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Specific and Quantitative Assessment of Naphthalene and Salicylate Bioavailability by Using a Bioluminescent Catabolic Reporter Bacterium

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A bioassay was developed and standardized for the rapid, specific, and quantitative assessment of naphthalene and salicylate bioavailability by use of bioluminescence monitoring of catabolic gene expression. The bioluminescent reporter strain *Pseudomonas fluorescens* HK44, which carries a transcriptional *nahG-luxCDABE* fusion for naphthalene and salicylate catabolism, was used. The physiological state of the reporter cultures as well as the intrinsic regulatory properties of the naphthalene degradation operon must be taken into account to obtain a high specificity at low target substrate concentrations. Experiments have shown that the use of exponentially growing reporter cultures has advantages over the use of carbon-starved, resting cultures. In aqueous solutions for both substrates, naphthalene and salicylate, linear relationships between initial substrate concentration and bioluminescence response were found over concentration ranges of 1 to 2 orders of magnitude. Naphthalene could be detected at a concentration of 45 ppb. Studies conducted under defined conditions with extracts and slurries of experimentally contaminated sterile soils and identical uncontaminated soil controls demonstrated that this method can be used for specific and quantitative estimations of target pollutant presence and bioavailability in soil extracts and for specific and qualitative estimations of napthalene in soil slurries.

A major issue in predicting the microbial degradation of nonpolar organic pollutants in natural environments concerns their bioavailability to bacteria and relative degradability. In complex environments such as soils, the distribution of pollutants among the solid, liquid, and gaseous phases is dependent on both the physicochemical properties of the pollutant itself and the type of the polluted matrix and determines whether or to what extent a compound is accessible to bacterial degradation. It has been shown that the degradation of polyaromatic hydrocarbons such as naphthalene and phenanthrene is controlled by the dissolved fraction of the compound (31, 34, 35). However, a significant portion of naphthalene in a polluted soil may not be readily bioavailable because of adsorption to soil particles (1, 20). For efficient bioremediation of soils polluted with polyaromatic hydrocarbons, it is important to develop rapid, specific, and quantitative methods to estimate the bioavailability of a particular compound and treatment efficacy to promote biodegradation.

Since the degradation of many pollutants, including naphthalene, is mediated by plasmid-encoded, specific, inducible catabolic operons (24), the expression of such catabolic genes can be used as a measure of the presence, availability, and biodegradation of a particular pollutant or a group of pollutants.

Gene expression can be quantified by the direct measurement of mRNA (9, 23), by the use of different types of gene fusions (11, 13, 29), by the measurement of specific enzyme activity, or by the analysis of protein synthesis patterns by two-dimensional polyacrylamide gel electrophoresis (15, 21). The application of the *lux* genes from various bioluminescent bacterial species to gene fusions has led to the possibility of rapidly monitoring gene expression in situ and on-line (5, 8, 25, 36). Bacterial bioluminescence in several different species has been characterized both genetically (17, 18) and physiologically (10). The bioluminescence reaction is catalyzed by a heterodimeric luciferase encoded by the *luxA* and *luxB* genes. The light reaction itself is dependent on O_2, reduced FMN, 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. Two different strategies have been used for the construction of *lux* gene fusions: one involving the *luxA* and *luxB* genes and another one involving the *luxCDABE* genes. While the former system makes the exogenous addition of the aldehyde substrate necessary, the latter is independent of such an addition in a compatible host. Recently, the construction of *luxCDABE* gene fusions to monitor the expression of the catabolic naphthalene degradation operons was reported (4, 14). The genes encoding the degradation of naphthalene are organized in two operons (37). The enzymes of the upper operon (naphthalene operon) catalyze the degradation of naphthalene to salicylate, while the enzymes of the lower operon (salicylate operon) are responsible for the degradation of salicylate to 2-oxo-4-hydroxy-pentanoate (38). The expression of both operons is positively regulated by the product of the *nahR* gene in the presence of salicylate (27). Upon exposure to either naphthalene or its degradation intermediate salicylate, both *nah* operons are expressed and the bioluminescent catabolic reporter strain emits increased levels of light that can be used as a measure of gene expression.

Light response is related to naphthalene degradation and

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has been shown to be a measure of naphthalene presence and bioavailability in chemically contaminated soils (4, 14). The objectives of the current work are to quantitatively relate the light response of a bioluminescent reporter strain to the concentrations of environmental contaminants and to develop and standardize assay procedures for monitoring the presence and bioavailability of contaminants in soil slurries.

MATERIALS AND METHODS

Organism. A bioluminescent catabolic reporter strain, *Pseudomonas fluorescens* HK44, a genetically engineered environmental isolate, was used in all the experiments conducted (14). 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.

Culture conditions. The organism was grown in 300-ml Erlenmeyer flasks containing 100 ml of liquid medium. The growth temperature and pH were maintained at 27°C and 7, respectively. The growth medium used was a yeast extract-peptone-glucose (YEPG) medium containing, in grams per liter, the following: yeast extract, 0.2; polypeptone, 2.0; glucose, 1.0; and NH_4NO_3, 0.2. Furthermore, 100 ml of 0.5 M phosphate buffer (pH 7) containing a K_2HPO_4-NaH_2PO_4 mixture per liter was added to YEPG medium. For some experiments, this medium was used without the addition of glucose (YEP medium). Glucose and the phosphate buffer were sterilized separately and added sterile to the medium. Cultures were grown with 14 mg of tetracycline per liter for positive selection of the *lux* transposon.

To investigate the possible effects of various media with and without a carbon substrate on the specificity of the bioluminescence response in the assay, we used two additional media: a mineral salts medium without a carbon substrate and Luria broth (LB) medium as a rich carbon substrate source. The mineral salts medium used contained, in grams per liter, the following: $MgSO_4 \cdot 7H_2O$, 0.1; and NH_4NO_3, 0.2. Furthermore, 100 ml of the phosphate buffer solution described above and 0.1 ml of a trace element solution per liter were added. The latter contained, in grams per liter, the following: MgO, 10.0; $CaCl_2$, 2.94; $FeCl_3 \cdot 6H_2O$, 5.4; $ZnSO_4 \cdot 7H_2O$, 1.44; $CuSO_4$, 0.25; H_3BO_4, 0.062; and $Na_2MoO_4 \cdot H_2O$, 0.49. LB medium contained, in grams per liter, the following: Bacto Tryptone, 10; yeast extract, 5; and NaCl, 10. It was slightly modified by the addition of 100 ml of the phosphate buffer solution described above per liter. Dilutions of YEP and LB media were made with mineral salts medium.

Bioavailability assays. An overnight culture grown in YEPG medium was prepared from a frozen stock of *P. fluorescens* HK44. From this culture, 5 ml was transferred to 100 ml of fresh medium and the optical density at 546 nm (OD_{546}) was monitored in 30-min intervals. At a predefined OD_{546} of 0.35, aliquots of the exponentially growing culture were transferred to the test vials. For the carbon starvation experiment, 30 ml of the exponentially growing culture was harvested at the same optical density as that described above and centrifuged at 7,741 × *g* for 10 min at 25°C. The pellet was resuspended in 30 ml of mineral salts medium without a carbon substrate and kept at 27°C on a shaker for 3 h.

Two milliliters of either an exponentially growing or a starved culture was added to 2 ml of test solution in sterile 25-ml mineralization vials (Pierce, Rockford, Ill.) with Teflon lids. Salicylate and naphthalene solutions were prepared in buffered mineral salts medium. The light output of the culture was measured in 15-min intervals with an Oriel digital display (model 7070), an Oriel photomultiplier (model 77340), and a liquid light cable. The light readouts were obtained as an amperometric signal and are presented in nanoamperes.

The experiments were conducted as two independent sets of three experiments each. Data from a representative set are shown as mean values of the triplicates with standard deviations. For the salicylate and naphthalene standard curves, all the values were corrected for the light output of the control without an inducing substrate. The corresponding standard deviations were calculated by taking the error propagation into account.

Soil experiments. For the soil experiments, a loamy sand with an organic matter content of 1.15% was used. The water content of the soil was 7.5 ± 0.3%.

(i) Preparation of soil extracts. Before contaminants were added to the soils, 10-g portions of soil were autoclaved at 121°C for 20 min in 25 ml of Corex glass centrifuge tubes (Corning Inc., Corning, N.Y.) with Teflon-sealed screw caps. The contaminants, either 69 μg of salicylate in 125 μl of mineral salts medium or 83 μg of naphthalene in 3 ml of mineral salts medium, were added to the soil and left for 1 h. To these salicylate- or naphthalene-contaminated soils either 9.875 or 7 ml of buffered mineral salts medium was added, respectively. The resulting slurries were incubated at 27°C on a shaker for 1 h. Then, the solid particles were allowed to settle after centrifugation at 7,741 × *g* for 10 min at 25°C. The soil controls were processed exactly as described above but without the addition of the contaminants. From the resulting clear supernatants, 2-ml aliquots were transferred to 25-ml mineralization vials with Teflon-sealed screw caps.

(ii) Preparation of soil slurries. Mineralization vials (25 ml) containing 2 g of soil were autoclaved at 121°C for 20 min. The contaminants, either 13.8 μg of salicylate in 25 μl of mineral salts medium or 16.6 μg of naphthalene in 0.6 ml of mineral salts medium, were added to the soil. To these salicylate- or naphthalene-contaminated soils either 1.975 or 1.4 ml of buffered mineral salts medium was added, respectively. The slurries were incubated in a shaker at 27°C for 1 h. Control slurries were prepared identically but without salicylate and naphthalene.

For the experiments with mineral salts medium involving salicylate and naphthalene, standard final concentrations of 3.45 and 4.15 mg/liter were used, respectively. These concentrations also represent the theoretical maximum aqueous concentrations of the target compounds to be expected for the soil extract and soil slurry experiments, provided no adsorption to the soil matrix takes place. Light measurements were made in 15-min intervals for an experimental set comprising mineral salts medium, soil extract, and soil slurry. After 1 h, the optical densities of the mineral salts medium and soil extract were compared after correction for the A_{546} of the soil extract.

Analytical procedures. All concentrations were measured in samples to which 2 ml of H_2O had been added instead of 2 ml of cell suspension. Soil extract samples were filtered through 0.2-μm-pore-size Teflon membrane filters prior to HPLC analysis. The HPLC system consisted of a model 5560 liquid chromatograph (Varian, Palo Alto, Calif.), a 25-cm VYDAC 201TP5 column (Separations Group, Hesparia, Calif.), and an LS-4 fluorescence spectrophotometer (Perkin Elmer, Norwalk, Conn.). The fluorometer was equipped with a 3-μl flow sample cell. The gradient condi-

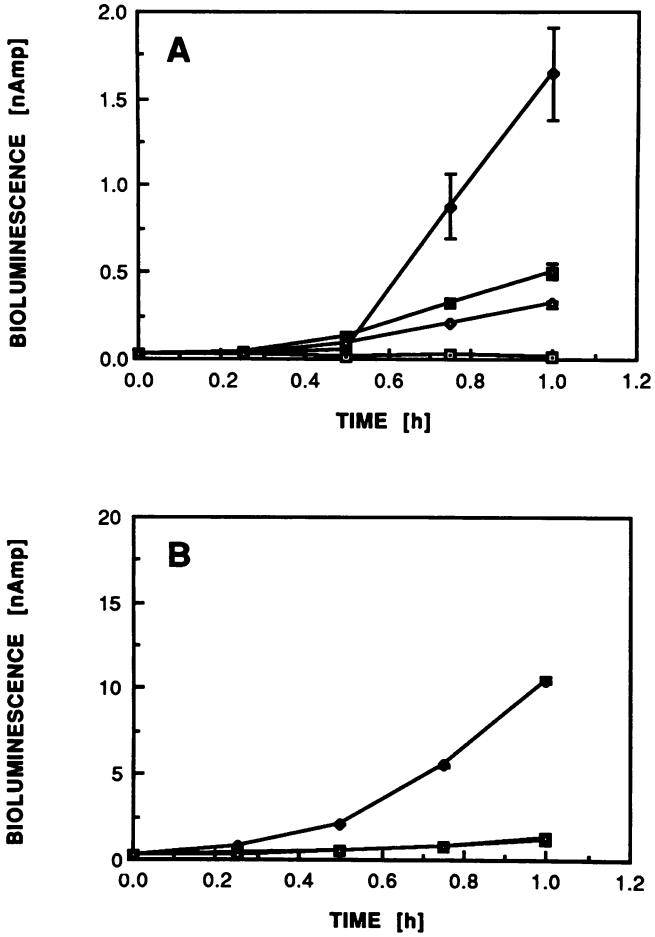

FIG. 1. Effect of the physiological state of the *P. fluorescens* HK44 culture on bioluminescence. Shown is the time course of *nahG-lux* gene expression after exposure to different inducing and noninducing substrates: ⊡, mineral salts medium without carbon substrate; ◆, 3.45 mg of salicylate per liter; ■, LB medium diluted 1:8; ◇, YEP medium diluted 1:4. (A) Culture carbon starved for 3 h in mineral salts medium. (B) Exponentially growing culture (the curves for mineral salts medium without carbon substrate, LB medium, and YEP medium are overlapping). Initial and final biomasses are presented in Table 1.

tions used involved a continuous gradient from 0 to 50% aqueous acetonitrile between minutes 1 and 2 and a second continuous gradient from 50 to 100% acetonitrile between minutes 10 and 19. At the end of the program used, the column was equilibrated for 3.5 min with H_2O. A flow rate of 1.5 ml/min was used, with sample injection occurring at 1 min in the gradient program. An excitation wavelength of 290 nm and an emission wavelength of 360 nm were used to quantify salicylate, and excitation and emission wavelengths of 272 and 330 nm were used to quantify naphthalene.

RESULTS

Exposure of carbon-starved cultures of *P. fluorescens* HK44 to noninducing carbon substrates other than salicylate resulted in a significant increase in *nahG* gene expression, measured as bioluminescence, compared with that in controls without any carbon substrate or with 3.45 mg of salicylate per liter (Fig. 1A). In contrast, exponentially grown cultures (Fig. 1B) did not exhibit such a difference. Starved cultures exposed to noninducing carbon substrates increased their biomass by about 50% during the 1-h assay period (Table 1). No growth occurred in the control without any carbon substrate or in the culture exposed to 3.45 mg of salicylate per liter as the only carbon substrate. In contrast, exponentially grown cultures did not exhibit significant differences in the final biomasses observed, indicating that the exposure to different substrates did not change the growth rate. The specific bioluminescence response, i.e., bioluminescence divided by biomass, to a given salicylate concentration was ca. 300% higher in exponentially grown cultures than in starved cultures after 1 h of incubation.

TABLE 1. Comparative final biomasses for carbon-starved and exponentially growing bioluminescent reporter strain cultures exposed to inducing and noninducing carbon substrates

Test condition	Biomass concn (OD_{546} ± SD) for the following culture[a]:	
	Starved	Growing
Mineral salts medium (control)	0.145 ± 0.002	0.359 ± 0.003
Salicylate (3.45 mg/liter)	0.145 ± 0.001	0.347 ± 0.008
LB medium, diluted 1:8	0.237 ± 0.004	0.361 ± 0.001
YEP medium, diluted 1:4	0.212 ± 0.004	0.367 ± 0.001

[a] Biomass concentrations were measured as OD_{546} 1 h after incubation. Inoculum densities at time zero were 0.145 for the carbon-starved culture and 0.175 for the exponentially growing culture.

Figure 2 illustrates the relationship between initial salicylate concentration and *nahG* gene expression, measured as an increase in bioluminescence 1 h after exposure of the exponentially grown culture to the inducing substrate. The light level of the control culture (no salicylate) was substracted from all the readings. At salicylate concentrations above 20 to 25 mg/liter, a saturation-type behavior was observed, with bioluminescence reaching a plateau at ca. 125 nA (Fig. 2A). Since the final biomass concentrations were the same for all the salicylate concentrations investigated, bioluminescence data can be directly compared and considered relative specific values. For concentrations of between 0.4 and 3.45 mg of salicylate per liter, a good linear relationship (r^2 = 0.991) between the concentration and the light response was observed (Fig. 2B).

A similar linear relationship (r^2 = 0.99) between bioluminescence and initial naphthalene concentrations of between 0.72 μg and 3.25 mg per liter was observed (Fig. 3). As with salicylate, the final biomass concentrations remained constant. Three independent experiments relating bioluminescence to naphthalene concentrations in test assays were conducted for concentrations ranging from 45 μg to 5.67 mg/liter. The linear regression coefficients varied from 0.951 to 0.993 for different concentrations. The slope of the regression lines maximally varied by 50% (Table 2) in independent experiments over similar concentrations but also depended on the concentrations used for a linear regression within the same data set. Relationships similar to those shown for salicylate and naphthalene could also be established after incubation of the reporter strain culture with the inducing substrate for as little as 15 min (data not shown). However, the differences in light output between the individual datum points for different substrate concentrations were smaller and the standard deviations were relatively larger, thereby making data separation, particularly at lower substrate concentrations, more difficult.

A comparison of the time course of the bioluminescence

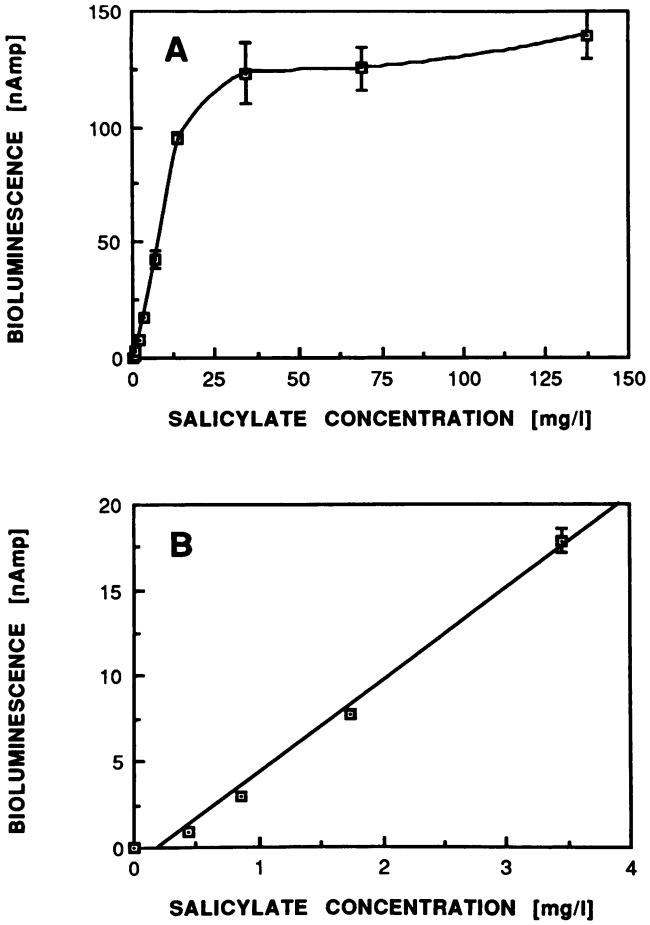

FIG. 2. Relationship between salicylate concentration and bioluminescence. Shown is *nahG-lux* gene expression in exponentially growing *P. fluorescens* HK44 1 h after incubation. Bioluminescence values are corrected for the response observed in the control (without salicylate). (A) Saturation-type behavior. (B) Linear range and regression fit. $y = -0.99524 + 5.3348x$; $r^2 = 0.991$.

response for mineral salts medium, soil extract, and soil slurry containing salicylate was conducted. The various samples were all expected to contain the same amount of salicylate per assay volume provided no adsorption to the soil matrix took place. The time courses for the light response in the various experiments are presented together with data for the corresponding controls without salicylate (Fig. 4). While the mineral salts medium (Fig. 4A) and the soil extract (Fig. 4B) exhibited similar bioluminescences, the response in the soil slurry was reduced by about 1 order of magnitude (Fig. 4C). However, the overall relative response pattern in the soil slurry was quite similar to the those observed for the mineral salts medium and the soil extract. The pHs were 7 for the mineral salts medium and between 6.8 and 6.85 for the soil extract and the soil slurry. For the mineral salts medium and the soil extract, similar final biomass levels were observed (Table 3). The light responses after 1 h of incubation for the different assay conditions, corrected for the control light output, are presented in Table 4, together with the corresponding analytically determined initial salicylate concentrations at time zero. The percentages provided are related to the light output and salicylate concentration measured in the mineral

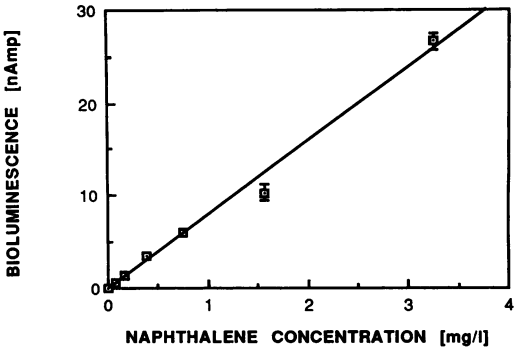

FIG. 3. Relationship between naphthalene concentration and bioluminescence. Shown is *nahG-lux* gene expression in exponentially growing *P. fluorescens* HK44 1 h after incubation. Bioluminescence values are corrected for the response observed in the control (without naphthalene). $y = -0.12125 + 7.9626x$; $r^2 = 0.990$.

salts medium. While the bioluminescence level in the soil extract sample corresponded to 111% ± 9.0% that in the control mineral salts medium, the analytically determined amount of salicylate was 84% ± 3.5% the corresponding control value.

An analogous experiment was conducted with naphthalene in mineral salts medium, soil extract, and soil slurry. Again, the various samples were expected to contain the same maximal amount of naphthalene provided no adsorption to the soil matrix took place. The bioluminescence time courses for the various experimental conditions with naphthalene are shown in Fig. 5. In contrast to the findings with salicylate, the bioluminescence response in the soil extract (Fig. 5B) corresponded to only 4.4% ± 0.2% (Table 4) that in the mineral salts medium control (Fig. 5A). This difference is also quantitatively reflected in the analytically determined initial naphthalene concentration in the soil extract, which corresponded to 3.9% ± 1.2% the amount found in the mineral salts medium control (Table 4). The biomass concentrations were the same in mineral salts medium and soil extract experiments (Table 3). The pH conditions were the same as those described above for salicylate. In the soil slurry, a reduction in the bioluminescence response of about 1 order of magnitude was found (Fig. 5C). However, the relative overall bioluminescence time course in the soil slurry was similar to that found for the soil extract.

DISCUSSION

The results presented demonstrated that catabolic gene expression with bioluminescent reporter strain *P. fluo-*

TABLE 2. Linear regressions obtained for the relationship between naphthalene concentration and bioluminescence of the reporter strain culture under defined assay conditions[a]

Expt	Concn range (mg/liter)	No. of datum points	Linear regression		
			Slope	*y* Intercept	r^2
A	0.07–0.75	5	8.246	0.041	0.992
B	0.095–0.725	5	12.902	−0.181	0.993
C	0.045–0.711	6	11.511	0.142	0.981
A	0.07–3.25	7	7.963	−0.121	0.990
C	0.045–5.66	9	8.413	1.237	0.951

[a] Bioluminescence was measured 1 h after incubation.

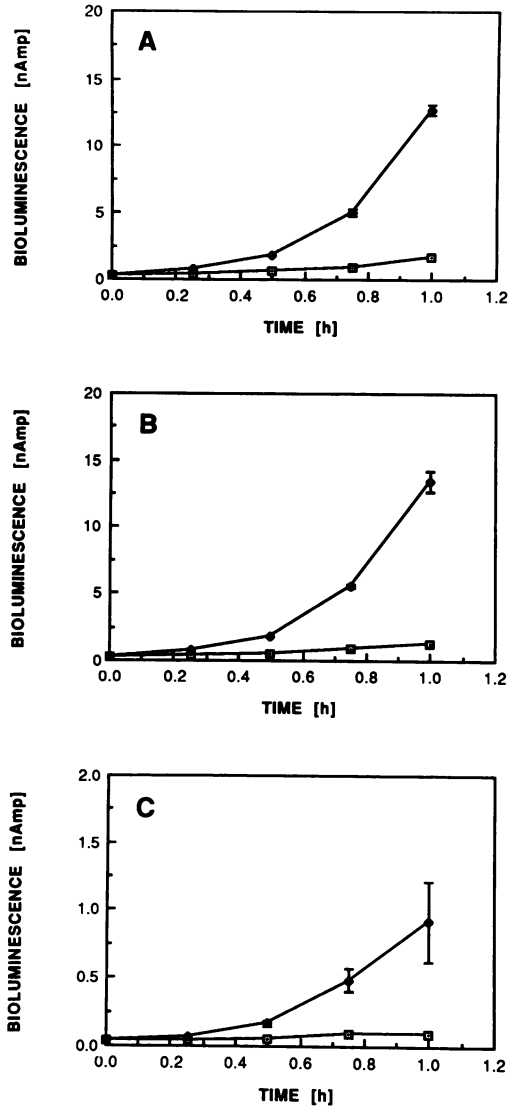

FIG. 4. Time course of salicylate-induced bioluminescence in growing *P. fluorescens* HK44. (A) Mineral salts medium with 3.45 mg of salicylate per liter. (B) Soil extract. (C) Soil slurry. In panels B and C, the soils received 6.9 mg of salicylate per kg, resulting in a theoretical maximum aqueous concentration of 3.45 mg/liter in the assay. The corresponding biomass concentrations and the analytically determined salicylate concentrations are given in Tables 3 and 4, respectively. Symbols: ◆, salicylate samples; ⊡, control samples (without salicylate).

rescens HK44, which carries a *nahG-luxCDABE* transcriptional fusion, can be used as a rapid, on-line method to quantitatively and specifically detect naphthalene and its degradation intermediate salicylate in defined and complex aqueous samples. This method provides for a potential in vivo assay for the presence, bioavailability, and biodegradability of these compounds under the conditions used.

Intrinsic regulatory characteristics of the naphthalene degradation pathway and the physiological aspects of the light reaction in the host metabolism must be considered when *lux* catabolic gene fusions are used. The experiments demonstrated that the physiological state of the test culture is a critical factor for the specificity of the bioluminescence

TABLE 3. Comparative final biomasses for salicylate- and naphthalene-exposed bioluminescent reporter strain cultures

Test condition	Biomass concn (OD_{546} ± SD) after exposure to[a]:	
	Salicylate	Naphthalene
Mineral salts medium (control)	0.353 ± 0.006	0.361 ± 0.001
Mineral salts medium (contaminated)	0.349 ± 0.006	0.359 ± 0.004
Soil extract (control)	0.363 ± 0.004	0.356 ± 0.004
Soil extract (contaminated)	0.356 ± 0.005	0.358 ± 0.002
Soil slurry (control)	ND	ND
Soil slurry (contaminated)	ND	ND

[a] Biomass concentrations were measured as OD_{546} 1 h after incubation. Inoculum densities at time zero were 0.175 for both salicylate and naphthalene experiments. ND, not determined.

response to the target substrates. While the use of resting, carbon-starved cells may have a practical advantage, under the conditions used exposure to substrates other than naphthalene and salicylate can result in significant growth of the culture and gene expression. This result can be explained by the low basal constitutive expression level observed for the naphthalene and salicylate operons on the closely related NAH7 plasmid in growing cells of *P. putida* (26) in the absence of an inducer. Such constitutive expression is probably not retained or is much lower in carbon-starved cells, as indicated by the almost unmeasurable bioluminescence in such cultures in the absence of a carbon substrate. Therefore, the low basal expression level together with the increase in biomass is responsible for the elevated bioluminescence levels observed with these substrates compared with the control. For practical purposes, such behavior may reduce the sensitivity and specificity of the assay significantly, because a response to a low target substrate concentration may not be distinguished from a bioluminescence increase caused by readily utilizable carbon substrates.

In contrast, an exponentially growing culture of *P. fluorescens* HK44 maintains a low basal constitutive expression level that is insensitive to increased bioluminescence caused by readily metabolizable carbon substrates. Furthermore, the use of a complex medium enables the cells to grow at a fast rate such that the addition of other rich substrates does not markedly affect the growth rate. The differences in bioluminescence intensity between starved and exponentially grown cultures might not only be a result of different gene expression levels but could also be an effect of the physiological cell status on the biochemical light reaction itself.

Under defined conditions, the relationship between initial substrate concentration and bioluminescence response exhibits a saturation-type behavior, as shown for salicylate. Over a limited concentration range of 1 to 2 orders of magnitude, a good linear relationship could be established for both salicylate and naphthalene. For the latter compound, a significant response could be observed to concentrations as low as 45 ppb, which does not necessarily represent the detection limit for the compound under defined conditions. These results demonstrate a satisfactory sensitivity of this reporter strain for environmental applications, in which naphthalene concentrations in the parts-per-million and parts-per-billion ranges are commonly found in contaminated samples (19, 28). However, the slope of the standard curves exhibited a certain variability in independent cell

TABLE 4. Comparison of the bioluminescence response of the reporter strain culture with initial inducing substrate concentrations

Test condition	Expt[a]			
	Salicylate		Naphthalene	
	Bioluminescence (nA ± SD)[b]	Initial salicylate concn [(mg/liter) ± SD][c]	Bioluminescence (nA ± SD)[b]	Initial naphthalene concn [(mg/liter) ± SD][c]
Mineral salts medium	11.05 ± 0.4 (100 ± 3.6)	3.45 ± 0.07 (100 ± 2.0)	55.71 ± 3.4 (100 ± 6.1)	4.15 ± 0.36 (100 ± 8.7)
Soil extract	12.28 ± 1.0 (111 ± 9.0)	2.90 ± 0.12 (84.1 ± 3.5)	2.41 ± 0.1 (4.4 ± 0.2)	0.16 ± 0.05 (3.9 ± 1.2)
Soil slurry	0.82 ± 0.29 (7.4 ± 2.6)	ND (ND)	0.27 ± 0.02 (0.48 ± 0.04)	ND (ND)

[a] Numbers in parentheses are percentages relative to the data for mineral salts medium.
[b] Measured 1 h after incubation and corrected for the response observed in the corresponding control (without salicylate or naphthalene).
[c] At time zero. ND, not determined.

preparations. For quantitation of the light response and for comparison among independent experiments, it may therefore be important to include standard samples with a known inducing substrate concentration for each test set. The studies conducted with extracts from experimentally contaminated soils showed that with this approach, at least a concentration range could be estimated for the contaminants. In the case of salicylate, the overestimation of the substrate concentration by use of the light response might be due to slight differences in the sample pH values that might affect salicylate transport into the bacterial cell or processes involved in the bioluminescence reaction itself. However, the light response describes the overall concentration pattern sufficiently for both salicylate and naphthalene. The low light response in the naphthalene-contaminated samples corresponds relatively well with the analytically determined concentration. It is well established that naphthalene strongly adsorbs to soils (2, 7), a fact that explains the large difference between the results obtained with mineral salts medium and soil extract samples for naphthalene versus salicylate. Although autoclaved soils, which have a chemical composition different from that of untreated soils, were used in these studies, the results can be considered a reasonable approximation for the application of this method to complex environmental samples. Experiments can also be conducted with soil slurries, but light quenching may make quantitation difficult unless a correction factor can be determined. However, qualitative information is achievable under these conditions if a control soil is available.

The results presented extend the various applications of whole-cell bioluminescence systems (30) to the assessment of the presence and bioavailability of specific compounds. A number of methods involving the use of whole cells in combination with, e.g., respirometric (22, 32) or calorimetric (12, 16, 33) measurements, for the quantitation of various chemical compounds have been reported. A general problem with whole-cell-based biosensors is their specificity, since the parameters used are often overall measures which cannot be related to a specific substrate in a complex mixture. However, substrate specificity is a prerequisite for application to environmental analysis, which commonly involves complex mixtures of frequently unknown composition. The bioluminescent reporter strain method presented here provides a specific analytical approach based on the genetic regulation of a specific, inducible degradation pathway. An important question with respect to environmental applications concerns the substrate range of the reporter strain. Since it has been shown that other polyaromatic hydrocarbon compounds are degraded through pathways that may converge at the level of the inducer salicylate (6), the range

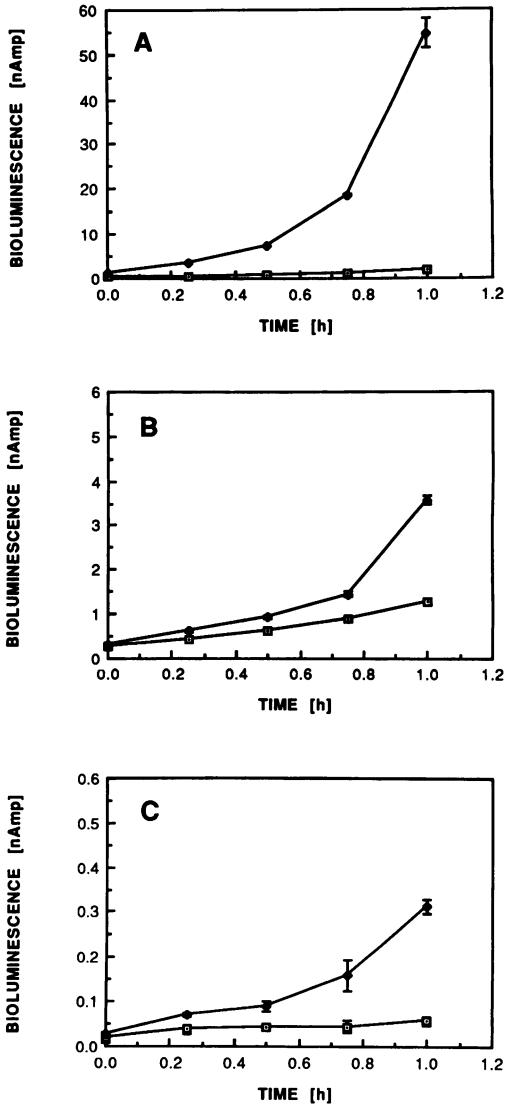

FIG. 5. Time course of naphthalene-induced bioluminescence in growing *P. fluorescens* HK44. (A) Mineral salts medium with 4.15 mg of naphthalene per liter. (B) Soil extract. (C) Soil slurry. In panels B and C, the soils received 8.3 mg of naphthalene per kg, resulting in a theoretical maximum aqueous concentration of 4.15 mg/liter in the assay. The corresponding biomass concentrations and the analytically determined naphthalene concentrations are given in Tables 3 and 4, respectively. Symbols: ◆, naphthalene samples; ⊡, control samples (without naphthalene).

of compounds to which this reporter strain responds may be extended in the future.

Not yet examined are possible effects of toxic compounds associated with polluted soils. The light response in native marine bioluminescent bacteria is affected by many toxic compounds; the fact forms the basis for commercial toxicity bioassays (3). However, the effects of such toxic compounds on the light response in *P. fluorescens* HK44 might be different from those observed in native bioluminescent bacteria. Additional studies are needed to determine the effects of environmentally relevant, potentially toxic compounds on the bioluminescence assessment of pollutant bioavailability and biodegradation. In addition, the construction and use of an isogenic strain exhibiting constitutive, noninducible bioluminescence should provide a usefull experimental control to assess such toxic compound effects on the light reaction.

The method presented describes a general approach to the determination of bioavailability in a biological system and provides a valuable tool for future monitoring, control, and optimization of bioremediation processes in complex systems such as soil slurries and wastewater.

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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. **Brusseau, M. L., R. E. Jessup, and P. S. C. Rao.** 1991. Nonequilibrium sorption of organic chemicals: elucidation of rate-limiting processes. Environ. Sci. Technol. **25:**134–142.
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, Fla.
4. **Burlage, R. S., G. S. Sayler, and F. Larimer.** 1990. Monitoring of naphthalene catabolism by bioluminescence with *nah-lux* transcriptional fusions. J. Bacteriol. **172:**4749–4757.
5. **Carmi, O. A., G. S. A. B. Stewart, S. Ulitzur, and J. Kuhn.** 1987. Use of bacterial luciferase to establish a promoter probe vehicle capable of nondestructive real-time analysis of gene expression in *Bacillus* spp. J. Bacteriol. **169:**2165–2170.
6. **Cerniglia, C. E.** 1984. Microbial transformation of aromatic hydrocarbons, p. 99–128. *In* R. M. Atlas (ed.), Petroleum microbiology. Macmillan Publishing, New York.
7. **Dzombak, D. A., and R. G. Luthy.** 1984. Estimating adsorption of polycyclic aromatic hydrocarbons on soils. Soil Sci. **137:**292–308.
8. **Engebrecht, J., M. Simon, and M. Silverman.** 1985. Measuring gene expression with light. Science **227:**1345–1347.
9. **Erickson, J. W., V. Vaughn, W. A. Walter, F. C. Neidhardt, and C. A. Gross.** 1987. Regulation of the promoters and transcripts of *rpoH*, the *Escherichia coli* heat shock regulatory gene. Genes Dev. **1:**419–432.
10. **Hastings, J. W., C. J. Potrikus, S. C. Gupta, M. Kurfurst, and J. C. Makemson.** 1985. Biochemistry and physiology of bioluminescent bacteria. Adv. Microb. Physiol. **26:**235–291.
11. **Heitzer, A., C. A. Mason, M. Snozzi, and G. Hamer.** 1990. Some effects of growth conditions on steady state and heat shock induced *htpG* gene expression in continuous cultures of *Escherichia coli*. Arch. Microbiol. **155:**7–12.
12. **Henrysson, T., and B. Mattiasson.** 1991. A dichloromethane sensitive biosensor based on immobilized *Hyphomicrobium* DM2 cells, p. 73–76. *In* H. Verachtert and W. Verstraete (ed.), International symposium on environmental biotechnology. Royal Flemish Society of Engineers, Ostend, Belgium.
13. **Jain, R. K., R. S. Burlage, and G. S. Sayler.** 1988. Methods for detecting recombinant DNA in the environment. Crit. Rev. Biotechnol. **8:**33–84.
14. **King, J. M. H., P. M. DiGrazia, B. Applegate, R. Burlage, J. Sanseverino, P. Dunbar, F. Larimer, and G. S. Sayler.** 1990. Rapid, sensitive bioluminescent reporter technology for naphthalene exposure and biodegradation. Science **249:**778–781.
15. **Matin, A.** 1990. Molecular analysis of the starvation stress in *Escherichia coli*. FEMS Microbiol. Ecol. **74:**185–196.
16. **Mattiasson, B., P. O. Larsson, and K. Mosbach.** 1977. The microbe thermistor. Nature (London) **268:**519–520.
17. **Meighen, E. A.** 1988. Enzymes and genes from the *lux* operons of bioluminescent bacteria. Annu. Rev. Microbiol. **42:**151–176.
18. **Meighen, E. A.** 1991. Molecular biology of bacterial bioluminescence. Microbiol. Rev. **55:**123–142.
19. **Middaugh, D. P., J. G. Mueller, R. L. Thomas, S. E. Lantz, M. H. Hemmer, G. T. Brooks, and P. J. Chapman.** 1991. Detoxification of pentachlorophenol and creosote contaminated groundwater by physical extraction: chemical and biological assessment. Arch. Environ. Contam. Toxicol. **21:**233–244.
20. **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.
21. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250:**4007–4021.
22. **Riedel, K., A. V. Naumov, A. M. Boronin, L. A. Golovleva, H. J. Stein, and F. Scheller.** 1991. Microbial sensors for determination of aromatics and their chloroderivatives. I. Determination of 3-chlorobenzoate using a *Pseudomonas*-containing biosensor. Appl. Microbiol. Biotechnol. **35:**559–562.
23. **Sayler, G. S., J. T. Fleming, B. Applegate, and C. Werner.** 1991. Nucleic acid extraction and analysis: detecting genes and their activity in the environment, p. 237–257. *In* E. M. Wellington and J. D. Van Elsas (ed.), Genetic interactions between microorganisms in the natural environment. Manchester Press, Manchester, United Kingdom.
24. **Sayler, G. S., S. W. Hooper, A. C. Layton, and J. M. H. King.** 1990. Catabolic plasmids of environmental and ecological significance. Microb. Ecol. **19:**1–20.
25. **Schauer, A., M. Ranes, R. Santamaria, J. Guijarro, E. Lawlor, C. Mendez, K. Chater, and R. Losick.** 1988. Visualizing gene expression in time and space in the filamentous bacterium *Streptomyces coelicolor*. Science **240:**768–772.
26. **Schell, M. A.** 1985. Transcriptional control of the *nah* and *sal* hydrocarbon-degradation operons by the *nahR* gene product. Gene **36:**301–309.
27. **Schell, M. A.** 1990. Regulation of the naphthalene degradation genes of plasmid NAH7: example of a generalized positive control system in *Pseudomonas* and related bacteria, p. 165–176. *In* S. Silver, A. M. Chakrabarty, B. Iglewski, S. Kaplan (ed.), *Pseudomonas*: biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.
28. **Shiaris, M. P., and D. Jambard-Sweet.** 1986. Polycyclic aromatic hydrocarbons in surficial sediments of Boston Harbour, Massachusetts, USA. Mar. Pollut. Bull. **17:**469–472.
29. **Silhavy, T. J., and J. R. Beckwith.** 1985. Uses of *lac* fusions for the study of biological problems. Microbiol. Rev. **49:**398–418.
30. **Stewart, G. S. A. B.** 1990. *In vivo* bioluminescence: new potentials for microbiology. Lett. Appl. Microbiol. **10:**1–8.
31. **Stucki, G., and M. Alexander.** 1987. Role of dissolution rate and solubility in biodegradation of aromatic compounds. Appl. Environ. Microbiol. **53:**292–297.
32. **Suzuki, S., and I. Karube.** 1979. Microbial electrodes for fermentation processes. Ann. N.Y. Acad. Sci. **326:**255–269.
33. **Thavarungkul, P., H. Hakanson, and B. Mattiasson.** 1991. Comparative study of cell-based biosensors using *Pseudomonas*

cepacia for monitoring aromatic compounds. Anal. Chim. Acta **249:**17–23.

34. **Wodzinski, R. S., and D. Bertolini.** 1972. Physical state in which naphthalene and bibenzyl are utilized by bacteria. Appl. Microbiol. **23:**1077–1081.
35. **Wodzinski, R. S., and J. E. Coyle.** 1974. Physical state of phenanthrene for utilization by bacteria. Appl. Microbiol. **27:** 1081–1084.
36. **Wolk, C. P., Y. Cai, and J.-M. Panoff.** 1991. Use of a transposon with luciferase as a reporter to identify environmentally responsive genes in a cyanobacterium. Proc. Natl. Acad. Sci. USA **88:**5355–5359.
37. **Yen, K. M., and I. C. Gunsalus.** 1982. Plasmid gene organization: naphthalene/salicylate oxidation. Proc. Natl. Acad. Sci. USA **79:**874–878.
38. **Yen, K. M., and C. M. Serdar.** 1988. Genetics of naphthalene catabolism in pseudomonads. Crit. Rev. Microbiol. **15:**247–268.