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Surveillance of Genetically Modified Microoganisms in the Environment

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ABSTRACT: The economic benefits of using genetically engineered microorganisms (GEMS)/GMMOs seems to be very high, especially in the improvement of agriculture, food stocks and in the regulation of environmental pollution due to pesticides and heavy metals. Surveillance of GMMs after addition and their survival in the environment is vital for the safety of our environment. It can be done by marking or tagging of genes in GMMOs conferring specific phenotypic characteristics which enable 'tracking' of the tagged organisms after their release into the environment. Use of molecular markers in the broadest sense refers to any chemical or molecule by which an organism or group of organisms can be easily identified. In this area certain workers utilized the role of whole genome sequencing to characterize and detect unknown and illegal genetically transformed bacterium in commercial microbial industrial products like enzymes and food products. Recently, luciferase gene responsible for emitting light has also been used for the tracking of genetically engineered microorganisms. This article is an attempt to review critically all the available methods (biochemical and genetic) in areas of surveillance of genetically engineered microorganisms (GEMS) and/or genetically modified microorganisms (GMMOs).

Keywords: Environment, Detection, GEMs, GMMOs, Molecular markers, Monitoring.

I. INTRODUCTION

The recombinant DNA molecular techniques are more useful in tracking genetically engineered microorganisms (GEMs) in the environment due to high rate of selectivity and sensitivity. The use of genetically modified microorganisms to obtain food enzymes (FE) by the food industry has rapidly increased [1, 2]. However, we need to have a sensitive, safe and cost-effective methods for the detection of GEMS/GMMOs before their release into the environment. Once in the environment they may cause drastic physio biochemical changes of the local soil microbial community [3, 4]. Unauthorized genetically modified bacteria in commercialized food enzyme (protease) have been notified [5]. In fact, several detection methods have been designed for the surveillance and enumeration of GMMs in the environment like direct plate count method, most-probable-number (MPN) method, microscopical count, serology, immune fluorescence and immune-radiography [6-9]. However, PCR based detection methods are generally accepted as the most sensitive and reliable method [10, 11]. A comparison of the direct plate counting method and MPN method with either multiple PCR or DNA dot blot were made for the detection of genetically modified *E. coli, P. putida*, and *A. oleivorans* harboring either the GFP gene or lacZ gene as an additional marker [12]. Their data demonstrated that the MPN-dot blot is highly efficient and is like MPN-PCR. These techniques fall into several groups as given below:

(i) Molecular Markers: Molecular markers that can easily differentiate the released GEMs from the natural population can be of following three types:

1. Functional genetic system based on a selective characteristic (e.g., emit light or resistance to antibiotics or the ability to utilize specific substrate), or a non-selective characteristic in form of a unique biomarker like synthesis of unique cell wall proteins.
2. Chromogenic markers like XylE [13] and lac YZ (beta glucuronidase) [14] markers. These markers give a distinctive colored appearance to the bacterial colonies
3. Short and specific oligonucleotide sequences (genes). Such sequences are detected by nucleic acid hybridization probes. These genes are incorporated into a plasmid which is then introduced into the host to be released into the environment. Addition of a new genetic material will hamper the viability and survival rate of the engineered microbes in the environment. This may be due to the maintenance of the marker system which impart an additional metabolic burden on the host. However, transferring the marker system on to the chromosomes may reduce the maintenance budget and expression of the controlled genes will be advantageous. Winstanley *et al.,* [13] has developed an effective system for XylE marker genes. By repressing the XylE genes bacteria containing this marker can be easily isolated on solid medium. In order to identify the target organisms, requires its activation at a temperature of 37°C for one hour. This was followed by the spraying of the substrate (catechol).

These colonies can be distinguished by the appearance of a dark yellow colour due to the formation of 2-hydroxymuconic semialdehyde. Thus, it will be useful in monitoring the metabolism of most aromatic hydrocarbons which are degraded via catechol pathway (Fig. 1).

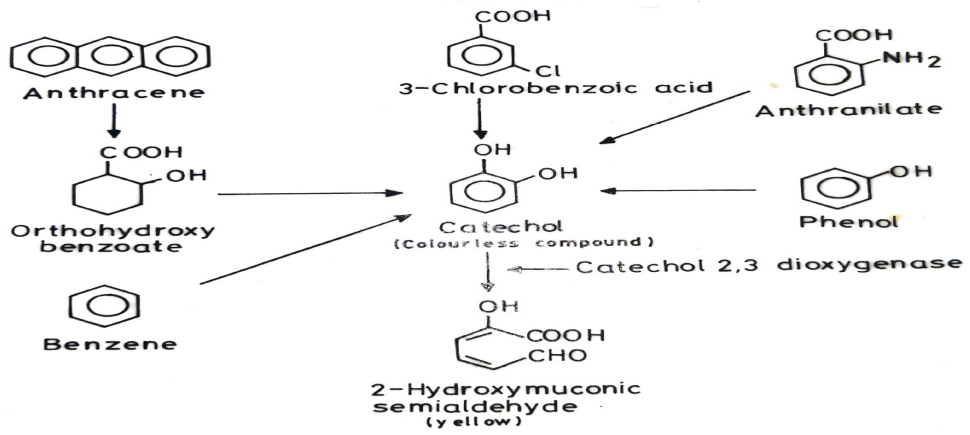

Fig. 1. Conversion of most aromatic hydrocrarbon through catechol (colourless compound) to 2-hydroxymuconic semialdehyde (yellow product) via catechol, 2, 3-dioxygenase.

The problem of lack of sensitivity and instability of plasmid constructs in other markers system was resolved by converting phenoxy acetate (PAA) to phenol by 2-4, dichlorophenoxyacetate (TFD) monooxygenase. Strains of *Pseudomonas aeruginosa* and *P. putida* containing plasmids were constructed [15]. They found that strains in which TFD monooxygenase was deregulated, colonies could be detected by applying aerosols with PAA. Under alkaline conditions it combines with phenol to produce a red antipyrine dye. This will enable in identifying colonies against the background population quite easily. This technique enabled detection of 10^3 cells/ml and this has a potential to be used for the detection of other environmental samples. However, this system applies to microorganisms specifically to bacteria which are unable to utilize phenols as carbon source. Another potential bacterial marker system that can be detected at very low levels uses the GUS (beta-glucuronidase gene from *E. coli*) system, which produces a fluorogenic product [14]. Shaw & Kado, [16] improved the technique further that uses visual markers of the lux operon cassette from *Vibrio fischeri*, in which the bacteria are identified by their ability to bioluminescence. This lux operon system has now been introduced into different types of bacteria, identifying them by a detectable bioluminescent phenotype [17].

A bioluminescent reporter plasmid for naphthalene catabolism (pUTK21) was developed by transposon (Tn 4431) insertion of the lux gene cassette from *Vibrio fischeri* into a naphthalene catabolic plasmid in *Pseudomonas fluorescence*. The insertion site of the lux transposon was the nahG gene encoding for salicylate hydroxylase. Strains harboring pUTK21 were responsive to aromatic hydrocarbon contamination and produced sufficient light to serve as biosensors of naphthalene exposure and reporters of naphthalene biodegradative activity [18].

(ii) Luminometry: Light emitted by organisms marked with lux genes can be quantified using luminometry. Cell number or biomass is directly proportional to the light emitted (luminescence). In the bacteria luminescence is because of the enzyme luciferase which in the presence of reducing oxygen and the substrate n-tetradecyl aldehyde, although other long chain fatty aldehydes e.g., dodecanal can act as substrate.

Luciferase

$$\underset{\text{Aldehyde}}{RCHO}+FMNH+O_2 \rightarrow \underset{\text{Fatty acid}}{RCOOH}+FMN+H_2O + \text{Light}$$

The use of bioluminescence as a genetic marker in biological systems has increased with the cloning of the lux genes from naturally bioluminescent organisms and genetic analysis of their components. Transgenic incorporation of the lux operon provided a non-labor intensive, sensitive detection method for monitoring of GEMs in the environment. As few as 5×10^{-1} CFU/g of soil in the inoculum were detected by this method [19]. Luminometry has been used extensively to detect the presence of bioluminescent recombinant *E. coli* in soil. However, the applicability of the lux system to long term monitoring of GMOs in the environment has been limited to an 8-week sampling period [20].

(iii) DNA Probes: Nucleic acid probe techniques are based on the reannealing of two complimentary, denatured single stranded DNA transferred to a filter. There are two methods of transferring the target DNA. (i) DNA can be transferred directly after extraction and purification (ii) transferred after restriction analysis and electrophoretic separation. Both these methods are quite cumbersome, and the screening of a few bacterial isolates can be achieved. Many workers have successfully used colony hybridization, where cells are cultured and lysed directly on to the filter and several thousand colonies can be screened simultaneously [21].

This technique of nucleic acid is well documented and can be effectively used to identify and detect bacteria in the environment provided suitable probes are available. Therefore, it is possible to use nucleic acid hybridization to detect the presence of specific nucleic acid sequences, oligonucleotides, functional recombinant genes directly in environment samples without first having to culture target bacteria. Of all the strategies available, colony hybridization has been the most successful. It has been widely used to detect a variety of microorganisms carrying specific characters [22]. For example, toluene degrading bacteria [23], PCB degrading bacteria [24] and mercury resistant bacteria [25] have been monitored using colony hybridization techniques. Quantitative assessment of transferring of traits within the indigenous population is possible through culturing of cells. However, it is difficult to detect target microorganisms or its nucleic acid when these organisms do not contain enough of the target genes. Therefore, we would like to design methods that enhances the detection limits. High specific-activity probes can be used in dot blot procedures to detect sub picogram levels of DNA [26]. However, this approach is limited when the target DNA comprises a very small fractions of the total DNA. Walia *et al.,* [27] successfully used two recombinant plasmid, pAW 6164 and pAW 313 as DNA probes to detect PCB degrading genotypes by colony hybridization and dot blot assays. It is suggested that combination of DNA probe and biodegradation assay be used for accurate assessment of bacteria or GEMs in the environment. Steffan and Atlas [28] used another strategy to screen large amounts of total DNA from a target sample. This technique is known as solution hybridization which relies on the specific hybrid formation between target DNA and radio-labelled single-stranded DNA probes. Once hybridization is over, nonhybridized probe along with unincorporated nucleotides is removed and the amount of double stranded radiolabeled hybrid is measured. This method can detect between 10^2 and 10^3 cells/gram soil sample [28].

(iv) Polymerase Chain Reaction: Amplification of DNA by polymerase chain reaction (PCR) allows detection of cells that are present in very low amount in the natural sample. This technique is generally used to increase the target sequences to enhance the relative concentration of the target DNA in a sample. By using this technique, Steffan and Atlas [10] showed as little as 0.3 picogram of target DNA which was equivalent to 100 target organisms in 100 g of soil against a background of 10^{11} non-target microorganisms (Table 1).

Table 1: Detection levels of different methods used for monitoring of GEMs.

Method	Target	Cell/mlor /g	Native Bacterial Population	References
Viable-non-selective-plating	Xy1E	10^3	10^6	Morgan *et al*., [29]
Viable selective plating	RP4-To1	10	10^6	Pickup *et al.* [30]
Bioassay	Xy1E	10^3	10^6	Morgan *et al.,* [29]
Bioluminescence	*Xanthomonas campestris*	$1.5x10^4$ cfu/g	-	Shaw *et al.,* [19]
Bioluminescence	luxAB	10^3 -10^4 /cm root	10^3-fold more sensitive	Prosser [6]
ELISA	XylE	10^3	10^6	Morgan *et al*. [29]
Luminometry	1ux	10^3	ND	Rattray *et al.,* [20]
DNA hybridization	Xy1E	10^3	10^6	Morgan *et al*., [29]
Solution hybridization	2,4,5-T	10^2-10^3	ND	Steffan& Atlas [28]
DNA hybridization MPN	Tn5	10-100	ND	Fredrickson *et al*., [31]
DNA probe	bacteriophage DNA	10^3 - 10^4	-	OgunSeitan *et al.* [32]
Polymerase Chain Reaction (PCR)	2,4,5-T	10^2(100 g)	10	Steffan & Atlas [10]
Fluorescent antibodies	Flavobacterium	20	ND	Mason & Burns [33]
Fluorescent oligonucleotide	16s RNA	3×10^5	10^8	Amman *et al.* [34]
Immunomagnetic capture technique	*Streptomyces lividens*	30% (Sterile soil) 4% Nonsterile soil	-	Wipat *et al*., [35]

Selenska *et al*., [36] had been able to amplify by PCR particular sequences of two genetically manipulated nitrogen fixing strains of *Enterobacter agglomerans* in DNA extracted from soil inoculated with 10^9 cells/ gram soil of each strain. The targeted sequences were detected at a time when conventional plating method no longer gave colonies of the studied bacteria. It is recommended to make use of combination of all available techniques like PCR in combination with solution hybridization plus highly specific probes to incorporate the advantages of each method, therefore, permitting highly sensitive detection tools for monitoring of target organisms that occur in extremely low numbers.

(v) Immunological Methods: The potential use of immunological methods like polyclonal or monoclonal antibodies offers a highly sensitive and specific means of identifying genetically engineered microorganisms. Antibodies of either type can be used to identify specific marker gene products or even intact microorganisms can be used as antibodies against which the appropriate antigen expresses itself. Enzyme linked immunosorbent assay (ELISA) has been used for the detection of recombinant bacteria, for instance *P. putida* in the presence of mixed populations [29]. In addition, direct counting along with epifluorescence microscopy has been one of the most reliable methods of determining cell number and biomass of soil bacteria in natural samples [37]. The commonly associated stains such as acridine orange and DAPI are non-specific. And if used in this technique would not be able to differentiate between a GEM and native bacteria. Although specificity can be achieved by coupling antibodies chemically to a fluorochrome fluorescent, there are several problems associated with it.

(a) There is a possibility that detection of an antigen if it expresses on the surface of cell would be difficult.

(b) In order to monitor and differentiate the GEM, genes encoding antigen should be stable. The survival of the GMMOs to be monitored depends upon its ability to exist and express itself.

(c) Certain factors may contribute to its reduction in specificity. These factors may be due to interference, cross-reactivity and inability to reach the target organism within a specified limit. A proper selection of release host may avoid such problems. Monoclonal antibodies prepared in laboratory against Flavobacterium P25, have been shown to be active even when the cell number is minimal, detecting roughly 20 bacteria per gram of soil [33]. Fluorescent monoclonal antibodies in conjunction with fluorescence microscopy was used specifically for the detection of 01 antigen of *Vibrio cholerae*. This procedure was highly sensitive method of assessing water quality as compared to standard cultural methods [38]. It is very difficult to enumerate introduced organisms that are present in low numbers. In such selective cases, enrichment techniques would probably enhance the number and cell biomass of viable engineered microorganisms within the total population. Morgan *et al.,* [38] developed a similar method in recombinant *Pseudomonas* using a monoclonal antibody raised against a strain specific domain of the flagellin subunits protein of flagella. In this method polystyrene magnetic beads (10 mm) were used. The surface of the beads was coated with the monoclonal antibody, MLV1, which is highly sensitive for the flagellin protein of *P. putida* PaW8. The PaW8 flagellin gene has been cloned & sequenced to identify the antigenic region [39]. The coated beads were mixed with lake water samples containing the target recombinant *Pseudomonas putida*, a complex was formed between bead and cell. This complex then could be recovered by attraction towards a strong magnet. Approximately 20% of the initial target population was recovered when cells were re-isolated by standard culture methods. This method is known to represent a single step in recovery and identification of specific engineered microorganisms. In case of spores of specific recombinant strains of *Streptomyces lividens* immunomagnetic capture technique has been used where recovery efficiencies range from 30% from inoculated sterile soil to 4% from non-sterile soil [36]. In addition, biosensor-based detection system may also play a vital a role in the monitoring of target microorganisms, for example surface plasmon resonance has been successfully used to measure sensitive antigen-antibody complex [40]. Sometimes techniques that are dependent on direct DNA extraction or lysis of total bacterial cell could not detect transconjugants. As these techniques failed to assess the presence or absence of recombinant marker gene, therefore, unable to distinguish viable bacterial hosts in which the gene might have carried.

(vi) Flow Cytometry: This technique along with fluorescence activated cell separating provides a fast and sensitive method of identification, separating, and enumeration of target microorganisms. Using fluorochromes with GEM specific antibodies allows the easy identification of engineered microorganisms. Then cell sorting function permits recovery of cells with specific cellular characteristics like positive binding to fluorescent antibodies. These sorted cells are recovered and enumerated before subjecting them to further analysis. Fluorochromes can also be attached to oligonucleotide probes for the monitoring of GEMS [41]. The coupling of fluorochromes with 16S RNA probes have permitted the single-cell identification of two bacterial species namely *Fibrobacter succinogenes* and *Methanosarcina acetivorans* from the mixed populations. This labelling methods has great potential and could be used as cytometric applications because of its specificity [34]. For this fixing the probes into the target cells are done using procedures like traditional microscopic staining methods. Further increase in fluorescent signals can be achieved by applying multiple fluorescent oligonucleotide probes. This method allows enumeration of cells without isolating them.

SUMMARY

Intentional release of GEMs and GMMOs into the environment makes it mandatory for the development of appropriate technologies for the surveillance of microorganisms and their gene products in different environmental samples. This article emphasizes on the sensitivities and limitation of current methods available for detecting GEMS in the environment. As of today, no single method of monitoring will be suitable for detecting GEMS in all possible habitats.

However, detection efficiency of GEMs/GMMOs can be improved by adding more specific PCR primers and targeting markers at the molecular level. It is suggested to use more of non-radiolabeled methods and microarray techniques over dot blot methods. This might be an important future direction for enumeration of GMMOs in the environment. Further, suitable modifications in the construct of GEMS are still required so that these could be detected and recalled if situation warrants. As newer and newer GEMS are being constructed and released into the environment, their impact on the changing scenario must be assessed as frequently as the need for their release.

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REFERENCES

[1]. Singh, R., Kumar, M., Mittal, A., and Kumar, P. (2016). Microbial enzymes: Industrial progress in 21st century3 *Biotech,* **6**, 1-15.

[2]. Liu, X. and Kokari, C. (2017). Microbial enzymes of use in industry. In Biotechnology of microbial enzymes; Elsevier: Amsterdam, The Netherlands.

[3]. Park, J. and Park, W. (2011). Phenotypic and physiological changes in *Acinetobacter* sp. Strain DR 1 with exogenous plasmid. *Curr. Microbiol,* **62**, 249-254.

[4]. Miyakoshi, M., Shintani, M., Terabayashi, T., Kai, S., Yamane, H. and Nojiri, H. (2007). Transcriptome analysis of *Pseudomonas putida* KT2440 harboring the completely sequenced IncP-7 plasmid pCARI. *J. Bacteriol.* **189**, 6849-6860.

[5]. Fraiture, M.A., Herman, P., De Loose, M., Debode, F. and Roosens, N. H. C. (2017). How can we better detect unauthorized GMO in the food and feed chain? *Trends in Biotechnology*, **35**, 508-517.

[6]. Prosser, J. L. (1994). Molecular marker systems for detection of genetically engineered micro-organism in the environment. *Microbiology,* **140**, 5-17.

[7]. McCormick, D. (1986). Detection technology: the key to environmental biotechnology, *Biotechnology* **4**, 419-422.

[8]. Henschke, R.B. and Schmidt, F. R. J. (1990). Plasmid mobilization from genetically engineered bacteria to members of the indigenous soil microflora in situ. *Curr., Microbiol,* **20,** 105-110.

[9]. Michelini, E., Simoni, P., Cevenini, L., Mezzanotte, L. and Roda, A. (2008). New Trends in bioanalytical tools for the detection of genetically organisms: an update. *Anal. Bioanal. Chem.,* **392**, 355-367.

[10]. Steffan, R.J. and Atlas. R.M. (1988). DNA amplification enhance the detection of genetically engineered bacteria in environmental samples. *Applied and Environmental Microbiology,* **54,** 2185-2191.

[11]. Lévy, R., Najioullah, F., Thouvenot. D., Bosshard, S., Aymard, M. and Lina. B. (1996). Evaluation and comparison of PCR and hybridization methods for rapid detection of cytomegalovirus in clinical samples. *J. Virol. Methods,* **62**, 103-111.

[12]. Yeom, J., Lee, Y., Noh, J., Jung, J., Park, J. and Seo, H., Kim, J., Han, J., Jeon, C. O., Kim, J. and Park, W. (2011). Detection of genetically modified microorganisms in soil using the most-probable-number method with multiple PCR and DNA dot blot. *Research in Microbiology*, **162**, 807-816.

[13]. Winstanely, C., Morgan, J.A.W., Pickup, R.W., Jones, J.G. and Saunders, J.R. (1989). Differential regulation of lambda pL and pR promoters by a cI repressor in a broad-host-range thermoregulated plasmid marker system. *Applied and Environmental Microbiology*, **5**, 771-777.

[14]. Jefferson, R.A. (1989). The GUS reporter gene system. *Nature* (London) **392**, 837-842.

[15]. King, R.J., Short, K.A. and Seildler, R.J. (1991). Assay for detection and enumeration of genetically engineered microorganisms which is based on the activity of a deregulated 2,4-dicholorophenoxyacette monooxygenase. *Appl. Environ. Microbiol.,* **57,** 1790-1792.

[16]. 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 nondisruptive manner. *Biotechnology,* **4,** 560-564.

[17]. Stewart, G.S.A.B. (1990). In vivo bioluminescence: new potentials for microbiology. *Letters in Applied Microbiology,* **10**, 1-8.

[18]. King, J.M.H., DiGrazia, P.M., Applegate, B., Burlage, R. Sanseverino, J., Dunber, P., Larimer, F. and Salyer, G.S. (1990). Rapid sensitive bioluminescent reporter technology for Naphthalene exposure and biodegradation. *Science,* **49,** 778-781.

[19]. Shaw, J.J., Dane, F., Geiger, D. and Kloepper, J.W. (1992). Use of bioluminescence for detection of genetically engineered micro-organisms released into the environment. *Applied and Environmental Microbiology*, **58**, 267-273.

[20]. Rattray, E.A., Prosser, J.I., Killham, K. and Glover, L.A. (1990). Luminescence-based non-extractive technique for situ detection of *Escherichia coli* in soil. *Applied and Environmental Microbiology*, **6**, 3368-3374.

[21]. Hanahan, D. and Meselson, M. (1980). Plasmid screening at high colony density. *Gene,* **10**, 63-68.

[22]. Sayler, G.S., and Layton, A.C. (1990). Environmental application of nucleic acid hybridization. *Annual Review of Microbiology*, **44**, 625-648.

[23]. Sayler, C.S., Shields, M.S., Tedford, E.T., Breen, A., Hooper, S.W., Sirotkin, K.M. and Davis, J.W. (1985). Application of DNA: DNA colony hybridization to the detection of catabolic genotypes in environmental samples. *Applied and Environmental Microbiology*, **49**, 1295-1303.

[24]. Pettigrew, C. and Sayler, G.S. (1986). The use of DNA: DNA colony hybridization in the rapid isolation of 4 chlorobiphenyl degradative bacterial phenotypes. *Journal of Microbiological Methods*, **5**, 205-213.

[25]. Diels, L. and Mergeay, M. (1990). DNA probe-mediated detection of resistant bacteria from soils highly polluted by heavy metals. *Applied Environmental Microbiology*, **56**, 1485-1491.

[26]. Holben, W.E., Jansson, J.K., Chelm, B.K. and Tiedje, J.M. (1988). DNA prove method for the detection of specific microorganisms in the soil community. *Applied and Environmental Microbiology*, **54**, 703-7115.

[27]. Walia, S., Khan, A. and Rosenthal, N. (1990). Construction, and application of DNA probes for the detection of polychlorinated biphenyl degrading genotype in toxicorganic contaminated soil environments. *Applied and Environmental Microbiology,* **56,** 254-259.

[28]. Steffan, R.J. and Atlas, R.M. (1990). Solution hybridization assay for detecting genetically engineered micro-organisms in environmental samples. *Biotechniques,* **8**, 316-118.

[29]. Morgan, J.A.W., Winstanely, C., Pickup, R.W., Jones, J.G. and Saunders, J.R. (1989). Direct phenotypic and genotypic detection of a recombinant pseudomonad population released into lake water. *Applied and Environmental Microbiology,* **55,** 2537-2544.

[30]. Pickup, R.W., Simon, B.M., Jones, J.G., Saunders, J.R., Carter, J.K., Morgan, J.A.W., Winstanely, C. and Raitt, P.C. (1990). Survival of laboratory and freshwater bacteria carrying an extra-chromosomal Xyl E gene in freshwater microcosms. In Bacterial Genetics in Natural Environments ed. Fry J.C. and Day M.J., pp 89-99 Landon: Chapman & Hall.

[31]. Fredrickson, J.K., Bezdicek, D.F., Brickman, E.J. and L. S.W. (1988). Enumeration of Tn5 mutant bacteria in soil by using a most probable number DNA hybridization technique and antibiotic resistance. *Applied and Environmental Microbiology*, **54**, 446-453.

[32]. Ogunseitan, A., Sayler, G.S. and Miller, R.V. (1992). Application of DNA probes to analysis of bacteriophage distribution patterns in the environment. *Applied and Environmental Microbiology,* **58,** 2046-2052.

[33]. Mason, J. and Burns, R.G. (1990). Production of a monoclonal antibody specific for a *Flavobacterium* species isolated from soil. Federation of European Microbiological Societies. *Microbiological Letters*, **73**, 299-308.

[34]. Amman, R.I., Binder, B.J., Olsen, R.J., Chisholm, S.W. Devereux, R. and Stahl, D.A. (1990b). Combination of 16S rRNA targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Applied and Environmental Microbiology,* **56**, 1919-1925.

[35]. Wipat, A., Wellington, E.M. and Saunders, V.A. (1991). System for Streptomyces Detection In: Genetic Interaction between Microorganisms in Natural Environments ed. Wellington E.M.H. and van Elsas, J.D. Oxford Manchester University Press.

[36]. Selenska, S., Schentzinger, S. and Klingmuller. W. (1992). Direct detection of DNA sequences in soil. In: Gene Transfers and Environment, ed. Gauthier, M.J. pp.3-7 Berlin: Springer Verlag.

[37]. Hall, G.H., Jones, J.G., Pickup, R.W. and Simon, B.H. (1990). Methods to study the bacterial ecology of freshwater environments. *Methods in Microbiology*, **23**, 181-210.

[38]. Morgan, J.A.W., Winstanely, C., Pickup, R.W. and Saunders, J.R. (1991). The rapid immunocapture of *Pseudomonas putida* cells from lake water by using bacterial flagella. *Applied and Environmental Microbiology*, **57**, 503-509.

[39]. Winstanely, C., Morgan, J.A.W., Saunders, J.R. and Pickup, R.W. (1992). The detection of marked populations of recombinant pseudomonads released into lake water. In: Gene Transfer and Environment, ed. Gauthier, M.J. pp. 15-20, Berlin, Springer-Verlag.

[40]. Saunders, J.R. and Saunders, V.A. (1991). Molecular technique for detecting and measuring the activity of genetically manipulated bacteria released into the environment, Monitoring of Genetically Manipulated Micro-organism in the Environment ed. Edwards, C. Milton Keynes: Open University Press.

[41]. Amman, R.I., Krumholz, L. and Stahl, D.A. (1990a). Fluorescent oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *Journal of Bacteriology*, **177**, 762-770.