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Physiological considerations of environmental applications of *lux* reporter fusions

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Abstract

The performance of the bioluminescent reporter bacterium *Pseudomonas fluorescens* HK44, which contains a *nahG-luxCDABE* fusion for naphthalene catabolism and bioavailability was investigated for environmental samples and mixed contaminants. For aqueous extracts from fuel hydrocarbon-contaminated soils, a reproducible bioluminescence response was obtained, which coincided with, but overestimated the presence of naphthalene. Therefore, the strain's bioluminescence response to mixed contaminants was investigated further. The strain showed a linear correlation between bioluminescence and the amount of JP-4 jet fuel present in an aqueous solution, representing a mixture of compounds including naphthalene. However, some non-inducing organic solvents such as toluene, *p*-xylene and acetone caused a significant bioluminescence increase as well. The analysis of *nah-lux* mRNA from cells exposed to toluene revealed that the bioluminescence response was not due to increased *nahG-luxCDABE* gene expression, whereas increased *lux* mRNA levels were found with exposure to naphthalene or JP-4 jet fuel. While different mixture combinations of solvents resulted in either additive or intermediate effects, the combination of naphthalene and solvent resulted in a synergistic effect on the bioluminescence response. The addition of *n*-decanal, a substrate for luciferase, showed that the cells were aldehyde-limited. If aldehyde was added, only the presence of naphthalene caused a significantly increased bioluminescence response over the control. The solvent effects were dependent on the physiological status of the reporter culture and were present in growing, but not in resting cell cultures. It was postulated that the increase in bioluminescence after exposure to solvents was due to changed fatty acid synthesis patterns affecting the aldehyde supply for the bioluminescence reaction. Exposure to toluene resulted in altered membrane fatty acid composition and release of fatty acids from the cells. Exposure to *n*-alkanes resulted in minor changes in the bioluminescence response, whereas, exposure to heavy metals or cyanide resulted in significant reductions in the overall bioluminescence. These results demonstrate the utility of bioluminescent reporter bacteria for environmental applications, as well as the need for adequate experimental controls in interpreting environmental sensing data.

Keywords: Bioluminescence; *Pseudomonas fluorescens*; Naphthalene

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1. Introduction

Bioluminescent (*lux*) transcriptional gene fusions have been used to develop light emitting reporter

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bacterial strains to sense the presence, bioavailability, and biodegradation of organic chemical pollutants such as naphthalene (Burlage et al., 1990, 1992; King et al., 1990; Heitzer et al., 1992), toluene (Applegate et al., 1997) and isopropylbenzene (Selifonova and Eaton, 1996). In general, the *lux* reporter genes are placed under regulatory control of inducible catabolic operons maintained in native or vector plasmids in the host strain. Analogous approaches have also been reported for inducible heavy metal detoxification and resistance systems including mercury (Taylor et al., 1992; Selifonova et al., 1993) and the heat shock (Van Dyk et al., 1994, 1995) and oxidative stress responses (Belkin et al., 1996). Implicit in the environmental use of such reporter strains is that the bioluminescent signal generated is directly related to the environmental inducer, most desirably in a quantitative manner.

A *lux* reporter strain, *Pseudomonas fluorescens* HK44 was used to detect fuel hydrocarbon contamination in shallow subsurface coral soil from the US Army Kwajalein Atoll Base (Siegrist et al., 1991). *P. fluorescens* HK44 which contains a *nahG-lux* fusion was found to function as a biosensor qualitatively detecting fuel hydrocarbons in the same two soil samples among 12 contaminated and uncontaminated samples examined. In applying whole cell *lux* biosensor organisms in environmental analysis such as this, a positive light response is indicative of a relatively specific genetic induction of the transcriptional *lux* gene fusion. Hence, light becomes a measure of specific chemical presence, its bioavailability, and by extension, a measure of the permissiveness of the environmental matrix to support biodegradation. However, the fidelity of the light response as a quantitative measure of specific chemical concentration in environmental samples may be open to interpretation because of uncertainties due to the complexity of the light reaction mechanisms, possible toxicants, and related but perhaps unknown non-specific inducers.

P. fluorescens HK44 harbors the reporter plasmid pUTK21 which contains a Tn*4431 lux*-transposon (Shaw et al., 1988) insertion in the *nahG* (salicylate hydroxylase) gene of the lower naphthalene degradation operon (Menn et al., 1993). The biochemical and molecular reactions involved in bioluminescence production are shown in Fig. 1. Exposure to either naphthalene or salicylate resulted in increased naphthalene gene expression and therefore, increased bioluminescence. In continuous cultures of *P. fluorescens* HK44, the magnitude of the bioluminescence response was correlated with the aqueous phase concentration of naphthalene under dynamic perturbation conditions (King et al., 1990). In addition, a linear relationship between bioluminescence response and pollutant concentration was found for both naphthalene and salicylate over a concentration range of two orders of magnitude (Heitzer et al., 1992). Reproducible bioluminescence responses were also observed in soil slurry samples which were experimentally contaminated with naphthalene as well as complex soil leachates and the water soluble jet fuel components (Heitzer et al., 1994).

Since these observations were more qualitative in nature, the first objective was to examine the light response of *P. fluorescens* HK44 to better define the quantitative response to mixed chemical contaminants in environmental samples. The second objective was to evaluate the influence of contaminant mixture components on cell physiology and the bioluminescent response. The results of these studies demonstrated that pollutant sensing in real contaminated samples is quantifiable. In addition, the net bioluminescent response is intrinsically limited by aldehyde substrate (fatty acid precursor) concentrations in the cell and is further influenced by solvents affecting fatty acid pools. These effects are documented by comparison of the bioluminescent response with *lux* mRNA and fatty acid synthesis patterns. These data point to factors which would need to be controlled or standardized when employing whole cell *lux* bioreporters for chemical sensing in environmental samples.

2. Materials and methods

2.1. Bacterium

Pseudomonas fluorescens HK44, a genetically engineered, bioluminescent catabolic reporter bacterium (King et al., 1990), was used in all the experiments. This strain carries a *nah-lux* reporter plasmid, pUTK21, which contains a transcriptional

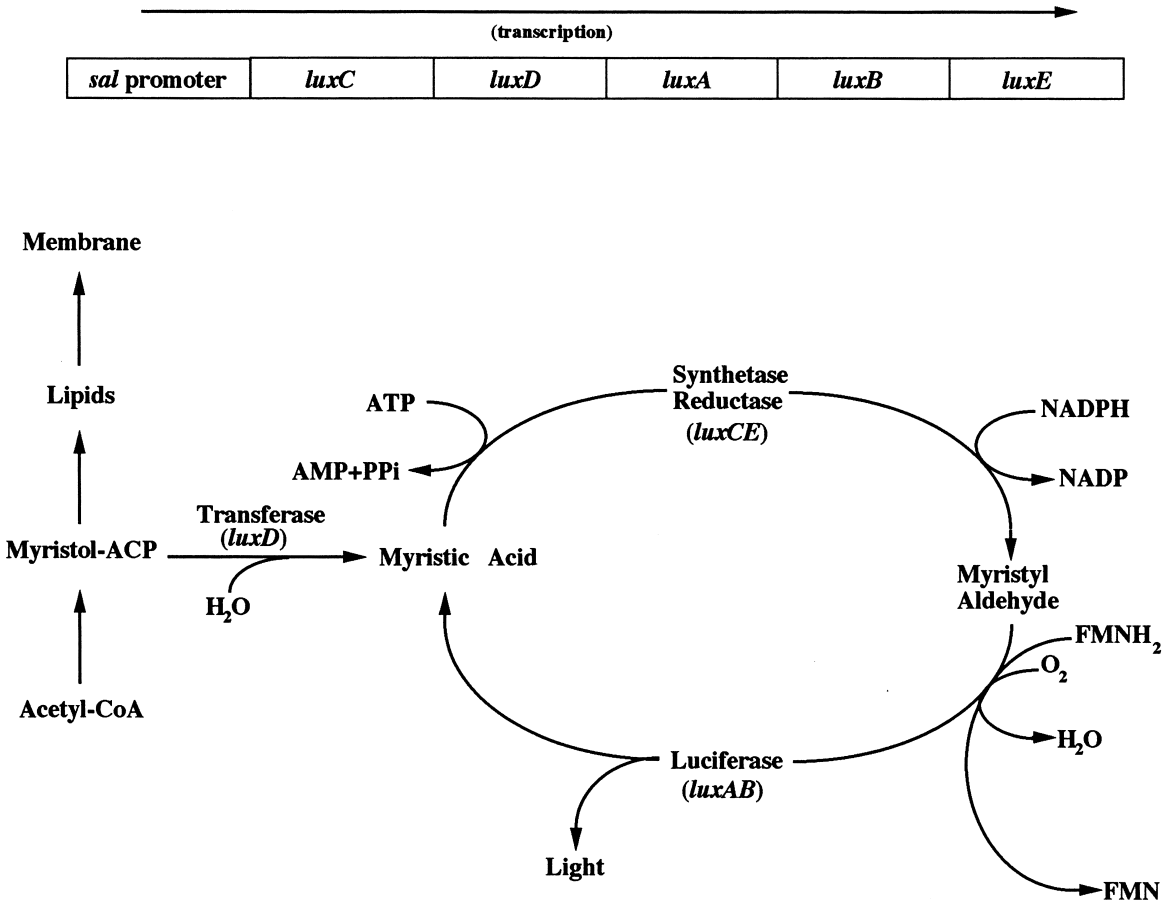

Fig. 1. Diagram of biochemical and molecular reactions involved in the bioluminescence response.

gene fusion between a *luxCDABE* gene cassette from *Vibrio fischeri* (Shaw et al., 1988) and the *nahG* gene (salicylate hydroxylase) of the salicylate operon (Menn et al., 1993). *P. fluorescens* HK44 produces light after exposure to salicylate or naphthalene and is capable of degrading both compounds.

2.2. Test sample preparation

Petroleum hydrocarbon-contaminated soil samples consisted of coral sand and were from cores taken at the US Army base at Kwajalein Atoll. Soil A was a control soil from the top of the core while soil B was contaminated with petroleum hydrocarbons. Soil extracts were prepared by mixing 10 g of soil and 10 ml dH_2O in 25-ml Corex glass centrifuge tubes with teflon-lined screw caps and shaking at 27°C for 1 h. The soil phase was separated by centrifugation at 7311×*g* for 10 min at 27°C and 2-ml aliquots of the aqueous phases were transferred to the test vials for assays.

Aqueous solutions saturated with JP-4 jet fuel, toluene, *p*-xylene or *n*-alkanes were prepared by adding 1 ml solvent to 20 ml sterile water in a 25-ml mineralization vial with a teflon-lined screw cap and mixing for 24 h on a rotary shaker. After phase separation, aliquots of the aqueous phase were transferred to test vials. A 1% (v/v) acetone solution was prepared in sterile water. Dilutions were prepared with water to give the following concentrations (based on water solubilities) for the test samples, in mg/l: 128, toluene; 1960, acetone; 44, *p*-xylene. For *n*-alkanes, the saturated solution was used without dilution. For JP-4, the saturated solution and dilu-

tions of 0.5, 0.25, 0.125 and 0.0625 were used. A saturated naphthalene solution was prepared by adding naphthalene crystals to sterile water and equilibrating with stirring for 24 h. Different naphthalene concentrations for a standard curve were obtained by preparing appropriate dilutions in dH_2O. Stock heavy metal and cyanide solutions of the following concentrations in mg/l were prepared in water: $CdCl_2$, 366; $HgCl_2$, 542; KCN, 172.

2.3. Bioluminescent sensing

The assays were conducted as described earlier (Heitzer et al., 1992): a culture from a frozen stock of *P. fluorescens* HK44 was prepared in 300-ml Erlenmeyer flasks containing 100 ml YEPG medium (Sayler et al., 1979) with 14 mg/l tetracycline. After 20–24 h, a subculture was prepared and at an optical density at 546 nm (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 with shaking except for the Kwajalein Atoll samples which were conducted at 27°C. Two experimental tests (I and II) were replicated in triplicate ($n=6$) for each soil and the bioluminescent response was compared to separate standard curves of bioluminescence over a range of naphthalene concentrations in water. The bioluminescent responses were compared to the standard curves and then compared to analytically determined naphthalene concentrations. Bioluminescence was monitored after an incubation time of 1 h unless otherwise indicated. For resting cell cultures, cells were harvested at an OD_{546} of 0.35 and resuspended in mineral salts medium (Stanier et al., 1966) without a carbon source. Cells were kept on a shaker for 3 h before adding in a 1:1 ratio with the test sample.

2.4. 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 and a digital display (models 77340 and 7070, Oriel, Stratford, CT). Since *P. fluorescens* HK44 contains a complete *luxCDABE* gene cassette, the strain produces and recycles the fatty aldehyde substrate for the light reaction. It is possible that the fatty aldehyde may be rate limiting. Consequently, aldehyde (*n*-decanal) can be added to saturate the light system for control purposes. The effect of *n*-decanal was investigated by adding 160 μl of a 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. The cell density was determined and used to normalize the bioluminescence values.

2.5. Naphthalene quantitation

Naphthalene concentrations were determined by high performance liquid chromatography (HPLC). Samples were injected manually with a glass syringe through a 0.2 μm pore-size metal frit for HPLC analysis. A model 5560 liquid chromatograph (Varian, Palo Alto, CA) in combination with an LS-4 fluorescence spectrophotometer (Perkin Elmer, Norwalk, CT) was used. The fluorometer was equipped with a 3 μl flow cell. A guard column (VYDAC 201TPB5, Separations Group, Hesparia, CA) was installed upstream of a 25 cm C_{18} reverse phase separation column (VYDAC 201TP54). Separation was achieved by the following gradient program: 100% H_2O from 0 to 1 min; continuous H_2O:acetonitrile gradient from 100:0 to 50:50 between min 1 and 2; 50:50 H_2O:acetonitrile for 8 min; ramp to 100% acetonitrile between min 10 and 19. At the end of the program 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. The excitation and emission wavelengths were 272 and 330 nm, respectively. Concentrations were determined by peak integration (HP3396A Integrator, Hewlett Packard, Avondale, PA) using a standard curve prepared from known amounts of naphthalene in acetonitrile.

2.6. Membrane lipid fatty acid analysis

Membrane lipid fatty acids were extracted and analyzed as described by Guckert et al. (1991) with

the same nomenclature. Three ml of a culture (OD_{546}=0.32) were centrifuged at 7311×*g* for 5 min at 4°C and washed once with 50 mM K_2HPO_4, pH 7.4. The cell pellets were kept frozen at −70°C until further processing. Released fatty acids were analyzed by extracting the supernatant of the cultures as previously described (Guckert et al., 1991). Samples were the same as above with a negative control consisting of only media.

2.7. mRNA analysis

After measuring bioluminescence at 1 h, the 4-ml culture contents of the test assay vials were frozen immediately by transferring them into pre-cooled centrifuge tubes on dry ice and ethanol. The samples were kept frozen at −70°C until further processing. RNA extraction was conducted as described by Oelmüller et al. (1990). For slot hybridization, RNA in concentrations of 10, 2.5, 0.5 and 0.25 μg estimated by UV absorbance (260 nm) measurements were loaded on nylon membranes (Biotrans+; ICN Biomedicals, Irvine, CA) using a Biodot SF apparatus (Bio-Rad, Melville, NY) and processed as described in Sambrook et al. (1989). A control filter membrane was prepared with samples that were treated with DNase-free RNase (Boehringer Mannheim, Indianapolis, IN). DNA standards (0.03 to 3 ng of *luxAB* dsDNA) were also loaded onto membranes and treated as the samples.

All the filter membranes were prehybridized for at least 4 h at 42°C in a solution containing 50% formamide, 5×SSPE (in g/l: 43.83 NaCl, 6.9 $NaH_2PO_4 \cdot H_2O$, 1.85 EDTA, pH 7.4), 5×Denhardt's reagent (in g/l: 1.0 Ficoll, 1.0 polyvinylpyrrolidone, 1.0 bovine serum albumin), 0.1% SDS and 0.1 mg/ml herring sperm DNA.

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 nucleotide 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 (5 ng/ml), 20 μl dNTP labeling mixture (Genius, Boehringer Mannheim), 10 μl reaction mix (10x) (Geneamp Perkin Elmer, Norwalk, CT), 1 μl Taq DNA polymerase and 66.5 μl H_2O. Thermocycler conditions for the 38 cycles were: 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C.

The filters were washed and the color was developed according to the manufacturer's protocol (Genius, Boehringer Mannheim). 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 imaging system and a Whole Band analysis software package (Millipore, Bedford, MA). 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 amounts of RNA loaded onto the filter membranes in order to obtain specific *lux*-mRNA levels for comparison. The data reported were obtained from three independent and prepared filter membranes and represent averages. Data were normalized to control values and expressed as relative *lux* mRNA levels.

3. Results

3.1. Contaminated soils

Strain HK44 was used to rapidly screen for fuel hydrocarbon presence and bioavailability in coral sand materials obtained from contaminated soils at the US Army base at Kwajalein Atoll. Two soils were initially examined based on preliminary data indicating no contamination (soil A) and high contamination (soil B). For all the samples investigated, the final HK44 biomass concentrations were identical and remained unaffected by the contaminants. Duplicate experiments were performed in triplicate and light response was calibrated by regression lines obtained for each separate standard curve: set I: $y=12.8436x+1.418$ with $r^2=0.992$ for 11 concentration samples over a range from 0 to 0.725 mg/l naphthalene and for set II: $y=11.5327x+1.8063$ with $r^2=0.983$ for 13 samples over a naphthalene concentration range from 0 to 0.711 mg/l. The ranges for the estimated naphthalene concentration were calculated using the intersection points of the upper and lower standard deviation limits of the measured

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

Replicate[a]	Soil[b]	Bioluminescence (nAmp)[c]	Estimated naphthalene concentration and range[d] (mg/l)	Analytically determined naphthalene concentration (mg/l)
I	A	1.75±0.12	0.026 (−0.003≤x≤0.052)	0.0
	B	2.75±0.08	0.104 (0.081≤x≤0.126)	0.015±0.009
II	A	1.79±0.08	0.001 (−0.015≤x≤0.028)	0.0
	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.
[b]Soil A was a low or uncontaminated surface coral soil, soil B was presumed to be a contaminated subsurface coral soil.
[c]Values are averages±standard deviations (S.D.).
[d]For each replicate a standard curve for the relationship between naphthalene concentration and bioluminescence was prepared by linear regression: replicate I: $y=12.8436x+1.418$; $r^2=0.9920$; $n=11$; naphthalene concentration range: 0–0.725 mg/l. Replicate II: $y=11.5327x+1.8063$; $r^2=0.9830$; $n=13$; naphthalene concentration range: 0–0.711 mg/l. The estimated concentrations were determined based on these equations. 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+S.D.) and lower (nAmp−S.D.) bioluminescence limits of the triplicate samples.

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 aqueous concentrations were close to zero, while for soil B the mean estimated concentrations were ~90 μg/l. Qualitatively the bioluminescent response reflected the aqueous phase naphthalene concentration as analytically determined by HPLC (Table 1). However, for the contaminated soils, the average aqueous naphthalene concentration was overestimated by a factor of five compared to HPLC analysis. This result may be due in part to the linear range for the bioluminescent response being quantitatively reproducible to a lower limit of 45 μg/l (Heitzer et al., 1992), which is threefold higher than the analytically determined aqueous concentration. A second factor affecting the bioluminescent response was the unknown effects of mixed contaminants on the function of the bioreporter strain.

3.2. Response to alternative contaminants and mixtures

To further evaluate the potential factors influencing bioluminescence, experiments were conducted with solvents, hydrocarbons and inorganics to examine the synergistic or antagonistic factors that may affect sensing in contaminated mixtures. The results are summarized in Table 2. Toluene (64 mg/l), *p*-xylene (22 mg/l) and acetone (980 mg/l) caused a significant bioluminescence increase as compared to a control (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 all cases, while significantly greater than the control, these light increases were less than 50% of that observed for naphthalene-induced bioluminescence (at 3.9 mg/l naphthalene). In contrast, the bioluminescence values after addition of *n*-decanal were not significantly different from each other for all these mixtures and were not significantly greater than the negative control. The only treatment which showed an increase in bioluminescence relative to the control after *n*-decanal addition was the treatment with naphthalene added. There was approximately a fourfold increase in light for this treatment. Cells for all treatments were aldehyde-limited since the addition of aldehyde increased the bioluminescence response 1–2 orders of magnitude (data not shown). Since no increase in relative

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

Compound[a]	Fold increase in relative bioluminescence ($x \pm$S.D.)	
	No *n*-decanal	With *n*-decanal
H_2O (neg. control)	1 ± 0.03[b]*	1 ± 0.00[c]
Naphthalene (pos. control)	27.7 ± 0.94[b]*	3.71 ± 0.20[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: naphthalene, 3.9 mg/l: 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]$n=4$. Values are relative to the negative control and are averages±standard deviations.
[c]$n=2$. Values are relative to the negative control and are averages±standard deviations. n.d., not determined.
*Indicates significantly greater than the control for $\alpha=0.05$, four degrees of freedom.

bioluminescence was observed for solvent treatments with added aldehyde, it suggested that solvents might influence the light response by affecting the aldehyde substrate level rather than the luciferase level. For 50% saturated aqueous solutions of *n*-decane, *n*-undecane and *n*-dodecane, only minor increases in the bioluminescence responses were found. The presence of either $CdCl_2$ (183 mg/l), $HgCl_2$ (271 mg/l) or KCN (86 mg/l) resulted in significantly decreased bioluminescence levels compared to the control.

It has been shown that HK44 responds qualitatively to fuel hydrocarbon mixtures in JP-4 jet fuel (Heitzer et al., 1994). This response was quantified by relating bioluminescence to a standard curve of dilutions of a JP-4 saturated water solution (Fig. 2). The dilution factors used refer to an aqueous solution, saturated with JP-4 constituents. Good linearity was found for the dilution range 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 $r^2=0.967$. For all samples investigated, the final biomass concentrations were identical and remained unaffected by the amount of JP-4 constituents.

Induction of bioluminescent response and *lux*-mRNA levels were compared for growing cultures of HK44 following a 30-min exposure to water (control), toluene (64 mg/l), naphthalene (3.9 mg/l) and an eightfold dilution of JP-4 saturated water (Table 3). A maximum light production (in the absence of *n*-decanal) was produced by naphthalene exposure, a 13.1-fold increase over the control. Significant increases in bioluminescence were observed for JP-4 constituents and toluene, 7.1-fold and 9.1-fold, re-

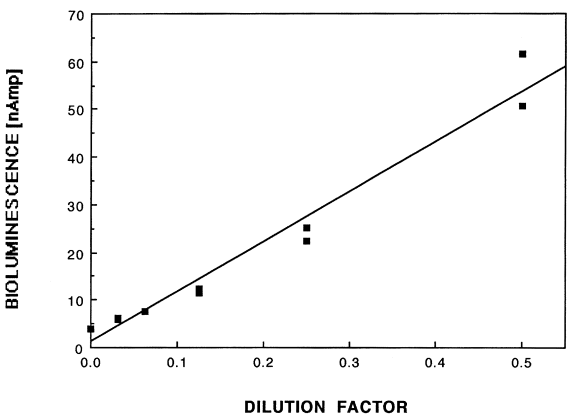

Fig. 2. Relationship between the amount of JP-4 jet fuel and the bioluminescence response of growing cultures of *P. fluorescens* HK44 after 1 h incubation. The amount of JP-4 is expressed as a factor of an aqueous solution, saturated with JP-4. $y=104.83x+1.2656$; $r^2=0.967$.

Table 3
Induction of the *nah-lux* reporter *P. fluorescens* HK44 after exposure (30 min) to different compounds as measured by bioluminescence and *lux* mRNA transcript level

Sample[a]	Relative *nah-lux* induction		
	Relative bioluminescence[b]		Relative mRNA transcript level[c]
	Without *n*-decanal	With *n*-decanal	*lux* mRNA
Control	1.00±0.04	1.00±0.06	1.00±0.22
Toluene	9.1±0.44*	0.79±0.13	0.99±0.68
Naphthalene	13.1±1.9*	3.4±0.5*	4.2±1.1*
JP-4	7.14±0.97*	1.26±0.03*	2.04±0.28*

[a]The control was water. Initial concentrations were 3.9 mg/l for naphthalene and 64 mg/l for toluene. An eightfold dilution of JP-4 saturated water was used.
[b]Bioluminescence values are relative to the control (divided by the control level). Values are averages±standard deviations.
[c]mRNA transcript levels are relative to the control (divided by the control level). Values are averages±standard deviations.
*Indicates significantly greater than the control for $\alpha=0.05$, four degrees of freedom.

spectively. In the presence of *n*-decanal there was no observable influence of toluene on bioluminescence compared to the controls, while naphthalene and JP-4 demonstrated a 3.44- and 1.26-fold increase in light production, respectively. These results indicate that the increase in bioluminescence upon toluene exposure was not due to induction. This interpretation was verified by mRNA data that showed no induction of *lux* mRNA in the toluene-exposed samples, while the naphthalene- and JP-4-exposed samples demonstrated a 4.2- and 2.0-fold increase in *lux* mRNA, respectively, as compared to the control (Table 3).

Since toluene did not induce the *nah-lux* transcriptional fusion, the influence of toluene on potential membrane perturbation and fatty acid biosynthesis was examined. The membrane fatty acid ratios for the unsaturated C_{16} *trans* to *cis* (I), the unsaturated C_{18} *trans* to *cis* (II) and the C_{17} cyclopropyl to unsaturated C_{16} *cis* fatty acids (III) for cells exposed to the three substrates are shown in Fig. 3. Exposure to toluene resulted in a pronounced increase in all three *trans* fatty acid ratios ranging from 7.5 C_{16} *trans* to 2.5 for cyclopropyl. Naphthalene caused a smaller and less significant increase for the ratios I and II (i.e. $\leq$2.5). The response observed after exposure to JP-4 constituents indicated an insignificant increase for all three ratios. Exposure to toluene also resulted in the release of C_{14}, C_{16}, and C_{18} molecular weight fatty acids into the supernatant (data not shown). These low molecular weight fatty acids were not detected in the negative control or the non-exposed cultures.

3.3. Physiological growth state effects

The effect of the physiological growth state of HK44 on the bioluminescence response after exposure to toluene (64 mg/l) and naphthalene (3.9 mg/l), either individually or as a mixture, is presented in Table 4. Bioluminescence values were normalized to optical density, since the final biomass levels were different for growing and resting cultures. The exposure of a growing culture to either toluene or naphthalene resulted in a 7.4-fold and 27.7-fold increase in bioluminescence, respectively, compared to the control. A mixture of naphthalene and toluene resulted in a bioluminescence level

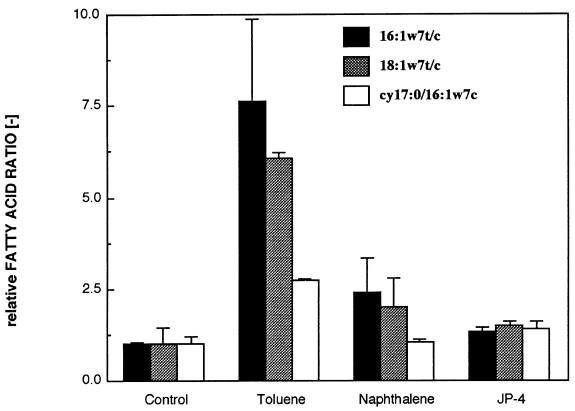

Fig. 3. Fatty acid ratios for 16:1ω7t/c (black bars), 18:1ω7t/c (grey bars) and cy17:0/16:1ω7c (open bars) after 1 h exposure. The toluene and naphthalene concentrations used were 64 mg/l and 3.9 mg/l, respectively, and the JP-4 jet fuel solution was an eightfold dilution of a saturated aqueous solution.

Table 4
Specific bioluminescence of *P. fluorescens* HK44 as a function of physiological growth state for different compounds (1 h exposure)

Physiological state	*n*-Decanal (+or−)	Specific bioluminescence (nAmp/OD)[a] Compounds[b]			
		Control	Toluene	Naphthalene	Naphthalene/toluene
Growing cells	−	15.1±0.2	110.6±2.3	414.9±13.1	1551.9±82.6
	+	1290.4±61.9	1242.2±251.1	4784.9±107.2	4860.7±0.0
	(Fold increase with decanal)	(86)	(11)	(11)	(3)
Resting cells	−	0.13±0.0	0.14±0.02	76.9±5.9	44.8±5.9
	+	100.9±8.1	110.7±3.1	826.1±1.5	644.7±0.7
	(Fold increase with decanal)	(776)	(792)	(11)	(14)

[a]Values are averages and standard deviations of three replicates.
[b]The control was water and the toluene and naphthalene concentrations used were 64 mg/l and 3.9 mg/l, respectively.

which was 100-fold greater than the control and also greater than the sum of the individual responses to both toluene and naphthalene. In contrast, the addition of aldehyde to growing cultures resulted in a different response pattern. The bioluminescence levels of both the control and toluene treatments were identical, while the level for naphthalene and naphthalene plus toluene were identical at ~3.7 times that of the control. The carbon-starved, resting culture exposure to toluene showed no bioluminescence, similar to the control. Naphthalene and naphthalene plus toluene produced a significant response in the absence of *n*-decanal for resting cultures. The addition of *n*-decanal to resting cultures resulted in an analogous bioluminescence response pattern as that seen for growing cells with *n*-decanal addition. In general, the bioluminescence responses found for the growing cultures were about an order of magnitude higher than that for the resting cultures. In addition, the bioluminescence levels increased by about one order of magnitude after the addition of *n*-decanal for both cultures.

4. Discussion

The performance of the bioluminescent catabolic reporter bacterium, *P. fluorescens* HK44, for naphthalene bioavailability and catabolic activity was investigated under environmentally relevant pollutant conditions demonstrating semiqualitative specificity for contaminated soils. Since refined petroleum products such as gasoline, diesel and jet fuels are complex mixtures containing a large number of aliphatic and aromatic hydrocarbons, with the naphthalene fractions in the range of 0.1 to 0.5 weight percent (Riser-Roberts, 1992), it was of interest to investigate the relationship between the total amount of naphthalene and bioluminescence. In addition, it was necessary to obtain an understanding of how other compounds present in these hydrocarbon mixtures might affect the performance of the reporter bacteria. In studies conducted with aqueous samples, HK44 exposed to water-soluble JP-4 jet fuel components demonstrated a good linear correlation between the relative amount of pollutant and the magnitude of the bioluminescence response. Similar observations were made earlier with aqueous solutions containing various amounts of naphthalene (Heitzer et al., 1992). However, when the bioluminescent response was related to the estimated concentration of naphthalene in the JP-4 saturated aqueous solution (Smith et al., 1981), the bioluminescent response averaged ~30-fold greater than if exposed to these naphthalene concentrations alone. These results suggested that other aqueous components may induce the *nah-lux* fusion or there was some other factor causing increased bioluminescence.

Further detailed analysis of various non-inducing individual solvents and simple solvent mixtures revealed that exposure of HK44 to compounds such as toluene, *p*-xylene, and acetone resulted in a significant bioluminescence response above background. Without further study one might have concluded that non-specific induction of the bioluminescence operon was occurring. Increased bioluminescence resulting from solvent exposure was

reported previously by White and Dundas (1970), examining the effect of anesthetics on light output from *Photobacterium phosphoreum*. Although their system was designed to look at the reduction of bioluminescence (i.e. toxic effects), they observed an increased light response with low concentrations of ethyl ether. More recently, Van Dyk et al. (1995) saw a synergistic effect using solvents (ethanol and pentachlorophenol) with heat shock *lux* fusions, which they attributed to increased transcription. However, there was no mRNA or aldehyde addition data to support their findings. Exposure to high concentrations of heavy metals and cyanide similar to levels found in manufactured gas plant soils caused a reduction of the bioluminescent response. These results show that other components which may be present in mixed contaminants can negatively effect the light response.

Although toluene had a strong effect on the bioluminescence response in growing reporter cultures, it did not increase the *lux* mRNA level. In contrast, samples containing either naphthalene alone or an eightfold dilution of JP-4 saturated water showed a significant *lux* mRNA level which correlated well with bioluminescence. When *n*-decanal was added to the test cultures, the results showed the same relative response pattern as the *lux* mRNA transcript level. Reported values for increases in *nah* mRNA levels after induction with salicylate were 15- to 30-fold (Schell, 1985). However, the salicylate concentration (500 mg/l) used for these induction studies of the *nah* system was more than two orders of magnitude higher than the amount of naphthalene used in this study. In general, the addition of *n*-decanal to either non-induced or induced cultures resulted in a significant increase in culture bioluminescence indicating an aldehyde-limited light reaction.

For environmental applications of *lux* bioreporters, both the chemical complexity of the environment and the physiological conditions of the organisms must be considered when interpreting the bioluminescent response. As indicated by Fig. 1, the biochemical reactions resulting in bioluminescence are dependent on a number of enzymatic activities, co-factors, and substrates which can be influenced by the environment or physiological status of the cell. Salicylate, the metabolic intermediate of naphthalene, induces the system by interacting with a regulatory protein encoded by the *nahR* gene (Schell, 1990), resulting in expression of the *luxCDABE* genes. Luciferase, encoded by the *luxAB* genes, catalyzes the bioluminescence reaction. This enzyme requires molecular oxygen, reduced flavin mononucleotides, and a substrate aldehyde, which is oxidized to the corresponding fatty acid with the reaction byproducts of light and water. The synthesis and recycling of the substrate aldehyde is catalyzed in an ATP- and NADPH-dependent reaction by the multienzyme fatty acid reductase complex, comprising a reductase, a transferase, and a synthetase encoded by the *luxC*, *luxD* and *luxE* genes, respectively (Meighen, 1991).

In naturally-occurring bioluminescent bacteria, the aldehyde substrate is obtained through reduction of myristic acid (Byers et al., 1987) derived from the fatty acid biosynthesis pathway (Fig. 1). Since HK44 contains the *luxC*, *luxD* and *luxE* genes in addition to the luciferase-encoding *luxAB* genes, an analogous reaction pattern can be postulated. It was reported that exposure to a number of different solvents caused significant changes in the fatty acid composition of bacterial membranes (Sikkama et al., 1995) resulting from changed synthesis patterns of fatty acids (Ingram, 1977, 1982; Pinkart et al., 1996). Consequently, the supply of fatty acid and the resulting aldehyde formation for the bioluminescence reaction could be affected, thereby providing a possible mechanistic explanation for the bioluminescence increases found after exposure to solvents. The basis for this interpretation is a low level basal expression of the transcriptional fusion under non-inducing conditions (Table 3) and intracellular pools of substrate for the light reaction affected by fatty acid synthesis.

In the studies conducted with *P. fluorescens* HK44, significantly increased levels of the *trans* to *cis* ratios of the C_{16}- and C_{18}-unsaturated fatty acids were found for toluene exposure and to a lesser extent also for the samples containing naphthalene and JP-4. In addition, low molecular weight fatty acids were released into the supernatant, which was reported to occur in studies conducted with *Escherichia coli* after exposure to toluene (DeSmet et al., 1978). It can be assumed, that such fatty acids have to be regenerated in order to maintain membrane integrity. The resulting synthesis of fatty acids

might provide increased amounts of substrate for the bioluminescence reaction (Byers et al., 1987). Based on this information, a causal relation between changes in fatty acid metabolism and increased bioluminescence after exposure of growing cultures to toluene can be postulated.

The bioluminescence response after exposure to solvents was also dependent on the physiological growth state of the reporter culture. While both growing cultures and resting cultures exhibited a significant bioluminescent response to naphthalene, only growing cultures were affected by solvents. This result is consistent with the hypothesis that the fatty acid pool is affected by the solvents causing membrane perturbation. Growing cells would be capable of significant levels of synthesis of fatty acids which could be shunted into the bioluminescence reaction. Exposure to non-inducing carbon substrates such as glucose or complex media including yeast extract and peptone caused a small but significant bioluminescence response in resting cultures as these compounds would allow the synthesis of fatty acids to be used in the bioluminescence reaction (unpublished data). Although resting cell cultures showed a higher signal-to-noise ratio (for the naphthalene treatment relative to the control) than the growing cell cultures, the bioluminescence increase upon addition of non-inducing carbon substrates precluded their use for accurate sensing of inducing substrates.

From a practical point of view, these findings provide a good basis for a specific understanding of bioluminescent reporter strains under environmental conditions. Since the bioluminescence response is proportional to the aqueous concentration of JP-4 and naphthalene, it allows a semi-quantitative analysis of samples containing these contaminants. To gain additional information, the relative bioluminescence response patterns in growing cultures before and after the addition of *n*-decanal can be used to discriminate between specific *nah-lux* gene expression and solvent effects.

Contaminated environmental systems include a number of compounds and environmental factors with unknown interactive effects on the bioluminescence response. The composition of pollutant mixtures as well as the presence of toxic compounds such as heavy metals or cyanides can significantly affect the performance of the reporter culture. Therefore, quantitative estimations of pollutant concentrations can be obtained with reporter strains only under controlled and non-inhibitory conditions. Under many environmental situations, results have to be realistically considered semi-quantitative or qualitative. This result is illustrated by these current studies conducted in aqueous extracts of soils contaminated with petroleum hydrocarbons, where a significant and reproducible bioluminescence response was demonstrated but overestimated the degree of soil contamination. However, this overall response did qualitatively coincide with the presence or absence of naphthalene. In addition, since bioluminescence is also an indicator that conditions are favorable for degradation in HK44, decreased bioluminescence due to the presence of inhibitors, such as heavy metals, can provide useful information concerning the degradability of compounds in a mixed waste sample. In this case, a standard addition of inducing substrate (salicylate) can be included in the environmental sample and decreased bioluminescence relative to the control (water with the same level of salicylate) may represent inhibitory compound(s) present in the sample.

The results of this investigation demonstrate that *P. fluorescens* HK44 can be applied in a semi-quantitative manner to rapidly detect aqueous phase fuel hydrocarbon contamination in soils. The specificity of the bioluminescent response is due to naphthalene constituents of the hydrocarbon mixture. The interpretation of these results extends the broad uses of *lux* gene fusion applications, particularly those employing an entire *lux* gene cassette (*luxCDABE*) but less likely to those that rely only on the luciferase (*luxAB*) and the addition of exogenous aldehyde. In applying the reporter strain for sensing bioavailable contaminants, it is necessary to have well standardized test conditions and insight into the cellular and physiological factors influencing bioluminescence in general.

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