in glass flasks containing rubber stoppers.

Operating conditions. Maintenance medium and either a waste stream solution or water were each continuously added at a flow rate of 2.5 ml/min, resulting in a constant total flow rate of 5 ml/min; consequently, 50% of the substrate concentration at the sensor tip was provided by the waste stream. Experiments were conducted at a constant, controlled temperature of 25°C and at pH 7.

To change the naphthalene concentration, the flow rates of the waste stream and maintenance medium pumps were varied while the total flow rate remained constant at 5 ml/min. A 25% saturated toluene solution was achieved by keeping the maintenance medium flow at 3.75 ml/min and the saturated aqueous toluene solution flow at 1.25 ml/min.

For studies using leachates of MGP soils, the setup was slightly modified. Between pump P2 and valve V3, a bypass containing a soil column flanked by two three-way valves was inserted. Sterile water was pumped through the stainless-steel soil column at a constant rate of 2.5 ml/min. A porous stainless-steel plate with a 20-μm pore size prevented significant loss of soil particles. The column was packed with 136 g (dry weight) of MGP soil. The aqueous volume (void volume) in the packed column was 31 ml.

Analysis and monitoring. Bioluminescence measurements were conducted on line with the equipment already described, and data were stored automatically on a personal computer using a software program written by Rod Bunn (University of Tennessee). For perturbation experiments, baseline bioluminescence in the absence of an inducer was subtracted prior to log transformation and further analysis of data.

Salicylate concentrations were determined spectrophotometrically at 296 nm by comparing the absorbance values with a standard curve derived from known amounts of salicylate. Naphthalene concentrations were determined by HPLC (Vista 5500 liquid chromatograph; Varian, Palo Alto, Calif.) in combination with UV detection at 254 nm (LC 90 UV spectrophotometric detector; Perkin Elmer, Norwalk, Conn.). A guard column containing C_{18} reverse-phase 5-μm packing material (VYDAC 201TPB5; Separations Group, Hesperia, Calif.) was installed upstream of the separation column. Separation was achieved with a 25-cm C_{18} reverse-phase column (VYDAC 201TP54; Separations Group) and a continuous aqueous acetonitrile gradient from 0 to 65% between min 1 and 2. At the end of the 11-min program, the column was equilibrated for 1.5 min with 1.5 ml of H_2O per min. A flow rate of 1.5 ml/min was used, with sample injection occurring at 0.4 min for manual injection or at 1 min for on-line automatic injection. Concentrations were determined by peak integration (HP3396A Integrator; Hewlett-Packard, Avondale, Pa.) by using a standard curve prepared from known amounts of naphthalene in acetonitrile.

Glucose concentrations were determined colorimetrically by using an enzyme assay kit (Sigma Chemical Co., St. Louis, Mo.). Dissolved-oxygen determinations were conducted in a 53 Biological Oxygen Monitor respirometer (Yellow Springs Instrument Co., Yellow Springs, Ohio). Calibration and measurements were conducted in accordance with the manufacturer's manual. Effluent solution samples were withdrawn with a directly connected syringe and transferred into the measurement cells.

Viable plate counts were conducted either after sampling at the biosensor effluent or, for the biosensor probe tip, after removal of the probe tip ferrule containing the cell-strontium alginate and dissolution of the matrix in 10 ml of sterile hexametaphosphate solution (50 mM) by gentle mixing for 5 min. Subsequent serial dilutions were conducted in a sterile 0.9% NaCl solution, and 50-μl samples were plated on YEPG agar with or without 14 mg of tetracycline per liter.

Colony hybridizations were conducted as described earlier (30). A PCR-generated, [α-^{32}P]dCTP-labeled, 1,717-base, single-stranded *luxAB* fragment was used as a gene probe to identify the reporter strain. The fragment starts at base 4254 in the *luxA* gene and ends at base 5971 in the *luxB* gene (3). The nucleotide sequence of the primer used for the PCR was GGGGGTTGTTATTCCAACAGC. Thermocycler conditions for the 38 cycles were 1 min at 94°C for strand melting, 2 min at 55°C for primer annealing, and 3 min at 72°C for strand extension.

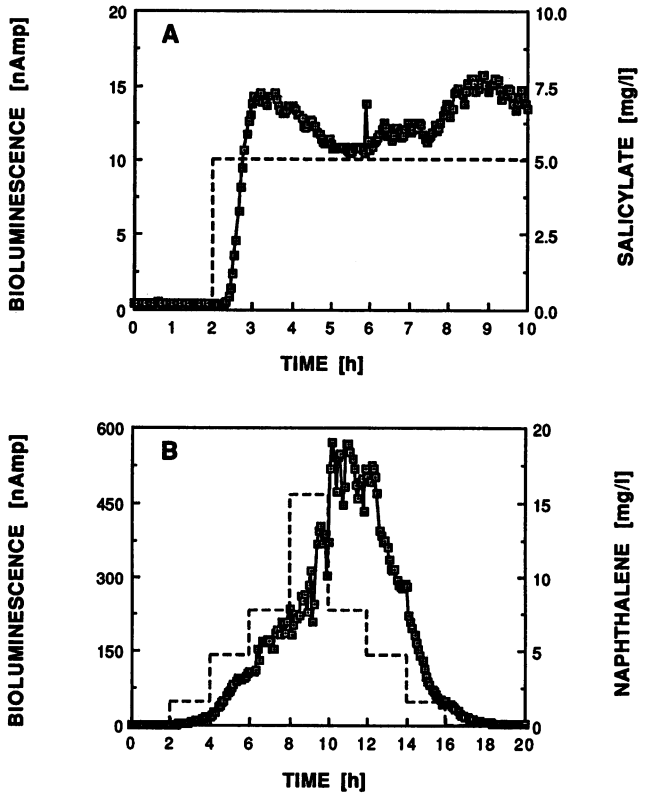


FIG. 2. Comparative bioluminescent sensor response to salicylate and naphthalene waste streams. (A) Biosensor response to a step change in salicylate concentration: ⊡, time course of the bioluminescence response; – – –, salicylate concentration (5 mg/liter) in the biosensor cell. (B) Biosensor response to gradual changes in naphthalene concentration: ⊡, time course of the bioluminescence response; – – –, naphthalene concentration in the biosensor cell. See the text for naphthalene concentrations; all biosensor substrate concentrations were diluted 1:1 in the sensor cell with respect to the waste stream concentration (flow rate, 5 ml/min).

RESULTS

The bioluminescence response of the biosensor to a step change in salicylate (10 mg/liter) in the waste stream is shown in Fig. 2A. The bioluminescence signal increased from a baseline level of 0.45 nA to a maximum level of approximately 15 nA. During constant exposure to salicylate, the bioluminescence signal fluctuated around a mean value of 12.9 nA with a standard deviation of ±1.5 nA for a 7-h measurement period with 140 measurements. The response time of the biosensor after exposure to the inducing compound varied between 8 and 15 min for 15.5 mg of naphthalene per liter and 5 mg of salicylate per liter, respectively. The response time was defined as the interval between exposure to the pollutant and the time when the bioluminescence response exceeded the sum of 3 standard deviations of the average baseline value prior to induction. It is interesting that at lower inducing substrate concentrations of 0.5 mg of salicylate per liter and 1.55 mg of naphthalene per liter the response times (24 and 8 min, respectively) were significantly longer than those measured at high concentrations.

The effects of different naphthalene concentrations on the bioluminescence response of the biosensor were investigated by simulating the event of a passing pollution plume in the waste stream. This was achieved by using gradual step changes in the biosensor cell naphthalene concentration from 0, 1.55, 5.16, 7.75, and 15.5 mg/liter in 2-h increments (Fig. 2B). The time course pattern of the bioluminescence response followed closely the gradual step changes in naphthalene concentration with a time delay. The bioluminescence response to short-term (90-s) exposure to salicylate (5 mg/liter) was a 115% increase over the background. Bioluminescence levels increased proportionally to the duration of salicylate addition over intervals of 23, 45, and 90 s (Table 1).

TABLE 1. Bioluminescence response of the biosensor after short-term exposure to a waste stream containing 10 mg of salicylate per liter

Duration of salicylate addition(s)	Maximum bioluminescence increase (%)[a]
0	0
23	26
45	48
90	115

[a] Percentage of the uninduced baseline response. The noise of the baseline was ±4.2%, expressed as a standard deviation of the average signal value.

The reproducibility of the biosensor response was investigated by using repetitive, defined 1-h exposures to either salicylate or naphthalene (Fig. 3). In Fig. 3A, the bioluminescence response to salicylate perturbations from 0 to 10 mg/liter in the waste stream is shown. Good reproducibility of the peak levels at 4.9 ± 0.5 nA was observed. The half-life, at 25°C, of the bioluminescence signal after a change from salicylate to

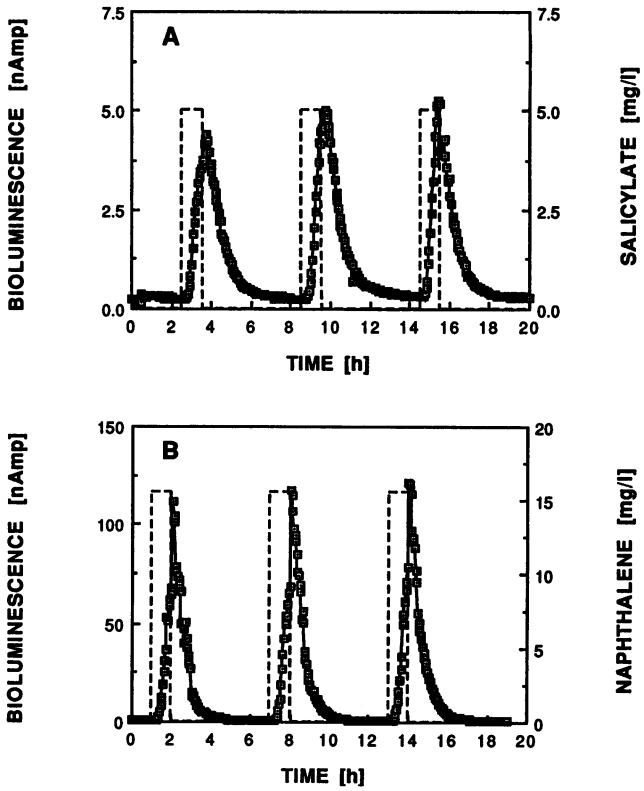


FIG. 3. Biosensor response to repetitive pollutant perturbations. Panels: A, salicylate; B, naphthalene. ⊡, time course of the bioluminescence response; – – –, pollutant concentration in the biosensor cell.

water was 24 to 30 min and was calculated on the basis of the formula half-life = ln $2/k_d$, where k_d is the specific bioluminescence decay rate. k_d was obtained from regression analysis of the linearized time course of the bioluminescence signal. For changes in the naphthalene concentration in the waste stream (0 to 31 mg/liter), similar results were obtained with significantly higher peak bioluminescence levels at 117 ± 5.1 nA, as illustrated in Fig. 3B. The half-life of the bioluminescence signal after a change from naphthalene to water was 0.4 h.

To investigate the specificity of the biosensor, the response to different carbon substrates (toluene, glucose, and YEPG medium) in the waste stream was tested and compared with the response to naphthalene. For both glucose (1.0 g/liter) and YEPG (1.0 g/liter), the bioluminescent response was insignificant (less than twofold) and the response times of 60 and 80 min were significantly longer than those observed for naphthalene, which varied between 8 and 24 min for 15.5 and 1.55 mg/liter, respectively (data not shown). Exposure to toluene resulted in no significant bioluminescence signal. After a step change from toluene-saturated water to pure water, the biosensor remained fully functional (results not shown).

An important operating parameter is the oxygen partial pressure in the biosensor system. Measurement in the effluent of the biosensor under noninduced conditions provided values of >90% saturation. Comparative studies conducted under induced conditions with a 10-mg/liter salicylate solution which was either degassed with helium or saturated with air revealed that the bioluminescence response was not affected, even at an effluent saturation level of 49%, which corresponds to a situation of an anaerobic waste stream (data not shown).

The practical utility of the biosensor system was investigated by using real, complex pollutant mixtures containing naphthalene. In Fig. 4A, the bioluminescence response to a waste stream of an aqueous solution saturated with JP-4 jet fuel is shown. After a response time of 24 min, the bioluminescence signal increased from a baseline level of 0.5 nA to 5 nA within 2 h after exposure to the JP-4 solution. Naphthalene was detectable at a concentration of 0.55 mg/liter in the effluent of the biosensor. Figure 4B illustrates the bioluminescence response to the aqueous leachate from an MGP soil. The bioluminescence increased after a response time of 21 min from a baseline level of 0.5 nA to a maximum of approximately 10 nA 2 h after exposure to the leachate. During the following 3 h, the bioluminescence level remained relatively constant (insert in Fig. 4B), corresponding to the constant naphthalene level of 0.6 mg/liter in the effluent of the soil column during the same interval.

The biosensor probe tip contained approximately 2×10^7 viable cells of the reporter bacterium *P. fluorescens* HK44 immediately after preparation at the beginning of an experiment. During the course of an experiment, reporter bacteria were released because of growth from the biosensor cell. Four days after the beginning of an experiment, the effluent cell concentration was approximately 3×10^4/ml. All colonies of the released cells were positive when probed during colony hybridizations with a *luxAB* gene probe. Experiments were conducted continuously over 14-day periods with the same probe tip without loss of dynamic function. Formation of a biofilm (visual and culturable) 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 (without immobilized cells).

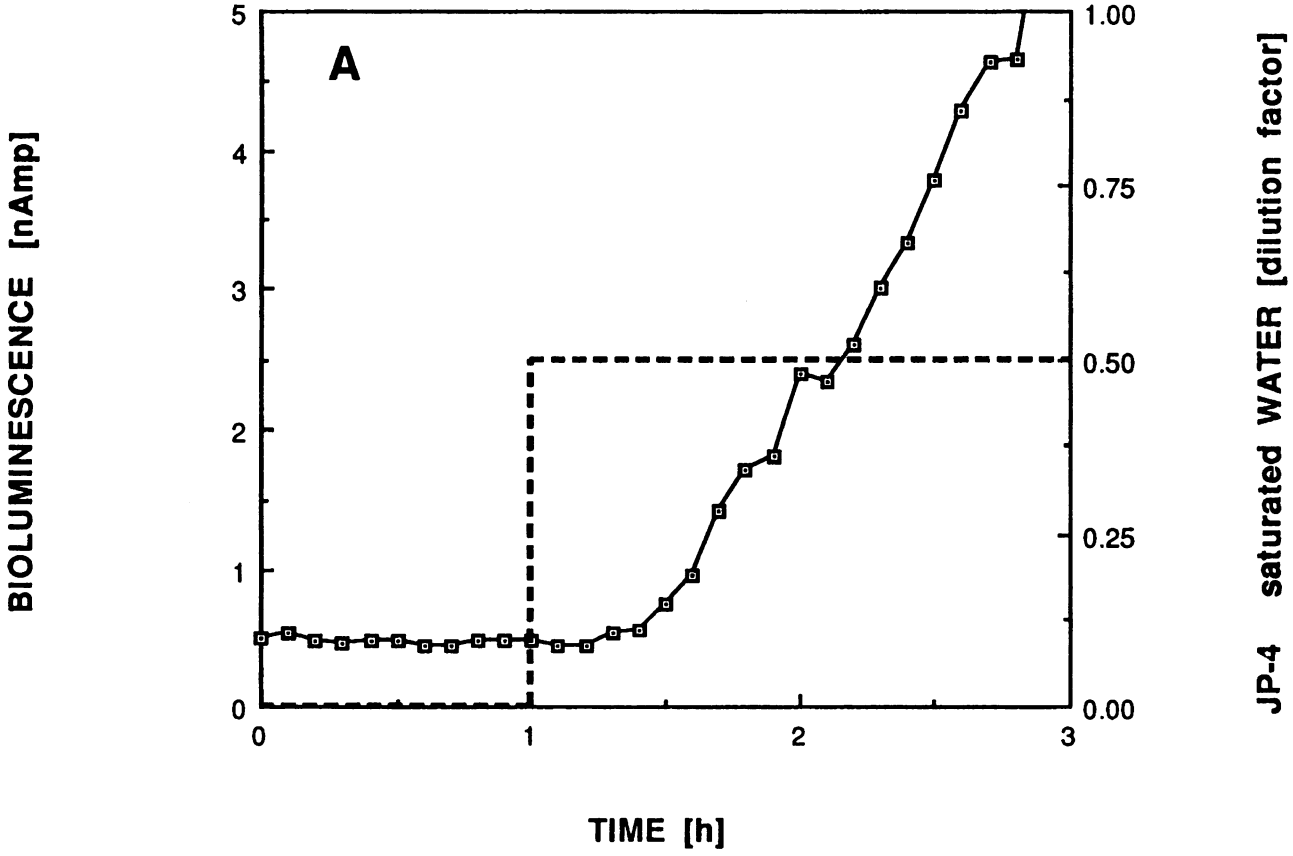
DISCUSSION

An optical whole-cell biosensor based on the bioluminescent catabolic reporter bacterium *P. fluorescens* HK44 was developed and designed around commercially available light detection equipment for rapid on-line monitoring of pollutant bioavailability and catabolic activity in waste streams, i.e., dirty samples (20). Therefore, a biosensor setup and configuration was developed in which a maintenance medium was provided at a high flow rate which resulted in a short mean residence time of 18 s in the measurement cell. The air-saturated maintenance medium provided carbon substrates, nutrients, and oxygen at a constant rate for the reporter bacteria immobilized in strontium-alginate, which was important for the bioluminescence reaction (10). Because of the high flow rate, the substrate-and-nutrient input rate exceeded the consumption rate of the reporter culture. In addition, the 1:1 flow ratio between the maintenance medium and the waste stream provided sufficient oxygen for the bioluminescence reaction for the worst-case scenario of an anaerobic waste stream.

Exposure of the biosensor to either naphthalene or its degradation product salicylate resulted in a rapid increase in the bioluminescence response. The data indicated also that the response time increased with a decreasing pollutant concentration, which can be explained by the fact that the rate of the bioluminescence increase varies with the pollutant concentration (11, 13). During continuous exposure to inducing pollutants, the signal remained at a higher level. The fluctuations of ±12.3%, expressed as a standard deviation of the average value observed over a 1-h interval with at least 20 measurements, could not be attributed to changes in the salicylate concentration (the baseline value was ±4.2%); therefore, such changes are due to biochemical processes involved in the bioluminescence reaction.

The performance of the biosensor during exposure to a pollution plume was simulated by changing the naphthalene concentration in the waste stream stepwise. The results demonstrated that the overall bioluminescence response reflected the naphthalene concentration profile very well. These observations were in accordance with earlier findings obtained with *P. fluorescens* HK44 immobilized in calcium-alginate beads, when exposure to different salicylate concentrations resulted in a linear relationship between maximum bioluminescence levels and the inducer concentration (11). The sensitivity of the biosensor was illustrated by using different short-term exposures to a constant-concentration salicylate solution. The maximum bioluminescence response was proportional to the salicylate exposure time, and salicylate addition for as short as 23 s resulted in a bioluminescence increase of 26%.

An important question concerned performance under repetitive-perturbation conditions. Good signal magnitude and response time reproducibilities were found for both substrates, salicylate and naphthalene. The half-life of the bioluminescence signal at 25°C, after replacement of the specific inducing substrate from the waste stream by water, varied between 24 and 30 min for naphthalene and salicylate, respectively. A number of factors could contribute to the decay of the bioluminescence response: (i) a reduced de novo luciferase synthesis rate, (ii) limited availability of reaction substrates such as O_2, aldehyde, reduced flavin mononucleotide, NADPH, and ATP, (iii) intracellular dilution by cellular growth, and (iv) intrinsic stability of the luciferase enzyme. Of all of these factors, modification of factor iv seems to be most appropriate for achievement of a more rapid decay of the bioluminescence signal. From the literature it is known that the in vitro half-life of the *V. fischeri* luciferase enzyme at 38°C is


A
BIOLUMINESCENCE [nAmp]
JP-4 saturated WATER [dilution factor]
TIME [h]
0
1
2
3
4
5
0.00
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0.50
0.75
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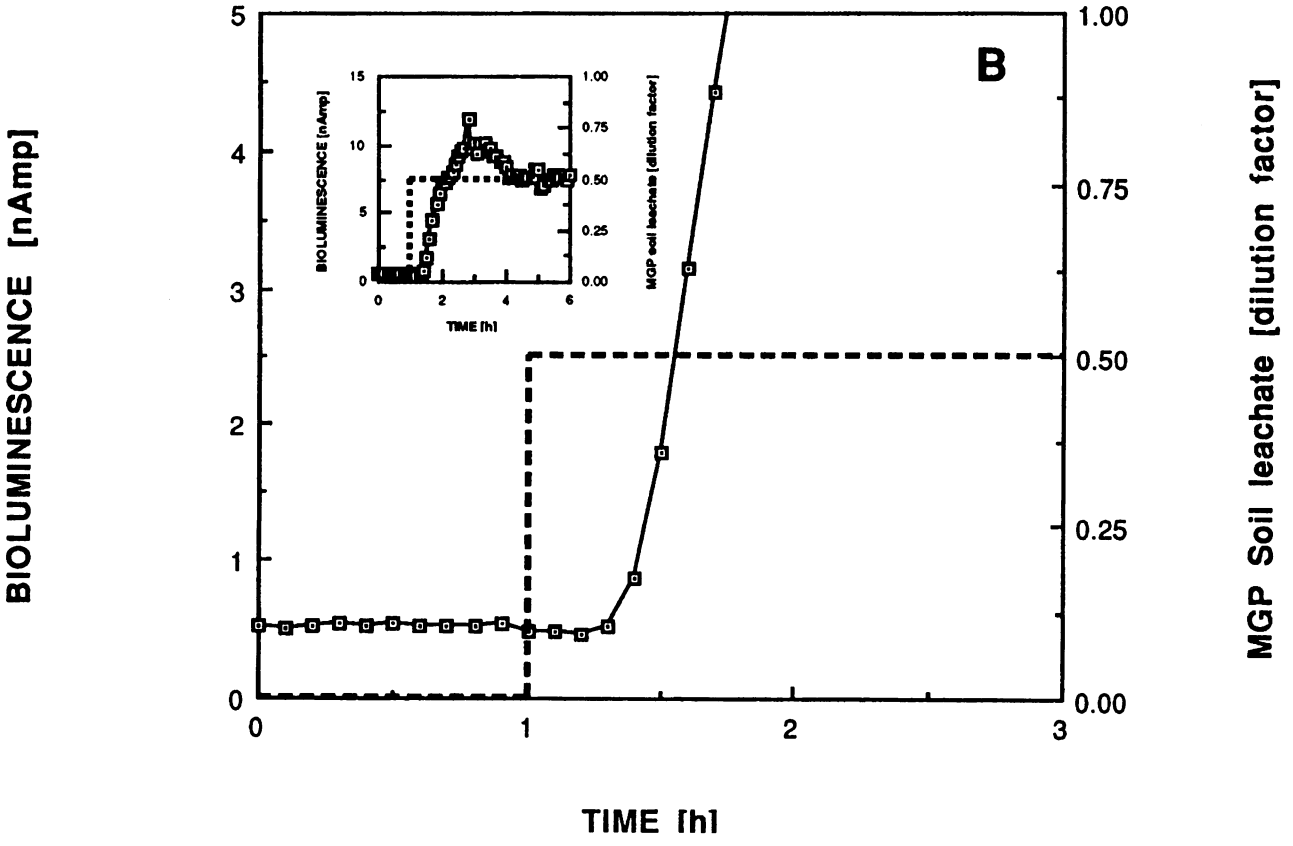
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FIG. 4. Biosensor responses to complex real pollutant mixtures containing naphthalene. (A) Step change in JP-4 jet fuel concentration, expressed as a dilution factor of an aqueous solution saturated with JP-4. (B) Step change in MGP soil leachate concentration, expressed as a dilution factor of the full-strength MGP soil leachate. The insert shows the response over a longer interval. ⊡, time course of the bioluminescence response; – – –, pollutant concentration profile in the biosensor cell represented as a step change from 0 to a 50% dilution with maintenance medium at 1 h. See the text for JP-4 and MGP details.

only about 1 min (29). Therefore, a transient increase of the biosensor temperature for a few minutes could accelerate the decay of the bioluminescence signal dramatically. However, it has been demonstrated that such short heat shocks in the superoptimum temperature range can significantly affect bacterial gene expression (12).

From a practical point of view, it was important to investigate the performance of the biosensor in the presence of solvents such as toluene or in the presence of readily degradable but noninducing substrates. Substrates such as glucose and peptone at high concentrations or the presence of toluene did not significantly interfere with the specificity of this biosensor. Although minor increases in bioluminescence were observed, the response to noninducing carbon substrates could be easily discriminated from the specific induction by naphthalene on the basis of response time. Mechanistically, the small bioluminescence increase after exposure to either glucose or YEPG medium could be explained by more efficient substrate diffusion into the alginate matrix because of higher aqueous-phase substrate concentrations. The resulting activation of bacteria deeper inside the strontium-alginate matrix would result in increased bioluminescence.

The experiments conducted with real pollutant mixtures such as JP-4 jet fuel and the MGP soil leachates demonstrate the utility of this biosensor under realistic environmental application conditions. In both situations, significant bioluminescence responses were observed. The response time (20 min) and magnitude indicated that the bioluminescence signal was due to specific induction and not the presence of readily degradable carbon substrates. In the latter case, a significantly longer response time would have been expected. This is also true for other polynuclear aromatic hydrocarbons, which may induce a light response with both a lesser magnitude and a greater time lag than either naphthalene or salicylate (24). In addition, the bioluminescence response coincided with the presence of naphthalene in both the JP-4-saturated water and the MGP soil leachate. Although the naphthalene concentration in the JP-4-saturated water was higher than that in the MGP soil leachate, the rate of bioluminescence increase showed an inverse relationship. This apparent contradiction might be due to the presence of toxic compounds in the JP-4 jet fuel. Such toxic compounds could affect the overall bioluminescence response adversely. Given the absence of a zero-naphthalene control for MGP soil or JP-4, these interpretations are somewhat speculative. It is well known that a number of solvents can reduce the bioluminescence response of *Photobacterium phosphoreum* in the Microtox assay (4). In addition, the experiments conducted with toluene indicated a slight reduction in the bioluminescence baseline response. However, further studies have to be conducted to investigate bioluminescence inhibition during induction of the catabolic system.

Whole living-cell biosensors have been reported for a number of compounds (34). Systems described for environmental pollutant monitoring were based on respirometric (15, 27) or calorimetric (14, 42) measurements, thereby making use of an overall physiological response of an organism after exposure to a pollutant. The advantages of whole-cell biosensors over enzyme biosensors are high stability and the information on physiological and biological parameters that can be obtained. Disadvantages include the longer response times due to the complex biochemical processes that are involved, as well as the higher diffusion resistances of cellular membranes and immobilization matrices. In addition, whole-cell systems exhibit reduced substrate specificities compared with enzymatic biosensors (34). However, the use of genetically engineered bacteria with *lux* fusions to the regulatory elements of inducible genes or operons largely overcomes the specificity problem and provides a general approach applicable to a large number of organic and inorganic pollutants for which degradation or detoxification pathways exist (5, 6, 16, 36, 41). Numerous plasmid-encoded catabolic systems for the degradation of organic pollutants have been described (31) and provide a good basis for the construction of a diversity of bioluminescent reporter bacteria.

The presence and concentration of a pollutant can be determined with greater accuracy by chemical analytical methods rather than with a bioluminescent reporter bacterium. However, the biosensor reported here provides additional unique information on bioavailability (13) and the specific catabolic activity of a naphthalene-degrading bacterium that can also be extended to phenanthrene and anthracene degradation in a contaminated sample or the environment (23, 30). Since both of these monitoring parameters are associated with living organism, they cannot be determined by using conventional chemical analytic methods alone. For comprehensive characterization of environmental samples, several different monitoring methods should be applied simultaneously to obtain balanced information on chemical or biological parameters effecting biodegradation.

ACKNOWLEDGMENTS

We thank Doug Fielden for modification of the biosensor measurement cell and construction of the soil column, John Sanseverino for providing MGP soil, Bruce Applegate for providing the *lux* gene probe, and Oren Webb for stimulating discussions.

This work was supported by the U.S. Air Force Office of Scientific Research (contract F49620-89-C-0023) and by the Office of Health and Environmental Research Subsurface Science Program, U.S. Department of Energy (grant DE-FG05-91ER61193).

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