

SURVIVAL AND ACTIVITY OF *LUX*-MARKED PHENANTHRENE-DEGRADING *PSEUDOMONAS STUTZERI* P16 UNDER DIFFERENT CONDITIONS*

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Abstract – Viable cell concentration and luminescence activity of *P. stutzeri* P16 *luxAB4* was measured by plate counting and luminometry in sterile and non-sterile soil adjusted to matric potential of -1500, -750 and -30 kPa. Results showed that matric potential had a significant effect on survival and activity of *P. stutzeri* P16 *luxAB4* in soil. *P. stutzeri* P16 *luxAB4* could survive better in Hutner's minimal medium supplemented with 1mg l⁻¹ phenanthrene than 1 mg ml⁻¹. However changes in viable cell concentration and luminescence activity of *lux*-marked and wild type strains of *P. stutzeri* P16 were significantly different in Hutner's medium, amended with high and low concentrations of phenanthrene. Inoculation of soil with high concentrations of phenanthrene caused an initial decline in the viable cell concentration of *P. stutzeri* P16 *luxAB4* that took a longer time than liquid medium. Changes in viable cell concentration were similar to changes in luminescence indicating the advantage of Bioluminescence-marker systems for monitoring the survival and activity of biodegradative genetically engineered microorganisms (GEMs) in polluted environments. Our results also showed the importance of influence of environmental factors such as matric potential and pollutant concentration on the fate of GEMs.

Keywords – Genetically engineered microorganisms, matric potential, biodegradation, luminescence

1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous organic compounds containing two or more aromatic rings and have been greatly considered because of their mutagenicity, toxicity and carcinogenicity. Many of them are recalcitrant and found in both aquatic and terrestrial environments as a result of fossil fuel utilization, combustion processes and chemical manufacturing [1].

There are many species of bacteria, fungi and microalgae capable of degrading PAH and other contaminants [2, 3, 4]. These bacteria are either members of the natural microbial population or genetically engineered microorganisms.

In spite of safer applications of naturally occurring microorganisms for bioremediation, they are not capable of complete degradation of a complex compound such as PAHs, and the introduction of a group of the bacteria might result in the competition among consortia. Therefore, the idea of application of GEMs for the biodegradation of pollutants such as PAHs has brought hope for better removal of pollutants from environments [5, 6, 7]. Several Biodegradative GEMs have been constructed, but there is still great concern about the risk of GEMs released into the environment. Problems such as gene transfer, ecological impact on biotic and abiotic factors, and monitoring the survival and activity of GEMs are some of the issues which have to be investigated for better application of GEMs in the environment [8, 9]. Several

*Received by the editors September 27, 2004 and in final revised form April 26, 2006

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biotic and abiotic factors can have an effect on the fate of biodegradative GEMs in soil or water including: moisture content of soil, PAH concentration, inoculation size and salinity [10, 11].

Monitoring the survival and activity of bacteria in terrestrial and aquatic ecosystems using bioluminescence marker systems have been reported [12, 13]. Therefore, this reporter system was used for our studies. Phenanthrene, a tricyclic aromatic hydrocarbon, is often used as a model substance for studies on the metabolism of carcinogenic polycyclic aromatic hydrocarbons because it is the smallest aromatic hydrocarbon to have a “bay-region” [14, 15, 16]. It has been shown that phenanthrene is not mutagenic or carcinogenic, but it is toxic to aquatic organisms [17, 18]. Therefore, in our studies, the fate of genetically engineered *P. stutzeri* P16 was investigated in soil at matric potentials of -30, -750 and -1500 kPa. The survival and activity of *P. stutzeri* P16 *luxAB4* were then further investigated in amended liquid culture and soil.

2. MATERIALS AND METHODS

a) Bacterial strains, growth, media

P. stutzeri P16 was obtained from Dr M.D. Aitken, University of North Carolina, North Carolina, USA.

b) Liquid medium experiment

All experiments were performed in sterile conditions. Phenanthrene was sterilized as described before and dissolved in 200 μ l Hutner's base medium in two sets of 250 ml flasks in triplicate to obtain a concentration of 1 mg ml⁻¹ and 1 mg l⁻¹. Overnight cultures of *lux*-marked and wild type strains of *P. stutzeri* P16 were prepared and centrifuged at 8000 g for 10 min. Pellets were then washed twice and resuspended in 50 ml Hutner's medium. Phenanthrene at a concentration of 1 mg l⁻¹ was added to sterile flasks before and at concentrations of 1 mg ml⁻¹ after the addition of Hutner's minimal medium for each set of flasks. Controls without phenanthrene, without inocula or both were considered in each sampling point.

c) Soil microcosms and inoculation

Soil microcosms consisted of 500 ml Duran bottles containing 50 g of soil. Soil was an insch series soil type. The soil characteristics were determined in the Department of Plant and Soil Science at the University of Aberdeen and were 57.7% silt, 11.51% clay, 3.75% organic matter, pH (CaCl₂) 6.2, and Base saturation 92.34. Soil was air dried for 48 h and passed through a 3.35 mm pore size sieve. Soil samples were adjusted to matric potential -30, -750, or -1500 kPa using equation: $J=aM^{-b}$, where **J** is the soil water potential, **a** and **b** are constants for a given soil [19] that for insch soil was $J=0.005613M^{-5.6509}$. After air drying of soils, they were packed into microcosms. A mixture of inocula and water were added to each microcosm to give the correct matric potential. Matric potential was continuously maintained by the replacement of water lost through evaporation. The pH of soil was adjusted to 7 by the addition of 30 mg Ca(OH)₂ per g of soil. Soil sterilisation was carried out by autoclaving the microcosms at 121°C for 1 h after the addition of Ca(OH)₂. Waterloss during the procedure was replaced and the process repeated twice further.

Inoculation was prepared as described before for the liquid experiment and introduced to sterile and non-sterile soil microcosms to give 10⁷-10⁹ cells g⁻¹ soil. The inoculated soil was then mixed thoroughly with a sterile spatula to ensure an even distribution of inocula. Microcosms were incubated at 25°C. Sterile phenanthrene was dissolved in acetone and added to sterile and non-sterile soil at concentrations of 1 mg kg⁻¹ and 1 g kg⁻¹ soil.

d) Enumeration of Viable Cell Concentration and Measurement of Luminescence Activity in Liquid Media

After the inoculation of the medium with wild type and lux-marked of *P. stutzeri* (10-fold), Lux-marked colonies of *P. stutzeri* P16 were counted on an LB medium containing antibiotics. [rifampicin ($25 \mu\text{g ml}^{-1}$) and tetracycline ($12.5 \mu\text{g ml}^{-1}$)]. The wild type strain was enumerated on LB medium without an antibiotic and MBM agar covered with phenanthrene as a control. Luminescence measurement was carried out by the addition of exogenous aldehyde substrate in ethanol (0.5 % v/v) to 1 ml using an Bio-Orbit 1251 luminometer.

e) Viable Cell Concentration and Luminescence Measurement of Introduced Microorganisms

At each sampling point, 1 g of soil was removed and suspended in 10 ml of sterile phosphate buffer (0.01 M, pH 7) using a mechanical shaker (speed 9). Viable cell concentration was determined by spotting 10 μl of serially diluted soil suspension onto an LB agar, supplemented with rifampicin ($25 \mu\text{g ml}^{-1}$) and cycloheximide ($25 \mu\text{g ml}^{-1}$). For measurement of luminescence activity, 1.4 ml of soil suspension were centrifuged (2000 rpm, 1 min) to remove soil debris and protocol was followed by the addition of 1 μl n-decyl aldehyde in ethanol (10 % v/v) to supernatants.

f) Quantification of Phenanthrene Degradation

A concentration of phenanthrene was measured in a 1 gram soil sample [11]. 20 ml pentane was added to 10 ml soil suspension and vortexed for 5 min. Three ml of pentane (upper layer) was removed for spectrophotometric measurement of phenanthrene content at 251 nm (A_{251}). Cuvette containing only phenanthrene was used as the control for the calibration of the spectrophotometer. Cuvette containing a known amount of phenanthrene in pentane was used as a standard to compare with cuvette containing phenanthrene was extracted from soil samples to indicate that UV spectra of the samples have no deviation from a published phenanthrene spectrum [20].

g) Statistical Analysis

Data were analyzed by the GLM (General Linear Model) procedure using the SPSS computer package version 9.0 for windows. One way analysis of variance was also used for multiple comparison of data. Data were log transformed to reduce the broad distribution of values.

3. RESULTS

a) Effect of Matric Potential on Viable Cell Concentration of *P. stutzeri* P16 luxAB4

Changes in viable cell number in non-sterile soil at matric potential of -750 kPa and -1500 kPa were greater than in sterile soil ($P < 0.05$) (Fig. 1.a). At -750 kPa, viable cell concentration significantly decreased in both sterile and non-sterile soil by 2 and 5 orders of magnitude respectively. Viable cell concentration was continuously decreased by 3 orders of magnitude in non-sterile soil at -30 kPa, while a small increase was observed from day 14 to day 18 in sterile soil (Fig. 1.a). The difference between viable cell concentration in sterile and non-sterile soil increased with time at -30kPa as a significant difference ($P < 0.05$) was observed in viable cell concentration between sterile and non-sterile soil.

b) Effect of Matric Potential on Luminescence Activity of *P. Stutzeri* P16 luxAB4

At -1500 kPa, changes in luminescence values were greater in non-sterile soil than sterile soil and in general, luminescence values in sterile soil were higher than non-sterile soil as significant differences were

observed between luminescence values of sterile and non-sterile soil at -1500 kPa (Fig. 1.b). No initial increase was observed in light output at any matric potential studied. Light output changed similarly at -750 kPa in both sterile and non-sterile soil until day 6, thereafter in non-sterile soil, luminescence values decreased more rapidly than sterile soil and reached a level which was undetectable by a luminometer after 32 days incubation at 25°C. Significant differences ($P < 0.05$) in luminescence values between sterile and non-sterile soil was observed at matric potentials of -1500 kPa and -750 kPa, but not at -30 kPa, in which light output decreased rapidly in both sterile and non-sterile soil as luminescence was undetectable at day 32 (Fig. 1.b).

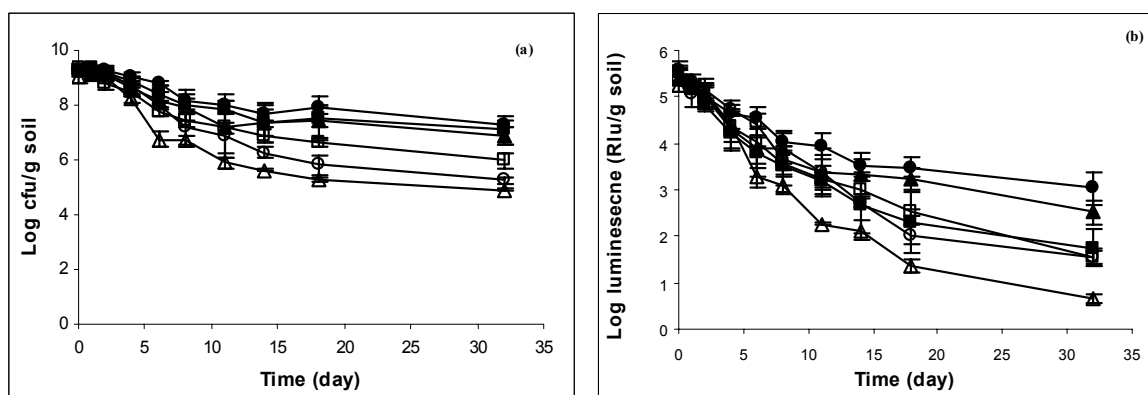


Fig. 1. Changes in viable cell concentration (a) and luminescence activity (b) of *P. stutzeri* P16 in soil adjusted to matric potentials of -1500 kPa (sterile (●), non-sterile (○)), -750 kPa (sterile (▲), non-sterile (Δ)) and -30 kPa (sterile (■), non-sterile (□)). Error bars indicate standard errors of mean (SEM). Where no bars are seen, SEMs were less than symbol size

c) Survival and Growth of *P. stutzeri* P16 *luxAB* in Liquid Medium Amended with High and Low Concentrations of Phenanthrene Comparing with its Wild Type

No significant changes in viable cell concentration of *P. stutzeri* P16 *luxAB4* was observed during the 10 day incubation in the presence of phenanthrene (1 mg l^{-1}) (Fig. 2.a). Viable cell concentration then decreased by more than 1 order or magnitude during further incubation until day 96 (Fig. 2.b). Addition of 1 mg l^{-1} phenanthrene had no significant effect on the viable cell concentration of *P. stutzeri* P16 *luxAB4*, as no significant differences ($P > 0.05$) were observed between viable cell concentration in the medium with and without phenanthrene. The Initial decrease was also observed for *P. stutzeri* P16 (wt) (Fig. 2.c). Changes in viable cell concentrations were significantly different between *lux*-marked and wild type strains of *P. stutzeri* P16 at a phenanthrene concentration of 1 mg l^{-1} (Figs. 2.a, 2.b, 2.c, 2.d).

At a concentration of phenanthrene (1 mg ml^{-1}), the initial decline in viable cell concentration was greater in medium inoculated with *lux*-marked *P. stutzeri* P16 than its wild type (Figs. 2.a, 2.c). Similar to low concentrations, significant differences were observed in viable cell concentration between medium supplemented with or without phenanthrene (1 mg ml^{-1}) for both *lux*-marked and wild type strains of *P. stutzeri* P16. However no significant differences observed in viable cell concentration between medium inoculated with wild type or *lux*-marked *P. stutzeri* P16, at a high concentration of phenanthren (1 mg ml^{-1}).

d) Luminescence Activity in Liquid Medium Amended with Phenanthrene (1 mg l^{-1} and 1 mg ml^{-1})

Similar to changes in viable cell concentrations at a phenanthrene concentration of 1 mg l^{-1} , there was a significant difference in luminescence between medium supplemented with or without phenanthrene (Figs. 2.e, 2.f). However, luminescence values increased from day 10 to day 30, was followed by a decline during further incubation until day 96 (Fig. 2.f). Also, an initial decline in luminescence values was

observed at a high concentration (1 mg ml^{-1}), which was not seen in the control. Statistical analysis showed significant differences ($P < 0.05$) in luminescence values between medium amended with high and low concentrations of phenanthrene.

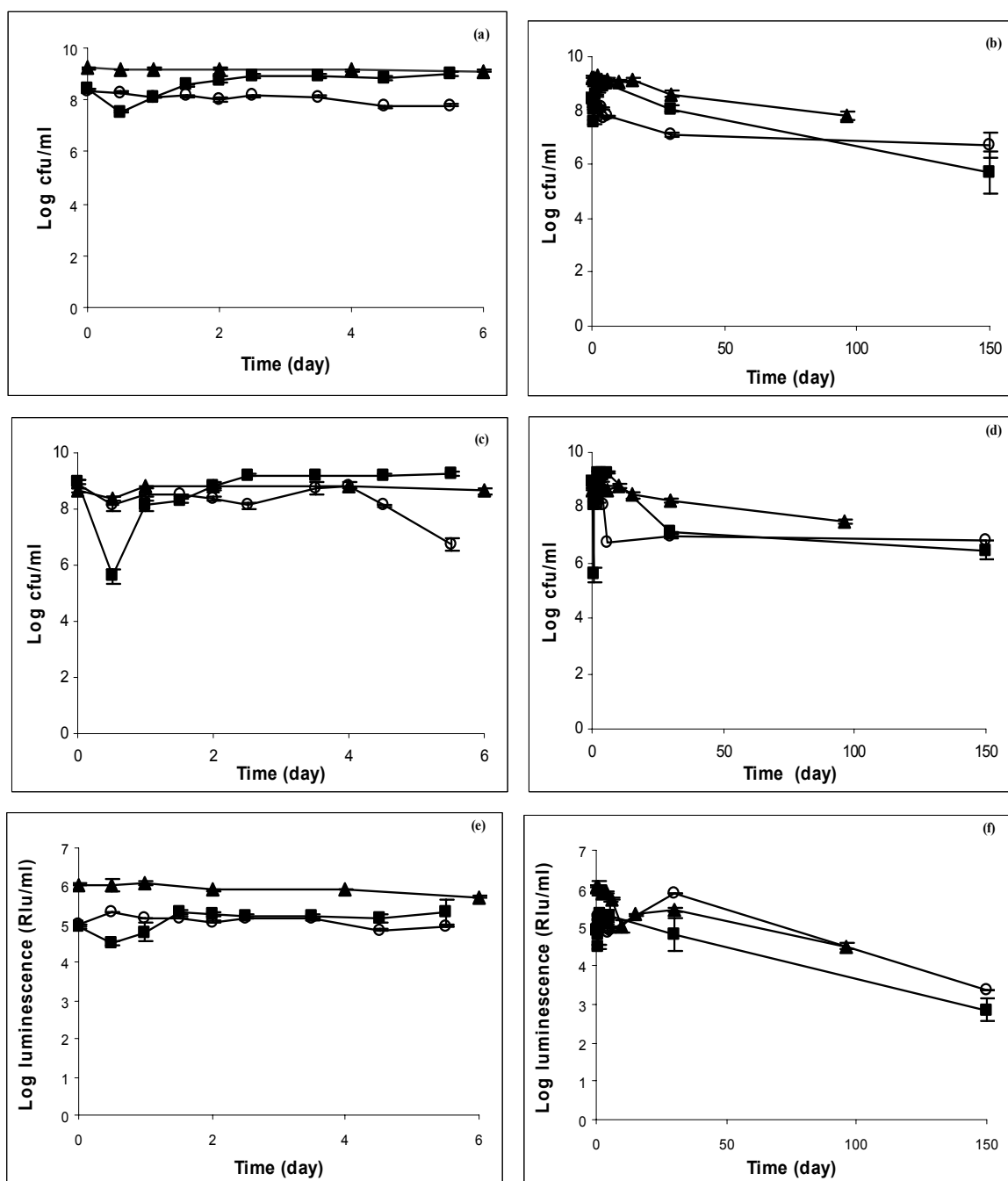


Fig. 2. Changes in viable cell concentration of lux-marked (a, b) and wild type (c, d) strains of *P. stutzeri* P16 in Hutner's minimal medium without phenanthrene (○) and amended with low (▲) and high (■) concentrations of phenanthrene during short (a, c) and long (b, d) incubation at 25°C. Changes in luminescence activity of *P. stutzeri* P16 *luxAB4* are shown in panels (e, f). Error bars indicate standard errors of mean (SEM). Where no bars are seen, SEMs were less than symbol size

e) Survival of *P. stutzeri* P16 *luxAB4* in Soil at Presence of Phenanthrene

Viable cell concentration decreased gradually in sterile and non-sterile soil amended with phenanthrene (1 mg kg^{-1} soil) during the first 20 days (Fig. 3.a), and then sterile soil viable cell concentration remained unchanged till day 40 (Fig. 3.b). With long incubation, bacteria survived better in sterile soil than non-sterile soil, as significant differences were observed between viable cell concentration of *P. stutzeri* P16 *luxAB4* in sterile and non-sterile soil (Fig. 3.b).

At a concentration of phenanthrene (1 g kg^{-1}), viable cell concentrations initially decreased in both sterile and non-sterile soil, but increased after 5 days incubation in sterile soil (Fig. 3.a). The difference between viable cell number in sterile and non-sterile soil became less as no significant difference ($P > 0.05$) was observed at this time. Viable cell concentrations in sterile soil were significantly higher than in nonsterile after further incubation of cells till day 110 (Fig. 3.b). There was significant difference ($P < 0.05$) in viable cell concentration in both sterile and non-sterile soil between soil amended with low and high concentrations of phenanthrene.

f) Luminescence Activity in Soil at Presence of Phenanthrene

Initial light output remained higher for 2 days at low concentrations of phenanthrene (1 mg kg^{-1} soil) in sterile soil (Fig. 3.c). Luminescence then decreased in both sterile and non-sterile soil with a longer incubation of *lux*-marked cells till day 80 (Fig. 3.d). Although at day 20 luminescence values in non-sterile soil were higher than sterile soil, no significant difference was observed in luminescence values between sterile and non-sterile soil during the 80 days of incubation of cells in soil microcosms amended with phenanthrene (1 mg kg^{-1} soil).

At a high concentration of phenanthrene (1 mg kg^{-1} soil), similar to changes in viable cell concentration, an initial decline was observed in luminescence values for both sterile and non-sterile soil (Fig. 3.c). Luminescence continued to decrease further in sterile soil, but not in non-sterile soil (Fig. 3.d), however there were no significant differences in luminescence between sterile and non-sterile soil. Statistical analysis showed that there were significant differences in luminescence values between soil microcosms amended with high and low concentrations of phenanthrene.

g) Phenanthrene Degradation in Sterile and Non-sterile Soil

Inoculation of sterile and non-sterile soil with *P. stutzeri* P16 *luxAB4* resulted in degradation of 91% of the added phenanthrene within 11 days (Figure 3.e). However the rate of degradation was much higher in non-sterile soil from day 1-5 than in sterile soil. Phenanthrene content in uninoculated soil was about 70–80% of added phenanthrene after 11 days incubation (Figure 3.f), as a significant difference ($P < 0.05$) was observed between the rate of degradation in inoculated and uninoculated (sterile and non-sterile) soil microcosms.

4. DISCUSSION

Statistical analysis showed that matric potential had a significant effect on viable cell concentration which was also observed for *E. coli* [13] and *P. fluorescens* [21]. The difference between viable cell concentration of *P. stutzeri* P16 *luxAB4* in sterile and non-sterile soil became greater with time.

These differences in viable cell number were less at -30 kPa compared to -750 kPa and -1500 kPa , indicating that the increase of moisture content increased substrate availability for introduced bacteria [22]. Matric potential also had a significant effect on luminescence in both sterile and non-sterile soil. The decline in activity bacteria at low soil water content was found to be due to the diffusional limitation of substrate supply and adverse physiological effects associated with cell dehydration [23].

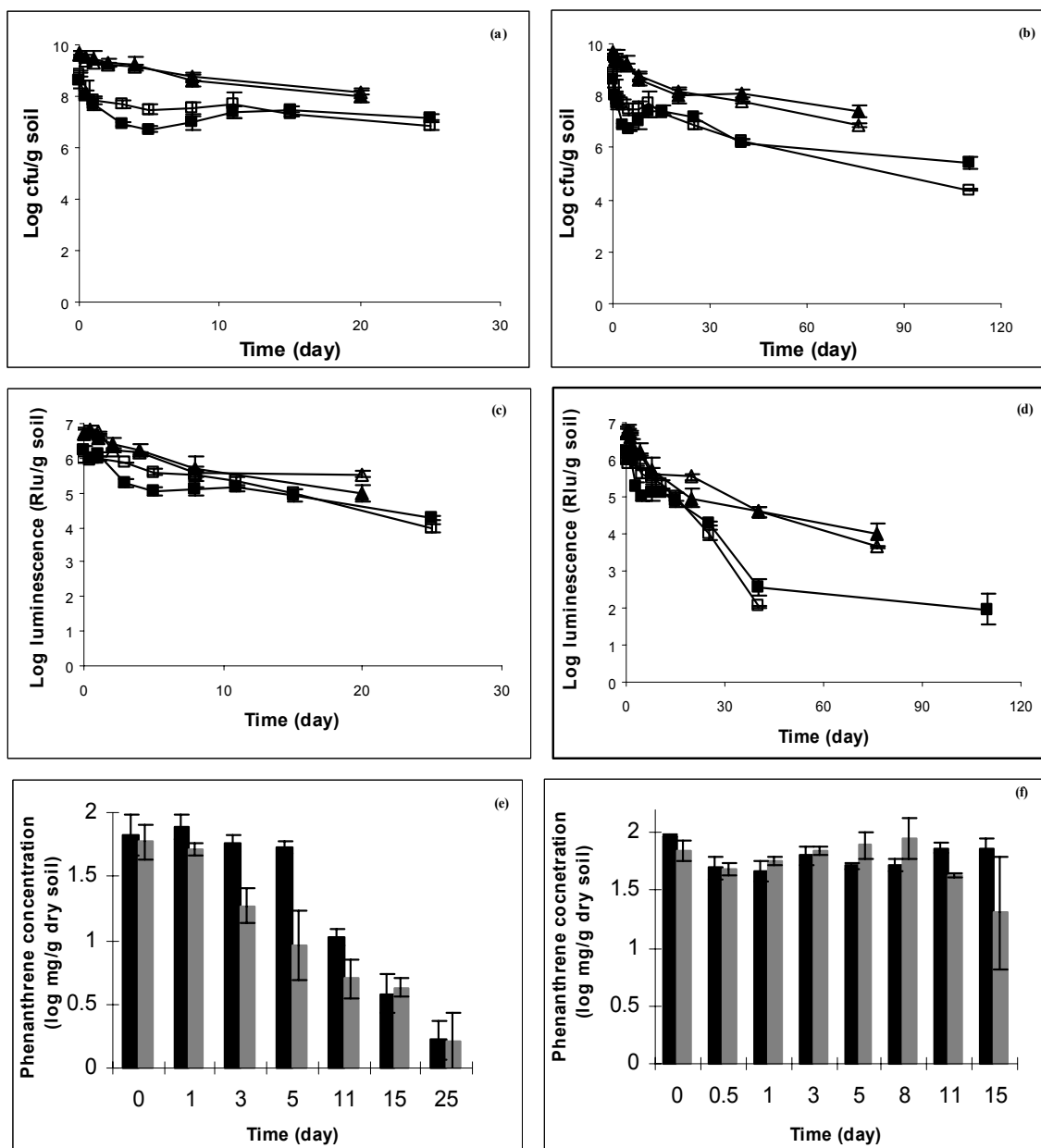


Fig. 3. Changes in viable cell concentration (a, b) and luminescence activity (c, d) of *P. stutzeri* P16 luxAB4 in soil amended with low (1 mg kg⁻¹ soil) (sterile (▲), non-sterile (△)) and high (1 g kg⁻¹) (sterile (■), non-sterile (□)) concentration of phenanthrene during short (a, c) and long incubation at 25°C. The rate of phenanthrene degradation in polluted soil (1 g phenanthrene per kg of soil) inoculated and not inoculated with *P. stutzeri* P16 luxAB4 are shown in panels (e) and (f) respectively. Error bars indicate standard errors of mean (SEM). Where no bars are seen, SEMs were less than symbol size

Changes in luminescence values were similar to changes in viable cell concentration, showing the usefulness of luminescence as a possible non-extractive method and an alternative to viable plate counting. In spite of the similarity between changes in luminescence activity and viable cell concentration, light output decreased to a greater extent in soil than viable cell concentration, which concurs with previous studies on *P. fluorescence* [21]. Results also showed that in absence of predators, cells were more active in drier conditions.

Viable cell concentration did not change significantly in flasks containing medium with or without phenanthrene (1 mg l^{-1}). It could be because phenanthrene was utilised at early hours by bacteria and the depletion of medium from phenanthrene made the environment similar to the medium without phenanthrene. Three reasons including: faster adaptation of GEMs, different inocula density, and negative and positive factors present in different habitats cause the inhibition of growth of indigenous bacteria and not genetically engineered *E. coli* JM10 and *Flaviminoas orizihabitans* KH1 in water microcosms amended with catechol [24].

An immediate decline in viable cell concentration after the inoculation of medium with high concentrations of phenanthrene (1 mg ml^{-1}) could probably be due to the toxic effect of a high concentration of phenanthrene as the most aromatic hydrocarbons are highly toxic for living organisms because the preferential partitioning of those compounds in cell membranes disrupt the membrane structure leading to cell death [25]. Although the presence of a high concentration of phenanthrene in the medium provided a greater energy source, cells could survive better in a low concentration than a high concentration for a longer period as significant differences were observed in viable cell concentrations between the medium supplemented with low and high concentrations of phenanthrene.

The Luminescence activity of *P. stutzeri* P16 *luxAB4* followed the same pattern as viable cell concentration, but decreased to a greater extent at the presence of high concentrations of phenanthrene, which could be due to a decline in the metabolic activity of the population through exhaustion of substrates and endogenous reserves.

The initial decline in the viable cell concentration was greater in soil than liquid medium in the presence of phenanthrene, but cells could survive longer in polluted soil than in liquid medium, possibly because the complexity of niche and environment reduces the toxicity effect of a compound on microbial survival [26]. Luminescence changes were very similar to changes in viable cell concentration, which again shows the usefulness of Bioluminescence-marker systems for tracking the GEMs in the environment.

Results showed that degradation of phenanthrene was enhanced by the introduction of bacteria into soil, especially in non-sterile soil that indicates the advantage of using microbial inocula for bioremediation of polluted sites compared to other methods. Only 70 % of an initial content of $88.5 \mu\text{g phenanthrene g}^{-1}$ dry soil was degraded after 14 days by landfarming [27], while in our studies degradation of 80% of initial concentration of 1 g kg^{-1} dry soil was observed after 5 days. The delay in degradation of phenanthrene in sterile soil could be due to interaction between organic and inorganic soil constituents. Phenanthrene mineralization was enhanced in the presence of dissolved humic acids and humic acid-clay complex [20]. Our studies supported previous works that pointed out the advantage of the application of the Bioluminescence-marker system in monitoring the survival and activity of GEMs in environment. This work also provided information that can be used for the further investigation on the fate of both genetically engineered and naturally occurring strains degrading PAH compounds with three rings or more.

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