REVIEW ARTICLE

Integrative approaches for assessing the ecological sustainability of *in situ* bioremediation

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Abstract

Application of microbial metabolic potential (bioremediation) is accepted as an environmentally benign and economical measure for decontamination of polluted environments. Bioremediation methods are generally categorized into *ex situ* and *in situ* bioremediation. Although *in situ* bioremediation methods have been in use for two to three decades, they have not yet yielded the expected results. Their limited success has been attributed to reduced ecological sustainability under environmental conditions. An important determinant of sustainability of *in situ* bioremediation is pollutant bioavailability. Microbial chemotaxis is postulated to improve pollutant bioavailability significantly; consequently, application of chemotactic microorganisms can considerably enhance the performance of *in situ* degradation. The environmental fate of degradative microorganisms and the ecological consequence of intervention constitute other important descriptors for the efficiency and sustainability of bioremediation processes. Integrative use of culture-dependent, culture-independent methods (e.g. amplified rDNA restriction analysis, terminal restriction fragment length polymorphism, denaturing/thermal gradient gel electrophoresis, phospholipid fatty acid, etc.), computational and statistical analyses has enabled successful monitoring of the above aspects. The present review provides a detailed insight into some of the key factors that affect the efficiency of *in situ* bioremediation along with a comprehensive account of the integrative approaches used for assessing the ecological sustainability of processes. The review also discusses the possibility of developing suicidal genetically engineered microorganisms for optimized and controlled *in situ* bioremediation.

Introduction

Anthropogenic activities aimed at industrial and agricultural advancement have resulted in the nonjudicious production and usage of chemical compounds. Consequently, the environment has become heavily contaminated with chemical pollutants that are toxic both to the environment and to human health (Travis, 2002; Ostroumov, 2003; Labie, 2007). However, with increasing awareness about the hazardous effects of these chemical pollutants, a polyphasic approach has been proposed to overcome this situation. This approach includes (1) stringent regulations for the production and usage of complex chemicals; (2) pretreatment and safer disposal of toxic chemical wastes; and (3) restoration of contaminated sites and environments (Robinson *et al.*, 2001; Felsot *et al.*, 2003). The first two approaches are of a preventive nature and concentrate on minimizing further damage, while the latter offers a curative mechanism. Several recent research activities have focused on the use of different physico-chemical and/or biological means for the decontamination of polluted environments (Udell *et al.*, 1995; Bonaventura & Johnson, 1997; Lodolo *et al.*, 2001; Scullion, 2006). These studies have led to a general acceptance of bioremediation as being an environmentally benign, efficient and economic measure for pollutant removal and restoration of contaminated sites (Watanabe, 2001; Paul *et al.*, 2005). Bioremediation methods are based on the exploitation of metabolic potential for attenuation of the toxic effects of the pollutant(s) by (1) transformation to lesser toxic products; (2) complete mineralization of pollutants; and (3) immobilization of the pollutant (Shannon & Unterman, 1993; Snellinx *et al.*, 2002; Lovley, 2003; Diaz,

2004; Parales & Haddock, 2004). Most of the living beings including plants and higher animals exhibit a minimal basal level of detoxification ability that is expressed via the above mechanisms; however, microorganisms have been studied in greater detail for carrying out the detoxification activities (Watanabe & Baker, 2000; Zhong & Zhou, 2002). Microorganisms in general, and bacteria in particular, harbor enormous metabolic diversity, allowing them to utilize the complex chemicals as energy sources (Diaz, 2004; Nojiri & Tsuda, 2005). Further, their ability to undergo rapid genetic evolution also enhances their chance to acquire new metabolic potential for degradation of the recently introduced xenobiotic chemicals (van der Meer, 1994; Janssen *et al*., 2005; Nojiri & Tsuda, 2005; Zhang *et al*., 2006; Phale *et al*., 2007).

Conventionally, studies on microbial degradation of chemical pollutants have followed a reductive approach based on the isolation and characterization of a single bacterial strain or a syntrophic bacterial consortium (which could bring together different degradative potentials) for carrying out the desired degradation under controlled laboratory conditions (Stolz & Knackmuss, 1993; Samanta *et al*., 1999). The other major thrust area of bioremediation studies has been the characterization of metabolic pathways and their respective molecular regulations (Haggblom, 1990; Arai *et al*., 2000; Solyanikova & Golovleva, 2004; Symons & Bruce, 2006). Some of the relatively recent studies have also attempted to address questions related to the finer details of the biodegradation process for example transcriptional regulation, kinetic behavior and the structure–function relation of the enzyme involved in the processes, etc. (Diaz & Prieto, 2000; Tropel & van der Meer, 2004; de Melo Plese *et al*., 2005; Svedruzic *et al*., 2005). The advent of whole-genome sequencing and related genomics methods has also given rise to new avenues for genome-wide screening of degradative genetic elements and regulatory sequences among the pollutant-degrading strains (Heidelberg *et al*., 2002; Golyshin *et al*., 2003; Rabus, 2005; Zhao & Poh, 2008). All these studies have provided insights that are of great significance for the development of bioremediation processes. However, the major concerns regarding the use of isolated microorganism(s) are as follows: (1) biases of culturing and enrichment methods usually overlook some fraction of microbial diversity that may have significant degrading potential and; (2) the kinetics of pollutant degradation under controlled laboratory conditions is rarely a true reflection of the *in situ* biodegradation (Thompson *et al*., 2005; Vinas *et al*., 2005b).

In principle, the ideal bioremediation technology needs to be implemented in a nonsterile natural environment(s) wherein the degradative microorganism(s)/potential(s) encounters a variety of biotic and abiotic factors (Ward & Brock, 1976; Dinkla *et al*., 2001; Kim & Graham, 2003; Thompson *et al*., 2005). The majority of these factors exert adverse effects on the efficiency of the degradation process via different action mechanisms. This realization has resulted in research programs that have been specifically designed to investigate the effects of environmental factor(s) (Hoyle *et al*., 1995; Lovanh *et al*., 2002). A recent review provides a detailed description of various types of microbial interactions with organic chemical pollutants in the soil and their consequences on the efficiency of the pollutant degradation (Semple *et al*., 2007). Alternatively, some of the studies have also suggested that not only do the environmental factors influence the bioremediation process but the technological intervention (for performing bioremediation) may also affect the environment. In light of the above understanding, studies for a descriptive assessment of the ecological sustainability of *in situ* bioremediation processes have emerged as a major area of environmental research. The need for such assessments has also been emphasized by environment-monitoring agencies such as the 'United States Environment Protection Agency' (USEPA). The major targets for assessment would be to evaluate the long-term ecological effectiveness of the process. In addition, monitoring programs would be required to address the following queries: (1) whether the attenuation of toxic substances is occurring according to expectations; (2) what is the environmental fate of the biodegradative microorganism/potential; and (3) whether the process is having any adverse impact on the environmental parameters (e.g. hydro-geological, geochemical, microbiological and other changes). Recently, one of the studies demonstrated successful use of 'Biomonitoring', an integrated tool for assessment of metal pollution bioremediation in an aquatic system (Zhou *et al*., 2008). The approach was based on the concurrent analysis of bioaccumulation of heavy metal pollutants, biochemical alterations of the contaminated site, bioremediation, morphological and behavior observation of site and degradative potential, toxicological assessment, population- and community-level approaches and an *in silico* modeling for predicting the fate of the remediation process. Previously, most of the studies on *in situ* bioremediation had focused only on the kinetics of pollutant degradation; however, with the advent of molecular ecology methods, there has been a paradigm shift to encompass community behavior as a complementary study for *in situ* bioremediation trials/studies (Purohit *et al*., 2003; Paul *et al*., 2006a). The findings of such studies have appropriately justified the need for assessment of the ecological aspects of *in situ* bioremediation processes as well as the advancement of corresponding assessment methods.

The main aim of the present review is to provide a comprehensive account of integrated approaches (based on application of methods and principles of different scientific disciplines such as biogeochemistry, toxicology, microbiology, analytical chemistry, molecular biology, genomics,

proteomics, microbial ecology, mathematics/statistics, bioinformatics, etc.) that have been implemented and/or would be implemented in future for systematic assessment of the ecological sustainability of the bioremediation processes. The assessment methods are also important in light of stringent regulations on *in situ* application of a biological treatment method. It would be imperative to mention that these assessments will acquire further significance if the bioremediation is to be carried out using genetically engineered microorganisms (GEMs). The major concern regarding the use of GEMs under a natural environment(s) is their uncontrolled proliferation and escape from targeted environments. The existing methodologies for generation of GEMs require substantial improvement for incorporation of genetic sequences that can specifically regulate the environmental behavior of GEMs. Towards the end of the review, we discuss briefly the theoretical development of a simple genetic circuit for generation of self-regulated suicidal GEMs that can be used for *in situ* bioremediation. The present review also discusses some of the environmental factors and their effects on *in situ* bioremediation processes in a factor vs. cause manner. The description of environmental factors in this review is rationalized by the idea that an insight into the different ecological factors can be of great help for overcoming the ecological limitations for successful bioremediation of contaminated ecosystems.

Ex situ and *in situ* bioremediation

In principle, *in situ* bioremediation targets pollutant removal or attenuation under natural environmental conditions by implementation of microbial metabolic potential without the need for excavation of the contaminated sample(s) (Fruchter, 2002; Farhadian *et al.*, 2007; Jorgensen, 2007), whereas *ex situ* bioremediation methods are marked by interventions to degrade chemical pollutants in excavated samples (Guerin, 1999; Carberry & Wik, 2001; Prpich *et al.*, 2006). Consequently, the *ex situ* bioremediation treatments are less economical in comparison with the *in situ* approaches. These two major methods of bioremediations are also significantly different in terms of experimental controls and consistency of the process outcome. As shown by a comparative study evaluating the performance of *ex situ* bioremediation and *in situ* bioremediation for decontamination of petroleum-contaminated soil, the biodegradation rate for the *in situ* biodegradation process was found to be more variable than the *ex situ* process (Carberry & Wik, 2001). The other significant advantage with the application of *ex situ* bioremediation method is its independence from the environmental factors that could adversely affect the efficacy of the process. Further, because *ex situ* bioremediation is carried out in non-natural environments, the process can be manipulated easily by physico-chemical treatments of the target pollutant before and/or during the degradation (Kim *et al.*, 2005). Table 1 provides a short list of some of the studies that have implemented alternative treatments for enhancing the pollutant-removal efficiency during *ex situ* bioremediation studies. Despite the above selective advantages of *ex situ* bioremediation methods, the *in situ* bioremediation approach constitutes the most commonly used treatment technologies for ecological restoration of contaminated environments (Bouwer *et al.*, 1994; Romantschuk *et al.*, 2000; Carberry & Wik, 2001; Jorgensen, 2007). A generalized % distribution of common treatment strategies for decontamination of soil and groundwater (shown in Fig. 1) indicates widespread application of *in situ*

Table 1. Use of alternative methods for enhancement of degradation efficiency during *ex situ* bioremediation

Pollutant	Class of contaminant	Scale of study	Alternative method to enhance the degradation efficiency	References
Phenol	Aromatic chemical	Laboratory scale bioreactor	Use of polymer beads [50 : 50 poly(butylenes terephthalate): poly ether glycol]	Prpich *et al.* (2006)
Nitrophenol	Aromatic chemical	Laboratory scale microcosms	Immobilization of degrading organism on carrier material	Labana *et al.* (2005b)
Low-molecular-weight PAHs	Aromatic chemical	Laboratory scale bioreactor	Application of two-phase bioreactor systems	Janikowski *et al.* (2002)
Low-molecular-weight PAHs	Aromatic chemical	Laboratory scale test	Use of spent mushroom compost as bulking agent	Lau *et al.* (2003)
Pentadecane	Aliphatic chemical	Laboratory scale test	Circulating electrolyte method	Kim *et al.* (2005)
Petroleum oil	Oil spill	Field scale study	Use of bark chips as soil-bulking agent	Jorgensen *et al.* (2000)
Gasoline	Oil spill	Field scale study	Use of poultry litter, coir pith and rhamnolipid biosurfactant	Rahman *et al.* (2002)
Arsenic, Iron	Heavy metals	Mine drainage based study	Immobilization of reducing microorganism on carrier material	Casiot *et al.* (2003)
Calcium, zinc, iron, manganese	Heavy metals	Field scale study	Use of bio-degradable metal chelating agents for example EDDA, IDSA, NTA	Tandy *et al.* (2004)

EDDA, [*S*,*S*]-ethylenediaminedisuccinic acid; IDSA, iminodisuccinic acid; NTA, nitrilotriacetic acid; PAHs, polycyclic aromatic hydrocarbons.

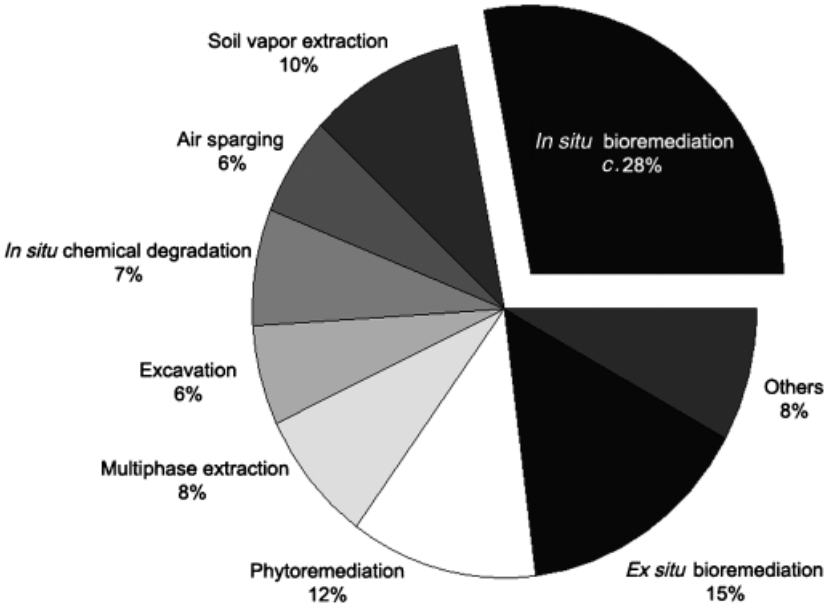

Fig. 1. A pie-chart representing the percentage distribution of common remediation technology used decontamination of polluted soil and ground water.

bioremediation. Among all remediation projects, about one in four make use of *in situ* bioremediation methods (Jorgensen, 2007). *In situ* bioremediation treatments do not require soil/sample evacuation and, therefore they are less expensive and, importantly, lead to lesser release of the volatile chemical pollutants adjoining nonpolluted sites. The other important facet of the *in situ* bioremediation process is its applicability to diverse environmental niches for example industrial sites, aquifers (Mandelbaum *et al.*, 1997), soil subsurface (Schmidt *et al.*, 2004), groundwater (Scow & Hicks, 2005; Farhadian *et al.*, 2007) and even in some of the extreme environments such as Arctic polar soil (Aislabie *et al.*, 2006), etc. This significance is further enhanced by the ubiquitous presence and activity of microorganisms, thereby providing the option of effective decontamination of even the remote microenvironments that are usually nonaccessible to methods falling under *ex situ* bioremediation.

Technically, *ex situ* bioremediation is carried out by a number of nonrelated methods such as 'slurry phase bioremediation' and 'solid-phase bioremediation,' wherein the degradation principles are driven by the physico-chemical nature of the pollutant(s) (Guerin, 1999; Janikowski *et al.*, 2002; Di Gennaro *et al.*, 2005). On the other hand, *in situ* bioremediation methods can be categorized into (1) biostimulation and (2) bioaugmentation (Omenn, 1992; Bouwer *et al.*, 1994; Romantschuk *et al.*, 2000; Jorgensen, 2007), and they focus on hastening the kinetics of pollutant removal. A comparative evaluation of the advantages and disadvantages of *ex situ* and *in situ* bioremediation may constitute an interesting study; however, for practical purposes, the selection between either of the above approaches is primarily determined by the 'physico-chemical features of the contaminated site', 'presence of cocontaminants,' 'type and concentration of the pollutant,' etc. For instance, it is suggested that the application of *ex situ* bioremediation methods may be more useful for remediation of (1) soil/sediments contaminated with high levels of recalcitratant pollutants, (2) clay-rich soil with low permeability of pollutant(s), (3) contaminated sites where environmental conditions are nonfavorable for biological processes and (4) where environmental release of microorganism(s) is not feasible because of regulatory reasons (Robles-Gonzalez *et al.*, 2008). Furthermore, it is important that the selection of remediation approach needs to be rationalized according to the expected outcomes of the process. A recent study performed for a biologically enhanced rate of tetrachloroethene (PCE) removal from a dense nonaqueous phase liquid (DNAPL) demonstrated that application of an active microbial culture capable of reductive dechlorination results in 4–13-fold enhanced degradation (Glover *et al.*, 2007). However, PCE degradation results in the formation of degradation products viz., trichloroethene (TCE), *cis*-1,2-dichloroethene (DCE), vinyl chloride (VC) and ethane, which have greater water solubility than the degradation substrate. The enhanced degradation by *in situ* bioremediation can result in increased contamination of lesser hydrophobic metabolites in the water resources in the vicinity of the source contamination (Kao *et al.*, 2003; Glover *et al.*, 2007).

The above studies clearly indicate that any generalization of an applicable bioremediation approach may be difficult to perceive. Further, the bioremediation technology may be rendered effective only by performing a detailed 'case-by-case' evaluation before the selection of an appropriate

bioremediation approach. It is also important to mention that, in spite of several differences, both the approaches share an elementary similarity of their dependence on microbial metabolism to break down the contaminant by utilizing them as a source of nutrients and energy, wherein the preferred resulting products are either carbon dioxide and water or a transformation product with a relatively attenuated toxicity.

In situ bioremediation strategies (biostimulation and bioaugmentation)

The underlining principle for *in situ* bioremediation is to hasten the pollutant degradation kinetics either by stimulation of the natural attenuation process (biostimulation) or via exogenous introduction of some efficient pollutant-degrading strain(s) (bioaugmentation) (Goulding *et al.*, 1988; Stephenson & Stephenson, 1992; Dzantor *et al.*, 1993; Knapp & Faison, 1997; Thomassin-Lacroix *et al.*, 2002). Some of the successful biostimulation approaches have been based on addition of nutrients (e.g. carbon, nitrogen, phosphorus, potassium, etc.) or electron acceptor/donors (e.g. acetate, nitrate, sulfate, glutamate, etc.) and gaseous formulations to the contaminated environment (Eguchi *et al.*, 2001; Thomassin-Lacroix *et al.*, 2002; Tang *et al.*, 2005; Garcia-Blanco *et al.*, 2007). Alternatively, pollutant removal rates have also be stimulated by generating an optimal balance of physical factors such as aeration, temperature and buffering of environmental pH by altering the redox state and electro-kinetic state of the contaminated sample (Vallejo *et al.*, 2001; Saito & Magara, 2003; Luo *et al.*, 2005). The recent past has also observed the use of non-conventional methods for biostimulation of the pollutant degradation process. In one such study, the application of quinonoid-enriched humic materials for regulation of the redox characteristics of soil led to the stimulation of the natural attenuation process (Perminova *et al.*, 2005). The humic material acted as a redox-buffering agent and, therefore it had a positive influence on the efficiency of the pollutant removal. Similarly, another biostimulation study used nonuniform 2D electric pulses to stimulate *in situ* bioremediation of 2,4-dichlorophenol (2,4-DCP) (Fan *et al.*, 2007). The findings of these studies point to the possibility of diverse applications of biostimulation for *in situ* decontamination of polluted environments. Further, biostimulation can be more effective if it is used in combination with bioaugmentation methods. Some of the recent studies have clearly demonstrated performance enhancement of biostimulation when used along with bioaugmentation of an efficient degradative strain (Thomassin-Lacroix *et al.*, 2002; Silva *et al.*, 2004; Olaniran *et al.*, 2006). Alternatively, some studies have also attempted to evaluate the performance of biostimulation methods in comparison with bioaugmentation and natural attenuation, wherein a common observation was that biostimulation may be sufficient for decontamination of pollutants; however, the kinetic efficiency of the biostimulation was relatively lesser as compared with the biostimulation process (Bento *et al.*, 2005; Smith *et al.*, 2005; Da Silva *et al.*, 2006). These observations suggest that biostimulation may be moderately effective for decontamination of polluted environments, and yet drawing such a conclusion would be nonjudicious. Therefore, similar to *ex situ* and *in situ* bioremediation approaches, the choice between biostimulation and bioaugmentation is also subjective to site-specific evaluations.

Characteristically, 'bioaugmentation' has been defined by (1) isolation, (2) characterization and (3) optimization of microorganism(s) for carrying out pollutant degradation in natural environments (Stephenson & Stephenson, 1992; Vogel, 1996). Another definition suggests that bioaugmentation is a rational rearrangement of the microbial richness leading to the dominance of microbial group(s) with specific catabolic traits necessary for the cleanup of the pollutant(s) (Dejonghe *et al.*, 2001). The selective advantage of bioaugmentation over other *in situ* bioremediation methods lies in its ability to hasten the rate of pollutant removal several fold over a relatively short time scale. This has also been corroborated by some of the studies that have evaluated the comparative performance of 'natural attenuation', 'biostimulation' and 'bioaugmentation' (Yu *et al.*, 2005; Da Silva *et al.*, 2006; Tongarun *et al.*, 2008). Conventionally, bioaugmentation studies have focused on exogenous introduction of efficient pollutant-degrading strain(s) or a bacterial consortium into the contaminated site for the decontamination purpose. Interestingly, a few of the studies have also attempted to evaluate the *in situ* biodegradation performance of microorganisms when they were bioaugmented along with the environmental matrixs (e.g. soil particle, sediment, etc.) for decontamination of contaminated site(s). In one such study, the activated soil biomass was inoculated into a pentachlorophenol-contaminated soil for enhancement of pollutant degradation (Barbeau *et al.*, 1997). The soil biomass used in the above study consisted of a microbial consortium that could degrade even a very high concentration of pollutant. The increased efficiency of degradation was explained on the basis of the beneficial effects of soil particles that acted as an immobilization matrix for the microbial cell during the bioremediation process. Apart from the above methods, quite a few other variants of bioaugmentation have also been developed and implemented successfully for remediation purposes. 'Cobioaugmentation' is one such variant wherein the process is rendered effective by exogenous introduction of multiple microbial strains with different metabolic potentials (Roane *et al.*, 2001; Pepper *et al.*, 2002; Li *et al.*, 2008). In one of the above studies, 3,5-dinitrobenzoic acid

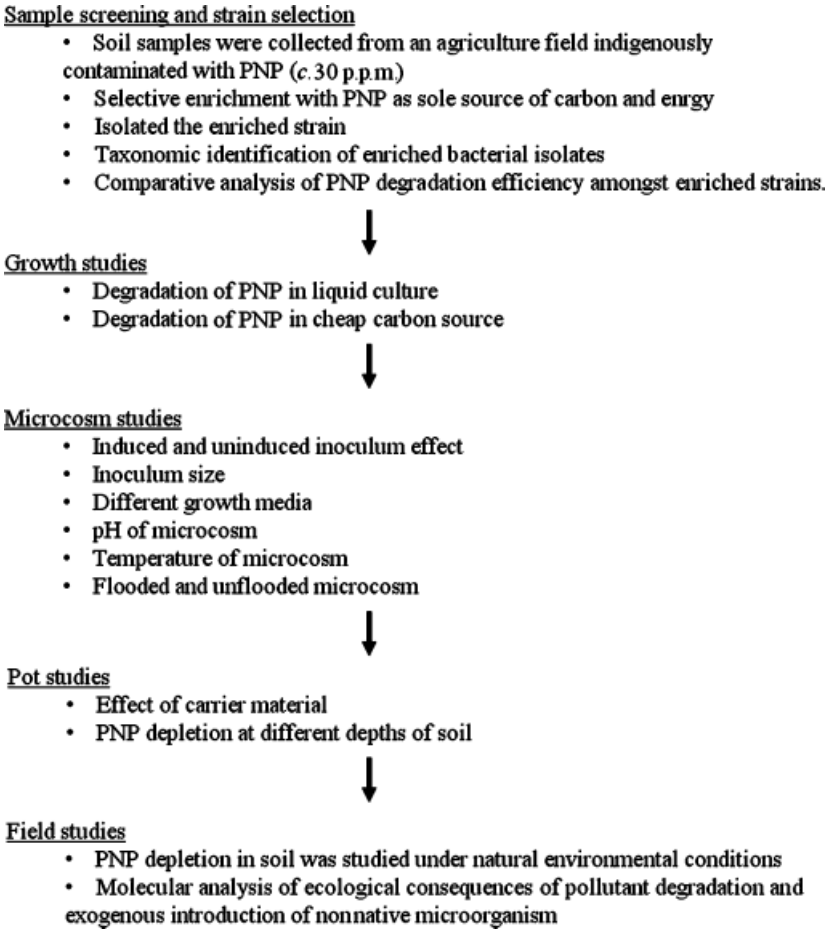

Fig. 2. Flow chart (based on a case study for *in situ* degradation of PNP) indicating different steps involved in development of a bioaugmentation based *in situ* bioremediation method.

(3,5-DNBA) bioremediation could be significantly enhanced by cobioaugmentation of a 3,5-DNBA-degrading microorganism (*Comamonas testosteroni* A3) and a biofilm-forming microorganism (*Pseudomonas putida* M9) (Li *et al.*, 2008).

These reports may suggest that bioaugmentation is a straightforward approach; yet the use of this technology has not become common largely due the limited success with isolation of efficient pollutant-degrading microorganisms, a process that is subjective to biases of culture-dependent approaches (Knapp & Faison, 1997; Singer *et al.*, 2005; Thompson *et al.*, 2005). Further, the development of an effective bioaugmentation process needs to address several issues pertaining to optimization of the process before its onsite application. The optimization usually includes 'scaling-up' and 'trails under natural environmental conditions'. Figure 2 shows a flow chart for different optimization procedures performed as part of a case study carried out for *in situ* degradation of *p*-nitrophenol (PNP) by bioaugmentation of a degrading strain viz., *Arthrobacter protophormiae* RKJ100 (Labana *et al.*, 2005a, b; Paul *et al.*, 2006a). Strain RKJ100 was earlier characterized for its ability to utilize PNP, 4-nitrocatechol (4-NC) and *o*-nitrobenzoate (ONB) as the sole source of carbon and energy (Chauhan & Jain, 2000; Chauhan *et al.*, 2000). Later, the strain could be used successfully for *in situ* bioremediation of PNP-contaminated soil(s) over different experimental setups (small-sized soil microcosm to naturally contaminated agriculture fields). The results obtained with strain RKJ100-mediated PNP bioremediation showed the real-time applicability of the bioaugmentation approach for decontamination of naturally contaminated ecological niches. However, unlike such successful studies, a large proportion of *in situ* bioaugmentation processes do not perform optimally, under the natural environmental conditions (Bouchez *et al.*, 2000). The incongruity can be attributed to the suboptimal performance and nonsustainability of the process under test environments, which in turn are the outcome of the impacts of different environmental factors and ecological interactions on the degradative microorganism. The limited success of *in situ* bioremediation methods has made it necessary to evaluate different environmental factors and their influence on the efficacy and sustainability of the *in situ* remediation process. It would be pertinent to mention that, by far, the

majority of the environmental factors exert a detrimental effect on the functionality of the degrading microorganisms. However, their effects are exerted via relatively lesser number of common action mechanisms for example reducing the accessibility of the target pollutant, enhancing the recalcitrance of the pollutant molecule and adversely affecting the survival and activity of the degrading microbial potential, etc. Therefore, a comprehensive assessment of various environmental factors and their likely impacts on the *in situ* bioremediation process is of great significance to confer optimal efficiency to the process.

Environmental factors and their effect on *in situ* bioremediation

It was not until the early 1980s that the bioremediation trials were performed under natural environmental conditions and, consequently, the influence of environmental factors on the remediation process was never expected. However, ever since the initiation of *in situ* bioremediation studies, it has been understood clearly that the process is influenced considerably by environmental factors such as the physicochemical nature of the contaminated environment, nutrient availability, presence, chemical nature and concentrations of cocontaminants, extent of contamination, community structure of the indigenous microbial communities, etc. (Leahy & Colwell, 1990; Morelli *et al.*, 2001; Moreels *et al.*, 2004; Coulon *et al.*, 2005, 2007). One of the studies reported that the *in situ* biodegradation pathway of triadimefon could be affected by the soil moisture content because the pollutant remains in soils with a low moisture content (Singh, 2005). In a similar study, Singh *et al.* (2003) evaluated the role of soil pH in *in situ* bioremediation of fenamiphos; the results from this study showed that bioremediation measures enhanced pollutant removal in soils with alkaline pH ($pH \geq 7.7$), whereas it remained slow in case of soils with acidic characteristics (pH 4.7–6.7). The effect of another environmental factor i.e. inorganic carbon (IC) content was evaluated during biodegradation of dimethyl phathalate (DMP). The results indicated that the rate of DMP degradation increased concurrently with the increased availability of IC (Yan *et al.*, 2002). Some studies have also been conducted to evaluate degradation of different compounds under specific ecological conditions (e.g. anaerobic, sulfate-reducing environment, methanogenic conditions, etc.) and these have demonstrated how these conditions are more conducive to degradation of some of the pollutant compounds (Lovley, 2000; Somsamak *et al.*, 2006). These reports clearly indicate how different environmental factors influence the efficiency and sustainability of the *in situ* bioremediation process. However, a generalization of such effects may be difficult because these factors have been found to influence the bioremediation process in a case-by-case manner, and yet their understanding could be of great significance for improving the efficiency of the *in situ* bioremediation process.

Also, it is important to recognize that different environmental factors affect the efficiency of the *in situ* bioremediation process is even more difficult because the environmental factors exert their effect(s) on the bioremediation process via contributory mechanisms. For example, one of the studies measuring *in situ* microbial degradative activity indicated that the effect of temperature variation on degradation efficiency was more pronounced in the case of soil with a high organic content and a low pH as compared with soil with a low total organic content and a higher pH (Pietikainen *et al.*, 2005). Another recent study reported that soil characteristics (e.g. soil organic matter, soil texture, particle size distribution, soil moisture, etc.) exerted a significant influence on hexadecane removal in an ozonization-mediated *in situ* bioremediation trial (Jung *et al.*, 2008). The influence of cosubstrate occurrence on the *in situ* degradation of the target pollutant have also been studied. One report indicated a positive influence of biosurfactant application on degradation of petroleum-associated hydrocarbons only when it was also supplemented along with gaseous toluene (Ortiz *et al.*, 2006). The positive influence was explained on the basis of the ability of toluene to cross-induce the biological activity for degradation of target hydrocarbons. The above studies indicate that any prior information/understanding of the possible impacts of different environmental factors can be of great significance for improving the overall efficiency and applicability of the *in situ* bioremediation processes.

Traditionally, environmental factors have been investigated in isolation and they have been placed in two subgroups: (1) biotic factors and (2) abiotic factors. However, the new approach considers the environmental factors in a nonisolated manner wherein biotic and abiotic factors are closely interlinked and exert their effect via related mechanisms (Sukul & Spiteller, 2001; Giacomazzi & Cochet, 2004). A detailed description of all the environmental factors is not the focus of the present review, and yet it is important to provide a brief account of some of the critical factors that are directly related to the sustainability and other ecological aspects of the *in situ* bioremediation process.

Biotic factors

Diverse indigenous life forms present within the contaminated environment constitute this subgroup and these are one of the most significant determinants of the success of the *in situ* bioremediation process (van Veen *et al.*, 1997; El Fantroussi & Agathos, 2005). Biotic factors largely exert their effect by reducing the survival, activity and migration of degradative microorganisms, wherein the above

alterations are a direct outcome of 'predation by protists', 'competition with autochthonous microorganisms', 'protozoan grazing' and 'other eukaryotic interactions' (van Veen *et al.*, 1997; Tso & Taghon, 1999; Rentz *et al.*, 2005). Among the above factors, the 'reduced survival of degradative microorganisms' has been found to be the most detrimental mechanism involved in nonoptimal performance of the *in situ* bioremediation process. It is postulated that maintenance of optimal effective inoculum is extremely important to improve the chances of successful bioremediation. Some of the *in situ* bioremediation studies have systematically demonstrated the importance of inoculum density for efficient pollutant removal (Ramadan *et al.*, 1990; Miethling & Karlson, 1996; Rousseaux *et al.*, 2003). To overcome the limitation of reduction in effective inoculum sizes, some of the studies have successfully utilized the methods of 'repetitive bioaugmentation' (Newcombe & Crowley, 1999; Bouchez *et al.*, 2000), preinduction and repeated inoculation (Gilbert & Crowley, 1998) or 'bioaugmentation with matrix immobilized cells' (Feng *et al.*, 1997; Moslemy *et al.*, 2002; Labana *et al.*, 2005a).

The importance of maintaining effective inoculum density was shown systematically in a study for bioremediation of atrazine-contaminated soil (Newcombe & Crowley, 1999). The contaminated field was divided into three plots that were inoculated once, four or eight times, respectively, with the atrazine-degrading bacterial consortium. After 12 weeks of inoculation, there was no degradation in soil that was inoculated only once. However, the other soils (inoculated four and eight times, respectively) had 38% and 72% atrazine mineralization. Labana *et al.* (2005a, b) studied the comparative effect of inoculum size on the rate of PNP degradation in a microcosm study, wherein a cell density of 2×10^8 CFU g^{-1} of soil was found to be most effective for pollutant degradation over a selected temperature range (20–40 °C). This study also demonstrated the significance of preinduction and cell immobilization of the degradative strain for carrying out the *in situ*-pollutant degradation (Labana *et al.*, 2005b). The other major consequence of cell immobilization was improved cell survival as indicated by enhancement of 2 log units of CFU survival as compared with the nonimmobilized cells during the bioremediation period. Another report also clearly demonstrated the application of immobilized cells for enhanced wastewater treatment in an activated sludge (Jittawattanarat *et al.*, 2007); the targeted activity (nitrogen removal from wastewater) showed an improvement of *c.* 25% as compared with the controlled sludge. These studies have indicated 'bacterial cell survival' as being one of the most important biotic factors determining the applicability and sustainability of *in situ* bioremediation.

Another biotic factor that can significantly affect the *in situ* bioremediation process is 'adaptation of degradative microorganism(s) towards environmental stresses' (Fiorenza & Ward, 1997; Rittmann *et al.*, 2001; Somova *et al.*, 2005). The *in situ* application of microorganisms exposes them to diverse stresses that can lead to a major decline in the survival of the degradative microbial strain as well as the efficiency of the pollutant removal (Pries *et al.*, 1994). One of the most common stresses encountered during *in situ* bioremediation is the elevated concentrations of target pollutant and cross-contamination of other nontargeted pollutants (Lee & Lin, 2006). Quite often, the concentration of the pollutant(s) at the source of contamination is high enough to kill the majority of the microorganisms; consequently, the role of bacterial adaptations to a higher pollutant concentration becomes extremely significant for successful implementation of *in situ* bioremediation (van der Meer *et al.*, 1992; van der Meer, 1994; Sikkema *et al.*, 1995; Sharma *et al.*, 2007a). Chemical pollutants (especially the organic compounds and solvents) are best-characterized microbial biocides, most of which affect the microbial cell survival by altering the biological membrane structure or by impairing the crucial biosynthetic pathways essential for the bacterial growth (Barrette *et al.*, 1989; Sikkema *et al.*, 1995; Bogdanov *et al.*, 1999). However, some microorganisms exhibit resistance towards these toxic chemicals and protect themselves against deleterious effects. The microbial ability to withstand chemical stress is attributed to different adaptive responses exhibited by tolerant microorganisms (Bradley & Fraise, 1996; Isken & de Bont, 1998). The molecular and biochemical mechanisms of bacterial resistance to toxic chemicals have been studied in detail for antibiotics, where the resistance is exerted by mechanisms for example enzymatic inactivation, passive exculsion from the cell or active efflux of the antibiotic molecule (Goessens, 1993; McManus, 1997; Charpentier & Tuomanen, 2000; Mitchell & Tuomanen, 2002). Bacterial resistance to biocidal chemicals including organic chemicals and solvents also follows a similar mechanism (Isken & de Bont, 1998; Ng *et al.*, 2002; Sardessai & Bhosle, 2002).

The toxicity of organic compounds to the bacterial cells is directly associated with the hydrophobicity of a compound(s) that regulates the concentration of accumulated chemical within the bacterial cell membrane (Jaworska & Schultz, 1994; Tanii, 1994; Dearden *et al.*, 1995). The extent of the microbial toxicity of organic compounds/solvents is expressed in terms of the log P_{ow} values (log value of the partitioning coefficient of the test compound in equal volumes of *n*-octanol and water). In general, organic solvents with a log P_{ow} value of 5 and above are considered safe for microbial cells, whereas those with a log P_{ow} of < 5 are considered to be microbicidal (Aono *et al.*, 2001). From the environmental toxicity aspect, the organic pollutants exhibit heightened toxicity to microorganisms, because they act in an additive manner if they are present in the form of

mixture(s) (Nirmalakhandan *et al.*, 1997), a condition that is quite often experienced with the contaminated environments. Therefore, microbial ability to adapt to and tolerate toxic organic pollutant(s) can be of great significance for bioremediation purposes. Several microorganisms have been isolated, identified and characterized for metabolic activities on different environmental pollutants; however, there are relatively few reports on the ability of microorganisms to tolerate very high concentrations of these chemicals (Asako *et al.*, 1999; Ramos *et al.*, 2002; Edvantoro *et al.*, 2003; Usami *et al.*, 2005). Many such studies of microbial tolerance towards organic compounds have focused largely on organic solvents for example phenol, benzene, toluene, styrene, *p*-xylene, cyclohexane, etc. (Li *et al.*, 1998; Kobayashi *et al.*, 2000; Okamoto *et al.*, 2003; Na *et al.*, 2005; Webber *et al.*, 2006). However, other reports have shown the occurrence of bacterial tolerance towards other organic compounds for example chlorophenol (Martinez *et al.*, 1999), hydroxybenzoate and nitrobenzoate (Sharma *et al.*, 2007a). The mechanism of bacterial tolerance has been presented in detail in the literature and majority of the adaptive responses are reported to be based on the morphological changes and/or the physiological changes (Button, 1991; Ramos *et al.*, 2002; Chavez *et al.*, 2006).

Because the toxicity of organic chemicals is exerted via their accumulation within the cell membrane, the primary mechanism for bacterial tolerance to these compounds attempt to minimize it by increasing the cell membrane rigidity and decreasing the chemical permeability. The same is achieved by alteration of the cell shape, cell surface area, cell membrane architecture and fatty acid composition of the cell membrane, increasing saturated fatty acids in the cell membrane, modification of phospholipid head groups, etc. (Kobayashi *et al.*, 1999; Ramos *et al.*, 2002; Sharma *et al.*, 2007a). Among the above changes, *cis* to *trans* isomerization of unsaturated fatty acids and alteration of the saturated : unsaturated fatty acid ratio are potentially the most significant for bacterial tolerance to organic compounds. A comparative characterization of benzene-induced fatty acid composition showed that the benzene-sensitive strain was unable to increase the ratio of saturated : unsaturated fatty acid, while the tolerant strain showed significant changes in the saturated : unsaturated fatty acid within the cell membrane (Gutierrez *et al.*, 2003). Similarly, Sharma *et al.* (2007a) reported an overall increase in the ratio of *trans* : *cis* unsaturated fatty acid in the total cell fatty acid analysis of a benzoate-tolerant bacterial strain when it was subjected to extremely high concentrations of nitrobenzoate or hydroxybenzoate. The effective contribution of phospholipid composition towards tolerance of *Pseudomonad* was shown in a study wherein all the test *Pseudomonad* altered the phospholipid composition and relative concentrations in response to their growth in the presence of toluene (in comparison with those grown on sodium succinate) (Fang *et al.*, 2000).

Apart from the cell membrane-associated alterations that lead to the bacterial tolerance to organic compounds and solvents, the other major mechanism of tolerance is 'extrusion of toxic compounds via efflux pumps in an energy-dependent process' (Kieboom *et al.*, 1998; Li *et al.*, 1998; Ramos *et al.*, 1998; Rojas *et al.*, 2001; Fernandes *et al.*, 2003; Rodriguez-Herva *et al.*, 2007). This mechanism leads to an active outward transport of the toxic compound from the bacterial cytoplasm into the external medium and consequently maintains effective concentration of the toxic compound within subtoxic levels. One of the initial studies indicating the physiological role of an efflux pump in the toluene tolerance of *P. putida* strain DOT-T1E showed that a random mutant (generated with transposon mutagenesis) was unable to tolerate elevated concentrations of toluene. The genetic analysis of the mutant confirmed that the knockout locus had a high sequence homology with the gene encoding for the drug exclusion efflux pump (Ramos *et al.*, 1998). In yet another report, site-directed efflux pump mutants of *Pseudomonas aeruginosa* were generated (three different sets of efflux pumps were selectively mutated) and tested for organic solvent tolerance. The experimental data from this study suggested that all the three sets of efflux pumps had important roles in the tolerance of the test strain towards organic solvents i.e. hexane and xylene (Li *et al.*, 1998). Several other studies have also established the role of efflux pumps as the most significant contributors towards bacterial tolerance to toxic organic compounds. The occurrence of alternative mechanisms such as vesicle-mediated entrapment of the toxic chemicals (Kobayashi *et al.*, 2000) and induction of stress-related chaperons that help in refolding of damaged proteins (Reva *et al.*, 2006) has also been indicated. Further analyses with transcriptomic and proteomic approaches have revealed the involvement of some novel mechanisms for bacterial tolerance to the organic compounds/solvents (Alsaker *et al.*, 2004; Santos *et al.*, 2004; Silveira *et al.*, 2004; Tomas *et al.*, 2004; Segura *et al.*, 2005; Volkers *et al.*, 2006).

The application of microbial strains to environmental niches with a high concentration of a toxic pollutant may be a challenging problem, because majority of the microorganisms cannot adapt and survive the toxic effects especially when the pollutants are present at very high concentration. Consequently, from the *in situ* bioremediation point of view, microorganisms with the ability of adaptation and tolerance towards higher pollutant concentrations have selective advantages in *in situ* bioremediation processes and they may be most useful for decontamination of source contamination sites (which are usually characterized by extremely high concentrations of toxic pollutants).

Table 2. Studies indicating nongeneralized effect of some of the abiotic environmental factors on the degradation efficiency of *in situ* bioremediation

Extrinsic factor(s)	Target pollutant	Effect on degradation efficiency	Putative mode of action	References
Pollutant concentration	Hydrocarbons	Inhibitory at high concentration	Lipophilic hydrocarbons accumulate in bacterial membrane leading to lose of membrane integrity	Sikkema *et al*. (1995)
			Only a few bacteria have been reported to adapt to high concentrations of lipophilic compounds	Sharma *et al*. (2007a, b)
Nonspecific pollutant	*p*-Nitrophenol	Cross induction leading to improved degradation	Presence of nonspecific by related pollutant allows a sustainable induction of degradative enzymes even with the diminishing amounts of target pollutant	Prakash *et al*. (1996)
Soil moisture	Linear alkylbenzene sulfonates	High soil moisture is inhibitory	High soil moisture leads to an increased diffusion of pollutant concentrate that is inhibitory to microbial population	Nielsen *et al*. (2004)
Soil pH	Nitrophenol	Optimal degradation at pH∼7.5	Most microorganisms exhibit optimal metabolic activity at neutral pH. Alternatively, soil pH also regulates the stability and solubility of nutrients and pollutant	Labana *et al*. (2005a)
	Polycyclic aromatic hydrocarbon	Degradation could occur at pH as low as 2.0	Degradation was carried out by indigenous microbial consortia consisting of functionally active bacteria and fungi	Stapleton *et al*. (1998)
Soil temperature	Hydrocarbons	Good degradation over 15–35 °C	Most of the characterized pollutant-degrading bacteria have been isolated from mesophilic environments wherein the metabolic activity is observable over a range of temperature	Leahy & Colwell (1990)
	Alkanes	Degradation at low temperatures (*c*. 5 °C)	Alkanes usually remain solid at lower temperatures, thereby they become nonaccessible at reduced temperatures; however, a psychrotroph viz., *Rhodococcus* sp. strain Q15 can assimilate even the solid form due to the alteration in cell-membrane architecture during growth at low temperatures	Whyte *et al*. (1999)

Abiotic factors

Any environment is constituted by a systematic assemblage of biotic and abiotic factors; therefore, abiotic factors play an equally significant role in the majority of environmental phenomena (Sukul & Spiteller, 2001; Jia *et al*., 2004). *In situ* bioremediation, being one such phenomenon, is hugely affected by the abiotic factors of the environment under intervention (Mergaert *et al*., 1992; Ferrari, 1996; Winkler *et al*., 2001; Giacomazzi & Cochet, 2004). The metabolic reactions involved in microbial degradation of chemical pollutants also follow the principles of 'enzyme catalysis' and, therefore, they exhibit optimal performance only over a very narrow range of physico-chemical parameters. Deviation from these optimal parameters results in reduced efficiency and sustainability of the degradation reaction. Relatively few microorganisms can withstand these deviations and they may carry out optimal degradation over a range of physico-chemical parameters (Eriksson *et al*., 2003). Alternatively, abiotic factors can also affect the ecological fate and distribution of pollutant chemicals, making them more recalcitrant for microbial degradation under natural environmental conditions (Leahy & Colwell, 1990; Master & Mohn, 1998; Bending *et al*., 2003). As indicated earlier, among all the abiotic factors pH, temperature, substrate (pollutant) concentration, moisture content, nutrient availability and the presence of metal ions have been studied in detail (Lowe *et al*., 1993; Semprini, 1995; Chenier *et al*., 2003). Such studies have indicated that diverse mechanisms of abiotic factors influence the efficiency of the *in situ* bioremediation process (Mukherji & Weber, 1998; Krasteva *et al*., 2001; Gourlay *et al*., 2005). A short list of studies on the systematic assessment of the impact of abiotic factors on *in situ* bioremediation is presented in Table 2. These studies suggest that most of the environmental factors do not follow a universal mechanism in influencing the efficiency of the *in situ* bioremediation process. One of the common mechanisms for abiotic factors and their influence on *in situ* bioremediation is 'reduction of pollutant bioavailability' (Blackburn & Hafker, 1993; Tabak & Govind, 1997; Loser *et al*., 1999). 'Pollutant bioavailability' is extremely important if remediation is to be carried out in a nonhomogenous micro-environment (e.g. soil, sediment, sludge and other aquatic bodies). According to most of the earlier definitions (as reviewed in detail by Semple *et al*., 2007), 'pollutant bioavailability' was explained only as a function of interaction of chemical compound(s) with the environmental matrix. However, now it is being increasingly considered as an outcome of a three-way interaction including the pollutant, the environmental matrix and microbial characteristics

(Grimm & Harwood, 1997; Park *et al.*, 2003; Parales, 2004). One of the microbial features that plays a pivotal role in pollutant bioavailability is 'chemotaxis', i.e. the ability of bacterial cell(s) to move towards the concentration gradient of the target chemical pollutant(s) (Grimm & Harwood, 1997; Samanta *et al.*, 2000; Hawkins & Harwood, 2002).

Bacterial chemotaxis for enhanced pollutant bioavailability

Microorganisms exhibit a wide array of behavioral adaptations that can be of great significance for *in situ* bioremediation purposes. Chemotaxis is one of the most important adaptations, because it allows increased bioavailability of the pollutant and thereby helps in maximization of pollutant degradation (Pieper & Reineke, 2000; Pandey & Jain, 2002; Parales & Harwood, 2002; Law & Aitken, 2003). Quite a few bacterial strains belonging to diverse taxonomic groups have been identified to exhibit chemotaxis towards environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs), nitroaromatic compounds (NACs), petroleum-associated hydrocarbons, explosives and their respective metabolic intermediates/transformants (Grimm & Harwood, 1997; Bhushan *et al.*, 2000, 2004; Samanta *et al.*, 2000; Gordillo *et al.*, 2007). During the initial chemotaxis studies, it was recommended to classify the bacterial chemotaxis on the basis of the 'chemoattractant' involved in the process (Parales *et al.*, 2000; Parales, 2004). On the other hand, a more common method for classification of chemotactic behaviors is based on the association of chemotaxis with metabolism. This method divides all chemotactic responses into two subgroups viz., (1) metabolism-dependent and (2) metabolism-independent chemotaxis. The latter is usually associated with pollutants that are cometabolically transformed to generate a lesser toxic product, whereas the former is associated with pollutants that are used as the source of metabolic energy (Grimm & Harwood, 1997; Samanta *et al.*, 2000; Pandey & Jain, 2002). Apart from the above chemotactic activities, a relatively recently observed form of chemotaxis is characterized by bacterial movement towards terminal electron donors/acceptor (Childers *et al.*, 2002; Methe *et al.*, 2003). Because electron donors/acceptors act as important components of the metabolic machinery, therefore, bacterial movement towards electron acceptor/donors may also be categorized under metabolism-dependent chemotaxis. Environmental microbiologists have generally acknowledged that *in situ* bioremediation processes can be considerably aided by implementation of bacterial strains that have a positive chemotaxis towards degradation substrates (Lopez-de-Victoria & Lovell, 1993; Parales *et al.*, 2000; Lovley, 2003).

Conventional studies for bacterial chemotactic behavior have focused on the phenotypic characterization of chemotactic responses in an *in vitro* environment, based on assays (e.g. drop-plate, swarm plate and capillary assay) that have been used successfully for qualitative determination of chemotaxis with a simple uniform medium (Harwood *et al.*, 1984, 1990; Shonnard *et al.*, 1992; Armitage, 2003). Further, it has been suggested that development of assays for quantitation of chemotactic response may bring about a significant improvement in the determination of chemotactic behavior as well as in the development of bioremediation technology (Marx & Aitken, 1999; Pedit *et al.*, 2002; Olson *et al.*, 2004). In one of the successful studies of quantitation of bacterial chemotaxis, Paul *et al.* (2006a) demonstrated chemotaxis of *Ralstonia* sp. SJ98 towards PNP in soil under conditions that were designed to mimic environmental conditions. This study provided a significant improvement for chemotactic assays by incorporating a 'flow cytometry'-based quantitation of the movement. The other major contribution of this study was that it could successfully demonstrate the occurrence of chemotaxis in a complex nonhomogenous medium like soil. The findings from this report further strengthen the idea of chemotactic bacteria overcoming the limitations of pollutant bioavailability even under natural environmental conditions. The development of assays for qualitative as well as quantitative characterization of chemotactic responses is expected to improve the *in situ* bioremediation methods significantly. However, concomitantly, it is also important to investigate the molecular mechanism(s) involved in the regulation of these chemotactic responses. With the advent of whole-genome sequencing and transcriptome analysis, it is being realized that the distribution of chemotaxis-related genetic elements is much wider than was initially expected (Joseph & Beier, 2007; Lange *et al.*, 2007; Li *et al.*, 2007a, b; Porter *et al.*, 2007). Earlier, some studies had characterized DNA fragments harboring genes associated with bacterial chemotaxis (Froyen *et al.*, 1997; Delgado *et al.*, 1998; Ditty *et al.*, 1998; Hauwaerts *et al.*, 2002). Based on the limited information available on the chemotactic response, it could be stated that regulation of bacterial chemotaxis is largely based on 'phosphorylation' and 'dephosphorylation' of transducer and effector proteins arranged in a cascade manner. Further, detailed understanding of the regulatory mechanism determining the chemotactic behavior may allow genetic manipulation of nonchemotactic strains (efficient pollutant degraders) to confer them with mobility and thus improve the chances of their successful application for *in situ* bioremediation.

Biofilms and biosurfactants: role in improving bioavailability

As stated earlier, microorganisms exhibit diverse behavioral adaptations for successful survival when exposed to

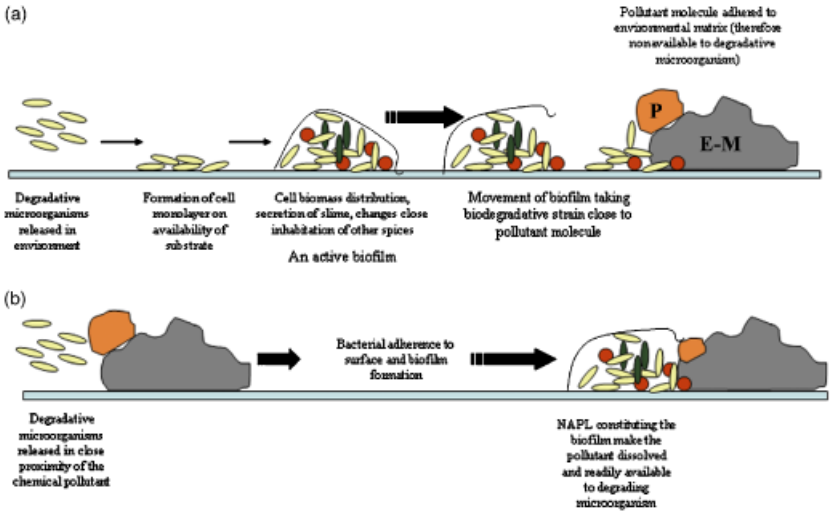

Fig. 3. Schematic representation of two of the most common methods for biofilm mediated enhance of pollutant bioavailability (consequently pollutant biodegradation).

environmental stresses during *in situ* bioremediation (Chavez *et al.*, 2006). Formation of a 'biofilm' and/or a 'biosurfactant' is one such adaptive response of microorganisms that can be successfully implemented for improvement of *in situ* bioremediation processes (Johnsen & Karlson, 2004; Paul *et al.*, 2006b). Most of the microorganisms under natural environmental conditions are found in multicellular aggregates that remain coated within a slimy material synthesized by some of the bacteria on adherence to a matrix or a substrate. This slimy coating is an important characteristic of the microorganisms present in the environment and has been identified as a 'microbial biofilm' (Costerton *et al.*, 1999; Guerrero *et al.*, 2002). Initial studies with microbial biofilms indicated their important role in microbial pathogenesis, wherein biofilms were reported to act for bacterial survival against the host's defense mechanism (Gristina & Costerton, 1985; Nickel *et al.*, 1986; Reed *et al.*, 1986; Passerini *et al.*, 1987). However, later, this phenomenon was found to be associated with almost all the *in situ* microbial activities related to community behaviors such as quorum sensing, bacterial signaling, etc. (Donlan, 2002; Filloux & Vallet, 2003; Pasmore & Costerton, 2003; Ward *et al.*, 2003). The role of the microbial biofilm in the improvement and enhancement of biodegradation was realized quite early by the initial studies, wherein the kinetics of biodegradation of chlorinated aliphatic compounds and polychlorinated hydrocarbons were hastened with the use of biofilm reactors (Fathepure & Vogel, 1991; Korde *et al.*, 1993). However, the same has been confirmed only by some of the recent systematic studies (Michel *et al.*, 2007; Schaule *et al.*, 2007).

Studies related to the physiological characterization of biofilm structure have demonstrated that more than 95% of the biofilm matrix is constituted of water or some non-aqueous phase liquid (NAPL); therefore, it forms a static yet mobile microbial environment (Sutherland, 2001; Hendrickx *et al.*, 2002; Solano *et al.*, 2002; Tsuneda *et al.*, 2003). A simple and straightforward consequence of the formation of such a static yet mobile environment is the ability of movement over long distances (on a microscopic scale) from the point of biofilm formation, resulting in improved pollutant bioavailability (Wick *et al.*, 2002). Another, mechanism of biofilm-mediated improvement of pollutant bioavailability is based on the dissolution of the pollutant chemical in the NAPL (Mukherji & Weber, 2001; Chu *et al.*, 2003). Figure 3 shows a stepwise representation of microbial biofilm formation and two of the most commonly accepted mechanisms for biofilm-based enhancement of bioavailability and biodegradation of chemical pollutants. Further, the mechanisms of the effect of biofilms on microbial degradation are still being investigated and it is expected that a detailed understanding of this phenomenon can lead to the development of considerably improved *in situ* bioremediation methods.

Another related microbial feature that has been identified to strongly influence the *in situ* bioremediation of chemical pollutants is the 'production of biosurfactants'. According to the common definition, biosurfactants (BS) are amphiphilic compounds produced on living surfaces (mostly microbial cell surfaces) and contain hydrophobic and hydrophilic moieties that reduce the surface tension (ST) and/or the interfacial tensions between individual molecules (Zhang & Miller, 1992; Lang & Philp, 1998; Benincasa *et al.*, 2004). The beneficial role of a biosurfactant in the biodegradation of different chemical pollutants was identified almost concurrently with the discovery of biosurfactants (Zhang & Miller, 1994; Iqbal *et al.*, 1995; Miller, 1995); however, details of the mechanism are still to be worked out. Sekelsky & Shreve (1999) reported a positive effect of biosurfactant application on the degradation of hexadecane by *P. aeruginosa*.

Similarly, another study reported an enhancement in wastewater bioremediation by addition of surfactins produced by a *Bacillus subtilis* strain (Moran *et al.*, 2000). Like biofilms, the biosurfactant-based enhancement of bioremediation is also mediated via improvement of pollutant bioavailability to the degradative microorganism(s) (Garcia-Junco *et al.*, 2003; Lu *et al.*, 2003; Chang *et al.*, 2004). The principle of ST suggests an inversely proportional relation between the ST and the spread of liquid substance on the surface. Therefore, by reducing the ST, biosurfactants maximize the interaction of dissolved chemical pollutants and the microbial cell surface. Although there may be a few other mechanisms involved in biosurfactant -mediated enhancement of bioremediation; their detailed understanding is subjective to future investigations. As per the present understanding and information, it could be easily summarized that the microbial strain(s) with the ability to synthesize adequate biosurfactants can be of great use for *in situ* bioremediation purposes.

Ecological sustainability of the *in situ* bioremediation process

One of the other major reasons for the limited application of *in situ* bioremediation has been the lack of measures to assess the ecological sustainability and consequences of the process. Assessment of the *in situ* bioremediation process may include a vast variety of observations; however, from the ecological sustainability point of view, it is most important to address the issues pertaining to (1) the efficiency of pollutant degradation kinetics; (2) soil/groundwater ecotoxicity of the residual pollutant; (3) the environmental fate of the degradative potential; and (4) the ecological consequences of a technological intervention. The need for such an assessment has paved the way for several studies that have focused on the development of method/approaches to perform the desired assessment. Further, some of the reports have also pointed to the use of integrated approaches for the successful assessment of the ecological sustainability of the *in situ* bioremediation process. The next section of this review provides a detailed account of some of the important approaches that have been implemented/will be implemented in the future for the assessment of the ecological sustainability and efficiency of the *in situ* bioremediation processes.

Efficiency of pollutant degradation kinetics

Conventionally, the efficiency of most of the *in situ* bioremediation processes has been evaluated in terms of the time-dependent end-point measurement of complete disappearance of the target pollutant(s) (Marvin-Sikkema & de Bont, 1994; Hageman *et al.*, 2004). Alternatively, the more logical variants of such studies attempted to assess the efficiency of the *in situ* biodegradation process on the basis of comparative kinetics of pollutant removal (Alvarez-Cohen & Speitel, 2001; de Melo Plese *et al.*, 2005). Such kinetics-based bioremediation studies have revealed several mechanistic insights into the pollutant degradation under *in situ* conditions. The kinetics-based *in situ* biodegradation studies have been considerably aided by the advancements in different analytical methods such as GC, GC-MS, HPLC, liquid chromatography-MS (LC-MS), ion chromatography, proton-nuclear magnetic resonance (1H-NMR), etc. (Combourieu *et al.*, 2001, 2004; Delort & Combourieu, 2001; Pieper *et al.*, 2002; Baroja *et al.*, 2005; Cledera-Castro *et al.*, 2005; Korenkova *et al.*, 2006). The effective monitoring of microbial degradation under *in situ* conditions is rather poor because in many cases the decrease in the pollutant concentration may be observed as an outcome of adsorbance of the pollutant to the environment matrix. In a study, proton high-resolution magic angle spinning NMR (1H HR MAS NMR) was implemented successfully to differentiate between pesticide (4-chloro-2-methylphenoxyacetic acid) molecules that were adsorbed to highly hydrated clay and the mobile pesticide molecules (Combourieu *et al.*, 2001). Similarly, in another study, a large-volume injection GC-MS (LVI-GC-MS) method was implemented for the detection and quantitation of organic compounds for example pentachlorobenzene, hexachlorobenzene, *o*-terphenyl and *m*-terphenyl in the extracted sample of the fly ash (Korenkova *et al.*, 2006). The *in situ* degradation of phenoxyalkanoic acid herbicides at three different environmental sites could be monitored quantitatively with HPLC analysis by Harrison *et al.* (1998). The contamination levels of the test herbicides decreased from 2000 to 10 mg L^{-1} only in the case of microbial degradation under the aerobic condition. Recently, HPLC analysis was used successfully in combination with IEC to achieve a comprehensive understanding of the kinetics of 2,4,6-trichlorophenol (TCP) degradation by a preacclimatized microbial consortium (Snyder *et al.*, 2006).

Among many other analytical methods that have been used recently for assessment of the *in situ* biodegradation, the most effective ones are based on spectroscopic analyses for example UV-Vis, fluorescence and fourier transform infrared (FTIR) spectroscopy. Spectroscopic methods have also been used for analysis of chemical pollutants that were not identified previously (Prieto *et al.*, 1999; Sun *et al.*, 2000; Weber *et al.*, 2000). The major advantage of spectroscopic methods is their ability for rapid monitoring of the degradation process along with identification of degradation intermediates produced during the metabolic process. UV-Vis spectroscopy has also been integrated successfully with mathematical modeling for determination of the substrate utilization process, microbial activity

products and biomass-associated products as indicators of the *in situ* load of different organic chemicals in a test sample (Carvallo *et al.*, 2007). The specific ability of FTIR spectroscopy to distinguish even among very similar chemical structures was used along with the GC-MS studies of biotransformation of 1,1-dichloro-2,2-bis(4-chlorophenyl) ethylene (DDE), a molecule that was earlier considered to be a dead-end product of dichloro-diphenyl trichloroethane (DDT) transformation. In this study, *Pseudomonas acidovorans* strain M3GY was identified for the ability to further transform DDE; however, the transformation products could only be identified using GC-MS-FTIR analysis (Hay & Focht, 1998). Such reports have clearly established the positive impact of different analytical methods on the bioremediation studies including *in situ* bioremediation studies/trials. The search for further development and advancement of analytical methods needs to be continued. It is also justified to acknowledge that most of these methods are based on the physico-chemical characteristics of the chemical compounds and their submolecular components and consequently the analytical methods canhave inherent advantages as well as disadvantages. Table 3 lists some of the most common methods (along with their respective prominent features) that have been implemented for the analysis of chemical pollutant(s) during *in situ* bioremediation studies.

It is equally important to mention that the analytical ability to evaluate the kinetic efficiency of pollutant removal during *in situ* bioremediation has also been aided considerably by the advancement of different pollutant extraction processes. The majority of the methods for pollutant extraction from environmental samples are based on the chemical characteristics of the target pollutant(s) and have been classified as 'exhaustive' and 'nonexhaustive' extraction methods (Shacter, 1984; Reid, 1986; Zambonin, 2003). On the basis of the chemical nature of the extraction treatment, they may also be classified as: (1) organic solvent extraction; (2) chemical oxidation extraction; (3) supercritical fluid extraction; and (4) aqueous sample extraction. Semple *et al.* (2007) have recently provided a comprehensive review of different extraction methods indicating their functional classification, target contamination, working principle, weaknesses and strengths. Further, recent studies for efficient pollutant extraction are targeting the physico-chemical nature (hydrophobicity, soil adsorption, aqueous solubility, etc.) of the pollutant(s) for developing the efficient extraction methods (Szolar *et al.*, 2004). The other common method for pollutant extraction focuses on the use of selective binding abilities to develop affinity chromatography-based extraction of chemical pollutants (Stevenson, 2000; Liu & Ding, 2001; Tudorache & Emneus, 2006).

Alternative methods for determining the pollutant degradation kinetics are based on indirect methods such as 'growth response of the pollutant-degrading strain'; 'appearance of degradation metabolites'; 'consumption of molecular oxygen (O_2)'; or 'evolution of carbon dioxide (CO_2)' biodegradation (Semprini, 1995; Barrena Gomez *et al.*, 2005; Esteve-Nunez *et al.*, 2005). With advancement in analytical chemistry methods, it has become feasible to monitor even the smallest difference in the pollutant quantities or determination of formation of a very small amount of a degradation intermediate at the site of bioremediation. However, this advancement has also neccessitated the need for (1) inefficient extraction of chemicals from environmental samples and (2) ability to distinguish between metabolic degradation vs. nonbiological removals. Different extraction methods perform differently under a given environmental

Table 3. Some of the useful analytical methods that have been implemented for determining the efficiency and sustainability of *in situ* bioremediation

Method	Comments
GC	Common method used for qualitative as well as quantitative analysis of complex mixture (such as intermediate of degradation process). Highly sensitive methods for identification of volatalizable compounds
HPLC	Works on the principle of solubility of compound(s) in polar and/or nonpolar solvents. The method supplements accurate identification of chemical compounds by generating their electro-magnetic absorption and emission spectrum
Thin layer chromatography (TLC)	One of the most frequently used methods for fast and reliable identification of a number of samples over a short time period. These methods still constitute major preliminary study for most of the *in situ* bioremediation
Respirometry	These methods work on an indirect basis for quantification of pollutant degradation according to the consumption of molecular oxygen or evolution of carbon dioxide
Spectroscopy	Spectroscopy methods include different methods for example UV-Vis spectroscopy, fluorescence spectroscopy, X-ray diffraction spectroscopy, Raman spectroscopy and high resolution electron diffraction spectroscopy
Nuclear magnetic resonance (NMR)	A highly accurate method for identification of compounds (including chemical pollutants and their microbial degradation products). The method is based on characterization of slightest differences in the quantum mechanic resonance of the atoms in a give molecule for ascertaining its identity
Solid-phase micro extraction (SPME) analysis	Determination of volatile and semi-volatile compounds during *in situ* biodegradation of complex mixtures such as oil spills
Quantitative structure relationships (QSBR)	QSBRs are core relations between molecular structure, activity and biodegradation; Biodegradability commonly use simple or multiple regression analyses and predicts biodegradability in terms of biodegradation rates and constants, half-lives, theoretical oxygen demand (ThOD), biochemical oxygen demand (BOD)

condition and, therefore may lead to an inconsistent conclusion about pollutant degradation (Hawthorne *et al.*, 2000). Similarly, the quantitation of the target pollutant (at the site of intervention) may vary as a consequence of nonbiological phenomena such as diffusion, wash-off, leaching, photolysis, adsorption to the substrate, etc. (Strand *et al.*, 2003). This situation has paved the way for improvement of chemical extraction methods and development of radioactive substance tracer techniques to discriminate biotic degradation from abiotic degradation.

Majority of the methods for pollutant extraction from environmental samples are based on the chemical characteristics of the target pollutant(s) and have been classified as 'exhaustive' and 'nonexhaustive' extraction methods (Shacter, 1984; Reid, 1986; Zambonin, 2003). On the basis of the chemical nature of the extraction treatment, they may also be classified as: (1) organic solvent extraction; (2) chemical oxidation extraction; (3) supercritical fluid extraction and (4) aqueous sample extraction. Semple *et al.* (2007) have recently provided a comprehensive review of different extraction methods indicating their functional classification, target contamination, working principle, weaknesses and strengths. Further, recent studies on efficient pollutant extraction are targeting the physico-chemical nature (hydrophobicity, soil adsorption, aqueous solubility, etc.) of the pollutant for developing efficient extraction methods (Szolar *et al.*, 2004). The other common method for pollutant extraction focuses on the use of selective binding abilities to develop affinity chromatography-based extraction of chemical pollutants (Stevenson, 2000; Liu & Ding, 2001; Tudorache & Emneus, 2006).

As mentioned above, to determine the *in situ* applicability and sustainability of the bioremediation process, it is also very important to distinguish between the metabolic vs. nonbiological degradation of the target pollutant. One of the most preferred technologies for such an evaluation has been 'stable isotope fractionation' (SIF) (Chu *et al.*, 2004; Meckenstock *et al.*, 2004; Morrill *et al.*, 2005; Vieth *et al.*, 2005; Lee *et al.*, 2007). The theoretical principle of SIF is based on the fact that the majority of the elements (e.g. carbon, hydrogen, oxygen, nitrogen, sulphur and chlorine) that constitute chemical pollutants have at least two stable isotopes (a heavy isotope and a light isotope) that can be distinguished easily on the basis of MS (Sturchio *et al.*, 2003; Somsamak *et al.*, 2006). Also, these compounds, consisting of the above elements, are a mixture of isotopomers wherein the percentage abundance of two isotopes is a fixed ratio for each element. For example, all the organic compounds comprise 99% molecules with ^{12}C whereas only *c.* 1.11% of the molecules have ^{13}C. Importantly, these isotopomers have identical physico-chemical characters, except for the slight difference in the 'quantum mechanical properties' that results in the lighter isotopomer forming relatively weaker chemical bonds. The lighter isotopomers are preferred reaction substrates for all the physico-chemical reactions because they require lesser activation energy (Mancini *et al.*, 2003; Sturchio *et al.*, 2003). On the other hand, in case of biological systems the bond cleavage reactions proceed via an alternative transition state(s) with the requirement of lesser activation energy; therefore, biological degradation can act on both types of isotopomers. A direct implication of this biotic activity is that the ratio of percentage abundance for heavier and lighter isotopes becomes altered (Rosell *et al.*, 2007). This property of the fixed percentage abundance ratio can be used as an indicator of the extent of biological degradation during *in situ* bioremediation. Further, this method can easily distinguish between metabolic degradation of pollutants and/or their nonbiological removal. One of the studies demonstrated that the application of 'stable carbon isotope fractionation' can be used for quantitative monitoring of *in situ* degradation of benzene and toluene in contaminated aquifers (Vieth *et al.*, 2005). Stable isotope fractionation has also been used for quantitative monitoring of the biodegradation of chemical pollutants in *ex situ* trials. VanStone *et al.* (2005) used this method to estimate the degradation of trichloromethane in a bioreactor-based experimental setup.

Technological advancements in the methods for qualitative and quantitative estimation of chemical pollutants and their degradation intermediates, along with improvement in the pollutant extraction methods, have led to accurate assessments of the efficiency of pollutant degradation kinetics. Further developments in these fields, along with the ability to distinguish between metabolic and nonbiological removal of pollutants, would lead to increased application and success of *in situ* bioremediation methods.

Assessment of soil and groundwater ecotoxicity

Assessment of the soil/groundwater ecotoxicity at different time points during the bioremediation process is another vital aspect for determining its applicability and ecological sustainability (Dodard *et al.*, 1999; Hubalek *et al.*, 2007). An ideal bioremediation process should result in an overall reduction in the ecotoxicity of the target environment on its completion. Most of the initial bioremediation studies did not conduct an ecotoxicity test; however, some of the later studies clearly indicated their significance. The ecotoxicity of hydrocarbon-contaminated soil was monitored over the period of the bioremediation trial as an important component of the bioremediation study (Dorn & Salanitro, 2000). The results obtained in the study showed that despite reduction of petroleum hydrocarbons in the soil, the ecotoxicity remained as high as it was at the beginning of the bioremediation. Another study on microbial degradation of

total petroleum (TPH) within contaminated soil resulted in the reduction of TPH by *c.* 65%. However, the ecotoxicity (as monitored by most common bioassays) did not decrease with the reduction of the pollutant concentration (Hubalek *et al.*, 2007). On the contrary, some of the studies have also indicated that the ecotoxicity of a contaminated site may sometimes increase as a consequence of the biodegradation activity. For example, it was observed that microbial degradation of higher chlorinated solvents resulted in accumulation of the degradation metabolites (i.e. lesser-chlorinated intermediates) in the subsurface water, which led to an overall increase in the ecotoxicity of the polluted environment (McDaniel *et al.*, 2004). Another example was observed in the case of microbial transformation of nitroaromatic compounds into their reductive degradation intermediates (nitroso and nitroamino compounds), which are even more toxic to different life forms (Dodard *et al.*, 1999). Therefore, it may be suggested that the ecotoxicity is not a straightforward outcome of the degradation of the target pollutant and it is important to monitor the ecotoxicity status of the bioremediation process.

Initially, the term 'ecotoxicity' was used to explain the hazardous effects of purified chemicals on different life forms as well as the environment (Cushman *et al.*, 1982; Macri & Sbardella, 1984; Neuberger, 1984). However, later, it has also been used with respect to complex environmental/industrial samples (e.g. soil, water, sediment, industrial effluent release, etc.) (Tabak *et al.*, 2003; Baun *et al.*, 2006). The improved ability to assess the toxicity of environmental samples along with the purified chemicals has been based on continued development of ecotoxicity assays and methodologies (Schultz *et al.*, 2002; Clement *et al.*, 2004; Joner *et al.*, 2004; Baun *et al.*, 2006). These methodologies may follow diverse principles depending on the class of the test chemicals or the environmental sample; however, for a detailed assessment, it is mandatory to measure the toxic effects of purified chemical(s) on members of different levels of the trophic chain i.e. producer (blue-green alga), primary consumer (crustaceans, amphibian, etc.), secondary consumer (fishes) and microorganisms (*P. putida, Photobacterium phosphoreum, Vibrio fischeri*, etc.) (Zeeman *et al.*, 1995; Pollack *et al.*, 2003; Licht *et al.*, 2004). Alternatively, some of the tests make use of specific organisms rather than assessing the toxicity against a number of different test organisms. One of the most commonly used methods for rapid measurement of chemical toxicity is based on the use of luminescent marine bacteria viz., *V. fischeri* (Richardson, 1996). This method performs a comparative detection and quantitation of bioluminescence in the presence and absence of the test chemical(s). Similarly, other common ecotoxicity assessment methods use shrimps (*Artemia* sp.), earthwarms (*Eisenia fetida*) and crustaceans (*Daphnia*) (Charrois *et al.*, 2001; Sobral *et al.*, 2001; Seco *et al.*, 2003; Nunes *et al.*, 2006). Other lesser common tests involve animal models like amphibian larvae and arthropods for testing the ecotoxicity of different test chemicals (Mouchet *et al.*, 2007).

Most of the biological assays used for monitoring the ecotoxicity e.g. (1) the fish 96 h-LC50 test, (2) the chronic fish test, (3) the acute crustacean test, etc. monitor the lethality of the test chemical/sample over a range of concentration at different time points during the test. Therefore, these may not be best suited for a high-throughput and quick evaluation of the ecotoxicity during bioremediation trials. Further, their use may also be limited because they are noneconomical and they require large sample volumes to determine the toxicity of the test samples. To address the need for alternative high-throughput methods, several recent studies have attempted to develop tests that are based on *in vitro* assays, biomarker-based assays, high-throughput chemical assays, etc. In one of the *in vitro* assays, a human cell line containing a heat shock protein (hsp70) promoter-linked selection marker gene *cat* (chloramphenicol acetyltransferase) was used to assess the toxic potential of chemical mixtures (Ait-Aissa *et al.*, 2003). The response of the hsp70 promotor towards different chemicals was measured by the *cat* test. Importantly, the results obtained with this method were found to be in close agreement with other toxicity assessment bioassays. Another recent report showed the development of a total environmental transcriptional analysis (microarray analysis-based method) for a rapid, cost-effective and high-throughput assay for the assessment of the ecotoxicity of multiple environmental samples. The above microarray incorporated DNA probes from 28 different environmental samples and it could be simultaneously hybridized with 64 different gene probes (Fredrickson *et al.*, 2001). Other studies have indicated the use of cellular biomarkers as being a cost-effectve and rapid means for assessment of ecotoxicity. In one of the recent studies, the immune response modulators viz., interleukins (IL-1, IL-2, IL-10), interferons (IFN-γ) and tumor necrotic factor (TNF-α), were used as sensitive biomarkers for the determination of the ecotoxicity of wastewater samples (Kontana *et al.*, 2008).

Alternatively, some of the studies have demonstrated the use of chemical methods to determine the ecotoxicity of test samples (Schultz *et al.*, 2002). Chemical assessment methods may well serve as complementary methods to the commonly used bioassays of ecotoxicity determination. This was found in one of the studies that evaluated the ecotoxicity of waste sludge using the chemical and biological methods (Mantis *et al.*, 2005). The chemical assessment was performed with a standard leaching test, whereas the acute toxicity to bioluminescence bacteria was used for the biological evaluation of the ecotoxicity of the process. Importantly, the results obtained from the biological assessment method found the treatment to be safe; however, the leaching test indicated

potential environmental risks. Therefore, it may be recommended that the ecotoxicity assessment of the bioremediation process should be evaluated by chemical as well as biological methods. Further, advancement of genomic, transcriptomic and proteomic tools will lead to the development of a new set of rapid and high-throughput ecotoxicity tests. These tests would target the alteration of the transcriptional response of stress-related genes in different test organisms as a direct indicator of the ecotoxicity of a chemical or an environmental sample.

Environmental fate of degradative potential

According to the information available in the literature, it could be readily inferred that the majority of the initial *in situ* bioremediation studies attempted to explain the efficiency of the process only on the basis of the kinetics of pollutant removal. However, later, this approach was expanded to include 'environmental fate of biodegradative organism' as one of the most important determinants for an efficient *in situ* bioremediation process (Atlas & Sayler, 1988; Awong *et al.*, 1990; Errampalli *et al.*, 1999; Backman *et al.*, 2004). A definition of environmental fate is difficult and tricky to envisage; however, the term has been used broadly to refer to the 'survival' and 'activity' of a bacterial strain under natural environmental conditions (Labana *et al.*, 2005a). A number of studies on *in situ* bioremediation have clearly indicated the need to monitor bacterial cell survival and activity with pollutant degradation (Schmidt *et al.*, 1995; Winkler *et al.*, 1995; Huertas *et al.*, 1998). Further, a few other studies have also established a positive correlation between 'bacterial cell survival' and the degradation of the target pollutant (Thomas *et al.*, 2000; Ambujom, 2001; Kang & Kondo, 2002). The positive effect of bacterial survival on the efficiency of *in situ* pollutant degradation was shown systematically in a study where degradation of 3-phenoxybenzoic acid (3-POB) in contaminated soil was carried out by *P. pseudoalcaligenes* strain POB310 and two modified *Pseudomonas* strains. The results obtained demonstrated that the modified strains had better survival under natural environmental conditions than strain POB310; the modified strains could completely degrade target pollutant whereas the degradation with strain POB310 was far less efficient under the most favorable conditions (Halden *et al.*, 1999). Similarly, several studies have justified the importance of sustained microbial activity for successful completion of the pollutant removal in *in situ* bioremediation trials (Thouand *et al.*, 1995; Park *et al.*, 2001; Labana *et al.*, 2005b). Some of the studies have indicated the positive effect of using preinduced microbial cells/inoculum for *in situ* biodegradation purposes (Santos *et al.*, 2003; Labana *et al.*, 2005b). With increasing understanding of the essential role of bacterial survival and activity in successful *in situ* bioremediation, it is now generally acknowledged that it is indispensable to monitor the survival and activity of degradative microorganisms to maximize sustained bioremediation under natural conditions. The need for monitoring bacterial survival and activity under natural environmental conditions has paved the way for development of scientific methods to accurately evaluate the bacterial fate after introduction into natural environments. Several methods ranging from plating and CFUs counts to cell sorting using flow cytometry and immunochemical methods have been implemented for the above purpose (Thomas *et al.*, 1997, 2000). All these methods offer some advantageous features as well as a few limitations and yet their justified use can be very beneficial for *in situ* bioremediation approaches. Table 4 provides a list of some of the important methods that have been commonly implemented for such monitoring. From the methodology point of view, these methods can be divided into two subgroups viz.: (1) culture-dependent methods and (2) culture-independent methods.

Culture-dependent methods: CFU counting and colony hybridization

In initial studies of *in situ* bioremediation, the survival of degradative microorganisms was monitored by conventional 'culture-dependent methods' such as CFUs count (Huertas *et al.*, 1998; Mannisto *et al.*, 2001; Park *et al.*, 2004). One of these studies evaluated the survival of different *Pseudomonad* strains in soil after they were exposed to a solvent shock. This evaluation was purely based on the CFUs count analysis. The result obtained showed that the test *Pseudomonads* were sensitive to toluene as indicated by a decrease of several log units of CFUs count per gram of soil for all the test strains (Huertas *et al.*, 1998). The studies with CFUs counts have been performed using both a nonselective medium and a selective/screening medium. The method largely made use of characteristic growth features of the target bacterial strain for example distinct colony morphology, antibiotic resistance and the ability to grow on unusual carbon sources. CFUs count is still one of the quickest and preferred methods for monitoring the ecological survival of the target microorganism (Labana *et al.*, 2005a; George *et al.*, 2008). Importantly, in several studies the results obtained with CFUs have been found to substantiate and corraborate the findings of molecular methods. However, a CFUs count may be difficult to perform if the target microorganisms do not exhibit a very distinct colony phenotype or if they cannot be distinguished easily from the background microbial communities. This limitation could be partially overcome with the use of 'colony hybridization', a technique that is carried out to discriminate among morphologically similar organisms. This method was

Table 4. Some of the regularly implemented methods for assessment of environmental fate of microbial potential used for *in situ* bioremediation and its ecological consequences

Method	Comments
Plating/CFU counting	A quick and easy method for estimation of bacterial cells survival during *in situ* bioremediation; subjective to errors based on the inherent biases of culture-dependent methods. It is also adversely affected by microorganisms acquiring 'viable but not culturable' (VBNC) state
BIOLOG	A method based on assimilation/hydrolysis of different carbon substrate for analysis of microbial activity. However, the successful use of this method is sensitive to inoculum sizes
Active cell staining	A culture-independent method for direct comparative enumeration of active microbial population during *in situ* bioremediation. However, the regular application of this method is limited due to the inability to distinguish the desired catabolic activity in the environmental background
Most probable number-PCR	A culture-independent method targets amount of DNA in environmental sample and results from this method can be correlated to the amount of bacterial cells present in the environment. Provides selective advantage based on the sequence specificity of PCR primers but it may not differentiate amongst live or dead bacterial cells
DNA : DNA hybridization	A culture-independent methods that estimates the abundance of a target gene fragments characteristic to degradative microorganism. This method is very similar to MNP-PCR in terms of advantages and limitations
Colony hybridization	A cultivation-based method that is one of the most frequently use method for monitoring the bacterial cell survival during *in situ* bioremediation studies. Although, the method allows a differentiation between live and dead cells but it is subjective to biases associated with culture-dependent method
Soil enzyme analysis	Soil enzyme analysis for constitutively expressed bacterial enzymes such as dehydrogenase, lipase etc. have been used in some of the *in situ* bioremediation studies as indicators of impacts of technological intervention on the indigenous microbial community
Immunochemical enumeration	If coupled with flow cytometry method, immunochemical enumeration constitutes a very strong method monitoring of survival and activity of bacterial cells used for *in situ* bioremediation. The high-affinity binding of bacterial cell surface antigen with antibody provides high degree of sensitivity to the method
FISH	Spatial and temporal monitoring of microbial cell based on the visualization of fluorescence. The method is based on specificity of DNA–DNA hybridization and ease of visual observation of emitted fluorescence. Despite these advantageous features, this method has not yet been very common amongst the *in situ* bioremediation studies due to high cost and labor required
Genome tagging	This method is based on integration of 'non-natural' DNA sequence(s) in the genome of the microorganism before *in situ* application, followed by PCR-based monitoring of integrated DNA sequence as an indirect measure of bacterial cell survival
Bacterial sensors	This method uses fusion constructs of a reporter gene to promoter element induced by the target compound, offer the possibility to characterize the biodegradability of specific contaminants present in a complex mixture without pretreatment of the environmental sample
Microarray	Rapid method for automated determination of transcriptional activity, allows justifications for the pollution-removal kinetics as well along with monitoring the bacterial cell survival. With rapid advancement in microarray technology, it is now feasible to monitor the transcriptional behavior of even the large bacterial community. A high-throughput method; it is being increasingly used for *in situ* bioremediation studies
Metabolic gene probing	Detect gene with function of interest, mRNA detection can reveal information about expression, limited to known genes activity cannot be inferred from the presence of genes alone
2D gel electrophoresis	Although a technically difficult method to standardize, it allows the analysis of comparative bacterial cell behavior during bioremediation at total proteome level
Amplified rDNA restriction (ARDRA)	A simple method for characterization of diversity and richness of the microbial community under analyses Further, this method allows the downstream confirmation by DNA sequencing. Quite a few *in situ* bioremediation studies have used this method for assessment of spatial and temporal changes in indigenous microbial population. Like all PCR-based method, ARDRA has also been reported to be subjective biases inherent to PCR amplification
Terminal restriction fragment length polymorphism (T-RFLP)	Offers high-throughput analysis of microbial community structure and its dynamics during *in situ* bioremediation studies. It has been one of the most frequently used methods for assessment of the ecological impacts of *in situ* bioremediation. However, the interpretation of T-RFLP data can be difficult and requires complementary analysis with multivariate statistical analysis to draw meaningful information. Another limitation associated with T-RFLP analysis is inability for downstream DNA sequencing, which might be necessary for some of the analyses
Denaturing/thermal gradient gel electrophoresis (D/TGGE)	Another PCR-based fingerprinting method that makes use of slight differences in denaturation profile of DNA fragments occurring as a consequence of base pair difference in the DNA sequence. It also offers the advantage of downstream DNA sequencing for confirmation of preliminary observation
Single strand confirmation polymorphism (SSCP)	Like D/TGGE this fingerprinting method also makes use of differential electrophoretic mobility of DNA strands with difference at nucleotide composition. This method is also one of the commonly used method for assessment of microbial community structure and dynamics during *in situ* bioremediation

developed almost three decades earlier when it was first used for screening a very large number of colonies of recombinant *Escherichia coli* clones to determine which hybrid plasmids contain a specified DNA sequence (Grunstein & Hogness, 1975). Subsequently, this method has been used for diverse applications including the screening of bacterial colonies derived from environmental samples (Layton *et al.*, 1994; Richards *et al.*, 1994; Ivanov *et al.*, 2004; Jjemba *et al.*, 2006). The method is based on the use of a selective DNA or RNA probe to bind with the DNA content of a bacterial cell and thereby distinguishing among the microbial cells on the basis of their molecular information. One of the major limitations of the comprehensive use of the colony hybridization method has been the selection of nucleic acid probes that can selectively distinguish among the positive colonies. Several efforts are being made to overcome this limitation by the development of specific DNA probes for targeting phylogenetically conserved genes (e.g. 16S rRNA gene) to distinguish among different taxonomic groups (Richards *et al.*, 1994; Wagner *et al.*, 1994; Schuppler *et al.*, 1995; Kowalchuk *et al.*, 1999). Alternatively, some of the recent studies have also used probes designed on the basis of a sequence of functionally relevant genes to monitor the environmental fate of biodegrading microorganisms (Jain *et al.*, 1987; Layton *et al.*, 1994; Labana *et al.*, 2005a).

Although colony hybridization could enhance the selectivity of the culture-dependent monitoring process, both of the above methods (CFUs count and colony hybridization) are characterized by the inherent limitations of 'culture-dependent methods'. Among these limitations, the non-amenability of *c.* 99% of microbial diversity to existing cultivation protocols represents a major limitation for the use of these methods to determine the environmental fate of microorganism(s) during an *in situ* bioremediation process. Another important feature limiting the application of cultivation-based methods is a microbial phenomenon termed as a viable but not a culturable (VBNC) state (Barer & Harwood, 1999). The term VBNC refers to a special physiological state of a microbial cell where it remains viable and efficiently performs almost all the metabolic activities; however, it becomes temporarily nonculturable (Barer & Harwood, 1999; Oliver, 2005). The temporary loss of culturability is often induced by some relatively ill-defined environmental stimuli (Ghezzi & Steck, 1999; Besnard *et al.*, 2002; Oliver *et al.*, 2005). Relatively less information is available on the mechanism of this phenomenon; however, it can be easily concluded that the VBNC state is a microbial adaptation response for survival against sudden changes in its microenvironment (Heim *et al.*, 2002; Ordax *et al.*, 2006). The mechanism is seemingly executed via alteration of the cell membrane fatty acid composition (Signoretto *et al.*, 2000; Day & Oliver, 2004). Several studies have attempted to determine the functional feature associated with the VBNC state of bacterial strain(s). Nebe-von-Caron *et al.* (2000) analyzed VBNC-based alteration in the cell functions using multi-color fluorescence flow cytometry and single-cell sorting. Other methods have largely attempted to detect the cellular features associated with the VBNC state. The basal level of transcriptional activity measured with reverse transcription PCR is one such feature that has been used to monitor the viability of the VBNC state over the time scale (Lleo *et al.*, 2000; Fischer-Le Saux *et al.*, 2002; Coutard *et al.*, 2005). With an everincreasing understanding of the VBNC state and its direct implication on reduced CFUs, it would limit the use of culture-dependent methods for monitoring of bacterial cell survival during *in situ* bioremediation.

Culture-independent methods: DNA hybridization, quantitative PCR and real-time PCR

The limitations of culture-dependent methods have resulted in increased use of culture-independent methods to determine the ecological fate of microorganisms under natural environments (Ellis *et al.*, 2003; Ahn *et al.*, 2005; Piskonen *et al.*, 2005; Ono *et al.*, 2007). This development has also been fueled by the advent of molecular methodologies for isolation of the microbial genomic DNA directly from the environmental sample without the need for culturing and isolation of bacterial strains (Dijkmans *et al.*, 1993; Paul *et al.*, 2005; Sharma *et al.*, 2007b). The DNA sample isolated directly from the environmental samples can be used for a variety of subsequent studies viz. DNA–DNA hybridization, DNA–RNA hybridization, PCR amplification of selective biomarker genes, quantification of a relevant gene, etc. (Plaza *et al.*, 2001; Chandler, 2002; Schneegurt *et al.*, 2003; Shen *et al.*, 2007). Hybridization experiments are straightforward methods to verify the presence of the target DNA fragment or microorganism and thereby interpret its survival under natural environmental conditions. Direct DNA hybridization is a relatively simple method that is primarily used to determine the presence vs. the absence of a target DNA sequence. A combination of the DNA hybridization method with densitometry analysis could also be used for determining the relative abundance of a test DNA sequence. DNA hybridization is commonly performed with two different methods: (1) dot/slot blot hybridization and (2) Southern blot hybridization. The former method is used for the quantitative detection and presence of a nucleic acid fragment or for determining the percentage relatedness of different DNA samples (Stolz & Tuan, 1996; Ji *et al.*, 2002) and, therefore, it is the preferred method for quantitation of target DNA in the environmental samples. Several improvements have been incorporated into the initial version of the DNA hybridization methods that have resulted in their application for diverse purposes such as differentiation of

microorganisms at subgenus and species levels (Wang & Wang, 1995; Pontes *et al.*, 2007). It has also been applied successfully for evaluation of the distribution of different catabolically significant genes among different microbial strains (Daly *et al.*, 1997; Vomberg & Klinner, 2000; Breitenstein *et al.*, 2001). DNA–DNA hybridization methods have also been successfully used to determine the ecological fate of the degradative microorganism(s) in *in situ* bioremediation studies (Iwamoto & Nasu, 2001; Jjemba *et al.*, 2006).

The culture-independent assessment of the fate of a target microorganism(s) has also been carried out by performing direct PCR using environmental DNA as a template and a microorganism-specific PCR primer set (Dijkmans *et al.*, 1993; Erb & Wagner-Dobler, 1993). A positive amplification of the target DNA indicates the survival of the corresponding microorganism. This method has been used extensively to track the survival of bacterial strains in *in situ* bioremediation studies (Whiteley & Bailey, 2000; Boye *et al.*, 2001). However, the results obtained from direct PCR reactions provide only qualitative information about the presence vs. the absence of the targeted DNA in the environmental samples. On the other hand, for a comprehensive understanding of bacterial cell survival, it is also very important to perform the quantitative assessment of microbial cell survival under *in situ* bioremediation conditions. This requirement can be fulfilled using quantitative PCR. Presently, there are four basic quantitative PCR approaches that have been used for monitoring microorganism(s) under *in situ* conditions: (1) quantitation with an external standard; (2) quantitation with an internal standard; (3) most probable number-PCR (MNP-PCR) and (4) competitive PCR (cPCR). The quantitative PCR works on the basis of a standard curve prepared according to the relationship between the amount of initial standard DNA template and the concentration of the final amplified product. The cPCR reaction is carried out such that the target template and the competitive standard are placed in the same reaction. Also, the primers are designed such that they can anneal with both the competitive standard and the target template; however, the amplified products are of different sizes. During PCR, the amplification occurs in a competitive manner. Because the amplification for both the fragments occurs under identical conditions, with the initial concentration of the target DNA being the only unknown factor, the initial concentration of template DNA can be calculated according to the mathematical relationship. A large number of recent *in situ* bioremediation studies have performed a competitive estimation of DNA (specific to the pollutant-degrading strain) as an integral part of their study (Lanthier *et al.*, 2000; Mesarch *et al.*, 2004; Biggerstaff *et al.*, 2007).

Further improvement in the quantitation of the DNA sample has been achieved using real-time monitoring of the PCR reaction using real-time PCR (RT-PCR). The methodology is based on the use of 'molecular beacons' (fluorescent-tagged single-stranded DNA primers) that undergo a conformational change when they anneal to the target DNA sequence during the PCR reaction. This conformational change leads to the release of fluorophore that is measured and used as the direct estimation of the amount of DNA template present in the reaction (Ong & Irvine, 2002; Mackay, 2004). The working methodology of RT-PCR is well established and has been reviewed thoroughly (Huggett *et al.*, 2005; Valasek & Repa, 2005; Kubista *et al.*, 2006; Cupples, 2008). The selective advantage of this method is its ability to quantify the DNA amount in real time during the amplification process. Unlike other quantitative PCR methods that measure the end-point amplicon quantities (which may be affected by quite a few PCR parameters other than the template concentration), RT-PCR tracks the comparative release of fluorophore units as an indicator of the number of template DNA molecules (Valasek & Repa, 2005). Several recent bioremediation studies have used RT-PCR as a conclusive method for determination of *in situ* survival of degradative strain(s) (Hristova *et al.*, 2001; Beller *et al.*, 2002; Kikuchi *et al.*, 2002; Labana *et al.*, 2005a; Van Raemdonck *et al.*, 2006).

Technological advancements in the molecular assessment of microbial survival and activity have significantly improved the understanding of the problems associated with the suboptimal performance of *in situ* bioremediation processes. This, along with development in the methods for assessing the *in situ* activity of microorganisms introduced into the environment, will enable environmental microbiologists to further improve the performance of *in situ* bioremediation.

Monitoring the activity of the degradative strain

Apart from the survival of the degradative strain, it is also extremely important that the microorganism(s) should remain active for carrying out the desired degradation reaction. This realization has resulted in an increased emphasis on the need for monitoring the degradation activity during *in situ* bioremediation (Selvaratnam *et al.*, 1995; Haroune *et al.*, 2002; Okeke & Frankenberger, 2003; Young & Phelps, 2005). Like most of the other biological systems, the biological activity during the degradation process can also be monitored by measuring the transcription and/or the translation product(s) of the genes that are involved in the degradation processes.

Analysis of the activity at the protein/transcript levels

The bacterial activity during *in situ* bioremediation (or other environmental phenomena) can be analyzed at the

level of proteins (King *et al.*, 1991; Heitzer *et al.*, 1992; Santos *et al.*, 2007; Zhao & Poh, 2008) or mRNA by direct analysis of transcription profile (Pichard & Paul, 1993; Jeffrey *et al.*, 1994). Specific enzyme activity can be measured by different enzyme assay approaches or by visualization of the whole-proteome profile on the 2D gel electrophoresis (Halden *et al.*, 1999; Gunsch *et al.*, 2006; Yoon *et al.*, 2007). Earlier, some of the studies also used 'Western blotting analysis' for determining the expression levels of significant enzymes involved in the bioremediation process (Richins *et al.*, 1997; Bott *et al.*, 2001). Despite the great potential of protein expression measurement-based methods for monitoring the microbial degradation activity during *in situ* bioremediation processes, its use has not become common due to the lack of effective methods for direct protein extraction from the environmental samples. Unlike methods for direct DNA/RNA isolation that have undergone significant improvement over the last few years (reviewed in detail by Streit & Schmitz, 2004; Rudi & Jakobsen, 2006; Herrera & Cockell, 2007; Shen *et al.*, 2007), there are not many efficient methods for direct extraction of protein samples from the environment(s). Direct protein isolation methods have been restricted by the relatively short half-life of microbial proteins and their susceptibility to structural disorganization when subjected to extraction procedures (Saleh-Lakha *et al.*, 2005).

Alternatively, the analysis of degradation activity (bacterial activity) during the *in situ* bioremediation process can also be carried out by measuring the transcriptional response of pollutant-degrading microorganisms (Wilson *et al.*, 1999). The common methods for such measurements are 'Northern blot analysis', 'reverse transcription PCR' and 'microarray analyses'. The basic principle of these methods is hybridization of a selective probe (quite often designed from the DNA sequence of genes involved in the degradation process) to mRNA samples extracted either from the degradative microorganism(s) or directly from the environmental sample. The standard methodologies of all these methods are well documented and reviewed in detail; however, a few variants of these methods have been developed recently to incorporate the ability of quantitation of transcriptional responses (Corkery & Dobson, 1998; Han & Semrau, 2004; Gunsch *et al.*, 2006). Among these methods, 'Northern blot analysis' and 'reverse transcription PCR' follow a reductive approach as they usually determine the transcriptional behavior of only a few selected genes or a gene set (Han & Semrau, 2004; Holmes *et al.*, 2005; Mahmood *et al.*, 2005), whereas 'microarray analysis' is a global/system biology approach that provides a detailed transcriptional response of all the genes of the selected microorganism (Chandler *et al.*, 2006; Butler *et al.*, 2007). Some of the recent *in situ* bioremediation studies have used 'high-density microarray' analysis successfully to evaluate the efficiency and activity of the *in situ* bioremediation process (Rhee *et al.*, 2004; Brodie *et al.*, 2006). The use of microarray analysis could provide a comprehensive picture of the compositions of biodegradation genes and the microbial community in contaminated environments. In one of the recent studies, He *et al.* (2007) generated a novel comprehensive microarray termed as 'GeoChip' that contains 24 243 oligonucleotide (50 mer) probes and covers $>10\,000$ genes in >150 functional groups involved in nitrogen, carbon, sulfur and phosphorus cycling, metal reduction and resistance and organic contaminant degradation. This GeoChip was successfully used for tracking the dynamics of metal-reducing bacteria and associated communities for an *in situ* bioremediation study. Apart from the above common methods for analysis of bacterial activity during *in situ* bioremediation, a few other alternative methods have also been used successfully. A brief description of some of these methods is given in the following.

Alternative methods for assessing survival and activity

Apart from the above methods, environmental microbiologists have also used some of the alternative methods for assessing the survival and activity of microorganisms under natural environmental conditions. FISH with targeted oligonucleotide probes has emerged as an invaluable molecular tool for assessment of the environmental survival of the degradative strain during the bioremediation process (Wagner *et al.*, 1994; Ficker *et al.*, 1999; Amann *et al.*, 2001; Aulenta *et al.*, 2004; Caracciolo *et al.*, 2005). From the methodology point of view, FISH is based on the sequence-specific *in situ* binding of a fluorescent-labeled probe to the target DNA/RNA, which results in the emission of measurable fluorescence (Thomas *et al.*, 1997). The most important characteristic feature of this method is it ability to monitor the target microorganism within an environmental sample without the need for culturability or DNA isolation. On the other hand, FISH suffers from the limitation of inefficient detection in the case of some of the very slow-growing bacteria and detection of the test microorganism in a sample of relatively small volumes. An extension of the conventional FISH method that enables improved bacterial detection within environmental samples is catalyzed reporter deposition-FISH (CARD-FISH) (Speel *et al.*, 1997). The most significant advantage of this method over FISH is its ability to detect the target bacterial cell within samples of very small volumes, samples with a low number of target bacterial cells, aquatic samples, etc. These samples are difficult to analyze with FISH, because with the small number of target bacterial cells, the hybridization signal intensity remains below the threshold detection limit. The CARD-FISH method was developed during the 1990s for

monitoring the intracellular distribution of single-copy DNA sequences with improved detection efficiency (Schmidt *et al.*, 1997; Buonamici *et al.*, 2000); however, later, it was used successfully for various purposes including tracking of bacterial cells in different test environments (Pernthaler *et al.*, 2002; Sekar *et al.*, 2003; Ferrari *et al.*, 2006). The basic working principle is based on the use of horseradish peroxidase (HRP)-labeled oligonucleotides and amplification of a fluorescent tyramide signal. Tyramines are phenolic compounds and HRP can catalyze dimerization of such compounds when they are present in high concentrations. However, at lower concentrations, the probability of dimerization is reduced, whereas the binding at or near the site of the peroxidase-binding site is favored. Numerous fluorescently labeled molecules deposit close to the hybridization site, which results in an enhanced FISH sensitivity (Pernthaler *et al.*, 2002). CARD-FISH analysis has been further improved by development of the mRNA-CARD-FISH methodology. The methodology of this process remains identical to CARD-FISH, but in this process, the detection targets are the mRNA molecule rather than the genomic DNA sequences (Pernthaler & Pernthaler, 2005). Thus, the method can be extremely useful for detection of bacterial activity for a desired function. However, like most of the other microbiological methods, CARD-FISH also has certain limitations for its universal application. The most commonly acknowledged limitation of the method relates to the use of the HRP molecule. Because penetration of HRP into bacterial cells requires permeabilization, a procedure that causes a high degree of cell loss in a species-dependent manner, it is only feebly used for detection of heterotrophic bacteria and a few other classes of slow-growing microorganisms (Pernthaler *et al.*, 2002; Sintes & Herndl, 2006). Therefore, it is rational to mention the need for further improvement of FISH and related techniques.

Another approach (including methods such as 'thymidine incorporation' and 'bromodeoxyuridine utilization') that has been used frequently for the systematic assessment of microbial survival and activity is based on the principle of incorporation of non-natural nutrients (radiolabeled amino acids, thymidine, bromouridine, etc.) in the DNA or the RNA of metabolically active microorganisms (Urbach *et al.*, 1999). Bromodeoxyuridine substrate utilization was developed to enable the identification of bacteria that grow in response to added substrate in the environment (Borneman, 1999). For identification of microorganisms that are actively involved in the metabolic process, the micro-environment is pulsed with bromodeoxyuridine. The metabolically active microorganisms utilize bromodeoxyuridine and incorporate it in to their nucleic content (Laird & Bodmer, 1967; Tice *et al.*, 1976). Later, the bromodeoxyuridine-labeled DNA is immunoprecipitated, followed using PCR amplification and sequencing of 16S rRNA gene-based identification. The metabolic activity of pollutant-degrading microorganisms has also been assessed by 'stable isotope probing (SIP)', a method that is based on selective labeling of DNA of the active microorganisms with stable isotopes (Radajewski *et al.*, 2000; Manefield *et al.*, 2002; Mahmood *et al.*, 2005; Andreoni & Gianfreda, 2007; Hatamoto *et al.*, 2007). During an SIP assessment, microorganisms are pulsed with the nucleic acid content of the metabolically active microorganism and become heavier due to the incorporation of a heavier isotope (^{13}C); later, it can be resolved from the nonlabeled (^{12}C) following CsCl density centrifugation. This DNA is subsequently identified on the basis of 16S rRNA gene sequencing.

The environmental fate of the pollutant-degrading potential can have a major effect on the efficiency as well as the ecological sustainability of the *in situ* bioremediation process. The use of conventional microbiological methods in combination with molecular tools has improved the ability of such an assessment considerably and, consequently, the effectiveness of the *in situ* bioremediation processes.

Ecological consequences of technological intervention

Monitoring the ecological consequences of any technological intervention that is directly or indirectly related to the environment (such as an *in situ* bioremediation process) is of utmost significance and it probably constitutes the most important aspect of the assessment of the ecological sustainability of a process. The scope for studying such ecological consequences encompasses several nonrelated phenomenon; however, for bioremediation technology development, a detailed analysis of the impact of the bioremediation process on the indigenous microbial community structure is most important (Wenderoth *et al.*, 2003; Roling *et al.*, 2004; Katsivela *et al.*, 2005; Vinas *et al.*, 2005a; Paul *et al.*, 2006a). The ideal remediation technology should not have any adverse effect on the total indigenous microbial community structure of the site under intervention (Iwamoto & Nasu, 2001; Mills *et al.*, 2003; Katsivela *et al.*, 2005). Traditional microbiology techniques and conventional microscopy methods are insufficient means to determine the microbial community structure. Most of the bacteria in the natural samples become nonamenable to the above methods due to their differential adherence to soil particles, sediments, water droplets and other surfaces such as plant –roots, etc. (Farrell & Quilty, 2002; Rodriguez-Navarro *et al.*, 2007). Development of methods based on the use of fluorescent dyes such as 4,6-diamino-2-phenylindole (DAPI) or acridine orange for direct staining of microorganisms within environmental samples could only offer a partial improvement of the above situation (Hesselmann *et al.*, 1999; Lozada *et al.*, 2004; Jjemba *et al.*, 2006). These methods are

based on the specific properties of the fluorescent stain (e.g. DAPI forms a fluorescent complex by attaching to the minor groove of the A–T-rich sequences of DNA) to detect the microbial cells even when present in the form of a complex with some substrate (Otto, 1994; Kapuscinski, 1995). Although such staining methods could aid in the enumeration of microbial cells, their use in environmental studies was limited because of their inability to distinguish microorganisms at different taxonomic levels.

The introduction of culture-independent molecular screening techniques made it feasible to determine the qualitative as well as the quantitative composition of the target microbial community (Morgan, 1991; Ranjard *et al.*, 2000; Dahllof, 2002). Further, these molecular methods also rendered the ability to characterize microorganisms according to their taxonomic status. The majority of these methods are based on the sequencing/fingerprinting analysis of some phylogenetically relevant genes (such as 16S rRNA gene) amplified from the total community DNA (Hur & Chun, 2004; McBurney *et al.*, 2006). Although the precise determination of the microbial community structure may be very difficult, sequencing and subsequent database match of the small subunit (SSU) rRNA clone libraries provides fundamental information about the composition as well as the diversity of complex microbial communities (Torsvik *et al.*, 1998; Theron & Cloete, 2000; Torsvik & Ovreas, 2002; Ward, 2006). Several environmental studies have determined the bacterial diversity of environmental niches by sequencing of the 16S rRNA gene library (Gonzalez *et al.*, 2000; Ellis *et al.*, 2003; Paul *et al.*, 2006a). In spite of its comprehensive ability to determine complex microbial community structures, construction and sequencing of multiple 16S rRNA gene libraries may be an expensive and laborious method. Therefore, it may not be very useful for comparison of complex communities that undergo spatial and temporal dynamics. This limitation associated with 16S rRNA gene library sequencing has resulted in continued attempts towards the development of high-throughput fingerprinting methods for quick and reliable determination of the community structure (Breen *et al.*, 1995; Busse *et al.*, 1996; Yang *et al.*, 2001; Collins *et al.*, 2006).

Some of the most common fingerprinting methods used for characterization of microbial community structure are: (1) serial analysis of ribosomal sequence tags (SARST); (2) oligonucleotide fingerprinting of the rRNA gene (OFRG); (3) rep PCR-genomic fingerprinting; (4) amplified rDNA restriction analysis (ARDRA); (5) terminal restriction fragment length polymorphism (T-RFLP); (6) denaturing/thermal gradient gel electrophoresis (D/TGGE); (7) single-strand conformation polymorphism (SSCP) and (8) automated ribosomal intergenic spacer analysis (ARISA) (Marsh, 1999; Kitts, 2001; Plaza *et al.*, 2001; Anderson & Cairney, 2004; Li *et al.*, 2006). All these fingerprinting methods have been widely used for determination of the microbial community structure; however, ARDRA, T-RFLP analysis, DGGE and SSCP are now used more frequently than other fingerprinting methods. The preferential use of these fingerprinting methods may be attributed to the selective inherent advantages associated with them (Table 4). The next section provides a descriptive account of the important features, work methodology and ecological applications of these fingerprinting methods.

ARDRA

ARDRA has been recognized as one of the most frequently used methods for the determination of the structure and the dynamics of microbial communities (Fernandez *et al.*, 1999; Gich *et al.*, 2000; Oravecz *et al.*, 2004). This method is based on PCR amplification of 16S rRNA genes from the total environmental DNA, followed by digestion with a few selected restriction endonuclease(s) that can provide an observable resolution among closely related microbial groups (Vaneechoutte *et al.*, 1992, 1995; Ingianni *et al.*, 1997; Jampachaisri *et al.*, 2005). However, the working methodology also requires an additional step viz. cloning of the 16S rRNA gene amplicon in a suitable vector before restriction digestion of the library clones to prevent the cross-contamination of 16S rRNA gene fragments of different microbial origins. Afterwards, the restricted clones are subjected to electrophoresis and categorized according to the restriction digestion pattern. Different restriction digestion pattern(s) obtained from the above electrophoresis are referred to as 'ribotypes' and they are considered as the 'operational taxonomic units' (OTUs). The identity of these OTUs is determined by sequencing of a few of the representatives of individual ribotypes. (Fig. 4a shows a schematic representation of the methodology used for ARDRA). ARDRA can also be used to determine community structure-related quantitative features such as 'community richness' and 'community evenness' by incorporation of 'rarefaction analysis' into the above method. 'Rarefaction analysis' is a statistical method to determine the number of 16S rRNA gene clones that must be sequenced for covering the entire diversity of the test environment (Friedrich *et al.*, 2002; Cottrell *et al.*, 2005). Similarly, several computer programs have been developed to determine the taxonomic identity of different OTUs according to algorithms that calculate sequence match and/or mis-match. 'DOTUR4' assigns sequences to OTUs using either the farthest, average or nearest-neighbor algorithm (Schloss & Handelsman, 2005). This method has been applied successfully for ARDRA analysis performed with the 16S rRNA gene library prepared from Scottish soil, Amazonian soils and the Sargasso Sea, wherein OTUs were assigned sequences at a very high statistical significance ($P > 0.05$). ARDRA

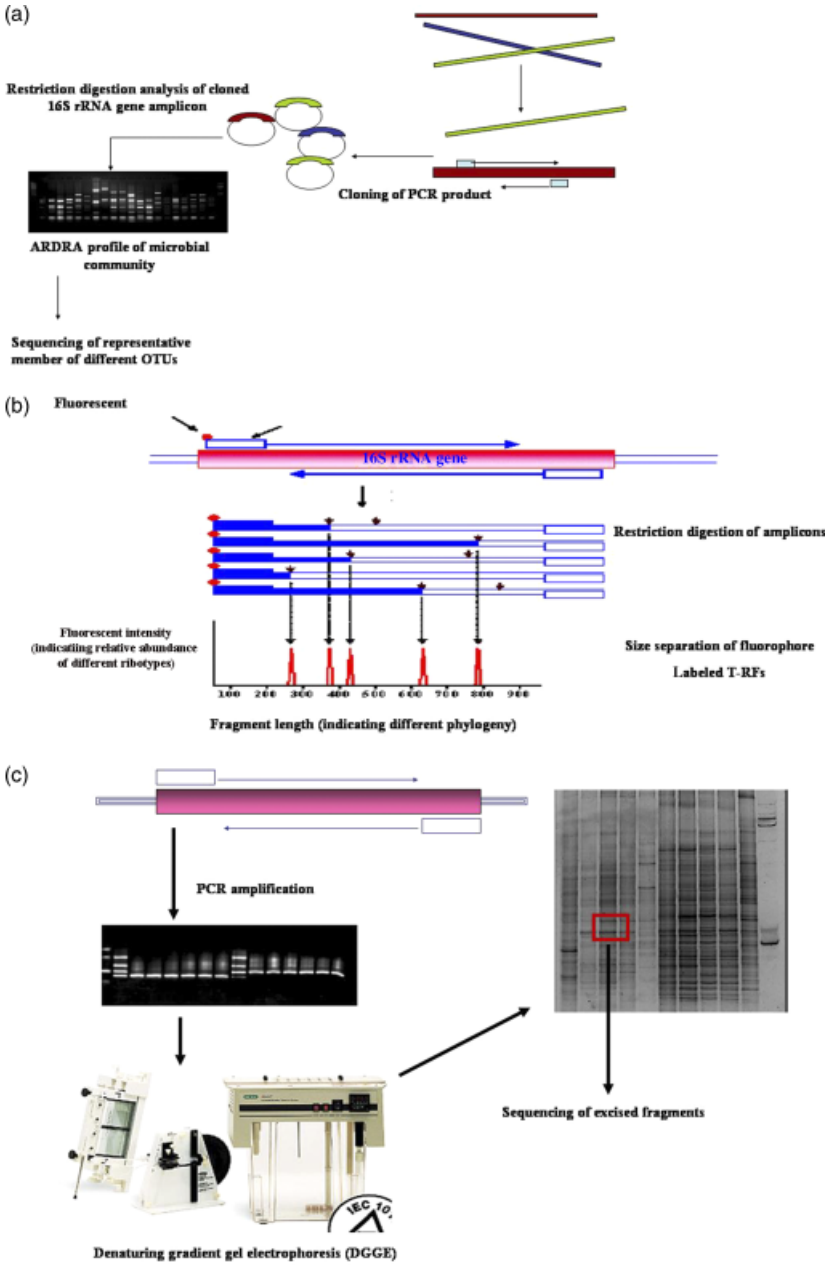

Fig. 4. Graphical representation work methodology of three most common methods: (a) amplified rDNA restriction analysis (ARDRA); (b) terminal restriction fragment length polymorphism (T-RFLP); (c) denaturing gradient gel electrophoresis (DGGE) implemented for assessment of microbial community structure and dynamics.

analysis, in combination with automated DNA sequencing and the above statistical method(s)/computer programs, can be of great use for assessment of the microbial community structure dynamics during different ecological phenomenon including *in situ* bioremediation. Because of this inherent feature (availability of clone for sequencing), ARDRA has been proposed to be potentially the most accurate among all fingerprinting methods. Some of the comparative studies that have analyzed the performance of different fingerprinting methods have showed ARDRA to be more reliable than random amplified polymorphic DNA (RAPD) analysis, amplified fragment length polymorphism (AFLP) analysis and recA-PCR analysis (Jawad *et al.*, 1998; Koeleman *et al.*, 1998; Watts *et al.*, 2001; Dherbecourt *et al.*, 2006).

From the application point of view, ARDRA has been used for the identification and characterization of diverse bacterial species (Vaneechoutte *et al.*, 1992, 1995; Hall *et al.*, 1999; Delgado & Mayo, 2004), determination of ecologically significant microorganisms in different environmental niches (Picard *et al.*, 2000; von der Weid *et al.*, 2002) and characterization of the total microbial community structure of natural and perturbed environments (Ovreas & Torsvik, 1998; Mohamed *et al.*, 2005; Sette *et al.*, 2007). Paul *et al.* (2006b) used ARDRA for the characterization of the total

microbial community structure in a pesticide-contaminated agricultural soil. This soil was having a consistently heavy load of PNP. ARDRA analysis, followed by sequencing of selected clones (representing different ribotypes), provided important information about the composition of the community that was dominated by microorganisms belonging to *Proteobacteria* and *Actenolycetes*. ARDRA has also been used sparingly for the assessment of microbial community structure dynamics. In one such study Gich *et al.* (2000) detected differences in activated sludge bacterial communities using samples from three activated sludge wastewater treatment plants. However, a relatively long technical procedure and dependence on DNA sequencing for extracting information about the taxonomic affiliation of the microorganisms have hindered the use of ARDRA as a common method for 'microbial community structure dynamics'.

T-RFLP analysis

As indicated above, the technical limitations associated with ARDRA (and other similar sequencing-dependent methods) have minimized their use as preferred methods for the assessment of microbial community structure dynamics. However, the increasing need for the determination of fluxes of microbial community structure has led to the increased requirement of high-throughput methods. This has paved the way for the development of an innovative-fingerprinting method viz., T-RFLP (Liu *et al.*, 1997; Lukow *et al.*, 2000; Marsh *et al.*, 2000; Kitts, 2001). Like other PCR-based fingerprinting methods, T-RFLP also depends on amplification of a target gene from environmental DNA sample(s). The method differs from all the other fingerprinting methods because it identifies microorganisms on the basis of the terminal restriction fragment (T-RF) (proximal to a fluorescently labeled primer) rather than the total digestion profile (Marsh, 1999; Dunbar *et al.*, 2000; Horz *et al.*, 2000). For performing the T-RFLP analysis, a target gene is PCR amplified using mixed community DNA (such as soil metagenome) and PCR primers that are usually labeled at the 5′ end with some fluorescent dye; this PCR amplification results in the generation of a pool of 5′ end-labeled amplicons. After amplification, the amplicons are digested and then size separated on an automated gel or capillary sequences. Out of several restriction fragment only those terminal fragments that carry the fluorescent tag at their 5′ end are identified. Further, the sizes are also assigned only to these terminal fragments (Marsh, 1999). Theoretically, T-RFLP analysis is based on the idea that the restriction fragments (including the terminal fragment) for any gene in different taxonomic groups are of different sizes; therefore, microorganisms can be distinguished on the basis of the length of their terminal fragment. Initially, this method was developed to use a radio-labeled primer and for application with clinical samples and to track medically important microbial populations (Cancilla *et al.*, 1992). However, with the advancement of the technology for fluorescent labeling of the phylogenetically conserved primers, the method was applied to various areas of microbiological research and finally the approach has emerged as a high-throughput method for comparative microbial community structure analysis (Brito *et al.*, 2006; Morales *et al.*, 2006). The methodology for T-RFLP analysis is quite well established and it has been studied in detail by several reviews (Osborn *et al.*, 2000; Kitts, 2001; Anderson & Cairney, 2004; Arias *et al.*, 2005; Dickie & FitzJohn, 2007). Figure 4b shows a schematic representation of the methodology and output profile of the T-RFLP analysis.

Because its introduction into microbial ecology, T-RFLP analysis has been used successfully for analysis of microbial communities from diverse environmental niches such as soils (Dunbar *et al.*, 2000; Buckley & Schmidt, 2001; Kuske *et al.*, 2002; Singh & Tate, 2007), marine sediments (Braker *et al.*, 2001; Luna *et al.*, 2004; Parkes *et al.*, 2007), bioreactors and chemostats (Guieysse *et al.*, 2001; Schmidt *et al.*, 2007). For *in situ* bioremediation studies, this method has especially been useful in the determination of the bacterial community dynamics structure during remediation processes (Song *et al.*, 2002; Macbeth *et al.*, 2004; Katsivela *et al.*, 2005; Paul *et al.*, 2006a). Importantly, the majority of such studies have targeted the complete microbial communities using the T-RFLP profile of the 16S rRNA gene; however, some of the T-RFLP studies have also attempted to selectively monitor the dynamics of functionally important subgroup(s) of microorganisms (Horz *et al.*, 2000; Miralles *et al.*, 2007). The use of T-RFLP analysis for assessment of bacterial subgroups is based on the use of primer sets that can specifically target the desired microorganism. Miralles *et al.* (2007) detected the dynamics of 'sulfate-reducing bacteria (SRB)' using primer sets that were designed on the basis of generic sequence features associated with 16S rRNA genes of SRB. Alternatively, T-RFLP analysis has also been performed with primers that can target environmentally significant catabolic genes (Siripong & Rittmann, 2007).

Although, over the recent past, T-RFLP analysis has developed as one of the most preferred approaches for assessment of the microbial community structure and its dynamics, the successful application of this method is subjective to rigorous standardization. Some of the reports have indicated the technical limitations of accurate determination of the structure of the microbial community (Egert & Friedrich, 2003; Lueders & Friedrich, 2003). In one such report, Pandey *et al.* (2007) indicated that T-RFLP profiles may vary as a function of a subtle difference in the molecular weight of the fluorophore used for labeling the PCR primers. Similarly, Kalpan & Kitts (2003) demonstrated a

variation in the true T-RFs length and the observed T-RF length, wherein the variation was explained on the basis of the differential purine content of the test fragment and the internal standard. A few other reports have also indicated that the accuracy of T-RFLP analysis is mainly influenced by the inherent biases of the associated PCR reaction (Lueders & Friedrich, 2003; Frey *et al.*, 2006). Another severe limitation of T-RFLP analysis is its inability to ascertain the identity of different T-RFs (which may be very important in some cases) by DNA sequencing. A common method used to overcome this situation is the combinatorial use of different fingerprinting methods (Anderson & Cairney, 2004; Smalla *et al.*, 2007) or use of high-throughput DNA fingerprinting methods such as D/TGGE that allow downstream DNA sequencing to determine the sequence identity (Muyzer & Smalla, 1998; Muyzer, 1999).

D/TGGE

D/TGGE incorporates the advantageous features of high-throughput fingerprinting methods and the ability to sequence the selected DNA fragments to determine the taxonomic status of different constituents of the complex bacterial communities (Heuer *et al.*, 1997). Like other DNA fingerprinting methods, D/TGGE also consists of direct extraction of nucleic acid (DNA or RNA), followed using PCR amplification of the target gene. Later, the amplicons are analyzed using electrophoretic separation on gradient gel(s) (for methodology, refer to Fig. 4c). Separation of amplicons is based on the decrease in the electrophoretic mobility of the partially melted DNA samples in the polyacrylamide gel containing a linear gradient of a denaturing agent or a linear temperature gradient (Borresen *et al.*, 1988; Takahashi *et al.*, 1990). DNA fragments of different microbial origins have different melting behaviors and, therefore, they stop at different gel position/denaturant concentrations (Muyzer, 1999). Ever since their development in the early 1990s, DGGE and TGGE have been used for an increasing number of microbial ecology purposes and they have also been used to characterize allelic variations, single nucleotide polymorphisms and point mutation(s) in different DNA samples (Lessa & Applebaum, 1993; Gelfi *et al.*, 1996). D/TGGE analyses have also been coupled with DNA hybridization analyses where taxon-specific probes are used to further elaborate upon the microbial identification generated by D/TGGE analysis (Heuer *et al.*, 1999).

Microbial ecology-related use of these methods has largely focused on the characterization of total microbial community structures as well as their dynamics (Gelsomino *et al.*, 1999; Whiteley & Bailey, 2000; Zhang *et al.*, 2005; Ziembinska *et al.*, 2007). MacNaughton *et al.* (1999) characterized the microbial community changes during biodegradation of polyaromatic hydrocarbons in experimental oil spills by phospholipid fatty acid (PFLA) analysis and DGGE targeting 16S rRNA gene. In this study, DGGE analysis showed major changes in the community structure that were not identified with PFLA analysis. D/TGGE analysis has also been used successfully for assessment of changes in the microbial community structure during *in situ* bioremediation processes (Whiteley & Bailey, 2000; Andreoni *et al.*, 2004). In another study, DGGE analysis was performed with the 16S rRNA gene amplified from total community DNA and RNA to determine the metabolically active fraction of the indigenous community in a pentachlorophenol-contaminated soil undergoing pollutant degradation (Mahmood *et al.*, 2005). D/TGGE, in combination with hybridization analysis with specific probes and sequencing of excised DNA bands, constitutes a very reliable method for studying the complexities and functional behavior of the microbial communities. Evidently, the method also overcomes the major limitations associated with other fingerprinting methods. However, for further applicability of this method, it is important to overcome the limitations caused by the formation of heteroduplexes, comigration of DNA fragments with different sequences, etc.

As indicated earlier, for successful implementation of an environmental intervention like *in situ* bioremediation, it is extremely important to evaluate the impact of the process on the native microbial community structure. Therefore, the above culture-independent methods have emerged as an indispensable tool for assessing the ecological sustainability of the *in situ* bioremediation process. However, at the same time, it is vital to understand that similar to culture-dependent approaches, the culture-independent molecular approaches also have biases and disadvantages in terms of precise quantitation of microbial biomass. Some of the major limitations include: (1) inability to distinguish among the DNA molecules extracted from live and dead cells, (2) nondistinguishment of the target and some of the very closely related microorganisms, (3) selective bias of the environmental DNA extraction method for some microorganisms over others, (4) nonuniversality of the general/universal PCR primers, (5) the common inherent limitations/biases of molecular steps for example PCR amplification, restriction digestion, etc. In general, the molecular methods have been more successfully used with gram-negative bacteria (probably due to the selective ease of DNA extraction). On the other hand, gram-negative bacteria are less preferred for culture-dependent approaches, because they are outgrown by fast-growing gram-positive bacteria and *Actenomycetes* during incubation. Therefore, it is important that the true evaluation of bacterial cell survival, activity and ecological consequences of the *in situ* bioremediation process can be performed only with application of a polyphasic approach using culture-dependent as well as culture-independent methods. Some of the studies

emphasizing the need for applying a polyphasic approach have used a molecular biomarker (i.e. the 16S rRNA gene sequence) along with cellular biomarkers (e.g. total phospholipid content, PLFA analysis) (Frostegard *et al.*, 1997; Hanson *et al.*, 1999; von Keitz *et al.*, 1999; Kozdroj & van Elsas, 2001).

PLFA as a microbial biomarker for assessing bacterial survival

PLFA molecules constitute an integral component of all cell types including bacterial cells, and their relative abundance within different organisms remains as a constant proportion of the total cellular biomass in nonperturbed natural environments (Guckert *et al.*, 1991; Kohring *et al.*, 1994). Hence, the PLFA pattern can be used as a biomarker for indicating the survival of different test bacteria under natural environmental conditions (Guckert *et al.*, 1991; Frostegard *et al.*, 1993, 1997; Smoot & Findlay, 2001). Furthermore, PLFA patterns can also be used for taxonomic identification of the bacterial species. From an ecological point of view, bacterial groups are characterized by considerable differences in certain PLFAs among them. In general, gram-negative bacteria are rich in monosaturated fatty acids and deficient in branched-chain fatty acids (Zelles, 1999). Similarly, other bacterial groups are identified by the abundance of other characteristic PLFA molecules. The use of PLFA analysis as a complementary method for the study of molecular phylogenetics has been demonstrated clearly in some studies, wherein PLFA analyses helped in identifying among diverse bacterial strains that could not be distinguished by 16S rRNA gene sequencing. In one such report, three strains viz., *Desulphobacter latus, Desulphobacter curvatus* and *Desulphobacter* sp., were grouped together using 16S rRNA gene sequence analysis; however, PLFA analysis correctly placed *Desulphobacter* sp. in a different group (Kohring *et al.*, 1994). Apart from the use of PLFA analysis for identification of microorganisms present within environmental samples, it has also been used successfully to determine the composition and dynamics of microbial community structures (Steger *et al.*, 2003).

Unlike the limitations observed with the PCR-primer-based biases, the PLFA-based culture-independent approach can identify the majority of the bacterial diversities consisting of gram-positive as well as gram-negative bacteria. The total community PLFA analysis of environmental samples collected from Osaka Bay, Japan, showed the predominance of gram-positive bacteria and *Actenomycetes* in the region that was heavily contaminated with organic chemical pollutants (Rajendran *et al.*, 1994). PLFA analysis has also been used to monitor the microbial community structure shift during bioremediation of an experimental oil spill (MacNaughton *et al.*, 1999). A time-based comparison of the microbial community within the contaminated and control plots was performed with PLFA analysis and DGGE. The contaminated plot was treated with nutrient amendment and/or inoculation of indigenous microbial flora. At the end of the bioremediation process, the DGGE profile indicated that the microbial communities of the contaminated and control plots were significantly different. However, the PLFA profile showed that the microbial community profiles of the treated plot were similar to those of the control plot at the end of the bioremediation experiment, an observation that can be better explained ecologically. These representative studies clearly indicate the importance and applicability of PLFA analysis as an important culture-independent method for the assessment of bacterial survival, community structure and dynamics in various ecological processes including *in situ* bioremediation. A recent advancement of PLFA analysis is its combination with stable isotope probing and development of SIP-PLFA for monitoring the functionally active bacterial community. For example, the methanotrophic bacterial community was analyzed in combination with transcriptional analysis (based on mRNA extraction and analysis) and SIP-PLFA analysis (Chen *et al.*, 2008). In this study, soil samples were spiked with ^{13}C-labeled methane, followed by subsequent analysis of ^{13}C-labeled PLFA profiles. The results identified 16 : 1 omega 7, 18 : 1 omega 7 and 18 : 1 omega 9 as the major labeled PLFAs. Most significantly, none of these PLFAs were previously known to be associated with known methanotrophic bacteria; hence, this application of SIP-PLFA analysis could identify the presence and involvement of novel methanotrophic bacteria in the test environment.

Such observations further emphasize the need to apply a polyphasic approach involving culture-dependent approaches as well as culture-independent approaches for assessment of various ecological aspects of *in situ* bioremediation. It is also important to use biochemical and celluar markers for example PLFA, fatty acid methyl ester (FAMEs), substrate-utilization (BIOLOG) analysis and chemotaxonomic characterization along with molecular phylogenetic assessment to gain a comprehensive understanding of the ecological sustainability of the *in situ* bioremediation process.

Data analysis of microbial community structure profiles

Microbial community structure determination is usually carried out on the basis of sequence variation in the 16S rRNA gene (the universal bacterial phylogenetic marker) (Hofle *et al.*, 2005). Use of the 16S rRNA gene provides an evident advantage for characterization of the complete microbial diversity; however, the universal presence of the 16S rRNA gene yields very complex data obtained with

community structure profiles. The complexity of these data is further increased because of the high degree of 16S rRNA gene sequence conservation and, therefore, it becomes extremely difficult to extract any meaningful information (Blackwood *et al.*, 2003; Abdo *et al.*, 2006). This situation has resulted in several efforts aimed at the development of automated methods (computer programs) for quick and reliable data analysis of the microbial community structure profiles. These data analysis methods can be broadly classified into three groups: (1) methods for ascertaining the microbial identity, (2) methods for evaluating the phylogenetic richness/evenness of the community structure and (3) methods for statistical validation of community structure data. The majority of the methods for determining the microbial identity are based on the principle of '*in silico*' comparison of the microbial community profiles, where 16S rRNA gene sequences deposited in the 'Ribosome Database Project' are used as the known reference. DNA sequencing-based identification is carried by performing global sequence alignments against DNA sequence databases such as GenBank, DDJB, EMBL, etc. Alternatively, local sequence alignment may also be performed to determine the sequence similarity or divergence with the community structure profiles. Nucleotide-specific BLAST (BLASTN) and CLUSTAL_X are the most frequently used programs for performing global and local nucleotide sequence alignments respectively (Thompson *et al.*, 1997). On the other hand, microbial identities against profiles generated with fingerprinting methods for example T-RFLP are ascertained using tools that compare *in silico* 'RDP' digestion profiles with those generated with the experimental procedure (Marsh *et al.*, 2000; Kent *et al.*, 2003). 'T-RFLP FRAGMENT-SORTER' and 'MICROBIAL COMMUNITY ANALYSIS (MICA)' are two of the most frequently used online softwares for determination of microbial community structures based on the experimentally generated T-RFLP profiles (Shyu *et al.*, 2007).

Cluster analysis of the microbial community structure data

Most of the microbial community structure determination studies generate data in terms of comparative signature sequences of the phylogenetic marker gene (e.g. 16S rRNA gene) and provide a comparable fingerprinting pattern. These results are usually enormous and, therefore, it is quite really difficult to draw any conclusions without further processing. A common and simple approach to overcome this is to perform a 'cluster' analysis of the results, because it provides first-hand and easy-to-understand information about the taxonomic relation of different microorganisms present in the analyzed samples (Fuhrman *et al.*, 1993; Bintrim *et al.*, 1997). The methodology of cluster analysis is relatively simple and straightforward. The resemblance and differences between all the samples are calculated and presented in the form of a matrix, which is then compressed for visualization in the form of a cluster. The visualization output is usually presented in the form of a dendogram that reveals the comparative relationships of different samples. Furthermore, any cluster analysis can be performed with a wide range of selection parameters or coefficients (which define the resemblance and differences). The most commonly implemented parameter is the 'similarity coefficient', which measures the association between two samples and continues to do so till all the samples are suitably placed in the output dendogram. In contrast to the similarity coefficient, a lesser common parameter for cluster analysis is the 'distance coefficient', which gives more weightage to the subtle dissimilarities among the test samples. Both the above coefficients take absolute values (including the relative abundance of individuals) into consideration, while some of the other coefficients use only the presence–absence for the clustering of different samples.

Among the similarity coefficients, the 'Jaccard Coefficient' (SJ) and the 'Dice Coefficient' (SD) are used most commonly however, a cluster analysis with these coefficients is strictly DNA fragment/Gel-band based. Therefore, they may not be the most suitable for analysis of the total microbial community structure where, most of the times, DNA fragments are not best resolved on the analytical gels. A possible solution to this situation is the use of sequence-based phylogenetic/clustering analysis methods. Several different algorithms have been formulated for calculating the sequence-based phylogenetic relationship of different microorganisms (Iushmanov & Chumakov, 1988; Morrison, 1996; Densmore, 2001; Bocci, 2006). Unlike gel-based methods, sequence-based analyses are more informative; on the other hand, they are more time-consuming and expensive. Two of the most commonly used phylogenetic approaches are based on either a 'maximum likelihood estimation' or a 'distance matrix' algorithm (Van de Peer & De Wachter, 1993; Olsen *et al.*, 1994; Retief, 2000). An important implication of performing phylogenetic analysis of the microbial community structure profiles is that it provides important information about the ecological functions of the microbial community. Table 5 lists some of the frequently used online search programs for performing microbial identification on the basis of 16S rRNA gene fingerprinting methods and characterization of the phylogenetic relationship of the constituent microorganisms. Cluster analysis of distributions and the dominance of different microbial groups can also provide significant insights into the mechanism of *in situ* processes. For example, cluster analysis of different microbial groups in a diesel oil-contaminated soil undergoing bioremediation indicated a high degree of 16S rRNA gene sequences among microorganisms that were enriched during the bioremediation

Table 5. A list of some of the common computer programs used for analysis of microbial community structure data

Computer program	World wide web link for direct access	Applicable for
GELQUEST	http://www.sequentix.de/gelquest/index.php	For analysis of all DNA fingerprinting analysis e.g. ARDRA, AFLP, T-RFLP or RAPD, minisatellites and microsatellites
GENEBASE PACKAGE	http://www.applied-maths.com/	Computer program for comparative sequence analysis
GELCOMPARE	http://www.applied-maths.com	For analysis DNA fingerprinting analysis for example ARDRA, AFLP, T-RFLP and RAPD
T-RFLP FRAGSORT	http://www.oardc.ohio-state.edu/trflpfragsort/whatisfragsort.php	Online program for analysis of T-RFLP profiles targeting eubacterial 16S rRNA gene
T-RFLP ANALYSIS PROGRAM (TAP)	http://rdp8.cme.msu.edu/html/TAP-trflp.html	Online program for analysis of T-RFLP profiles targeting eubacterial 16S rRNA gene
MICA: T-RFLP ANALYSIS	http://mica.ibest.uidaho.edu/trflp.php	Online program for analysis of T-RFLP profile targeting a few phylogenetically conserved genes of eubacterial and archeal origin
BAS 2500 IMAGE ANALYSIS SYSTEM	http://www.fujifilmlifescienceusa.com	Image analysis program for graphical analysis of profiles generated with microarray, DGGE, TGGE, 2D gel electrophoresis etc
BLAST	http://www.ncbi.nlm.nih.gov/blast/Blast.cgi	Online program for global sequence alignment analysis for preliminary establishment of taxonomic status of different DNA/protein sequences
3CON (TREECON)	http://bioinformatics.psb.ugent.be/psb/Userman/treecon_userman.html	A software package for the construction and drawing of evolutionary distance trees
PHILIP	http://evolution.genetics.washington.edu/phylip.html	Software program for inferring phylogenies

process (Menezes Bento *et al.*, 2005). The results obtained with the cluster analysis corroborated the degradation function of enriched organisms. Another study pertaining to the cluster analysis of 16S rRNA genes clearly demonstrated how ecological stresses can selectively affect a particular group of microorganisms, while some of the other microorganisms are not affected adversely (Seghers *et al.*, 2001).

Over the last 8–10 years, several algorithms have been developed to perform cluster analysis, with each having certain advantageous features. Therefore, for detailed cluster analysis of fingerprinting profiles as well as DNA sequences, several clustering methods are recommended to decipher the true clustering patterns of microbial patterns within the test environment. Apart from the above cluster analyses, other effective methods are based on multidimensional scaling that represent sample(s) a point(s) two or three-dimensional plot. Multidimensional scaling analyses are best suited for monitoring time-dependent changes and may include a large number of samples. Some researchers also classify multidimensional analyses under statistical methods. For *in situ* bioremediation studies, multidimensional analyses are best suited; however, they may be used more effectively along with cluster analysis methods.

Statistical analysis of microbial community structure data

As stated above, due to the enormous complexity of the microbial community structure profile, it is possible that these profiles may be interpreted wrongly. However, to overcome this problem, quite a few statistical tests have been developed and used as complementary methods to *in situ* bioremediation (Rees *et al.*, 2004; Hartmann *et al.*, 2005). Further, use of these statistical methods provides justified explanations for the dynamics of the microbial community structure (Dollhopf *et al.*, 2001; Fromin *et al.*, 2002; Abdo *et al.*, 2006). Some of the recently developed statistical analyses also attempt to address environmental functions that usually have independent sources of variability and therefore may be very difficult to analyze (Kah *et al.*, 2007; Lucas & Jauzein, 2008; Wu *et al.*, 2008). The major advantage with the use of statistical analysis lies in their ability to summarize results in an easy-to-interpret manner. Figure 5 shows a study performed to monitor the microbial community structure dynamics (in an *in situ* bioremediation study) using T-RFLP analysis. The T-RFLP profiles showed only subtle 'spatial' and 'temporal' differences in the community structure, whereas the statistical analysis could further elaborate upon these subtle differences. Most of the studies carried out for statistical assessment of microbial community structure data have used methods that are based on 'multivariate statistical analysis' (MVS) (Christensen *et al.*, 2005; Rudi *et al.*, 2007), whereas a few studies have also used alternative methods based on an 'artificial neural network' (Olden *et al.*, 2006).

One of the most efficient methods for statistical analysis of the microbial community structure is 'principal component analysis' (PCA) (Wang *et al.*, 2004). PCA is a multivariate ordinate analysis that attempts to recover the underlying structure of the data by projecting in a lesser dimensional space (Daffertshofer *et al.*, 2004). Other common methods used for multivariate statistical analysis of

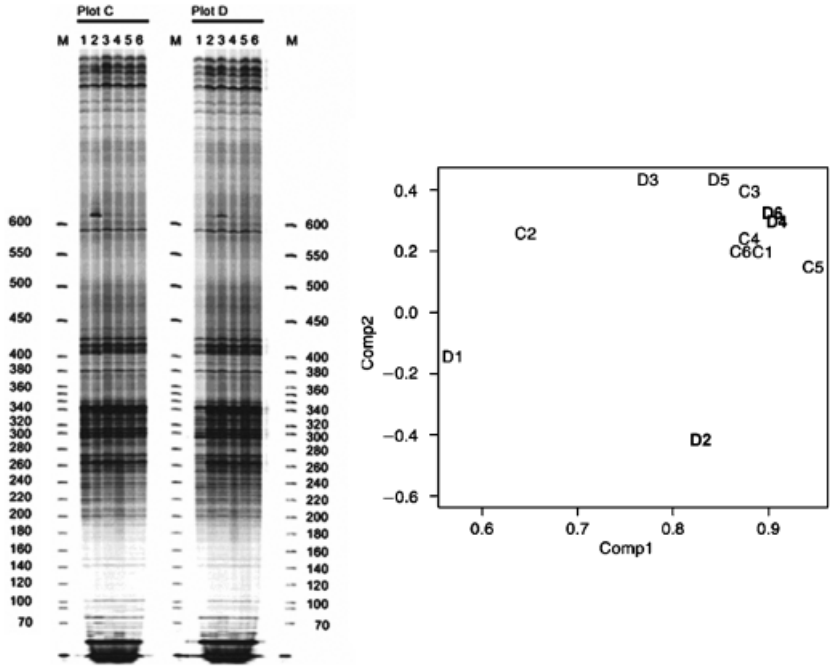

Fig. 5. Non-scalar principal component analysis (PCA) of terminal restriction fragment length polymorphism (T-RFLP) analysis, showing the 3-dimensional distribution of microbial community structure (expressed by individual quadrate points in the PCA graph). The T-RFLP electropherograms shows only minor difference in microbial community structure at different time points, whereas the PCA analysis shows significant difference (e.g. time points D2 and D3) (adapted from Paul *et al.*, 2006a).

microbial community structure data are 'correspondence analysis' (CA) and 'canonical correspondence analysis' (CCA). These methods also attempt to recover the underlying structure of the data set; however, they use algorithms different from those of the PCA analysis. CA and CCA have been used very successfully to determine the specieswise weightage of a microbial community (Cordova-Kreylos *et al.*, 2006; Sapp *et al.*, 2007). Some of the studies have also used CCA analysis to examine the association of environmental factors with the microbial community structure (Yannarell & Triplett, 2005). Another study reported a strong influence of lake temperature and the presence of phytoplanktons on the microbial community composition on the basis of the CCA analysis of the above factors and DGGE (Sapp *et al.*, 2007). Assessment of *in situ* bioremediation can also be aided considerably by performing such statistical analyses on the microbial community structure and dynamics data. Similarly, Allen *et al.* (2007) subjected the microbial community structure of petroleum-contaminated sediments to CA analysis, which revealed that various hydrocarbon-degrading microorganisms and sulfate-reducing bacteria, along with iron-reducing bacteria, were the major microbial populations contributing towards degradation of petroleum-associated PAHs. Apart from the above MVS-based methods, calculation of 'diversity indices' is another statistical approach that has been used frequently for evaluation of the richness and/or the evenness of the microbial communities. Diversity calculation is probably most useful for a comparative assessment of the spatial and temporal changes in the community structure of environments undergoing *in situ* decontamination of chemical pollutants (Humphries *et al.*, 2005).

It can be concluded from the above examples that intergrative applications of different methods of culture-dependent approaches, analysis of biochemical and cellular phylogenetic markers, culture-independent molecular approaches, molecular microbial ecology, along with the use bioinformatic methods and statistical approaches, have significantly enhanced the ability for precise determination of the microbial community structure and dynamics during important environmental processes such as *in situ* bioremediation.

Suicidal genetically engineered microorganisms (S-GEMs) for *in situ* bioremediation

Rational combination of genetic elements from different microorganisms in one recipient i.e. development of genetically modified microorganism(s) (GEMs) was proposed as a useful strategy for achieving enhanced bioremediation capabilities (Jain & Sayler, 1987; Timmis & Pieper, 1999; Pieper & Reineke, 2000). Further, with the advancement of whole-genome sequencing methods, information about a large number of catabolic genes as well as regulatory genetic elements became readily available. This information has been used for the successful development of GEMs with improved degradation ability (Brim *et al.*, 2000; Lorenzo *et al.*, 2003). In spite of the significantly improved ability for the development of GEMs, their application for *in situ* bioremediation has remained extremely limited due to the

unforeseen risks associated with their release into the environment. One of the most commonly anticipated risks is a 'horizontal gene transfer' to the native microbial population (Urgun-Demirtas *et al.*, 2006). Alternatively, the GEMs released may proliferate within the new environment to adversely affect the equilibrium of the microbial community diversity. Therefore, environmental microbiologists have strongly recommended the incorporation of genetic circuit(s) into GEMs, which may restrict their proliferation to only the environment(s), where they perform the targeted degradation (Alexander, 1984; Lorenzo *et al.*, 2003; Urgun-Demirtas *et al.*, 2006).

One of the common methods used to restrict the proliferation of GEMs is 'bacterial containment systems' that work on the basis of either distinguishing bacterial phenotypes in targeted and nontargeted environments (Ford *et al.*, 1999) or selective killing of GEMs when they proliferate out of the targeted environment (Torres *et al.*, 2003). GEMs designed with the 'killing-based bacterial containment systems' are often referred to as 'S-GEMs' and use a 'killer gene' for inducing cell death in response to environmental changes. Recent studies with whole-genome sequencing have identified several 'killer genes' as well as 'killer–antikiller gene pairs' such as *hokW-sokW, ccdB-ccdA, pemK-pem1*, *parE-parD*, etc. Initially, these genes/gene sets were identified for their role in a plasmid addiction system (i.e. selecting the plasmid-free bacterial cells from a population dominated by plasmid-bearing cells). Some of these genes have been used for the construction of efficient 'S-GEMs' that have been used for environmental purposes e.g. *in situ* bioremediation. The containment may occur via a 'repressible' pathway, such that in the absence of environmental induction (e.g. a chemical pollutant), there is repression of the killer gene, leading to killing activity. An improved bacterial containment system especially for use during *in situ* bioremediation can be developed by rendering constitutive expression of the killer gene and pollutant-inducible expression of the corresponding antikiller gene. Thus antikiller gene would be produced to negate the effects of the killer gene only as long as the pollutant is present in the environment while the absence of the pollutant will lead to rapid killing of the S-GEMs. A simplified scheme of the construction of 'S-GEMs' and the action mechanism is shown in Fig. 6. This novel 'bacterial containment system' for developing S-GEMs and their use for *in situ* bioremediation can lead to increased use of the process for decontamination of a polluted environment. Further, it is postulated to minimize the need for assessment of the 'environmental fate' of the degradative microorganism.

Concluding remarks

With an everincreasing understanding of the hazardous effects of chemical pollutants, there has been an upsurge in research activities for the development of strategies that might be used for decontamination of polluted environments. Application of the microbial metabolic potential is accepted as a safe and efficient tool for the removal of several chemical pollutants. Among the existing methodologies, *in situ* bioremediation has been proposed as being possibly

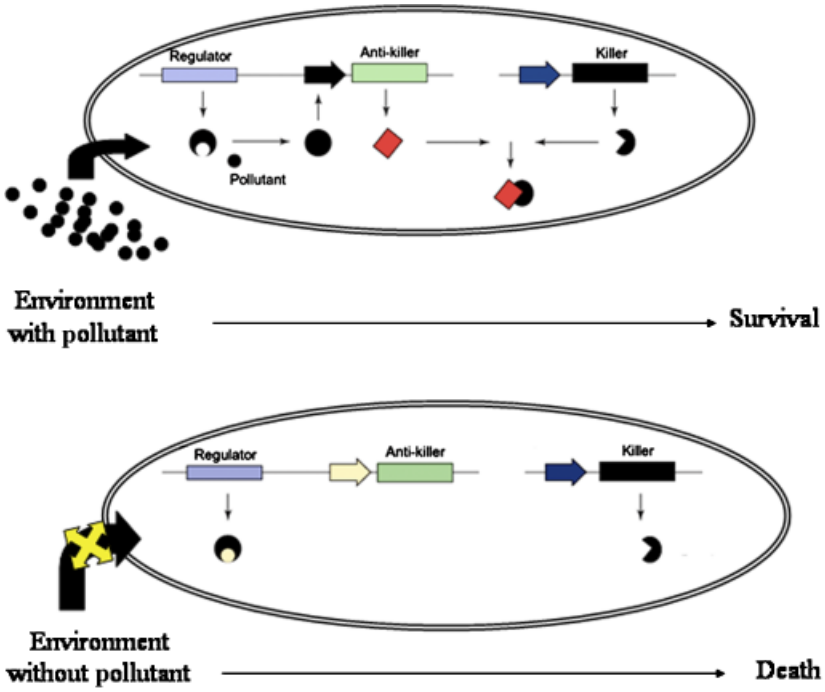

Fig. 6. Representation of bacterial containment systems designed for the safe introduction of GEMs into the environment (adapted from Paul *et al.*, 2005). Bacterial containment system in which the killer gene is constitutively expressed and its cognate anti-killer gene is positively regulated by environmental signals: (a) the anti-killer is synthesized only in presence of environmental pollutant and bacteria survive; (b) in absence of pollutant the anti-killer is not synthesized leading to a killer mediated cell death.

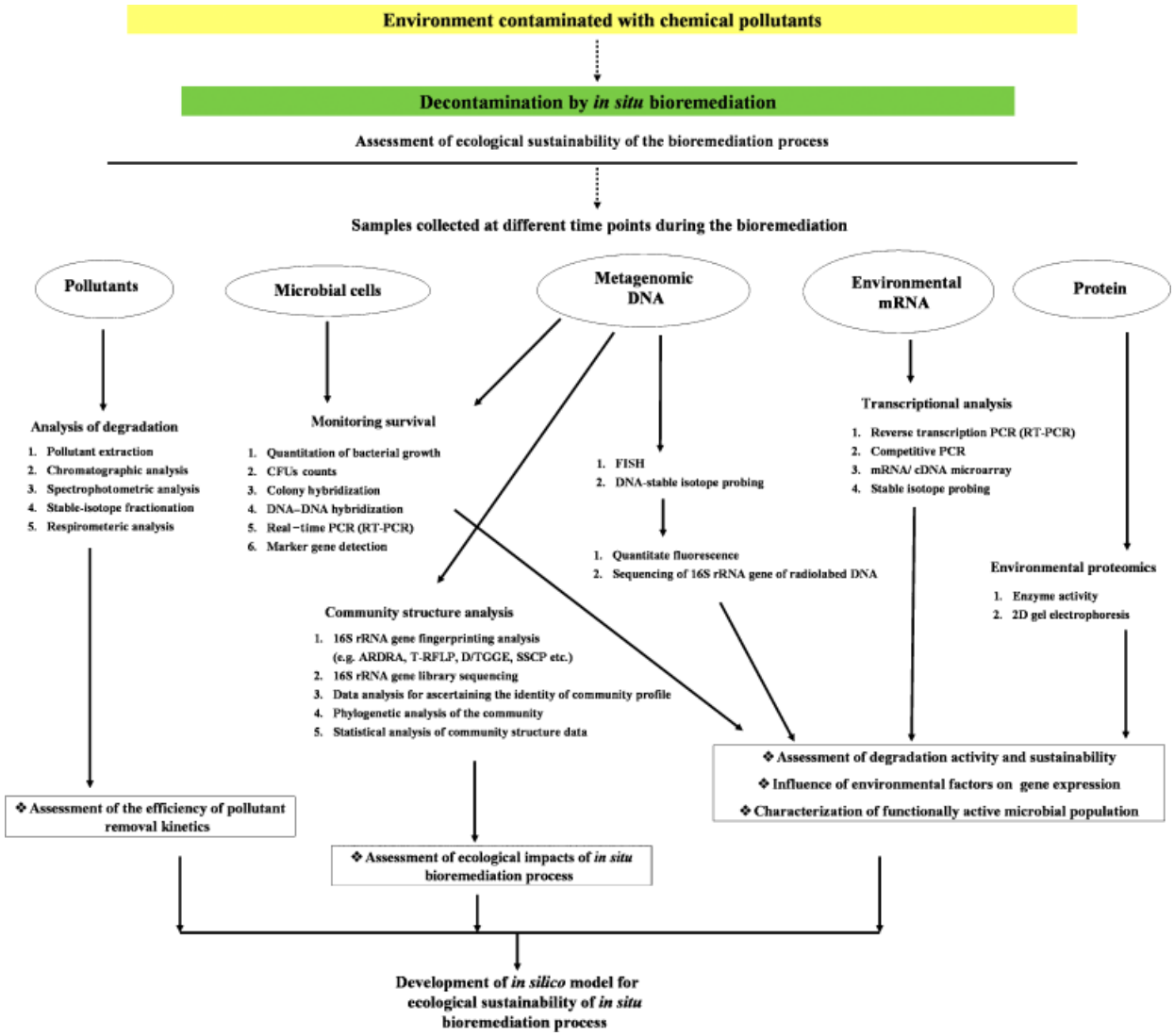

Fig. 7. Flow chart representing different approaches used for assessment of the ecological sustainability of the *in situ* bioremediation process.

the most potent measure. However, the complexities associated with the use of microorganism(s) under natural environmental conditions have quite often resulted in the nonoptimal performance of these processes. The nonoptimal performance of *in situ* bioremediation has led to several studies attempting to investigate the possible methods to overcome the above limitations. Preliminary observations in most of these studies indicate reduced sustainability of the process as the major cause for nonoptimal degradation. Consequently, it is recommended that for successful implementation of the *in situ* bioremediation process, monitoring of the ecological sustainability is needed. Such an assessment requires concurrent application of principles and technologies from diverse scientific areas in an integrated manner. Figure 7 shows a simplified flow chart of the different approaches used to assess the ecological sustainability of the *in situ* bioremediation process. Use of such integrated methods can also help to address issues pertaining to the ethical aspects of microbial release into the non-native environments.

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