Effect of oxygen on bioluminescence emitted by the biosensor *Pseudomonas putida* HK-44 in presence of phenanthrene

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**ABSTRACT**

*Pseudomonas putida* HK-44, a bioluminescent reporter strain with a *nah-lux* fusion, can be used to assess the presence of polynuclear aromatic hydrocarbons by stimulating the light production upon inducing the naphthalene dioxygenase system. However, this assay can be affected by the presence of oxygen. The aim of this work was to evaluate the effect of different oxygen tensions on luminescence emission by HK-44 in presence of phenanthrene. The relationship between light emission and bacterial cell density was positively correlated (P<0.05). Light emission produced by the strain HK-44 was enhanced in the presence of 2 ppm of phenanthrene and a maximum light emission was observed at the highest oxygen tension, this is because the bioluminescence reaction requires oxygen. Data fitted to a Stern-Volmer model at longer periods of time (starting from 150 min) with phenanthrene were observed to have decreased bioluminescence (Kₚ) at 2 to 10% oxygen tensions, which indicated a possible luminescence quenching by the oxygen. However, at 20% of oxygen saturation, bioluminescence of biosensor was not affected, therefore this biosensor is reliable for polycyclic aromatic hydrocarbons (PAHs) detection under this oxygen saturation conditions.

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**INTRODUCTION**

Polycyclic aromatic hydrocarbons (PAH) are compounds composed of carbon and hydrogen with fused benzene rings in linear, angular and cluster arrangements. Some PAHs are heterocyclic molecules with the substitution of one carbon of an aromatic ring by nitrogen, oxygen or sulphur (Münchnerová and Augustin, 1994). Many PAHs, especially those highly condensed with four or more rings and their metabolites, have a variety of mutagenic and carcinogenic effects on microorganisms, plants and Animals; and are classified as compounds which pose significant risk to human health (Kalf et al., 1997). Studies on the biodegradation of PAH have paved the way for further analyses of the biological disposal of these compounds.

Adapted or isolated bacteria or lower fungi have been used to degrade various, generally low-condensed PAH (Amézcua-Vega et al., 2003; Cerniglia, 1992; Cerniglia and Gibson, 1979; Cerniglia et al., 1982, 1984; Wilson and Jones, 1993; Chávez-Gómez et al., 2003). However, PAHs are hydrophobic and have a strong tendency to be sorbed to soil, such that their biodegradation is often controlled by their bioavailability. Thus, quantifying PAH
bioavailability is important for both risk assessment and remediation technology selection.

Biosensors such as bioluminescent reporter bacteria represent a potentially rapid, specific and quantitative approach to characterize PAH contamination and identify trends in the concentration and bioavailability of these compounds, but they are affected by the oxygen tensions. These evaluations are often based on the emission of a signal (for example, light or fluorescence) by reporter bacteria, upon exposure to a pollutant that triggers the expression of a catabolic gene fused with the reporter gene (Heitzer et al., 1998; Jaspers et al., 1999). Thus, signal intensity can be correlated to catabolic gene expression, which is in turn correlated to the bioavailable concentration of the inducer. Common reporter genes include _lux_, coding for luciferase enzymes which is an enzyme that produces bioluminescence from adenosine triphosphate (ATP) hydrolysis, and _gfp_, which codes for green fluorescence proteins (D’Souza, 2001).

Several bioreporter strains have been engineered to assess aromatic hydrocarbon bioavailability and biodegradation by fusing _lux_ with catabolic genes such as _tod_ (which codes for toluene dioxygenase and is induced by toluene) and _nah_ (which codes for naphthalene dioxygenase and is induced by naphthalene or salicylate) (Sayler et al., 2001, Shingleton et al., 2001). These strains are typically produced using a plasmid in which _lux_ is kept in a promoter that recognizes the aromatic hydrocarbon of interest. When these microbes metabolize the organic pollutant, the mechanisms of genetic control change toluene dioxygenase synthesis and produce light, which can be detected by luminometers. Such bacteria have led to the possibility of rapidly monitoring gene expression and bioavailability. However, bioluminescence data can be confounded by environmental and physiological factors that influence the intensity of bioluminescence (Neilson, 1999). For example, bioluminescence requires the presence of Nicotinamide adenine dinucleotide phosphate (NADPH), oxygen, reduced riboflavin phosphate (FMNH₂) and aldehydes (Heitzer et al., 1998; Jaspers et al., 1999; Meighen, 1991). Therefore, investigating the effect of environmental factors on bioluminescence is important to delineate the capabilities and limitations of such strains to serve as environmental bioreporters. Heitzer et al. (1992) and Sticher et al. (1997) observed a linear pattern between the concentration of substrate and luminescence, using _Pseudomonas putida_ HK44 and _Escherichia coli_ DH5 respectively, while Rattray et al. (1990) with _E. coli_ and Meikle et al. (1994) again with _P. putida_ noticed an effect of cellular density on luminescence.

Oxygen availability is an important environmental factor influencing the dynamics of aerobic processes in PAH-contaminated soil (Chung and Alexander, 1999). The concentration of oxygen affects PAH biodegradation kinetics due to its role as co-substrate for PAHs ring oxidation and as electron acceptor for microbial respiration (Hurst et al., 1996). Furthermore, oxygen is both a key ingredient for luciferase-catalyzed light production upon ATP hydrolysis, as well as a luminescence inhibitor at high concentrations (Neilson et al., 1999). However, other emission processes (fluorescence) of aromatic compounds that are taken in lower time compared to our work where biological species are used, the luminescence of complex- aromatics (excimers) are quenched by the oxygen. It was found that oxygen tension concentrations from 2 to 4 mg L⁻¹ inhibited both the degradation and the intensity of luminescence due to the _luxCDABE_ in _P. putida_ RB 1353 _nah_ gene expression. Nevertheless, the effect of oxygen tensions on luminescence intensity in response to the presence of PAHs has not been addressed in the scientific literature.

This paper therefore describes laboratory experiments conducted to evaluate how variations in oxygen tensions affect luminescence emission by the _lux_ bioreporter of _P. putida_ HK-44 in the presence of phenanthrene.

**MATERIALS AND METHODS**

**Bacterial strains, media and reagents**

The reporter strain _P. putida_ HK-44, which has a _nah-lux_ fusion (courtesy: Gary Sayler, UT, TN) was used to investigate the effect of oxygen concentration on bioluminescence response to phenanthrene, a model PAH. The construction of this strain has been described elsewhere (King et al., 1990). Naphthalene dioxygenase was selected as a model PAH degrading enzyme system not only because it is widely spread among naphthalene-degrading soil bacteria but also because its relaxed substrate specificity, allows co-oxidation of numerous aromatic hydrocarbons such as phenanthrene, anthracene, biphenyl, toluene and fluorene (Resnick et al., 1996). This microorganism is bioluminescent when it degrades naphthalene and other PAH compounds that yield salicylate, a metabolite that induces _nah_. HK-44 also has a kanamycin resistance cassette, which allows it to grow in the presence of this antibiotic that is added to the growth medium to preclude contamination by other strains.

HK-44 was pregrown in a modified agar and then inoculated in a medium with mineral salts, namely; _Luria Broth_ (LB) (Neilson et al., 1999). Composition of this medium in 1 L of deionized water was: 10 g of polypeptone, 5 g of yeast extract, 10 g of NaCl and 100 mg of kanamycin. The pH was adjusted to 7 using a Jenway 3020 pH meter. Likewise the bacterial strain was exposed to tetracycline. The mineral salts (MSB) added were; KH₂PO₄ = 1.5 g, Na₂HPO₄ = 0.5 g, MgSO₄ = 0.2 g,
NH_4Cl = 2.5 g, FeCl_3 = 3^4 g and CaCl_2 = 0.013 g.

Growth conditions

The strain was incubated in LB at 30°C with constant shaking at 120 rpm. Cell density was determined spectrophotometrically at 550 nm in a Perkin-Elmer Lambda 3A spectrophotometer. Cultures were inoculated at a density of 10^6 CFU ml^{-1} from a 30 h preculture in the same medium as reported by Neilson et al. (1999). They were allowed to grow until they reached the desired growth stage, according to a previously determined growth graph. Cells were grown in LB prior to transfer to a solution polluted with phenanthrene which was dissolved in acetone for a final phenanthrene concentration of 2 ppm (Neilson et al., 1999). n-decyl aldehyde that allowed the expected light production to occur was then added by 4 ml (Hill et al., 1993).

After removal from the LB, the cells were washed in triplicate in a saline solution (0.85% NaCl) with 30 ml of culture (per flask), and incubated at the shaking and temperature conditions mentioned previously.

Luminescence measurements

Samples were analyzed individually for luminescence emission in the experimental units in 4 ml glass scintillation vials with 350 µl of culture mixture, shaken vigorously in a Vortex during two minutes for homogenization and was then sealed with septa. The samples were quantified in a Turner Design TD20/20 0302 luminometer under the following operating conditions: Late Time: 2 s; Integration Time: 20 s; Replicates: 3; Sensitivity Level: 49.4%; Mode: standard. The luminometer was connected to a computer allowing continuous register over long periods of time.

The samples were conserved in scintillation vials and immediately counted for 20 s, generating relative values expressed as luminescence relative units for all assay times. Incubations longer than 6 h were avoided because the counts were high compared to samples that were read immediately. Luminescence values obtained at each sampling time were plotted as a function of time.

Effect of luminescence emission at different oxygen tensions

The experiment was designed to determine the effects of oxygen tension on phenanthrene degradation and luminescent emission. Oxygen tension was adjusted by a daily supply of both oxygen and nitrogen to obtain a final oxygen tension of 20, 10, 5, 2 and near to 0% (Henry’s constant for oxygen = 760 atm L mole^{-1}). Oxygen tension was determined by direct injection of 1 ml, to a Gow-Mac 550 chromatograph equipped with a thermal conductivity detector and a Alltech CTRI steel column. Conditions were; oven temperature (30°C), injector temperature (40°C), and detector temperature (100°C). Helium was used as transporting gas at a flow rate of 45 ml min^{-1}. Data were processed and plotted to obtain the CO_2 instantaneous production.

The experiment allowed evaluating the effect of different initially adjusted oxygen tensions on phenanthrene removal and bioluminescence emission. Sterile samples were added to a glass scintillation vial and purged with sterile O_2 and N_2 (99.9%) at constant flow and a specific time. Figure 1 shows the bioluminescence detection process at different oxygen tensions.

Determination of quenching constants (K_q)

In order to verify the existence of the bioreporter luminescence quenching by the oxygen, the quenching constants (K_q) were calculated through the Stern-Volmer equation. Therefore a plot of I/I against the oxygen concentration (quencher) gave a straight line to validate the model. The quenching constants, K_q were obtained (Table 1).

\[
\frac{I_o}{I} = 1 + K_q[D]
\]  

Where: I_o = Bacterial luminescence intensity in the absense of oxygen; I = bacterial luminescence intensity in the presence of oxygen; K_q = quenching constant; and [D] = quencher tension (oxygen).

RESULTS AND DISCUSSION

Bacterial luminescence could help detect changes in the patterns of expression of induced genes under adverse conditions. The potential of bioluminescent cells to serve as environmental bioreporters may depend on their inherent nature to survive under physiological fluctuations due to changes in substrate reporters may depend on their inherent nature to survive under physiological fluctuations due to changes in substrate resources, pH, temperature and oxygen in the deployed environment.

Kinetic profile of bioluminescence response

The growth curve of P. putida HK-44 was characterized by an initial growth phase, followed by a stability period and a decline or death period (Figure 2). A set of normalized initial luminescence levels was established within 54 h. From the beginning of the experiment and until 28 h, P. putida HK-44 reached its maximum luminescent response and gradually declined from there.
Figure 1. Flow diagram showing bioluminescence detection at different oxygen tensions.

**Table 1.** Quenching constants ($K_q$) with and without phenanthrene.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>With phenanthrene $R^2 (K_q)$</th>
<th>Without phenanthrene $R^2 (K_q)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.03-0.34</td>
<td>0.39-0.00</td>
</tr>
<tr>
<td>15</td>
<td>0.10-0.03</td>
<td>0.82-0.19</td>
</tr>
<tr>
<td>30</td>
<td>0.80-0.34</td>
<td>0.81-0.05</td>
</tr>
<tr>
<td>45</td>
<td>0.12-0.03</td>
<td>0.99-0.06</td>
</tr>
<tr>
<td>60</td>
<td>0.68-0.15</td>
<td>0.52-0.06</td>
</tr>
<tr>
<td>90</td>
<td>0.46-0.02</td>
<td>0.89-0.12</td>
</tr>
<tr>
<td>120</td>
<td>0.86-0.08</td>
<td>0.82-0.13</td>
</tr>
<tr>
<td>150</td>
<td>0.10-0.17</td>
<td>0.96-0.20</td>
</tr>
<tr>
<td>210</td>
<td>0.10-0.39</td>
<td>0.82-0.20</td>
</tr>
<tr>
<td>270</td>
<td>0.99-0.59</td>
<td>0.92-0.20</td>
</tr>
<tr>
<td>330</td>
<td>0.96-0.81</td>
<td>0.80-0.17</td>
</tr>
<tr>
<td>390</td>
<td>0.95-0.94</td>
<td>0.87-0.20</td>
</tr>
<tr>
<td>450</td>
<td>0.97-1.07</td>
<td>0.90-0.19</td>
</tr>
</tbody>
</table>
Bioluminescence peak max.

Figure 2. Growth curve of *P. putida* HK-44, according to maximum bioluminescence response. IDM = Initial dry matter.

until the end at 54 h.

During the initial growth (28 h), cells were transferred to a solution with 2 ppm of phenanthrene (Figure 2). Bioluminescence viability were determined by transferring an aliquot of the dissolved bead suspension on tetracycline (100 mg L$^{-1}$) containing Luria/agar medium, and monitoring the cells continuously until maximum light emission was reached, which typically took 28 h. After about 32 h, the bioluminescence output leveled off and the magnitude of the bioluminescent response differed, depending on the induction time and cell concentration in phenanthrene.

**Bioluminescence reaction rate with and without phenanthrene**

Assays to determine the bioluminescence response of *P. putida* HK-44 were performed at different oxygen tensions and the values recorded were plotted against the respective oxygen tension, using the phenanthrene concentration of 2 mg L$^{-1}$. After induction, the effect of cell concentration on the bioluminescence response indicates a linear increase in luminescence with time (7.5 h) similar to the previous experiment, where emitted luminescence increased with time.

Bioluminescence was monitored during 15-30 min and the reaction rate of luminescence emission was calculated within 7.5 h post induction period (450 min) for all the assayed times (Figures 3 and 4). The biosensor response time after exposure to different treatments (added with phenanthrene) was around 60 min. Heitzer et al. (1992) reported a response time of 8 min for the same strain in presence of different salicylate concentrations. The enzymatic activity of luciferase from HK-44 was favoured in presence of phenanthrene. The bioluminescence levels were lower in the absence of phenanthrene. As regards the presence of this compound, maximum bioluminescence values were 58 (LRU) with phenanthrene and 1.38 (LRU) without phenanthrene. Sayler (1990) established that the increase in bioluminescence of *P. putida* HK-44 is enhanced when this microorganism is exposed to the organic compound of interest, and it is known that high aromatic characteristic of phenanthrene may allow the interaction with *P. putida lux* mainly at saturated oxygen tensions of 20%. However, there are limitations to the use of a lux-based reporter system for this kind of work, for example, oxygen is essential for bioluminescence emission.

**Bioluminescence reaction rate as a response to different oxygen tensions**

Oxygen tensions were adjusted to 0, 2, 5, 10 and 20% in
the headspace. The oxygen tension levels were roughly stable along all of the induction time and the variations among treatments were lower than ±1% (P<0.005). Although microaerophilic conditions were established (<2%), it was not possible to reach 0% of oxygen tension (oxygen tension measured in the headspace was of the order of 0.1-0.01%). Likewise, response time and bioluminescence...
magnitude varied depending on oxygen tension and phenanthrene availability. As shown for induction with phenanthrene at different oxygen tensions, bioluminescence depleted as a response to oxygen tension in treatments and the light magnitude ranged between 1.38 and 58 (LRU). Aldehyde availability can affect the bioluminescence emitted by HK-44. However, aldehyde was not limiting, since addition of decanal prior to sample measurement did not enhance bioluminescence readings. Therefore, the bioluminescence assay described here using biosensor is appropriate to discern the presence of polynuclear aromatic hydrocarbon during aerobic degradation. A pattern in the magnitude of the maximum light at the highest oxygen tension was observed. For instance, the response of 58 (LRU) was consistent with the higher oxygen tension of 20% (Figure 4). Oxygen was probably limiting, because the bioluminescence reaction requires oxygen. Simpson et al. (2001) also verified this oxygen limitation behavior in the bioluminescence experiments with toluene, suggesting a steady state of luciferase in the cell.

The results allowed us to determine the behavior of induction time and cell density during the bioluminescence assays. Biomass and luminescence were not significantly correlated until 32 h, but in the 48th hour, both variables turned out to be highly significant (0.005). During the induction phase of bioluminescence, the emitted light intensity increased with time. Values obtained from bioluminescence expression during the early response phase could yield significantly low values.

The values of the bioluminescence intensities were substituted in the Stern-Volmer equation type, starting from the value of the slopes of the straight lines plotted at different oxygen tensions in the presence and absence of phenanthrene to obtain the quenching constants \( K_q \). The Stern-Volmer model was adjusted to a straight line above the quenching constants \( K_q \) observed mainly at oxygen tensions of 2, 5 and 10%, which explains the positive \( K_q \) value. That indicates their effect under this low oxygen tensions in the luminescence intensity, even though the \( O_2 \) could be necessary for the \( nah \) naphthalene dioxygenase activity (Sayler et al., 2001; Shingleton et al., 2001). The \( K_q \) constants without phenanthrene were maintained during all the adjustments carried out (2–20%).

**Conclusion**

It was observed that at oxygen tensions between 2, 5 and 10%, the bioluminescence response of *P. putida* HK-44 decreased as the oxygen tension increased in the presence of phenanthrene. However, at 20% of oxygen saturation and phenanthrene, bioluminescence was enhanced for a time greater than 150 min.

The bioluminescence produced by *P. putida* HK44 is a sensitive analytic technique with a potential to be used in the detection of polluted environments with PAHs. However, their use as a biosensor can be said to reliable in the detection of PAHs under normal conditions of oxygen saturation (20%).

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**REFERENCES**


