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Development of an acute and chronic ecotoxicity assay using *lux*-marked *Rhizobium leguminosarum* biovar *trifolii*

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G.I. PATON, G. PALMER, M. BURTON, E.A.S. RATTRAY, S.P. McGRATH, L.A. GLOVER AND K. KILLHAM. 1997. A soil isolate of *Rhizobium leguminosarum* bv. *trifolii* was marked with a *lux* CDABE gene cassette to enable the expression of bioluminescence. The suitability of the bacterium as a soil pollution biosensor was assessed using acute and chronic assays. Bacterial bioluminescence responded sensitively to the metals studied. The order of sensitivity was found to be Cd > Ni = Zn > Cu for the acute test and Cd > Ni = Zn = Cu for the chronic test. The sensitive response of the biosensor highlighted its potential for use as an indicator of soil pollution.

INTRODUCTION

There is a need to develop rapid, relevant and sensitive soil ecotoxicity bioassays. The Royal Commission Report (1996) highlighted the need to consider microbial parameters for soil ecotoxicity assessment. To assess soil pollution, a wide range of microbial assays may be carried out including growth inhibition (Trevors *et al.* 1981), microbial biomass (Brookes and McGrath 1984), respirometry (Montgomery 1981), survival of specific organisms (Chaudri *et al.* 1992), viability of cells (Williamson and Johnson 1981), mycorrhizal inhibition (Killham and Firestone 1983) and wider ecological effects (Bitton and Dutka 1986).

Giller *et al.* (1989) identified *Rhizobium leguminosarum* bv. *trifolii* as a particularly sensitive indicator of soil pollutants. This has been demonstrated using nodulation assays (Giller *et al.* 1989) and growth inhibition studies (Chaudri *et al.* 1992). While these methods demonstrate the sensitivity of *Rh. leguminosarum* bv. *trifolii*, they are time consuming, requiring a large number of replicates, and a period of incubation sufficient for colonies to develop for enumeration.

The use of bioluminescence-based biosensors has become widely adopted as a sensitive method in microbial ecotoxicity assessment (Steinberg *et al.* 1995). The Microtox assay, which uses the naturally bioluminescent marine bacterium, *Vibrio fischeri*, has become internationally adopted as a microbial biosensor, but the choice of bacterium is ecologically inappropriate for soil testing. Paton *et al.* (1995a) were able to demonstrate the sensitivity and rapid response of an ecologically relevant *lux*-based biosensor using the soil rhizobacterium *Pseudomonas fluorescens*.

This paper describes the work carried out to *lux* mark a soil isolate of *Rh. leguminosarum* bv. *trifolii* and to assess the sensitivity of the bacterium to metal-contaminated solutions using acute and chronic ecotoxicity screening assays. The paper then discusses the feasibility of using these assays for soil ecotoxicity assessment.

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MATERIALS AND METHODS

Bacterial strains and culturing conditions

Rhizobium leguminosarum bv. *trifolii* isolate F6 (obtained from IACR-Rothamsted) was routinely grown in yeast extract mannitol glutamate medium (YEMG) (Johnston and Wood 1987) at 25°C. Antibiotic resistance characterization showed that this isolate was spectinomycin-resistant at a concentration of 100 μg ml^{-1}. *Escherichia coli* was routinely cultured in LB broth at 37°C (Amin-Hanjani *et al.* 1994). Where appropriate, media were supplemented with kanamycin (Km) (40 μg ml^{-1}), spectinomycin (Sp) (100 μg ml^{-1}) and ampicillin (Amp) (25 μg ml^{-1}). *Escherichia coli* HB101 (pRK2013) (Km^r) and HB101 (pUCD607) (Km^r, Amp^r) were used as helper and donor strains, respectively. Plasmid pUCD607 contains the *Vibrio fischeri lux*CDABE genes under the control of the

constitutive tetracycline resistance gene promoter (Shaw and Kado 1986).

Construction of *lux*-marked *Rhizobium leguminosarum* bv. *trifolii* F6

A triparental mating was performed by mixing cells of *Rh. leguminosarum* bv. *trifolii* F6, HB101 (pUCD607) and HB101 (pRK2013) in late exponential phase, which had been grown in the appropriate antibiotic-containing media. The cells were mixed by transferring 50 μl from each culture to 5 ml of sterile KCl (0·85%). The bacterial suspension was filtered through a sterile 0·22 μm Whatman cellulose acetate filter membrane under vacuum. The filter membrane was then placed, cell side uppermost, on YEMG agar plates (Johnson and Wood 1987) using sterile tweezers and incubated 30°C for 5 h to allow mating to occur. Following incubation, the filters were shaken with 5 ml of sterile 0·85% KCl. Aliquots of 100 μl were plated onto a selective medium, and incubation continued at 25°C until colonies of transconjugants appeared. Transconjugants containing pUCD607 were confirmed by detecting luminescence.

Acute toxicity testing with *Rh. leguminosarum* bv. *trifolii* F6 pUCD607

Stock solutions of metal pollutants were made up by weighing 555·555 mg of Zn, Cu, Cd and Ni as sulphate salts in volumetric flasks. Aliquots of 200 ml of sterile H_2O (dd) were added to each flask before 1 ml of 1 mol l^{-1} HNO_3 was added. The volume was made up to the 500 ml mark with sterile H_2O (dd). The stock solution was then transferred to a sterile acid-washed glass Duran bottle and stored at 4°C.

Standard test solutions were prepared for Zn, Cu, Cd and Ni at concentrations of 0, 0·5 1, 2, 5, 10, 14 and 25 mg l^{-1}. Samples were adjusted to pH 5·50 ($\pm$ 0·04) with 0·01 mol l^{-1} HCl or 0·01 mol l^{-1} NaOH and stored at 25°C.

A culture was grown in 100 ml of YEMG with 100 mg l^{-1} spectinomycin in a 250 ml Erlenmeyer flask shaken at 180 rev min^{-1} at 25°C until a cell concentration of 10^8 cfu ml^{-1} was obtained. The required volume of cell suspension was removed from the Erlenmeyer flask and centrifuged at 7550 *g* in a Micro Centaur MSC for 1 min. The supernatant fluid was discarded and the pellet was resuspended in 0·1 mol l^{-1} KCl. The assay procedure as described by Paton *et al.* (1995b) was then carried out using a Bio-orbit 1251 luminometer.

Chronic toxicity testing with *Rh. leguminosarum* bv. *trifolii* F6 pUCD607

A cell suspension of *Rh. leguminosarum* bv. *trifolii* was inoculated into 30 ml of YEMG and incubated on an orbital shaker at 150 rev min^{-1} at 28°C for 3 d. Ten ml aliquots were transferred to 25 ml Corning centrifuge tubes and centrifuged at 28°C for 10 min at 3000 *g*. The supernatant fluid was discarded and the cells resuspended in 10 ml of sterile deionized water. The washing procedure was repeated twice with fresh sterile water before the washed cells were resuspended in 10 ml of sterile water.

Test solutions of the metals were made by diluting the stock solutions as required, filter sterilizing the solution through a 0·22 μm cellulose acetate membrane, adding a 10 ml aliquot of the metal solution to 90 ml of YEMG and adjusting the pH to 6·8 by the addition of HCl or NaOH. The test solutions were inoculated with 30 μl of the washed cell suspension to give a concentration of *ca* 10^6 cfu ml^{-1}, and incubated at 28°C on an orbital shaker at 105 rev min^{-1} for 72 h. Cell viability was assessed at 0, 5, 10, 27 and 72 h as described by Miles and Misra (1938), with quarter strength Ringer's solution as the diluent on YEMG plates. Bioluminescence was measured as described by Paton *et al.* (1995a).

ANALYSIS OF RESULTS

Results were analysed using analysis of variance on the statistical package Genstat 5 Rel 2.2 (Genstat 5 Committee 1991). Significant differences were assessed using the least significant difference (LSD) test at the $P \leqslant 0{\cdot}05$ level. The effective concentration 50 (EC_{50}) is the concentration of metal at which there was a 50% decline in the measured response (either bioluminescence or cfu ml^{-1}) when compared against the non-metal contaminated standard.

EC_{50} values for metals were calculated using the plotted graph of bioluminescence/cfu ml^{-1} as a percentage of the maximum against metal concentration. Negative exponential curves were fitted by non-linear regression techniques to triplicate data sets for each metal. The decline in bioluminescence was described by the exponential curve, $Y = a + br^x$, where $Y =$ % bioluminescence, $X =$ metal concentration, $a =$ lower asymptote, $b =$ range of possible Y-values and $r =$ rate parameter. Curves were fitted using Genstat 5 Rel 2.2 and EC_{50} values were calculated by solving the equation for values of $Y = 50\%$ for each of the three replicates of the individual metals.

To enable a comparison of the different techniques and pollutants used in these experiments, mean EC_{50} and LSD were calculated using a one-way analysis of variance. Restricted maximum likelihood (REML) was used to calculate pooled estimates of variance, for pairwise tests of significance.

RESULTS

Acute toxicity response of *Rh. leguminosarum* bv. *trifolii* F6 pUCD607

The bioluminescence response of *Rh. leguminosarum* bv. *trifolii* F6 pUCD607 was sensitive to the metals tested. The

EC_{50} values (Table 1) were 0·06 mg l^{-1} for Cd, 0·42 mg l^{-1} for Cu, 0·48 mg l^{-1} for Ni and 0·94 mg l^{-1} for Zn. All of these values were significantly lower than those recorded for the Microtox assay as performed by Paton *et al.* (1994). The *lux*-based assay using *Rh. leguminosarum* bv. *trifolii* was more sensitive to Cd than the *lux*-based assay using *Ps. fluorescens,* although *Rh. leguminosarum* bv. *trifolii* was less sensitive to Zn, Cu and Ni.

Chronic toxicity response of *Rh. leguminosarum* bv. *trifolii* F6 pUCD607

The sensitivity of the bioluminescence response of *Rh. leguminosarum* bv. *trifolii* F6 pUCD607 to metals was found to increase with time (Table 2). The EC_{50} value for the *lux*-based assay for Zn decreased from 1·71 mg l^{-1} for 5 h to 0·44 mg l^{-1} after 10 h. There was no significant difference between 10, 27 or 72 h. The EC_{50} values for Cu and Ni were most sensitive at 72 h with values of 0·51 mg l^{-1} and 0·32 mg l^{-1}, respectively. There was no significant difference in the EC_{50} value for Zn, Cu and Ni at 72 h. *Rhizobium leguminosarum* bv. *trifolii* was most sensitive to Cd, and the EC_{50} value declined from 0·14 mg l^{-1} after 5 h to 0·01 mg l^{-1} at 72 h.

The chronic assay, based on the measurement of cfu ml^{-1}, was also found to be sensitive to metal pollutants (Table 2). The EC_{50} value for Zn dropped from 2·99 mg l^{-1} after 5 h to 0·37 mg l^{-1} after 27 h but did not significantly change thereafter. The EC_{50} value for Ni dropped from 2·68 mg l^{-1} at 5 h to 2·42 mg l^{-1} at 10 h and continued to drop to 0·82 mg l^{-1} at 27 h and 0·28 mg l^{-1} at 72 h. The response to Cd was also found to decline with time from 2·72 mg l^{-1} at 5 h to 0·13 mg l^{-1} at 72 h. The assay was found to be most sensitive to Cu with an EC_{50} of 0·01 mg l^{-1} at 5 h, remaining unchanged at 10 and 27 h. At 72 h, there was no evidence of culturability of *Rhizobium* in any of the Cu contaminated samples and therefore it was not possible to predict an EC_{50} value.

At 5 and 10 h, the *lux*-based technique was more sensitive to all metals except Cu. At 27 h, the *lux*-based method was more sensitive to Cd and Ni than the cfu enumeration technique. There was no significant difference between the two techniques for Zn, although the cfu enumeration procedure was more sensitive for Cu than the bioluminescence measurement method. At 72 h, there was no significant difference between the two techniques for Zn and Ni, although the *lux*-based method was more sensitive to Cd, and the cfu method remained more sensitive for Cu.

Table 1 EC_{50} values (mg l^{-1}) of four metals for a range of acute bioluminescence-based biosensors

	Rhizobium trifolii F6 pUCD607	*Pseudomonas fluorescens* pUCD607*	Microtox*
Zn	0·94[a]	0·09[b]	2·35[c]
Cu	0·42[d]	0·09[b]	1·89[e]
Cd	0·06[b]	0·17[f]	9·78[g]
Ni	0·48[d]	0·28[h]	4·35[i]

Values followed by the same superscript letter were not significantly different ($P \leqslant 0·05$) as determined by ANOVA for assay and pollutant.
*As Paton *et al.* (1994).

DISCUSSION

The sensitivity of *Rh. leguminosarum* bv. *trifolii* to the metal pollutants tested (Chaudri *et al.* 1992) makes it an ideal biosensor for acute soil ecotoxicity testing at environmentally relevant pollutant concentrations. Typical solution concentrations in sewage sludge amended soils may be in the range of 0·1–0·4 mg l^{-1} for Cu, 0·1–3·6 mg l^{-1} for Ni and 0·5–26·1 mg l^{-1} for Zn (Campbell *et al.* 1995). The differences in response of *Rhizobium* and *Ps. fluorescens* to metal pollutants may reflect the different ecological niches that these organisms occupy in the soil. The application of both of these biosensors, *Rh. leguminosarum* bv. *trifolii* and *Ps. fluorescens*, could provide the basis for a battery of microbial biosensors for soil pollution monitoring.

Table 2 EC_{50} values (mg l^{-1}) for four metals as assessed by chronic assay using *Rhizobium leguminosarum* bv. *trifolii* F6 pUCD607 (EC_{50} values determined by bioluminescence and cfu ml^{-1})

	5 h		10 h		27 h		72 h	
Time	*lux*	cfu	*lux*	cfu	*lux*	cfu	*lux*	cfu
Zn	1·71[a]	2·99[b]	0·44[c]	1·86[a]	0·37[c]	0·32[c]	0·36[c]	0·28[c]
Cu	2·22[d]	0·01[e]	0·91[f]	0·01[e]	0·84[f]	0·01[e]	0·51[c]	ND
Cd	0·14[g]	2·72[h]	0·09[i]	0·41[c]	0·05[i]	0·26[c]	0·01[e]	0·13[g]
Ni	2·11[j]	2·68[k]	0·45[c]	2·42[l]	0·72[m]	0·82[n]	0·32[c]	0·28[c]

Values followed by the same superscript letter were not significantly different ($P \leqslant 0·05$) as determined by ANOVA for assay and pollutant.
ND, Not determined.

The results of the chronic assay after 72 h (the point at which Chaudri *et al.* (1992) compared the response of metal-tolerant and non-tolerant strains of *Rh. leguminosarum* bv. *trifolii*) is an appropriate end point for the chronic assay. The bioluminescence measured corresponded with the cfu measurement for both Zn and Ni, although bioluminescence was more sensitive to Cd than the cfu method. The cfu method, which was very sensitive to Cu, required a 48 h incubation period (for colonies to form to enable enumeration). The bioluminescence technique offered a rapid and convenient method to assess growth inhibition of *Rhizobium*. While this is an ideal method for routine analysis, the procedure is not as sensitive to Cu as the cfu technique. In the case of Cu, cfus were found to decline rapidly after commencement of the experiment, although bioluminescence remained high. This suggests that although there were fewer cells, those that remained viable were metabolically more active and exhibited greater bioluminescence. In the case of Cd, the opposite observation was found in that the decline in bioluminescence was greater than the decline in cfus.

The level of bioluminescence did not correspond to colony counts (cfus). This may suggest that particular metals (for example Cu) may induce the *Rhizobium* to enter the viable but non-culturable state (VBNC). This phenomenon has been associated with environmental stresses including temperature (Wolf and Oliver 1992) and nutrient limitation (Binnerup *et al.* 1993; Turpin *et al.* 1993) but has yet to be linked with metal stress. If *Rhizobium* and other soil bacteria enter a VBNC state as a result of metal stress then microbial ecotoxicity tests that rely on culturability may be inappropriate to assess the microbial activity and diversity of contaminated soils. It may be essential to use *lux*-based detection systems (Duncan *et al.* 1994), activity stains (Kogure *et al.* 1979) or molecular techniques (Brauns *et al.* 1991) to assess cell viability in metal-contaminated environments, but it is also important to consider that cells detected by these techniques may be non-culturable.

CONCLUSION

The use of bioluminescence-based soil biosensors offers a rapid and sensitive technique for soil ecotoxicity assessment. The ability to insert the *lux* genes into a range of terrestrial bacteria enables ecological relevance to be combined with a convenient screening tool. By combining the *Rh. leguminosarum* bv. *trifolii* and *Ps. fluorescens lux*-based assays for soil toxicity assessment it may be possible to develop a microbial battery test system to assess the acute and chronic response of bacteria from different ecological niches to pollutants in the soil system. The use of *Rh. leguminosarum* bv. *trifolii* as a chronic assay also suggested that cells may enter a VBNC stage in a metal-contaminated environment. If certain pollutants induce soil bacteria to become viable but non-culturable then molecular techniques such as DNA probes and genetically marked micro-organisms may offer the best opportunity to comprehensively assess the effect of contaminants on the soil microbial community. However, the ecological relevance of non-culturability in soils also needs to be assessed.

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REFERENCES

Amin-Hanjani, S., Meikle, A., Glover, L.A., Prosser, J.I. and Killham, K. (1993) Plasmid and chromosomally encoded luminescence marker systems for detection of *Pseudomonas fluorescens* in soil. *Molecular Ecology* **2,** 47–54.

Binnerup, S.J., Jensen, D.F., Thordalchristensen, H. and Sorensen, J. (1993) Detection of viable but non-culturable *Pseudomonas fluorescens* DF57 in soil using a microcolony epifluorescence technique. *FEMS Microbiology Ecology* **12,** 97–105.

Bitton, G. and Dutka, B.J. (1986) Introduction and review of microbial and biochemical toxicity screening procedures. In *Toxicity Testing Using Microorganisms* ed. Dutka, B.J. and Bitton, G. Vol. II, pp. 1–8. Boca Raton, FL: CRC Press.

Brauns, L.A., Hudson, M.C. and Oliver, J.D. (1991) Use of the polymerised chain reaction in detection of culturable and non-culturable *Vibrio vulnificus* cells. *Applied and Environmental Microbiology* **57,** 875–878.

Brookes, P.C. and McGrath, S.P. (1984) Effects of metal toxicity on the size of the microbial biomass. *Journal of Soil Science* **35,** 341–346.

Campbell, C.D., Warren, A., Cameron, C.M. and Hope, S.J. (1995) Use of a soil protozoan assay to assess the bioavailability of metals in a long term sewage sludge treated soil. In *Heavy Metals in the Environment* ed. Wilken, R.-D., Forstner, U. and Knochel, A. Vol. 2. Hamburg, September 1995. Edinburgh: CEP Consultants.

Chaudri, A.M., McGrath, S.P. and Giller, K.E. (1992) Metal tolerance of isolates of *Rhizobium leguminosarum* bv *trifolii* from soil contaminated with past applications of sewage sludge. *Soil Biology and Biochemistry* **24,** 83–88.

Duncan, S., Glover, L.A., Killham, K. and Prosser, J.I. (1994) Luminescence-based detection of activity of starved and viable but non-culturable bacteria. *Applied and Environmental Microbiology* **60,** 1308–1316.

Giller, K.E., McGrath, S.P. and Hirsch, P.R. (1989) Absence of nitrogen fixation in clover grown on soil subjected to long term contamination of heavy metals is due to survival of only ineffective *Rhizobium*. *Soil Biology and Biochemistry* **21,** 841–848.

Johnson, A.C. and Wood, M. (1987) Deionised distilled water as a medium for aluminium toxicity studies of *Rhizobium*. *Letters in Applied Microbiology* **4,** 137–139.

Killham, K. and Firestone, M.K. (1983) Vesicular-arbuscular mycorrhizal mediation of grass response to acidic and heavy metal depositions. *Plant and Soil* **72,** 39–48.

Kogure, K., Simidu, U. and Taga, N. (1979) A tentative direct microscopic method for counting living marine bacteria. *Canadian Journal of Microbiology* **25,** 415–420.

Ministry of Agriculture Fisheries and Food/Department of the Environment (1993) Review of the rules for sewage sludge application to agricultural lang. Soil fertility aspects of potentially toxic elements. Report of an independent scientific committee. 89 pp. London: MAFF Publications.

Miles, A.A. and Misra, S.S. (1938) The estimation of the bactericidal power of blood. *Journal of Hygiene, Cambridge* **38,** 732–748.

Montgomery, H.A.C. (1981) The determination of biochemical oxygen demand by respirometric techniques. *Water Research* **1,** 631–637.

Paton, G.I., Campbell, C.D., Cresser, M.S., Glover, L.A., Rattray, E.A.S. and Killham, K. (1994) Bioluminescence-based ecotoxicity testing of soil and water. *OECD International Workshop on Bioremediation,* Tokyo, Japan. pp. 547–551.

Paton, G.I., Campbell, C.D., Glover, L.A. and Killham, K. (1995a) Assessment of bioavailability of heavy metals using *lux* modified constructs of *Pseudomonas fluorescens*. *Letters in Applied Microbiology* **20,** 52–56.

Paton, G.I., Palmer, G., Kindness, A., Campbell, C.D., Glover, L.A. and Killham, K. (1995b) The use of luminescence-marked bacteria to assess the toxicity of malt whisky distillery effluent. *Chemosphere* **31,** 3217–3224.

Royal Commission on Environmental Pollution (1996) No. 19, Sustainable Use of Soils. February 1996, Cm 3165, 260 pp. London: HMSO

Shaw, J.J. and Kado, C.I. (1986) Development of a *Vibrio* bioluminescence gene-set to monitor phytopathogenic bacteria during the ongoing disease process in a non-disruptive manner. *Biotechnology* **4,** 560–564.

Steinberg, S.M., Poziomek, E.J., Englemann, W.H. and Rogers, K.R. (1995) A review of environmental applications of bioluminescence measurements. *Chemosphere* **30,** 2155–2197.

Trevors, J.T., Mayfield, C.I. and Innis, W.E. (1981) A rapid toxicity test using *Pseudomonas fluorescens*. *Bulletin of Environmental Contamination and Toxicology* **26,** 433–437.

Turpin, P.E., Maycroft, K.A., Rowland, C.L. and Wellington, E.M.H. (1993) Viable but non-culturable Salmonellas in soil. *Journal of Applied Bacteriology* **74,** 421–427.

Williamson, K.J. and Johnson, D.G. (1981) A bacterial bioassay for the assessment of wastewater quality. *Water Research* **15,** 383–391.

Wolf, P.W. and Oliver, J.D. (1992) Temperature effects on the viable but nonculturable state of *Vibrio vulnificus*. *FEMS Microbial Ecology* **101,** 33–39.