

## Influence of Matric Potential on Survival and Activity of Genetically Engineered *Ralstonia eutropha* H850Lr

M. Mashreghi\*

Department of Biology, Faculty of Sciences, Ferdowsi University of  
Mashhad, Mashhad, Islamic Republic of Iran

### Abstract

Although the application of biodegradative genetically engineered micro organisms (GEMs) for bioremediation is very promising, the risks of their release should be assessed before their introduction into the environment. *Lux*-marked *Ralstonia eutropha* H850Lr (formerly *Alcaligenes eutrophus* H850Lr) was introduced into sterile and non-sterile soil microcosms at matric potentials  $-2.11$ ,  $-30$ ,  $-750$ , and  $-1500$  kPa. Viable cell concentration and luminescence activity of *R. eutropha* H850Lr was measured by plate counting and luminometry respectively. *R. eutropha* H850Lr could survive better in non-sterile soil at  $-30$  kPa than other matric potentials. Luminescence values were closely related to viable cell concentrations indicating the usefulness of a Bioluminescence-marker system for tracking the above bacterium in the environment. Statistical analysis showed a significant effect of matric potential on viable cell concentrations. The luminescence activities in the soil environment emphasize that these factors should be considered during application of GEMs in the natural environment.

**Keywords:** Genetically engineered micro organism; Matric potential; Bioluminescence; Survival; *Lux* genes

### Introduction

Genetically engineered micro organisms (GEMs) and or indigenous micro organisms have been used for bioremediation of environmental pollutants [5,7,19]. The success of GEMs in the contaminated environment depends on the survival and subsequent activity of the newly introduced species. These, in turn, depend on biotic and abiotic factors which are variable in time and space [3,10,12,20,22,24].

Pollutants mostly have a complex chemical structure; therefore micro organisms may not be able to degrade it

completely, although introduction of micro organisms in consortia might help to catabolize soil pollutants. However, independent requirements of such associations may make it difficult to apply them to environments. It is therefore of major interest to introduce a GEM with a well-regulated catabolic pathway which can be applied to polluted areas for effective pollutant degradation [4].

There are potential problems associated with the deliberate release of GEMs into the environment. The released GEMs may have the capacity to reproduce, spread beyond the initial point of release, and transfer

\* E-mail: mmashreghi@science1.um.ac.ir

their novel genetic information to the indigenous microbial population. Such information is therefore needed to properly evaluate and assess the fate of GEMs in the environment. A major method of acquiring this information involves the marking of a microbial model with an easily detectable phenotype to monitor its survival/or gene transfer [11,23,25]. Luminescence-based techniques enable detection through measurement of light [13,16,21].

Of particular relevance to the introduction of microbial inocula to soils will be the effect of water stress on survival and activity of the introduced organisms. Rattray *et al.* (1992) reported that a *lux*-marked *E. coli* can survive better in soil at higher matric potential; however similar studies by Meikle *et al.* (1995) on *P. fluorescence* showed that increasing the water stress did not have significant effect on activity of *P. fluorescence* compared with a non-soil microbe such as *E. coli*.

In this study the effect of water potential and matric stress on survival and activity of *lux*-marked *Ralstonia eutropha* H850Lr has been investigated. The ability of this organism to degrade polychlorinated biphenyls (PCBs) congeners containing four or more chlorines has previously been reported [1]. *Ralstonia eutropha* H850Lr was used in this study because of having two important characteristics appropriate for investigation on the fate of biodegradative GEMs in environments. First carrying genes for PCBs degradation and second containing bioluminescent monitoring systems allowed for measuring its survival and activity in soil at different conditions.

## Materials and Methods

### Bacterial Strain

A rifampicin resistant strain of *R. eutropha* H850Lr, genetically engineered to express genes for luminescence was used in all studies. Details of its construction, light output characteristics and growth rate comparing to its wild type are provided by Van Dyke *et al.* (1996). This strain produces luciferase, which bears chromosomally integrated *luxA* and *B* genes (the structural genes for luciferase). Light output was determined after addition of exogenous aldehyde substrate, as the construct lacks functional *luxC*, *D* and *E* genes.

### Media, Growth Conditions, and Maintenance

*R. eutropha* H850Lr was routinely grown in tryptic soya broth (Oxoid) and Luria-Bertani (LB) medium

(tryptone, 10 g; yeast extract, 5g; glucose, 1g; distilled water, 1 liter; pH 7) at 30°C. Stock cultures of strain were maintained at -70°C in 20% (v/v) glycerol.

### Soil

A sandy loam soil from the Insch soil series; organic carbon, 2.13%; organic nitrogen 0.19%; pH 7, following amendment with Ca(OH)<sub>2</sub> was employed throughout this study. The soil was sieved to collect the fraction of particle size less than 3 mm. It was air dried and then stored at 4°C.

### Soil Inoculation and Soil Microcosms

Inocula were prepared by plating exponentially grown cells of *R. eutropha* H850Lr from batch cultures grown in LB broth at 30°C. Cells were washed twice in 0.01 M phosphate buffered saline (PBS, pH 7) and resuspended in a volume of buffer sufficient to give the required cell concentration. Cells were then incubated overnight at room temperature (22-25°C) before inoculation into soil.

Soil microcosms consisted of 500 ml screw capped bottles containing 50 g dry weight soil. Soil samples were adjusted to matric potentials of -30, -750, and -1500 kPa using equation:  $J = aM^{-b}$  where *J* is the soil water potential *A* and *B* are constants for a given soil, *M* is the soil moisture content (g water/ g soil) [8]. The equation for the Insch soil was found to be:  $J = 0.005613M^{-5.6509}$ . With the use of the equation the water content of each soil sample at matric potentials of -30, -750 and -1500 kPa would be 0.22, 0.12 and 0.11 ml water g<sup>-1</sup> air dried soil respectively. Matric potentials were maintained by addition of water lost through evaporation.

### Sterile vs. Non-sterile Soil

Soil was sterilized by three times autoclaving for 1 h at 121°C over 3 consecutive days. Six replicate microcosms were set up for each matric potential, inoculated with bacterial strain, also an uninoculated microcosm was conducted as a control. Experiments involving sterile soil were terminated if and when soil became contaminated.

### Soil Sampling and Enumeration of Introduced Cells

Sampling was at set times *i.e.* 0, 1, 2, 4, 6, 9, 12, 15, 30 up to 95 days for both sterile and non-sterile soil. To determine concentrations of viable bacterial cells in soil, soil was thoroughly mixed and 1 g dry weight soil was

taken and suspended in 10 ml phosphate buffer (15 mM, pH 7), shaken for 10 min using a stuart wrist-action shaker. Serial 10-fold dilutions of the soil suspensions were then plated in triplicate on TSA plates supplemented with  $50 \mu\text{g cm}^{-3}$  rifampicin and  $50 \mu\text{g cm}^{-3}$  cyclohexamide. Plates were incubated at  $30^\circ\text{C}$  for 1-2 days prior to enumeration.

### Measurement of Bioluminescence

Bioluminescence was measured by luminometry (Bio-orbit, Turku, Finland) placed in an eppendorf tube and centrifuged at 2000 rpm and  $25^\circ\text{C}$  for 1 min. The luciferase substrate n-dacanal (10  $\mu\text{l}$  of 10% (v/v) suspension in ethanol) was added to 1 ml of supernatant in a luminometer cuvetes and incubated at  $25^\circ\text{C}$  for 5 min. Bioluminescence was expressed in relative light units (RLU) per 10 s.

### Statistical Analysis

Data were analyzed by GLM (general linear model) procedure using the SPSS software package (9.0 for windows 4.0). The SPSS package was also used for one-way analysis of variance. Data were log transformed prior to analysis. Differences were considered to be significant at the  $P < 0.05$  level.

## Results

### Survival of *Ralstonia eutropha* H850 Lr

Following inoculation into sterile soil at  $-2.11$  kPa, an initial increase up to 2 order of magnitude was observed in viable cell concentration (Fig. 1a) and remained in that level for up to 50 days. However, in non-sterile soil at  $-2.11$  kPa viable cell concentration decreased by 1 order of magnitude by day 30, approximately 100 fold less than in sterile soil. In sterile soil viable cell concentration increased slightly at early stages after inoculation at matric potential  $-30$  kPa (Fig. 1b) and from day 20 it started to decrease, showing that although viable population were still large, it was still lower than that observed in initial inocula. In non-sterile soil following inoculation, viable cell concentration after a slight increase at early days, decreased by 1 order of magnitude by day 80 and reached the same cell number as observed in sterile soil (Fig. 1b). An initial increase in the early days was also seen in sterile soil at matric potential  $-750$  kPa and  $-1500$  kPa which was greater than  $-30$  kPa and lower than that at  $-2.11$  kPa (Fig. 1c-1d). Viable cell concentrations in both matric potential ( $-1500$  kPa and  $-750$  kPa) decreased after day

20, however still  $10^7$  cells were alive after 80 days incubation. In non-sterile soil at  $-750$  kPa viable cell did not change during 80 days incubation (Fig. 1c). In spite of small fluctuations in viable cell concentrations at  $-1500$  kPa, significant change was not observed in non-sterile soil at this matric potential (Fig. 1d).

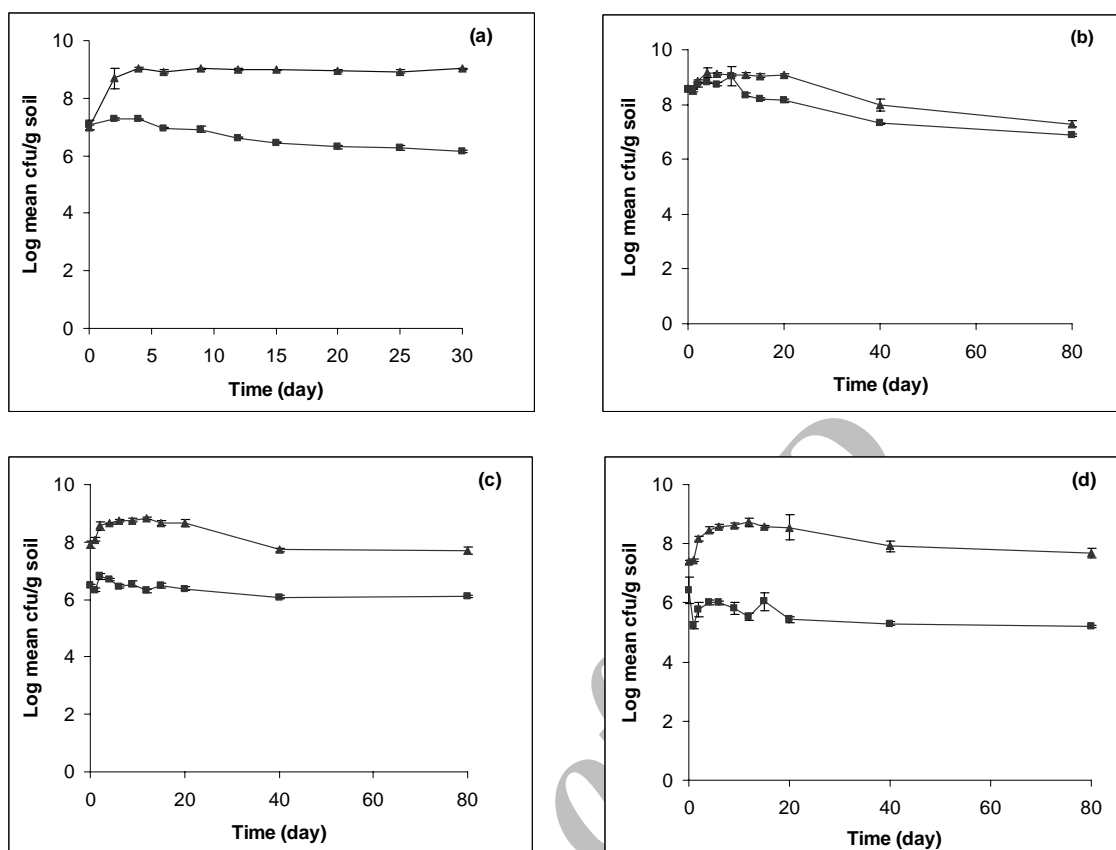
### Luminescence Activity of *Ralstonia eutropha* H850 Lr

Luminescence initially increased in sterile soil and then was relatively constant for 40 days at a level that was higher than initial values (Fig. 2a). However, in non-sterile soil, luminescence gradually decreased by 2 orders of magnitude at this matric potential ( $-2.11$  kPa). In both sterile and non-sterile soils at  $-30$  kPa, light output increased for the first days of incubation and then decreased rapidly by 2 orders of magnitude up to day 40, after which luminescence remained constant (Fig. 2b). An initial increase was observed at  $-750$  kPa in luminescence values of both sterile and non-sterile soils, in spite of lower light output at  $-30$  kPa (Fig. 2c). At  $-750$  kPa, in non-sterile soil, luminescence decreased more rapidly than in sterile soil and differences between sterile and non-sterile soil was more apparent. At  $-1500$  kPa despite greater decline in initial inoculum size, light output in sterile soil changed in similar manner to  $-30$  kPa and  $-750$  kPa (Fig. 2d). In non-sterile soil, luminescence activity changed in very similar manner to viable cell concentration and differences between luminescence values in sterile and non-sterile soil was greater than  $-30$  kPa and  $-750$  kPa.

### Statistical Analysis

GLM procedure was used to calculate the significance of the effect on viable cell concentration and luminescence of three factors, autoclaving, time and their interactions (Table 1). The data shows that all factors had a significant effect on both viable cell concentration and luminescence.

Despite the significance of all three factors (matric potential, time and autoclaving) on viable cell concentration and luminescence, the interactions of the three factors (autoclaving  $\times$  time  $\times$  matric potential) did not have any significant effect on viable cell concentration. The effect of the interactions of autoclaving  $\times$  time and autoclaving  $\times$  time  $\times$  matric potential were less significant than other factors and their interactions (Table 1). Viable cell concentrations were less sensitive to the effect of interactions between time  $\times$  matric potential than other factors and their interactions.



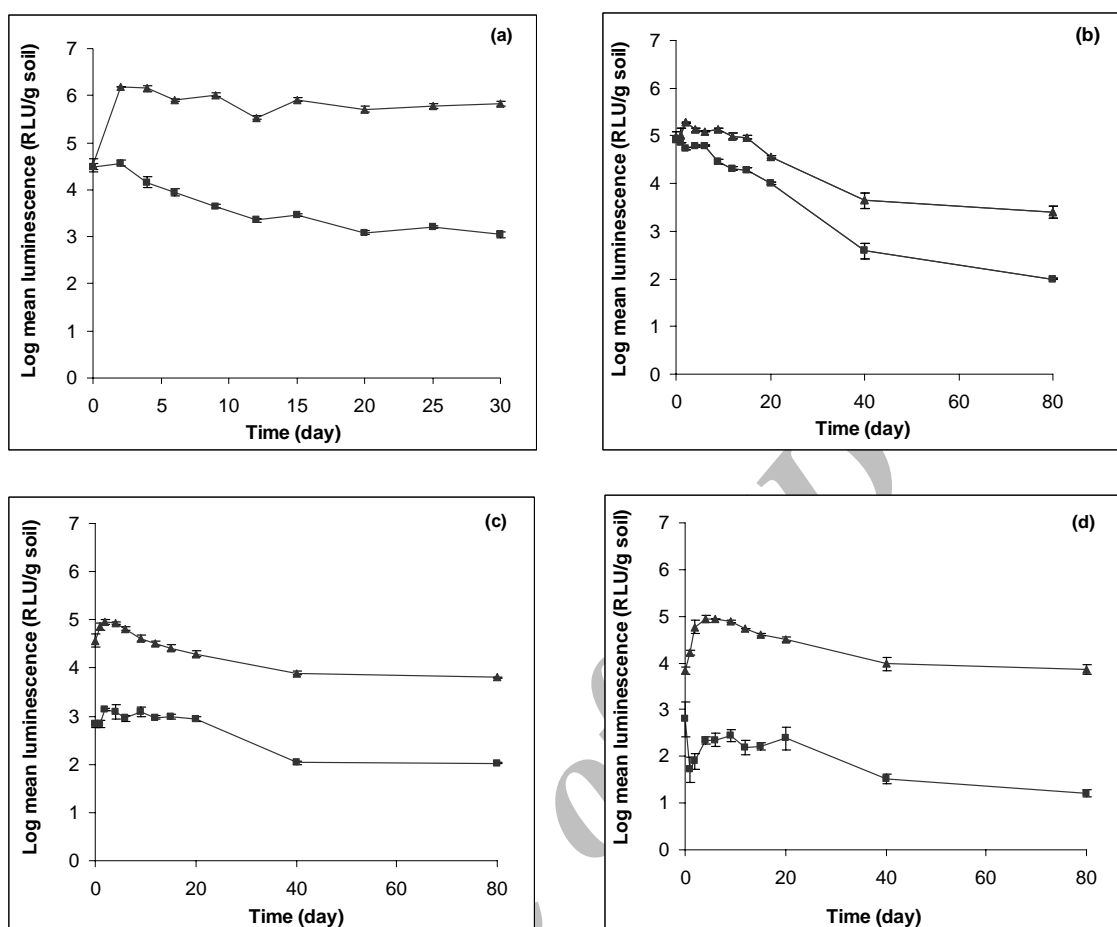
**Figure 1.** Changes in viable cell concentration of *Ralstonia eutropha* H850Lr in sterile (▲) and non-sterile (■) microcosms following incubation at (a)  $-2.11$  kPa, (b)  $-30$  kPa, (c)  $-750$  kPa, and (d)  $-1500$  kPa. Bars on graph indicate standard errors of the mean (SEM). Where no bars are seen, SEMs were less than the symbol size.

### Discussions

The results obtained in this study suggest that matric potential had a significant effect on the viable cell concentration of *R. eutropha* H850Lr following inoculation into soil, which support previous studies on the effects of matric potential by Rattray *et al.* (1992) and Meikle *et al.* (1995). It is found that, because of higher moisture content at  $-2.11$  kPa, and therefore availability of more nutrients, *R. eutropha* H850Lr survived better comparing with lower matric potentials. However, the decrease in viable cell concentrations in sterile soil was more obvious at  $-2.11$  kPa and  $-30$  kPa soil than  $-750$  kPa and  $-1500$  kPa suggesting that matric potentials lower than  $-750$  would probably have the same effect on the survival of this particular microbe. In non-sterile, *R. eutropha* H850Lr could survive better at  $-30$  kPa than at  $-2.11$  kPa with a high moisture content, possibly because of better conditions for this GEM at this matric potential provided in the soil microcosms. The significant difference in viable cell

concentration observed between sterile and non-sterile soil and also among the matric potentials studied in non-sterile soil could be explained by the grazing activities of indigenous protozoa as a significant component in the survival and establishment of bacterial inocula [6]. Also the predatory activities of protozoa are considered to be affected by the matric potential of the soil, since protozoa are dependent upon water for their dispersal and movement through soil [17]. It has been observed that the largest protozoan populations are found to exist in water saturated soils, and the lowest concentrations in dry soil [2].

The prokaryotic *lux* genes encoding luciferase have been used as markers to examine viable but nonculturable (VBNC) state as well as the survival rate of allochthonous bacteria in the environment [26,27]. Eberl *et al.* (1997) showed that by employing a luciferase-marked derivative of *P. putida* KT2442 in combination with a highly sensitive low-light imaging system, live and dead cells could be distinguished.



**Figure 2.** Changes in luminescence in sterile (▲) and non-sterile (■) microcosms inoculated with *R. eutropha* H850 Lr and adjusted to matric potentials (a) -2.11 kPa, (b) -30 kPa, (c) -750 kPa, and (d) -1500 kPa. Bars on graph indicate standard errors of the mean (SEM). Where no bars are seen, SEMs were less than the symbol size.

**Table 1.** Values of probabilities calculated using GLM procedure to assess the effects of matric potential, time and autoclaving soil on viable cell concentration, and luminescence in soil microcosms inoculated with *Ralstonia eutropha* H850Lr

	Viable Cell Concentration	Luminescence
Autoclaving	$P < 3.54 \times 10^{-11}$	$P < 3.54 \times 10^{-11}$
Time	$P < 3.54 \times 10^{-11}$	$P < 3.54 \times 10^{-11}$
Matric Potential	$P < 3.54 \times 10^{-11}$	$P < 3.54 \times 10^{-11}$
Autoclaving × Time	$P < 3.54 \times 10^{-11}$	$2.7 \times 10^{-10}$
Autoclaving × Matric Potential	$P < 3.54 \times 10^{-11}$	$P < 3.54 \times 10^{-11}$
Time × Matric Potential	$3.54 \times 10^{-11}$	$P < 3.54 \times 10^{-11}$
Autoclaving × Time × Matric Potential	0.106	$5.01 \times 10^{-9}$

Luminescence values were closely related to viable cell concentrations, showing the advantage of bioluminescence-marker systems compared to other methods for measuring of activity such as respirometry

and dehydrogenase activity. It is concluded that matric potential is an important factor which has to be carefully considered for better application of GEMs in the environment. For example in a range of matric

potentials from saturated condition to dry condition in non-sterile soil, *R. eutropha* H850Lr could survive better at -30 kPa. Also bioluminescence marker systems used for monitoring the genetically engineered *R. eutropha* H850 Lr proved to be an appropriate marker system for assessing the activity of above GEM even in the presence of indigenous micro organisms. However, in spite of their proficiency in detecting culturable bacteria in natural environments, techniques using luminometry to measure *in situ* activity and potential activity of introduced micro organisms are limited in their ability to confirm the VBNC state. The reason for this is that *in situ* detection of *lux*-encoded luciferase activity is dependent on energy reserves within cells, but energy reserves of bacteria in the environment may be too low to allow *in situ* detection of high energy requiring enzyme systems. Therefore, to overcome a green fluorescence protein (GFP) from *Aequorea victoria* as a marker can be used to verify the VBNC state of *R. eutropha* H850 Lr isolated from the environment. GFP is a very good marker system that can be easily detected by the conventional antibiotic resistance, fluorescence colony counting, and measuring direct fluorescence with spectrofluorometry or epifluorescence microscopy. In addition, the green fluorescent phenotypes are detectable in all growth phases even under starved conditions. Unge *et al.* (1999) developed a dual marker system for simultaneous quantification of bacterial cell numbers and their activity with the *luxAB* and *gfp* genes. Their dual marker system allowed simultaneous monitoring of the metabolic activity and cell number of a specific bacterial population *in situ* in environmental samples.

### References

1. Bedard D.L., Haber M.L., May R.J., and Berennan M.J. Evidence for novel mechanisms of polychlorinated biphenyl metabolism in *Alcaligenes eutrophus* H850. *Appl. Environ. Microbiol.*, **53**: 1103-12 (1987).
2. Darbyshire J.F. Effect of water suction on the growth in soil of the ciliate *Colopoda steinii* and the bacterium *Azotobacter chroococcum*. *J. Soil Sci.*, **27**: 369-376 (1976).
3. Dighton J., Jones H.E., Robinson C.H., and Beckett J. The role of abiotic factors, cultivation practices and soil fauna in the dispersal of genetically modified microorganisms in soils. *Appl. Soil Ecol.*, **5**:109-131 (1997).
4. Dwyer D.F., Rojo F., and Timmis K.N. Fate and behaviour in an activated sludge microcosm of a genetically engineered microorganism designed to degrade substituted aromatic compounds. In: Sussman M., Collins C.H., Skineer F.A., and Stewart-Tull D.E. (Eds), *The Release of Genetically Engineered Microorganisms*. London, San Diego: Academic Press Ltd, pp. 77-88 (1988).
5. Guerin W.F. and Boyd S.A. Maintenance and induction of naphthalene degradation activity in *Pseudomonas putida* and *Alcaligenes* sp. under different culture conditions. *Appl. Environ. Microbiol.*, **61**: 4061-4068 (1995).
6. Habte M. and Alexander M. Protozoan density and the coexistence of protozoan predators and bacterial prey. *Ecology*, **59**: 140-146 (1978).
7. Hickey W.J., Searles D.B., and Focht D.D. Enhanced mineralization of polychlorinated biphenyls in soil inoculated with chlorobenzoate-degrading bacteria. *Appl. Environ. Microbiol.*, **59**: 1194-1200 (1993).
8. Marshall T.J., Holmes J.W., and Rose C.W. Soil physics. 3rd Edition. Cambridge University Press, Cambridge (1996).
9. Meikle A., Amin-Hanjani S., Glover L.A., Killham K., and Prosser J.I. Matric potential and the survival and activity of a *Pseudomonas fluorescens* inoculum in soil. *Soil Biol. Biochem.*, **26**: 747-755 (1995).
10. Park K.S., Sims R.C., Dupont R.R., Doucette W.J., and Matthews J.E. Fate of PAH compounds in types: influence of volatilization, abiotic loss and biological activity. *Environ. Toxicol. Chem.*, **9**: 187-195 (1990).
11. Pickup R.W. and Saunders J.R. Detection of genetically engineered traits among bacteria in the environment. *Trends in Biotechnol.*, **8**: 329-335 (1990).
12. Postma J., Hok-A-Hin C.H., and Oude Voshaar J.H. Influence of the inoculum density on the growth and survival of *Rhizobium leguminosarum* biovar *trifolii* introduced into sterile and non-sterile loamy sand and silt loam. *FEMS Microbiol. Ecol.*, **73**: 49-58 (1990).
13. Rattray E.A.S., Prosser J.I., Killham K., and Glover L.A. Luminescence-Based nonextractive technique for *in situ* detection of *Escherichia coli* in Soil. *Appl. Environ. Microbiol.*, **56**: 3368-3374 (1990).
14. Rattray E.A.S., Prosser J.I., Glover L.A., and Killham K. Matric potential in relation to survival and activity of a genetically modified microbial inoculum in soil. *Soil Biol. Biochem.*, **24**: 421-425 (1992).
15. Ryder M. Key issues in the deliberate release of genetically-manipulated bacteria. *FEMS Microbiol. Ecol.*, **15**: 139-146 (1994).
16. Shaw J.J., Dane F., Geiger D., and Kloepper J.W. Use of bioluminescence for detection of genetically engineered microorganism released into environment. *Appl. Environ. Microbiol.*, **58**: 267-273 (1992).
17. Sleigh M. *The Biology of Protozoa*. Edward Arnold, London, pp. 273-274 (1973).
18. Van Dyke M.I., Lee H., and Trevors J.T. Survival of *lux*-marked *Alcaligenes eutrophus* H850 in PCB-contaminated soil and sediment. *J. Chemical Technol. Biotechnol.*, **65**: 115-122 (1996).
19. Vanderberg P.A. Degradative bacteria. In: Levin M.A., Seidler R.J., and Regul M. (Eds) *Microbial Ecology: Principals, Methods and Application*. New York: Mc Graw-Hill, pp. 435-453 (1992).
20. Volkering F., Breure A.M., Stekenburg A., and van An del J.G. Microbial degradation of polycyclic aromatic hydrocarbons: effect of substrate availability on bacterial

- growth kinetics. *Appl. Microbiol. Biotechnol.*, **36**: 548-552 (1992)
21. Ritchie J.M., Campbell G.R., Shepherd J., Beaton Y., Jones D., Killham K., and Artz R.R.E. A Stable Bioluminescent Construct of *Escherichia coli* O157:H7 for Hazard Assessments of Long-Term Survival in the Environment. *Appl. Environ. Microbiol.*, **69**: 3359-3367 (2003).
  22. Richard J., Whittington D., Marshall J., Nicholls P.J., Marsh I.B., and Reddacliff L.A. Survival and Dormancy of *Mycobacterium avium* subsp. *Paratuberculosis* in the Environment. *Appl. Environ. Microbiol.*, **70**: 2989-3004 (2004).
  23. Waddell, T.E., and Poppe C. Construction of mini-Tn10luxABcam/Ptac-ATS and its use for developing a bacteriophage that transduces bioluminescence to *Escherichia coli* O157:H7. *FEMS Microbiol. Lett.*, **182**: 285-289 (2000).
  24. Turnbull, G.A., Morgan J.A., Whipps W.J.M., and Saunders J.R. The role of bacterial motility in the survival and spread of *Pseudomonas fluorescens* in soil and in the attachment and colonisation of wheat roots. *FEMS Microbiol. Ecol.*, **36**: 21-31 (2001).
  25. Campbell G.R., Prosser J.I., Glover L.A., and Killham K. Detection of *Escherichia coli* O157:H7 in soil and water with multiplex PCR. *J. Appl. Microbiol.*, **91**: 1-7 (2001).
  26. Duncan S., Glover L.A., Killham K., and Prosser J.I. Luminescence-based detection of activity of starved and viable but nonculturable bacteria. *Appl. Environ. Microbiol.*, **60**: 1308-1316 (1994).
  27. Oliver J.D., McDougald D., Barrett T., Glover L.A., and Prosser J.I. Effect of temperature and plasmid carriage on nonculturability in organisms targeted for release. *FEMS Microbiol. Ecol.*, **17**: 229-238 (1995).
  28. Eberl L., Givskov M., Poulsen L.K., and Molin S. Use of bioluminescence for monitoring the viability of individual *Pseudomonas putida* KT2442 cells. *FEMS Microbiol. Lett.*, **149**: pp. 133-140 (1997).
  29. Unge A., Tombolini R., Molbak L., and Jansson J.K. Simultaneous monitoring of cell number and metabolic activity of specific bacterial populations with a dual *gfp-luxAB* marker system. *Appl. Environ. Microbiol.*, **65**: 813-821 (1999).