Bioremediation: towards a credible technology

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Context

Bioremediation is the technological process whereby biological systems are harnessed to effect the clean-up of environmental pollutants. Currently, microbial systems are most widely employed in bioremediation programmes, generally in the treatment of soils and waters contaminated with organic pollutants. Micro-organisms have a huge metabolic repertoire that enables them to degrade a panoply of organic pollutants and in many cases the complex biochemistry and molecular biology of the catabolic pathways involved have been unravelled (e.g. Gibson, 1984; Franz et al., 1987; Evans & Fuchs, 1988; Burlage et al., 1989; Abramowicz, 1990; Assinder & Williams, 1990; Chaudhry & Chapalamadugu, 1991; Cerniglia, 1992; Knackmuss, 1996). Despite valuable basic knowledge on the mechanisms of pollutant biodegradation, bioremediation has yet to be accepted as a routine treatment technology and the environmental industry is wary of applying bioremediation for the treatment of contaminated sites.

Bioremediation has the potential to treat contaminants on site with relatively little disturbance to the contaminated matrix. Furthermore, micro-organisms offer the possibility that organic pollutants can be completely mineralized to inorganic materials (e.g. \( \text{CO}_2, \text{H}_2\text{O}, \text{Cl}^-, \text{NO}_3^- \)), making bioremediation an attractive treatment strategy. In contrast, removal of contaminated material to landfill sites, or extraction of contaminants using physical processes such as soil washing, do not destroy the contaminants present, but simply concentrate the contaminated material in a different location. Why then, if bioremediation offers benefits over other technologies, has it not been more widely adopted to treat environmental contamination? One reason is that physical treatments are rapid and their outcome is generally predictable in the short term; they are also relatively inexpensive. Bioremediation too, can be relatively cheap, but methods to confirm efficacy on a field scale have either been unavailable or simply not applied routinely. The unpredictability of bioremediation, meanwhile, stems from a lack of understanding of the behaviour of microbial populations in natural environments and how physical, biological and chemical factors interact to control their activity against environmental pollutants. With this realization, has come a different philosophy towards bioremediation; the focus of bioremediation research has shifted from isolation and construction of ‘superbugs’ to determining the factors that limit pollutant transformations and mineralization in natural environments. It is now clear that access of micro-organisms to pollutants in situ is a critical factor in determining the success of bioremediation. Consequently, methods are being developed to enable predictions regarding the feasibility of bioremediation based on pollutant bioavailability and biodegradation. With knowledge of the causes of unsuccessful bioremediation, methods can be formulated to overcome these limitations. Assessing the feasibility of bioremediation within a predictive framework and confirming its efficacy in the field is the focus of this review.

Assessing bioremediation potential: a problem of bioavailability

It is relatively simple to demonstrate biodegradation of specific compounds in a contaminated environmental sample. Spiking a sample of contaminated soil with a pollutant chemical of interest and monitoring its loss and the appearance of degradation end products in comparison with sterilized control samples often demonstrates rapid biodegradation. Conversion of \(^{14}\text{C}-\)labelled compounds to \(^{14}\text{CO}_2\) is undoubtedly the best evidence that a microbial community has the ability to mineralize an organic compound, but is insufficient to demonstrate convincingly that bioremediation will be successful. For example, added naphthalene and phenanthrene were rapidly degraded in a polycyclic aromatic hydrocarbon (PAH)-contaminated soil, but levels of indigenous naphthalene and phenanthrene were not reduced appreciably (Erickson et al., 1993). Comparable observations have been made in other soils and with different chemicals (Steinberg et al., 1987; Doelman et al., 1990; Weissenfels et al., 1992; Beurskens et al., 1993). Reduced bioavailability results from the interaction of pollutants with both organic and inorganic...
components of the soil matrix. Access of micro-organisms or their enzymes to the pollutant molecules is constrained and with increasing contact time ('aging') the proportion of the pollutant that becomes biologically unavailable increases. A number of mechanisms are involved: diffusion limitation due to sequestration of the pollutant in micropores (< 1 μm diameter; Steinberg et al., 1987; Wu & Gschwend, 1986) or humic and mineral flocs (Kan et al., 1994); binding to soil minerals by ionic or electrostatic interactions (e.g. Knaebel et al., 1994); oxidative covalent coupling of the pollutant with soil organic matter via enzymic or chemical catalysis (e.g. Bhandari et al., 1996); and partition/dissolution of the pollutants into the soil organic matter (e.g. Kan et al., 1994).

There is consensus that the last of these is quantitatively the most important in immobilizing and reducing the bioavailability of organic pollutants, particularly in organic-rich soils and sediments (Pignatello & Xing, 1996). However, as the soil organic matter content decreases (less than 0.4% organic carbon; Carmichael et al., 1997), catalytic effects of soil minerals may result in a greater proportion of pollutant immobilization via oxidative covalent coupling with soil organic matter (Bhandari et al., 1996). Clearly, several mechanisms operate in consort to influence bioavailability, and different mechanisms will predominate in any given situation (Pignatello & Xing, 1996).

The phenomenon of pollutant 'aging' in soils has been investigated by examining adsorption and desorption isotherms which, under ideal, equilibrium conditions, should be identical. Experimental measurements typically indicate that desorption occurs at a much lower rate than would be predicted from a simple equilibrium model (e.g. Kan et al., 1994; Pignatello & Xing, 1996). Furthermore, a discontinuity in the desorption of pollutants from a soil or sediment is often observed. Following initial rapid desorption of a fraction of the bound contaminant, the remaining material desorbs at a considerably reduced rate. Kan et al. (1994) demonstrated that equilibrium for adsorption of naphthalene to soil was achieved within 24 h, but with increasing adsorption time up to 30 d, the amount of 'desorption-resistant' material increased from 15 to 23%. It has been suggested that this is due to slow diffusion of pollutant molecules from soil micropores (Steinberg et al., 1987; Wu & Gschwend, 1986) or through soil organic matter that is in a glassy liquid state (Carroll et al., 1994; Pignatello & Xing, 1996). It has however been noted that if diffusion kinetics are the cause, then desorption should be affected to the same extent as desorption (Kan et al., 1994). This is not the case and it has been recently demonstrated by the use of laboratory-constructed homogeneous mineral/organic matter matrices lacking tortuous, diffusion-limiting micropores that similar 'two-phase' desorption behaviour occurs (Hunter et al., 1996). Whilst this is difficult to explain in the framework of equilibrium kinetics, it is feasible that the activation energy for desorption may be greater than the activation energy for adsorption (Cornelissen et al., 1997; Pignatello & Xing, 1996).

At present, we do not fully understand the mechanisms involved in reducing bioavailability, but it is certainly a key factor in determining the feasibility of bioremediation. Bosma et al. (1997) recently commented that '... a critical assessment of bioremediation data reveals that the intrinsic microbial activities limit bioremediation in only a few cases. In most cases, mass transfer limitation prevented the full exploitation of the microbial degradative potential'. While reduced bioavailability may lead to failure of bioremediation, it can also be responsible for reduced toxicity of pollutant residues.

**Can bioavailability be measured and its effect on bioremediation predicted?**

Bioavailability has been estimated using microbial bioassays (Heitzer et al., 1992), differential solvent extraction techniques (Kelsey et al., 1997), analysis of desorption thermodynamics and kinetics (Cornelissen et al., 1997), and a combination of desorption, transport and biodegradation kinetics (Bosma et al., 1997).

**Bioassay.** A microbial bioassay for assessment of bioavailability has been developed for naphthalene and its degradation intermediate salicylate (Heitzer et al., 1992). This system is based on a recombinant *Pseudomonas fluorescens* strain (KH44) containing a plasmid-encoded nabG–luxCDABE transcription fusion. In response to induction by naphthalene or salicylate, nabG and the lux operon are co-expressed, resulting in bioluminescence. When a whole-cell bioassay was tested in aqueous extracts of soil artificially contaminated with naphthalene, the reduction in aqueous phase concentration (approx. 96% by HPLC) due to sorption to the soil matrix corresponded well with the reduction in bioluminescence determined by the bioassay. However, in soil slurries the bioassay was compromised due to luminescence quenching (Heitzer et al., 1992). The system was further developed to produce a flow-through biosensor that enabled on-line monitoring of naphthalene and salicylate in process waters (Heitzer et al., 1994). Although the biosensor showed a reproducible response to pulsed concentrations of naphthalene and salicylate, the response time was slow (8-24 min) and dependent on the concentration of the analyte. Further limitations of the bioassay included a long signal decay period (approx. 30 min) and sensitivity to the presence of toxic substances. Rather than bioavailability, it was essentially aqueous phase pollutant concentration that the biosensor responded to and significant refinement would be required to compete with more traditional measurement techniques.

**Differential extraction.** A simple approach to determining the bioavailability of aged residues of atrazine and phenanthrene has been adopted by Kelsey et al. (1997) and is based on the differential extraction of pollutants from soil using a range of solvents. Up to 11 different extraction regimes were compared for their ability to reflect the degree of pollutant uptake by earthworms, and the degree of bacterial mineralization in sterilized...
soils containing aged pollutants and inoculated with specific pollutant-degrading pure cultures. Recovery of atrazine with 1:1 methanol:water for example, was similar to the degree of bacterial mineralization of the herbicide, whereas n-butanol extraction efficiency mirrored the level of phenanthrene mineralization. This preliminary study was limited in the scope of pollutants and soil tested and it is likely that empirical determination of the appropriate extractant for any particular soil/pollutant system will be required. Nonetheless, because the methodology is straightforward it could prove an attractive means to assess bioavailability.

**Modelling approaches.** The above methods may be of use in the empirical determination of bioavailability, but do not by themselves allow the likely success of bioremediation to be determined. Potentially more valuable in this respect is the development of mathematical models that can explain biodegradation behaviour in relation to both biological and abiotic factors such as pollutant sorption and diffusion.

Rigorous kinetic and thermodynamic analyses have recently been applied to the problem of desorption of pollutants from soil (Cornelissen et al., 1997) and the effect of desorption on biodegradation rates (Bosma et al., 1997). The desorption of chlorobenzenes, polychlorinated biphenyls (PCBs) and PAHs was studied in laboratory-contaminated and field-aged sediments (Cornelissen et al., 1997). The pollutants were found to occur in three ‘compartments’. The pollutants desorbed from one of the compartments rapidly, from a second slowly and from a third very slowly. From 8 to 54% of the pollutant was present in the slowly desorbing fraction, with the exact value depending on the particular chemical and sediment. Up to 47%, though more typically 0.5–16%, of some contaminants was found to be present in the very slowly desorbing compartment (Cornelissen et al., 1997). The latter can equate to significant quantities of pollutant in a contaminated soil and its occurrence in this form has considerable implications for the time that may be required to effect satisfactory bioremediation. The very slow desorption phase could only be measured in reasonable time scales if desorption was carried out at elevated temperatures, and values obtained for the fraction of very slowly desorbed material at 20 °C and 60 °C were generally in good agreement. This approach offers the possibility that not only the amount of pollutant in differentially desorbing pools can be determined, but also the time taken for complete desorption to occur under field conditions can be estimated. This may be invaluable in determining the length of time required for bioremediation if it is limited by mass transfer of pollutants from a sorbed phase to solution phase, as is generally the case with aged pollutant residues (Bosma et al., 1997; Carmichael et al., 1997).

The biodegradation of a contaminant in situ is a function of both the catabolic activity of the micro-organisms present and transport of the contaminant to microbial cells with the ability to degrade the contaminant. It is the latter that is probably the most important factor in determining the success of a bioremediation programme. By considering both of these factors, a parameter termed the bioavailability number (Bn) has been derived (Bosma et al., 1997). The Bn is equal to a first-order exchange constant k (k controls the rate of desorption/diffusion of a sorbed contaminant), divided by the product of the maximal transformation rate (q max) and the reciprocal of the half-saturation constant for contaminant transformation (K m) (equation 1). This is essentially the ratio of the exchange constant and the first-order rate constant applicable to Michaelis–Menten kinetics (equation 2) at substrate concentrations much less than K m (equation 3).

\[
Bn = \frac{k}{q_{\text{max}} K_m^{-1}}
\]

\[
q = \frac{d_{\text{max}} [S]}{(K_m + [S])}
\]

When [S] is much lower than K m this approximates to

\[
q = (q_{\text{max}}/K_m) [S]
\]

The Bn is hence a measure of the importance of mass transfer relative to the intrinsic catabolic activity of the microbial cell or population. Thus, for values of Bn < 1 desorption and diffusion (i.e. mass transfer) are more important than biodegradation in determining the rate of transformation of a pollutant. At values of Bn > 1, the reverse is true and the rate of transformation is not limited by desorption and diffusion. In other words, if mass transfer (k) is large relative to biodegradation (q max/K m) bioavailability is not likely to limit biodegradation. The value of k can be calculated for different modes of mass transfer (e.g. linear diffusion vs radial diffusion vs dissolution) using experimentally determined values for distribution coefficients and dissolution rate constants (Rijnaarts et al., 1990; Bosma et al., 1997). Values of q max and K m can be determined by measurement of initial biotransformation rates at different added substrate concentrations in soil slurries. The parameters can then be determined graphically (e.g. using a Lineweaver–Burk-type plot).

This approach was used recently to investigate the importance of mass transport and hence bioavailability on α-hexachlorocyclohexane (α-HCH)-degradation in soil slurries (Bosma et al., 1997). The exchange constant was calculated from α-HCH desorption data using a first-order model (Rijnaarts et al., 1990) and values for K m and q max were determined using Lineweaver–Burk plots (Bachmann et al., 1988). The Bn for this system was calculated at 0.016–0.030 under different mixing regimens that affected mass transfer. Both values were however less than one and hence biodegradation was likely to be sorption limited. When the biodegradation process was modelled using an expression that takes account of both mass transfer and biodegradation, the fit with experimental data was extremely good (Bosma et al., 1997). This relatively simple method may therefore prove useful in predicting the applicability of bioremediation if it can be shown to be valid over a wide range of conditions.

The same authors also developed an expression relating
the exchange coefficient and the quantity of pollutant transformed to satisfy cell maintenance requirements to the threshold concentration below which no further biodegradation would be observed. High and low values for substrate fluxes required to satisfy maintenance energy were estimated at $10^{-11}$ µg s$^{-1}$ for copiotrophs and $10^{-14}$ µg s$^{-1}$ for oligotrophic bacteria, using published data on the maintenance energy requirements for a range of bacteria (e.g. Chesbrough et al., 1979; Bouillot et al., 1990; Tros et al., 1996a, b). Exchange coefficients representing a range of mass transfer conditions from the diffusion of hydrophilic compounds in water to the diffusion of hydrophobic compounds in soils were also calculated. With this information, threshold pollutant concentrations expected under different mass transfer conditions and with bacterial populations with various maintenance energy requirements were determined (Bosma et al., 1997). The range of low maintenance energy systems with high mass transfer to high maintenance energy systems with low mass transfer exhibited threshold concentrations that varied from $10^{-6}$ µg L$^{-1}$ to greater than $10^4$ µg L$^{-1}$, respectively. It was apparent from this analysis that under certain conditions residual concentrations of pollutant, even in microbiologically active environments, can be very high and this is consistent with the levels of residual pollutant often measured in the field.

The fundamental importance of bioavailability in determining the success of bioremediation is becoming more widely appreciated (e.g. Tabak et al., 1994; Bosma et al., 1997). The development of methods that can potentially predict the extent of bioavailability based on relatively simple measurements and calculations is likely to have a significant impact on decisions to bioremediate. Certainly, more research is required to determine if predictions made from kinetic and thermodynamic data are widely applicable and to extend their utility to complex mixtures. As we increase our understanding of the mechanisms involved, it is likely that predictions will become more reliable and the efficacy, and hence the reputation, of bioremediation will undoubtedly be improved. Continued isolation of particular organisms with the ability to catabolize specific pollutant chemicals may yet reveal much regarding the biochemical and genetics of biological transformations of novel xenobiotics and may even prove useful in 'end-of-pipe' processes for the treatment of well-defined wastestreams. However, improvement of the efficacy of bioremediation of contaminated matrices in the environment will require a redistribution of research effort, away from such studies and towards a greater understanding of the limitations on microbial transformations in situ.

**Can bioavailability limitation be overcome?**

The prospects for predictable evaluation of bioremediation efficacy are improving, but it is not clear that this knowledge will allow development of more effective means to treat organic pollutants biologically in contaminated soils and sediments.

A number of strategies for improving mass transfer have been suggested. Diffusion and desorption of pollutants are temperature-dependent (Cornelissen et al., 1997) and this is the basis of physico-chemical treatments such as thermal desorption and steam-stripping. In the context of bioremediation it has been suggested that the use of composting systems, where elevated temperatures are maintained, should improve mass transfer and hence bioremediation rates (Pignatello & Xing, 1996); rapid degradation of chlorophenols in contaminated soil by composting has been demonstrated accordingly (Laine & Jørgensen, 1996; Jaspers et al., 1997). Physical grinding or mixing to disaggregate soils has been suggested as a means of alleviating mass transfer limitation, and there is some evidence that this is effective (e.g. Rijnaarts et al., 1990). The cost involved in large-scale application of physical disruption to soils to the degree required to improve mass transfer substantially may, however, be prohibitive. The most widely investigated method for improving bioavailability of sorbed pollutants is treatment with surfactants. Surfactant-enhanced biodegradation of sorbed and poorly soluble chemicals is probably due to decreasing the distribution coefficient ($K_d$) for the pollutant in a soil–water system, i.e. increasing the equilibrium concentration in the aqueous phase relative to the sorbed phase. This would require the surfactant to be provided at a concentration greater than the critical micelle concentration ($C_{MC}$; Alexander, 1994; Deitsch & Smith, 1995), unfortunately requiring the use of substantial amounts of surfactant that can be both costly and toxic to micro-organisms. There is however evidence that mineralization rates can be increased even at surfactant concentrations well below the $C_{MC}$ (Aronstein et al., 1991). Recent evidence suggests that although high surfactant concentrations do lower $K_d$, concentrations below the $C_{MC}$ increase the mass transfer exchange coefficient (equivalent to $k$ in equation 1; Deitsch & Smith, 1996). In essence, this means that in abiotic systems the equilibrium concentration in the aqueous phase does not change, but the time for equilibrium to be reached is reduced. Consequently, enhanced rates of mineralization can be observed at surfactant concentrations below the $C_{MC}$ despite no increase in the aqueous equilibrium concentration being noted in abiotic control systems.

In a number of cases however, surfactant addition has had no effect or been detrimental (see Liu et al., 1995 for a summary) due to toxicity of the surfactant at high concentrations (e.g. Aronstein et al., 1991) or retardation of surface attachment of bacterial cells to the hydrophobic contaminant (e.g. Efroyimson & Alexander, 1991). Furthermore, the identity of the surfactant and its concentration are critical. One study compared the effects of seven surfactants (anionic and non-ionic) on desorption of phenanthrene, of which five had no effect on desorption of the PAH from sterile soil (Aronstein et al., 1991). The effect of the remaining two non-ionic surfactants on the mineralization of phenanthrene and biphenyl was quite different in mineral and...
organically rich soils. It is interesting to note that one of the surfactants, Alfonic 810-60, did not increase the apparent aqueous concentration of phenanthrene at equilibrium in sterile slurries of the mineral soil. Despite this, mineralization was markedly enhanced, supporting the view that it is the mass transfer coefficient and not $K_D$ that is affected by surfactant addition.

On the basis of pollutant- and soil-specific effects (Aronstein et al., 1991; Providenti et al., 1995), it seems likely that a universally applicable surfactant treatment to promote the biodegradation of poorly bioavailable contaminants will be elusive. Consequently, surfactant-enhanced bioremediation is likely to rely on ad hoc rather than proprietary solutions. A need for case by case evaluation has considerable cost implications for bioremediation and the formulation of generic treatment practices would reduce the unit cost. The development of reliable, widely applicable practices will be essential if bioremediation is to compete successfully with engineering solutions. In order to achieve this, it is important to understand processes rather than rely on empirical observations, and the causes of reduced bioavailability are now being rigorously investigated.

Field demonstration of the efficacy of bioremediation

One of the greatest challenges faced by advocates of bioremediation is proof that a chosen treatment is effective under field conditions. Concentration of contaminant compounds and residue toxicity are the most common criteria specified by legislative bodies to define an end point for successful bioremediation. Chemical analyses (e.g. GC-MS) and toxicological assays (e.g. Microtox) can be applied to accurately identify and quantify organic contaminants or assess residual toxicity following treatment. Nevertheless, a major factor hindering the acquisition of statistically valid proof of bioremediation is to compete successfully with engineering solutions. In order to achieve this, it is important to understand processes rather than rely on empirical observations, and the causes of reduced bioavailability are now being rigorously investigated.

**Poorly biodegradable components of complex mixtures**

The overall biodegradation of contaminants that comprise a complex mixture of compounds with different susceptibilities to biodegradation can be assessed by measuring the ratio of the degradable components to a poorly degradable component in the mixture. Crude oil is an excellent example of such a contaminant mixture and this approach has been used successfully to confirm biodegradation of weathered oil spilled from the Exxon Valdez (Bragg et al., 1994). Crude oil biodegradation studies have commonly used the ratio of the linear alkane n-heptadecane ($n$C$_{17}$) to the more resistant branched alkane pristane (or alternatively n-octadecane: phytane ratios) to evaluate the extent of biodegradation (e.g. Fayad et al., 1992; Pritchard & Costa, 1991). A decrease in the ratio of the n-alkane to the branched alkane is taken as evidence of crude oil biodegradation. However studies of beach sediments from Prince William Sound, Alaska, demonstrated that even the branched alkanes were readily degraded, resulting in anomalously high n-C$_{17}$:pristane ratios in highly degraded oils (Bragg et al., 1992). For this reason, 17α(H)21β(H)-hopane, a minor component of the North Slope crude spilled at Prince William Sound, was used to index the biodegradation of the more labile components of the oil (Bragg et al., 1994). 17α(H)21β(H)-hopane is a pentacyclic triterpane (Fig. 1) and was chosen as a conserved internal marker because is known to be poorly biodegradable. Hopanoids are membrane lipids found in many bacterial taxa (Rohmer et al., 1984) but they exhibit stereochemical differences to those occurring in crude oil and can be distinguished readily. Comparisons of fertilizer-treated beach plots with untreated plots using the ratio of either the total GC-detectable hydrocarbons, total PAH or total resolvable hydrocarbons to the hopane concentration provided convincing evidence that bioremediation had been effective (Fig. 1; Bragg et al., 1994).

![Fig. 1. Changes in the ratio of total GC-detectable hydrocarbons (TGCDHC) to hopane during the bioremediation of the Exxon Valdez oil spill.](image-url)
Whilst assessment of crude oil biodegradation has been the main application of this approach to date, it can be applied to other complex mixtures. PCBs, for example, are normally found as a mixture of congeners with similar physico-chemical properties but different biological fates and are therefore amenable to this approach. 3,4-3’4’-tetrachlorobiphenyl is more readily biodegradable than 2,3,6-3’4’-pentachlorobiphenyl and the ratio of the two congeners has been used to monitor bioremediation of PCBs in river sediments (Harkness et al., 1993). There is potential for 2,3,6-3’4’-pentachlorobiphenyl to be transformed by 3-, 3’- and 4’-dehalogenation reactions, compromising its use as a conserved tracer. This is because anaerobic dechlorination has been shown to result in preferential dehalogenation at meta- and para-positions in PCB molecules (Bedard & May, 1996). However, independent studies have demonstrated that the meta and para chlorines of the 2,3,6-3’4’-substituted congener are not susceptible to reductive dehalogenation in anoxic sediments (Bedard et al., 1996).

**Analysis of degradation products**

Analysis of metabolic products or intermediates of contaminant metabolism in situ is not universally applicable as many intermediates may be short-lived or only accumulate to very low levels. Some features of metabolic products applicable in the diagnosis of successful bioremediation are: an unequivocal bio-chemical relationship with the parent compound; no exogenous sources of contamination; and biological and chemical stability under in situ conditions (Beller et al., 1995). Degradation products of trichloroethylene (TCE) metabolism meet some of these criteria in that biological degradation produces cis-1,2-dichloroethylene whereas abiotic TCE transformation results in the formation of 1,1-dichloroethylene (Kästner, 1991).

Anaerobic biodegradation of the alkyl substituted components of BTEX (benzene, toluene, ethylbenzene, xylenes) is also known to produce characteristic by-products under denitrifying and sulphate reducing conditions (Beller et al., 1992; Evans et al., 1992) and this has been exploited recently under field conditions (Beller et al., 1995). Benzylsuccinate, benzylfumarate and corresponding homologues accumulate during anaerobic degradation of BTEX in laboratory incubations (Fig. 2). These compounds could be detected in samples from an anaerobic aquifer contaminated with gasoline, implying that anaerobic transformations of the BTEX had occurred in the aquifer (Beller et al., 1995). Furthermore, in an aquifer spiked with BTEX-contaminated water (containing bromide as a conservative tracer) it was possible to demonstrate that the accumulation of the metabolic products followed the disappearance of the corresponding contaminant hydrocarbons. When BTEX became undetectable, the concentration of the metabolic products began to decrease.

This approach is not fail-safe in determining biodegradation at field sites, as work on pentachlorophenol degradation has illustrated. Both photolytic (Steiert & Crawford, 1986) and biodegradative reactions (Apajalahti & Salkinoja-Salonen, 1987) produce the same intermediates. It may thus be impossible to distinguish these reaction mechanisms under field conditions, and unequivocally associate disappearance of pentachlorophenol with a biological process, simply by detection of putative metabolic products.

**Geochemical indicators of bioremediation**

Except in fermentation and disproportion reactions, the oxidation of organic compounds is coupled to the reduction of exogenous electron acceptors. In soil and sediment environments these are usually O$_2$, NO$_3^-$, Fe$^{III}$, Mn$^{IV}$, SO$_4^{2-}$ or CO$_2$. Depletion of these species or accumulation of their reduced products in contaminated relative to uncontaminated material may be indicative of organic pollutant metabolism (Borden et al., 1995). This can be achieved most convincingly if the concentrations are compared with levels of a conservative tracer (e.g. chloride or bromide) that has similar physico-chemical properties to the analyte of interest, but is not susceptible to biological reduction. Indirect evidence of this nature is far from definitive unless reduction in contaminant concentrations can be related to changes in oxidant concentrations and a mass balance constructed. In the field, these objectives are often difficult to meet and geochemical analysis of redox-sensitive species at best provides supplementary evidence for biodegradation.
Stable isotopes

A rapid method that is used to monitor the progress of bioremediation is measurement of CO$_2$ production. While this gives an indication of increased rates of breakdown of organic matter it does not necessarily follow that the CO$_2$ originates from the target pollutant. The advantage of using stable isotope analysis is that it is possible to determine the source of CO$_2$ evolved during a remediation process. This is feasible because there is very little isotopic fractionation associated with the aerobic mineralization of organic matter by microorganisms (Chapelle et al., 1988; Jackson et al., 1996). Whilst potentially a very useful technique to rapidly assess the biodegradation of contaminants, it is only applicable if the $\delta^{13}C$ of the endogenous organic matter and the contaminant are measurably different.

A good example of the potential of this approach has been published recently (Jackson et al., 1996). The $\delta^{13}C$ ratio of crude oils is typically $-29\%$ to $-32\%$. In contrast, organic carbon in soils and sediments dominated by C-4 plants exhibits a $\delta^{13}C$ signature of between $-14\%$ and $-17\%$. Thus it has been possible to clearly distinguish the rate of CO$_2$ production from hydrocarbon degradation and from mineralization of endogenous organic matter in oil-contaminated salt marsh sediments dominated by Spartina (Jackson et al., 1996). In addition, kinetic constants calculated using the $\delta^{13}C$ data for CO$_2$ were statistically no different from those calculated from data on alkane degradation. That hydrocarbon degradation had occurred was further indicated by reductions in the ratio of labile hydrocarbons to hopane.

The carbon isotope ratio of CO$_2$ can be readily and inexpensively determined. This method of assessing the biodegradability of organic pollutants under field conditions therefore has considerable promise for adoption in bioremediation studies (e.g. Aggarwal & Hinchee, 1991; Landmeyer et al., 1996; Aggarwal et al., 1997). Its application, however, will be limited to situations where the $\delta^{13}C$ of the contaminant and endogenous organic matter are known and distinct.

A role for molecular biology in assessing bioremediation?

The ability to isolate and enumerate bacteria from contaminated sites capable of degrading a particular pollutant is one line of evidence often used to support the feasibility of bioremediation. This is particularly true if an increase in the population of degradative bacteria occurs following implementation of a treatment to stimulate biodegradation. Culture-based methods underestimate both qualitative and quantitative measures of microbial populations by orders of magnitude. This has led to the development and application of nucleic-acid-based techniques to study the ecology and diversity of micro-organisms in nature. Since a more complete understanding of microbial ecology will undoubtedly be required to gain maximum benefits from bioremediation, it is not surprising that molecular biological methods are now being employed to study bioremediation. This can be viewed as analogous to the isolation of bacteria with appropriate catabolic properties from a polluted site, as a means of implying that competent degradative populations are present at the site, and thus that bioremediation potential exists.

Molecular biological methods have already been used successfully to evaluate the bioremediation potential of a contaminated site (Fleming et al., 1993). The expression of a catabolic gene (nahA) involved in the initial oxidation of naphthalene to 1,2-dihydroxynaphthalene was investigated at a manufactured gas plant site contaminated with PAHs. RNA was extracted from the contaminated soil and mRNA for the nahA gene quantified using an RNase protection assay (Belin, 1996). The extracted RNA was hybridized in solution with a radioactively labelled antisense nahA RNA probe transcribed from a cloned nahA gene. Treatment of the hybridized RNA preparation with RNase preferentially degrades single-stranded RNA leaving probe RNA:mRNA hybrids intact. The amount of radiolabelled probe RNA:mRNA hybrid is quantified in relation to known amounts of target RNA analysed using the assay. Comparable samples of the contaminated soil were used to determine the frequency of nahA genes in a cultured fraction of the bacterial population by a colony hybridization protocol. Residual naphthalene concentrations and the rate of radiolabelled naphthalene catabolism were also measured. Comparison of the data revealed a good correlation between nahA gene expression, as determined by quantification of nahA mRNA, and the other three parameters. The ability to monitor expression of specific catabolic genes may be of great importance in determining the feasibility of bioremediation in situ, particularly since samples can be taken from the site and processed with minimal disturbance due to storage and transport or alteration of conditions in the sample, as occurs with culture based techniques.

More recently, a similar approach has been adopted to examine the expression of lignin peroxidases (Lamar et al., 1995) and manganese-dependent peroxidases (Bogan et al., 1996) in soil inoculated with Phanerochaete chrysosporium. These enzymes are believed to be important in the degradation of organic pollutants by white-rot fungi. In these studies, reverse transcriptase competitive PCR was used to quantify different levels of the gene transcripts and transcription of the fungal genes was shown to be related to the degradation of contaminant PAHs (Bogan et al., 1996). This was in turn related to the activity of manganese peroxidase in extracts of the soil.

Although these examples demonstrate the feasibility of using molecular methods to monitor bioremediation, routine practical application is perhaps a little way off. A number of factors will determine how widely adopted these approaches will be for routine evaluation and monitoring of bioremediation programmes (Brockman, 1996).
pathways and molecular genetics involved in the catabolism of a relatively small number of intensively studied pollutants by a relatively small group of microorganisms. It may have been assumed from this knowledge that the catabolic diversity of micro-organisms could be harnessed to solve a wide range of pollution problems. On this basis, initial predictions for the benefits of bioremediation were overstated with the consequence that it was never likely to live up to expectations.

It is now clear that it is not our knowledge of pollutant catabolism that limits the success of bioremediation, but rather a restricted understanding of the interplay between the biotic and abiotic factors that determine the outcome of any particular remedial strategy. Treating bioremediation as a natural bioengineering process that takes account of these interactions, coupled with rigorous quantitative assessment of the outcome of remedial treatments is required to ensure bioremediation is successful and to raise the credibility of the technology.

References


