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Natural attenuation: What does the subsurface have in store?

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Abstract

Throughout the world, organic and inorganic substances leach into the subsurface as a result of human activities and accidents. There, the chemicals pose direct or indirect threats to the environment and to increasingly scarce drinking water resources. At many contaminated sites the subsurface is able to attenuate pollutants which, potentially, lowers the costs of remediation. Natural attenuation comprises a wide range of processes of which the microbiological component, which is responsible for intrinsic bioremediation, can decrease the mass and toxicity of the contaminants and is, therefore, the most important. Reliance on intrinsic bioremediation requires methods to monitor the process. The subject of this review is how knowledge of subsurface geology and hydrology, microbial ecology and degradation processes is used and can be used to monitor the potential and capacity for intrinsic bioremediation in the subsurface and to verify degradation *in situ*. As research on natural attenuation in the subsurface has been rather fragmented and limited and often allows only conclusions to be drawn of the site under investigation, we provide a concept based on Environmental Specimen Banking which will contribute to further understanding subsurface natural attenuation processes and will help to develop and implement new monitoring techniques.

Introduction

Human activities have profound effects on the Earth including its subsurface which is an important source of clean drinking water for humankind. Unacceptable pollution ranges from single compounds in less than a microgram per litre to highly complex mixtures with up to grams per litre per compound. The former can result from the use of pesticides in agriculture (see Ralebitso et al. 2002, this issue) while an example of the later is landfill leachate, which contains mixtures of organic (e.g., benzene, toluene, ethylbenzene and xylene (BTEX), chlorinated hydrocarbons, pesticides, medicals) compounds and inorganic (e.g., heavy metals, macro-components) compounds (Christensen et al. 2001). In general, it is difficult and too expensive to remediate a subsurface environment. However, the toxicity, mass and/or mobility of the contaminant(s) can be reduced without human intervention when suitable conditions prevail. The processes involved are dilution, dispersion, volatilization, precipitation, ion exchange, sorption, transformation and degradation which collectively are referred to as natural attenuation (Christensen et al. 2001). Microorganisms are the principal mediators of the natural attenuation of many pollutants, such as organic molecules, metals and inorganic nitrogen compounds (Christensen et al. 2001; Lovley 2001). They transform or mineralise pollutants thereby decreasing their masses and toxicities in contrast to most other components of natural attenuation. Reliance on intrinsic bioremediation (the microbiological component of natural attenuation) requires information on: whether it can occur (potential); whether it is actually occurring at a significant rate (verification); and how it will behave in the future (capacity). In this review, we describe how knowledge

of subsurface geology and hydrology, microbial ecology and degradation processes is used and can be used to monitor intrinsic subsurface bioremediation, and what their pitfalls are. We develop a concept based on Environmental Specimen Banking (Paulus et al. 1996) that will contribute to further understanding subsurface natural attenuation processes and so help to develop (and implement) new monitoring techniques.

Determining the potential for intrinsic bioremediation

Laboratory assays

The US National Research Council stipulates that three criteria should be met in documenting microbial metabolism of contaminants *in situ* to evaluate the efficacy of the process: (1) documented loss of contaminants from the site; (2) laboratory assays showing that microorganisms from site samples have the potential to transform the contaminants; and (3) evidence that the biodegradation potential is actually realized in the field (NRC 1993). Confirmation of criterion 2 is, in general, attempted by measuring decreases in contaminant concentrations in laboratory microcosms spiked with contaminants relevant to the site under investigation. These assays are also often used to determine degradation parameters for mathematical modelling purposes. In the literature, however, one encounters cases where criteria 1 and 3 are satisfied while criterion 2 is not. Research by the group of Christensen (Lyngkilde & Christensen 1992; Nielsen et al. 1995) showed a decrease in benzene, toluene, ethylbenzene and xylene (BTEX) in the anaerobic leachate plume of the Vejen landfill which confirmed criterion 1. Also criterion 3 was fulfilled, as the concentrations decreased relative to a conservative marker (chloride) and the decreases could not be explained by sorption. However, in laboratory microcosms only toluene, and sometimes xylene degradation was observed, often after long lag periods. Criterion 2 could not be verified for benzene. In contrast, Phelps et al. (1994) showed that measurements of microbial activity, based on the utilization of isotopically-labelled substrates in laboratory incubations with (uncontaminated) homogenized sediment, overestimated *in situ* activities, as determined by groundwater analysis, up to a million times.

The inability of laboratory assays to reflect the field situation can have several causes. A general problem in subsurface research is that the obtained sample and/or the volume used in an assay is unrepresentative for the environment from which the sample was obtained. Biomass content is low in the subsurface and microbiological properties can vary by many orders of magnitude, often exhibit a log normal distribution and are related to the geological context (Brockman & Murray 1997; Chandler & Brockman 2001). Culturing-independent methods have shown that microbial communities in groundwater are very different from those in sediment (Röling et al. 2000, 2001). Also geological, hydrological and geochemical properties are not constant in space. Individual properties show continuity over certain distances while different properties are spatial autocorrelated over certain distances. This complicates statistical analyses because classical methods rely on sample independence so the use of geostatistics in sampling has been advocated (Brockman & Murray 1997; Chandler & Brockman 2001) to evaluate the degree of spatial dependence of a property or the spatial correlation of properties as a function of distance between the samples. Geostatistics require special design considerations, as well as large numbers of samples, but provide important information for the design of improved sampling strategies and can be used to predict values at not-sampled locations. Subsurface heterogeneity calls for acquisition of coupled information at similar scales from the same sample (Chandler & Brockman 2001).

Another problem with laboratory assays of subsurface samples is post-sampling changes in microbial activities and community structure (Rochelle et al. 1994; Haldeman et al. 1994, 1995; Chandler et al. 1997; Brockman et al. 1998). Sampling can effectively be equivalent to passing a plough through the soil which causes major chemical and biological changes. It is often useful to work with intact cores (e.g., Lynch & Panting 1981). It has been shown that cell counts remained relatively constant while the numbers of culturable aerobic bacteria increased by up to six orders of magnitude for subsurface paleosol samples incubated for 224 days in a laboratory at the *in situ* temperature of 15 °C (Brockman, et al. 1998). Microbial activities, as measured by ^{14}C-glucose mineralisation, were enhanced. Also analysis of 16S rDNA clone libraries revealed obvious changes in microbial community structure in another experiment which involved incubation of subsurface paleosol samples under *in situ* conditions (Chandler et al. 1997). In these studies, changes were noted for not only homogenized samples but also intact core samples, although to a lesser extent. This indicated that minor changes in chemical

or physical properties of subsurface sediments result in major changes in the activity and composition of the microbial community (Chandler et al. 1997; Brockman et al. 1998). Both growth and resuscitational processes are considered responsible for post-sampling stimulation of subsurface microorganisms (Haldeman et al. 1995; Brockman et al. 1998). Perturbation possibly leads to an increased availability of limiting nutrients by redistribution of cells and solid-phase nutrients, improved gas exchange and moisture movement. Isolates with greater abilities to utilize substrates and higher growth rates were recovered after storage of subsurface volcanic rock (Haldeman et al. 1994). The availability of more labile substrates after sample perturbation can interfere with the degradation of more recalcitrant pollutants. Since in subsurface environments nutrient fluxes and biomass are far lower than in surface environments, the post-sampling resuscitation and growth responses may require protracted periods of time to manifest themselves. Therefore, Brockman et al. (1998) proposed that assays which measure *in situ* microbial activities should be done as soon as possible after sample acquisition especially when the samples are homogenized in the field. However, determination of the potential for contaminant degradation requires long incubation times (in itself a disadvantage) and thus might be affected by post-sampling changes. The measurement of isotopically-labelled end-products produced from radio-labelled substrates facilitates faster determination of contaminant degradation than residual substrate concentration measurement in assays with unlabelled substrates.

The enumeration of specific contaminant-degrading bacteria in samples also indicates degradation potential but, besides being labour intensive and time consuming, culturable microorganisms, in general, represent only a small fraction of the total microbial community (Amann et al. 1995). Thus, results from laboratory assays should be interpreted with care and these measures of potentials need to be extended and combined with other determinations (Chapelle et al. 1996).

Culturing-independent molecular methods

Culturing-independent molecular techniques are, currently, rapidly advancing our understanding of microbial community structure and bioremediation in the subsurface (Brockman 1995; Stapleton et al. 1998; Madsen 2000; Chandler & Brockman 2001). Polymerase Chain Reaction (PCR) amplification of nucleic acids extracted from environmental samples is at present the most powerful cultivation-independent technique. PCR facilitates the sensitive and fast detection of low amounts of specific gene fragments. This is of importance for monitoring purposes as subsurface environments are, in general, characterized by low biomass which releases low amounts of nucleic acids upon extraction (Chandler & Brockman 2001).

Specific information on the potential for natural attenuation of a certain contaminant can be obtained by assessing the functional genes which are responsible for its degradation (Brockman 1995; Stapleton et al. 1998). The development of probes which target functional genes depends on the availability of sequences, which strongly relates to the availability and diversity of catabolic strains (Biodegradative Strain Database; bsd.cme.msu.edu) and the scientific interest they have received. Most sequence information relates to aerobic degradation of BTEX and polycyclic aromatic hydrocarbons (PAHs) with the genes involved (such as toluene dioxygenase, naphthalene dioxygenase, catechol dioxygenase and protocatechuate dioxygenase) derived from 1 to 24 species, mainly *Pseudomonas* spp. (May 2001 search in Genbank via Entrez; www.ncbi.nlm.nih.gov/entrez). A factor which further complicates probe design is that a single pollutant can be degraded by several different pathways and functionally equivalent proteins show limited nucleic acid sequence identity (Biocatalysis/Biodegradation Database, at umbbd.ahc.umn.edu/index.htlm, with links to GenBank). Many probes used currently are biased towards *Pseudomonas*.

In contrast to functional genes, the 16S ribosomal RNA gene is present in all microorganisms and contains evolutionary well-conserved regions (Head et al. 1998). This has allowed the development of primers for PCR and has resulted in a large database of 16S rRNA sequences (currently $>$ 40000 sequences). By comparing obtained sequence data to the database, microbiologists can get insight of which microorganisms are present in a certain environment and in which specific processes they might play a role (i.e., potential). Unfortunately, it is often the case that phylogenetic information cannot be unambiguously related to specific processes and *vice versa*. Phylogenetically divergent microorganisms perform similar processes in natural attenuation such as iron reduction (Lonergan et al. 1996) and aerobic degradation of aromatic pollutants (Anderson & Lovley 1997). In contrast, current knowledge suggests the involvement of a single phylogenetic group for some important

subsurface processes such as anaerobic degradation of BTEX by *Azoarcus/Thauera* spp, *Geobacteraceae* and *Desulfobacteria* under denitrifying, iron-reducing and sulphate reducing conditions, respectively (Spormann & Widdel 2000). However, these phylogenetic groups also include non-BTEX degraders.

For many sequences no closely related cultured microorganisms are known. Until recently, linking 16S rDNA information to contaminant degradation was dependent on culturing studies, but new nucleic acid-based techniques, such as stable isotope probing (Radajewski et al. 2000) and bromodeoxyuridine labelling (Urbach et al. 1999), allow specific microbial processes and functions to be related to individual members of microbial communities in a cultivation-independent manner. Both techniques rely on the synthesis of labelled DNA by microorganisms which grow in response to a specific stimulus and the subsequent separation of this labelled DNA from the pool of total DNA. These techniques will contribute to the development of new probes for the detection of specific catabolic species.

The specific assessment of potential for intrinsic bioremediation based on molecular biological detection will either require a large number of independent PCR reactions with specific primers or sensitive fingerprinting techniques. Oligonucleotide microchips will become important in the future although the application to microbial communities is still in its infancy (Guschin et al. 1997; Chandler & Brockman 2001). Several 16S rDNA-based techniques have been used to fingerprint microbial communities (Head et al. 1998). The most widely used is denaturing/temperature gradient gel electrophoresis (DGGE/TGGE). In DGGE/TGGE 16S rDNA fragments, resulting from a single PCR reaction, are separated based on differences in their melting behaviour (Muyzer & Smalla 1998). A new, promising fingerprinting technique is Terminal Restriction Fragment Length Polymorphism (T-RFLP) in which $3'$ or $5'$ fluorescently-labelled PCR products are digested with restriction enzymes and separated using automatic sequencing technology. Although the requirements of expensive equipment and reagents have probably so far limited its adoption, T-RFLP offers some important advantages over other fingerprint techniques. Its resolution is higher and direct reference can be made to the 16S rDNA sequence database (Tiedje et al. 1999; Marsh et al. 2000). Phylogenetic inference is, however, most effective when only a single bacterial division or smaller group is addressed and is far less useful when the entire bacterial community is profiled (Dunbar et al. 2001).

Whether potential is monitored with a large number of independent PCRs or with fingerprinting techniques, as microorganisms can be present in or transported to environments where they lack suitable substrates or electron acceptors for activity (Ludvigsen et al. 1999; Röling et al. 2001), molecular analysis should be combined with field data or laboratory assays (e.g., redox assays) to assess the potential for intrinsic bioremediation more precisely. With specialized computer software, fingerprints can be databased and subjected to multivariate statistical analyses (e.g., Röling et al. 2000; Dunbar et al. 2001). Computer-assisted analysis allows the comparison of different profiles with each other and the establishment of relationships between fingerprints and environmental conditions. By performing cluster analysis on DGGE fingerprints of *Archaea* and *Bacteria* communities in an anaerobic landfill leachate plume, Röling et al. (2001) were able to relate microbial community structure to pollutant concentration and redox processes. Artificial neural networks (ANNs) deal better than multivariate statistical techniques with data structures that are complex, non-linear, fuzzy, probabilistic and inconsistent, such as fingerprints. (Noble et al. 2000; Moschetti et al. 2001). Sensitivity analysis on ANNs distinguished which input parameters (phospholipid fatty acids representing microbial communities) determined the output, the sediment and the worm burrow types from which the microbial communities were isolated (Noble et al. 2000). Likewise, ANNs can be used to find sets of bands in fingerprints that indicate potential for contaminant degradation. This requires a large database of fingerprints and corresponding information on environmental parameters from a large number of sites.

Verification of intrinsic bioremediation

Reliance on intrinsic bioremediation requires that field data should confirm that the potential for intrinsic bioremediation is expressed *in situ* (NRC 1993). Quantitative data can be used in mathematical modelling to estimate how long it might take before the contaminant concentrations are lowered to acceptable levels.

Verification by molecular biological methods

While measurements directed at functional genes reveal the potential for degradation of a certain compound, the actual occurrence of activity *in situ* can be determined by detecting messenger RNA (mRNA) transcribed from functional genes. As mRNA is short-lived, rapid on-site freezing is required, to avoid sampling artefacts (Wilson et al. 1999).

Direct, quantitative hybridization of extracted mRNA with probes has shown that mRNA levels of genes involved in PAH degradation correlated with ^{14}C-PAH mineralization rates in soils at town gas manufacturing sites (Fleming et al. 1993). Direct hybridization requires a relative high level of mRNA ($\geq 10^4$ targets) to be present (Brockman 1995) which can be a problem with relatively low biomass-containing subsurface samples. Reverse transcriptase (RT) PCR is capable of detecting low amounts of mRNA. Quantitative PCR methods have been applied successfully to subsurface environments. These have included Most-Probable-Number-PCR to determine biodegradative gene numbers at a jet fuel contaminated site (Chandler & Brockman 1996) and TaqMan-PCR to enumarate *Geobacter* rRNA and rDNA in aquifer sediment (Stults et al. 2001). Of the quantitative PCR methods, real-time 5′ fluorogenic exonuclease, or TaqMan PCR, appears most promising. It is capable of determining numbers of templates over a wide range of concentrations with higher detection sensitivity, speed and dynamic range. It suffers from the influence of contaminating humic acids in nucleic acid extracts, which is a general problem in PCR but is enhanced in TaqMan PCR due to fluorescence quenching and autofluorescence. Replicate dilutions and replicate analysis improve TaqMan accuracy (Stults et al. 2001). Quantitative PCR is further complicated by preferential extraction of nucleic acids and preferential PCR amplification (von Wintzingerode et al. 1997; Chandler & Brockman 2001). Also, expression may show low constitutive levels and expression of a single gene in a degradative pathway cannot easily be extrapolated to the overall activity of the pathway (Madsen 2000). Therefore, quantitative PCR methods directed at mRNA provide semi-quantitative data on activity and need to be combined with other approaches.

Although it is often stated that the analysis of 16S ribosomal RNA provides information on *in situ* activity (e.g., Madsen 2000), it must be realized that even non-growing microorganisms possess considerable numbers of ribosomes and, thus, rRNA. Based on studies of a large number of marine isolates and laboratory strains such as *E. coli*, Kemp (1995) derived an equation relating growth rate to the ratio of RNA to DNA. As rRNA comprises about 80% of the total RNA, this relation (Figure 1) shows clearly that for the subsurface, where the growth rates are probably well below 0.01 h^{-1} (Whitman et al. 1998), the amount of rRNA is merely indicative of presence, but not activity.

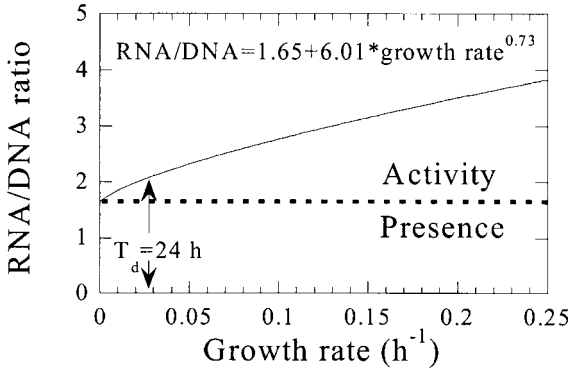

Figure 1. Relationship between growth rate (μ) and RNA/DNA ratio as determined by Kemp (1995). The area between the solid line (RNA/DNA ratio as calculated from the equation) and the dotted line (RNA/DNA ratio at $\mu = 0$ h^{-1}) indicates activity, the RNA/DNA ratio below the dotted line indicates microbial presence only. The RNA/DNA ratio coincident with a doubling time of one day is indicated specifically.

Verification by chemical methods

Chemical methods based on measurements of changes of concentrations of contaminants, metabolic end products and/or co-reactants along a flow path allow rapid verification of intrinsic bioremediation. The use of internal conservative tracers enables verification by determining the loss of contaminants relative to the persistence of less biodegradable, but similarly transported, compounds. Two examples are the already discussed decrease of BTEX compounds relative to chloride in landfill leachate plumes (Lyngkilde & Christensen 1992) or decrease in oil components relative to the recalcitrant, crude oil component 17α(H),21β(H) hopane (Bragg et al. 1994). Measurements of substrates do not to distinguish between mineralisation and transformation. These contrast measurements which show increased concentrations of the mineralization products CO_2 and CH_4 along a flowpath. Changes in the concentrations of electron acceptors can also indicate the occurrence of intrinsic bioremediation. However, measurements of oxidized or reduced redox species can neither be related to

the degradation of specific compounds in mixtures of pollutants nor indicate whether the contaminants are mineralized or transformed. Redox processes, their assessment and problems associated with redox characterization, are treated in more detail by Christensen et al. (2000). Verification of intrinsic bioremediation by the above methods can be complicated by subsurface heterogeneity and the simultaneous occurrence of other attenuating processes on substrates (e.g., sorption) or products (e.g., precipitation, volatilisation). Parameters which relate to these factors, such as pH, should also be assessed and included in modelling approaches to draw correct conclusions on intrinsic bioremediation (van Breukelen, personal communication). When groundwater flow rates are known, degradation constants can be inferred and used for reactive transport modelling (Van Breukelen, personal communication).

The application of stable isotope analysis to verify intrinsic bioremediation relies on differences in the ratio's of light to heavy isotopes for different carbon sources and the preferential utilization of substrates which contain light isotopes by microorganisms. An isotope with a lower mass has a slightly lower activation energy than a heavier isotope and this result in a relatively higher rate of reaction. As a result, the fraction of heavier isotope containing residual substrate increases and the products of microbial action are slightly depleted in heavier isotopes. The most widely used isotope is carbon. The degree of carbon fractionation depends on the source of the substrate and the redox conditions (Conrad et al. 1997; Meckenstock et al. 1999) but a small enrichment in the carbon isotope value ($< 2‰$) will often be difficult to use as a suitable indicator of *in situ* biodegradation. Recently, hydrogen isotope analysis of substrates has been proposed as a more reliable method to validate intrinsic bioremediation due to the very large isotopic enrichment in ^{2}H ($> 60‰$). This may relate to the fact that the mass difference between ^{12}C and ^{13}C is only 8% while for ^{1}H and ^{2}H it is 50% and results in significantly hydrogen isotope fractionation (Ward et al. 2000). However, even in the absence of substantial fractionation, carbon isotopes can be used when the $\delta^{13}C$ values of endogenous organic matter and the contaminant are known to be different. Then, the carbon isotope ratio of the mineralization product CO_2 provides an estimate of the relative contributions of different carbon sources to product evolution. For hydrocarbons, simultaneous measurement of radiocarbon (^{14}C) contents of CO_2 can be used to determine their sources when $\delta^{13}C$ values of the different sources overlap or methanogenesis occurs. Fossil hydrocarbons are ^{14}C free while more recent natural carbon sources contain ^{14}C (Conrad et al. 1997).

The presence of intermediary metabolites provides information on *in situ* degradation of specific compounds when an unequivocal and unique biochemical link with the parent compound exists, no other sources for the particular metabolite are present and the released product exhibits biochemical and chemical stability under *in situ* conditions. Preferably, it should be an intermediate of mineralization rather than a product of co-metabolism (Beller 2000). Detection of intermediary metabolites requires knowledge on the biochemistry of pollutant degradation which depends on the availability of cultures and enrichments. As an example, alkyl-succinates are released during anaerobic degradation of alkanes, BTEX and PAHs and can be detected in the field (Beller 2000). Compared to monocultures, knowledge of degradation by microbial associations is limited (Zwolinski et al. 2000) although various parts of a multi-step degradation pathway can be mediated by different microbial components of an association. The points at which intermediates are released and subsequently used by other members of the association may be determined by examining the energetics of different transformations in the pathway. Compounds in which a significant amount of energy is invested are unlikely to be released (Zwolinski et al. 2000).

Capacity for intrinsic bioremediation

Together with knowledge of the occurrence and rate of intrinsic bioremediation, it is important to know whether it will be able to reduce the level and/or spread of pollution to an acceptable degree within a certain timeframe. Determination of the capacity for intrinsic bioremediation has received relatively little attention. It requires knowledge of interactions between microorganisms and their geological and hydrological environment, especially the availability of substrates, electron acceptors and nutrients.

Substrate bioavailability and utilization

Substrate bioavailability is determined by the interactions of pollutants with both organic and inorganic (negatively-charged clay particles) matter which can render them less accessible for biodegradation (Alexander 1994; see Ralebitso et al. in this issue for a more

extensive discussion, including methods for determining bioavailability). Sorption and sequestration are influenced by a variety of factors such as organic matter content, mineralogy, pH, temperature and pollutant type. In particular, PAHs sorb well. With increased contact time the proportion of pollutant that becomes unavailable for biodegradation increases. This influences degradation negatively since the rates at which microbial cells can convert chemicals relate to the rates of mass transfer to the cells (Bosma et al. 1997).

Threshold substrate concentrations are the concentrations at which the population size does not increase due to the fact that all the energy from metabolism is used for cell maintenance. Threshold values are substrate- and species-dependent (Alexander 1994). Also, substrate utilization is influenced by the presence and concentrations of other substrates.

Redox buffering and bioavailability of electron acceptors

The redox environment sets the boundaries for attenuation of many compounds (Christensen et al. 2000). The activities of attenuating microorganisms are often restricted to certain redox environments and degradation processes occur at different rates in different redox environments. For example, the reductive dechlorination of chloroethenes only occurs under methanogenic conditions (Bradley 2000). The ability to maintain certain redox conditions and to buffer the spread and level of pollution is a function of the capacity of pollutants and natural sources in the subsurface to donate electrons (reducing capacity; Heron & Christensen 1995) and the capacity of the subsurface, and sometimes pollutants (e.g., chlorinated organic compounds, metals), to accept electrons (oxidation capacity; Heron et al. 1994). Reducing and oxidation capacity can be determined by measuring the concentrations of available solid and aqueous electron acceptors and donors.

Aqueous electron acceptors are readily used by microorganisms. In contrast, while Fe(III) is often the major electron acceptor in the subsurface (Heron & Christensen 1995), its presence as solid Fe(III) minerals hinders microbial attack and accessibility depends on the type of Fe(III) mineral (Lovley 2001). Iron reducers either need to establish direct contact with Fe(III) or use intermediates, such as humic acids, as electron transport shuttles. Humic acids fit into spaces where microorganisms cannot access. The addition of humic acids or chelators of iron to microcosm experiments often increases degradation considerable (Lovley 2001). Thus, iron bioavailability can be a major controller of the degradation.

Nutrient bioavailability

Nitrogen and phosphorous can become growth and rate limiting when the contaminant functions as a carbon source. Even in natural groundwaters *N* and, especially, *P* are often limiting (Chapelle 1993). Interestingly, some important subsurface microorganisms, such as iron reducing *Geobacteraceae*, are capable of nitrogen fixation (Bazylinski et al. 2000) and are, thus, able to overcome ammonium and nitrate shortage in groundwater. Like Fe(III), *P* is present in minerals. Microorganisms attached to minerals produce a very reactive microenvironment at the mineral surface which accelerates the dissolution of silicate minerals via the production of organic ligands (metabolic by-products, extracellular enzymes, chelators and simple and complex organic acids) or releases *P* secondary to Fe(III) reductive dissolution (Bennett et al. 2000, 2001). Released limiting *P* offers the colonizing microorganisms a competitive advantage. The progression of mineral weathering is influenced by its nutritional potential. The most unstable silicate mineral will weather first. A near positive correlation between microbial colonization and weathering rate has been recorded (Bennett et al. 2001). The sediment composition, therefore, may reflect early destruction of biologically-valuable minerals (Bennett et al. 2001) as well as capacity for degradation.

What limits the rate of intrinsic bioremediation?

From the above, it is clear that microbial activity and, hence, the rate of intrinsic bioremediation, is dependent on the bioavailability of substrates, electron acceptors and nutrients, which are related to environmental conditions.

Also properties of microbial associations can limit degradation. Protozoan grazing is, in general, enhanced in polluted environments due to a higher density of preys (the contaminant- degrading microorganisms) and, thus, can constrain the natural attenuation rate. Predation relates to the physical and chemical characteristics of the aquifer, including the redox conditions, soil characteristics, groundwater velocity and contaminant properties (Kota et al. 1999). In interactions between microorganisms in contaminant degradation, a single species type species could be rate controlling. For example, in redox mapping by thermody-

namic calculations involving hydrogen concentrations (Postma & Jakobsen 1996), it is assumed that the fermenting, hydrogen producing microorganisms are rate controlling and not the hydrogen-consuming, electron acceptor-reducing microorganisms. This assumption is based on the low concentrations of hydrogen and its high turnover.

Statements in microbial ecology on rate limitation by a certain factor, such as in the above example, often lack scientific proof. In the areas of microbial physiology and biochemistry it has been recognized for many years that a low concentration of a certain factor is insufficient to draw the conclusion that this factor, or the process producing it, is rate limiting (Fell 1997). Studies employing Metabolic Control Analysis (MCA) have revealed that often the flux through a metabolic pathway is not controlled by a single factor, but rather that control is distributed over several parameters (enzymes, substrates). Basically, MCA is a sensitivity analysis, which is supported by powerful theorems, for the establishment of the control of an enzyme or metabolite concentration on flux through a metabolic pathway (Fell 1997). This can be achieved by changing these parameters slightly (*in silico*, *in vitro* or *in situ*) and measuring the effect on the flux after the system has again achieved steady-state conditions. The control coefficient is expressed as standardized partial derivates, and lies between 0 and 1. A value of 0 indicates that the investigated parameter has little influence on the flux while a value of 1 indicates that the flux is controlled completely by the parameter (Fell 1997). An ecological variant of MCA will contribute to a better understanding of which factor(s) control degradation and, thus, should be monitored specifically. In this variant, (groups of) microorganisms represent the enzymes in a metabolic pathway while substrates, intermediates, co-reactants and nutrients represent metabolites.

Resource-ratio theory predicts that a change in ratios of growth-limiting factors during biodegradation influences competition for these resources and selects for a microbial association with a different optimum substrate affinity, maximal growth rate and cell decay rate (Smith 1993; Head & Swannell 1999). A change in a microbial association can lead to a change in contaminant degradation. Molecular methods, based on the detection of specific regulatory proteins, can be used to monitor which factor(s) limits degradation. Such methods have been described for marine environments (e.g., Lindell & Post 2001) but not yet for subsurface environments.

Secondary effects of natural attenuation

Attenuation of pollutants may have secondary effects which influence natural attenuation and its acceptability, these secondary effects should be evaluated. Biological transformation can result in products with higher toxicities than the parent molecules. For example, vinyl chloride, produced from trichloroethane by reductive dechlorination under methanogenic conditions, is only slowly degraded under other redox conditions which are usually present downstream of the source of the pollutant (Bradley 2000).

Microbial activities change not only water and sediment chemistry but also the physical properties of aquifers. Dissolution of minerals, such as Fe and P for metabolic activity and growth, can increase secondary porosity (Bennett et al. 2000, 2001). Under oxic conditions, excess carbon dioxide production promotes calcite and dolomite dissolution via acidity, and so produce secondary porosity (Bennett et al. 2000). In anoxic groundwater, carbonate and bicarbonate accumulate and become saturated with calcite. This results in its precipitation and cementation of pores which reduces permeability (Bennett et al. 2000). A change in porosity affects the spread of pollution due to changes in groundwater flow.

Subsurface specimen banking

As discussed above, monitoring technique development still requires much fundamental and applied multidisciplinary research. Integration of knowledge on the biochemistry, genetics, physiology and ecology of subsurface microorganisms, and their relationships to subsurface geology, hydrology and chemistry, is required. Intrinsic bioremediation is only well established for aerobic BTEX attenuation and it is still difficult to extrapolate predictions of potential beyond the site of investigation (Hoyle & Arthur 2000). A factor which complicates subsurface research is that it is both difficult and costly to obtain samples of acceptable quality for research purposes. Therefore, a more effective use of currently taken subsurface samples and the data obtained for these samples is required.

During the last two decades Environmental Specimen Banking (ESBs) programmes have been established to collect and store large numbers of representative, mainly biotic environmental samples (e.g., tissues) under chemically stable conditions for periods of at least several decades (Paulus et al. 1996).

The main function of ESB is retrospective monitoring of ecotoxicological aspects for which the potential has been proven. For example, historical trends of toxaphene in fish from Canadian lakes were determined after exceptional high concentrations in fish tissue were noted (Kerry et al. 1993). The origin of a pneumonia-epidemic was determined 15 years after the outbreak when an antibody-based method was developed and applied to stored serum samples (Gunter 1997). Likewise, banking of large numbers of subsurface specimens (sediment, groundwater) will be very useful with respect to enhancing fundamental knowledge of microbial communities and processes in the subsurface, testing general hypotheses on relationships between microorganisms, microbial activities and environmental parameters, translating new knowledge into monitoring techniques and validating new techniques.

The organization of Subsurface Specimen Banks (SSBs) can be similar to that of ESBs, where quality assurance and registration play key roles (Kerry et al. 1993; Paulus et al. 1996). Representativeness and precision are the central concepts of quality assurance. Stringent, well documented methods cover the whole chain from sampling in the field to distribution of the samples for analysis. The registration covers a detailed description of the sampling area, the choice of representative sampling locations and observations made in the field of the samples (origin data), steps and deviations in Standard Operation Procedures, storage location, and details of all determinations on the sample (analysis data). All of these data are stored in a database which allows the researcher to put analysis data in their biological and chemical perspectives and to look for specific information to select samples for further research. Requests for samples for analysis have to be justified and, when approved, a sample is released. Data of the analysis have to be provided to the ESB and are subsequently added to the database, enhancing its value.

Optimal conditions for long term storage of subsurface samples

ESBs aim to conserve the chemical status (especially organic contaminants and heavy metals) of samples which are well maintained in a frozen state. Long-term conservation of subsurface material should aim to suspend activities and maintain physical, chemical and biological properties as near as possible to the *in situ* state. To monitor microbial presence and activity, DNA and RNA analysis, in particular, will play an important role and so DNA and RNA should be well preserved.

Results of research on the effects of various storage conditions on conservation are compiled in Table 1 and show that cryopreservation in liquid nitrogen is the most suitable. One report (Zelles et al. 1991) claimed changes in microbial parameters (heat output after glucose addition, ATP content, adenylate energy charge and hydrolysis of fluorescein diacetate) during 20 month of storage over liquid nitrogen (−140 °C). However, during thawing in this study the samples were maintained at −18 °C for 5 days, followed by 4 days at 4 °C, before analysis. This short-term storage at higher temperatures might have induced the changes. Cryobiological research has shown that the rates of most reactions decrease exponentially with decreasing temperature. It is recommended that cells are cooled to below the eutectic point (lowest solidifying point of a component); at least to −135 °C (Karlsson & Toner 1996). At −196 °C there is insufficient thermal energy for significant chemical changes in biological samples (Karlsson & Toner 1996; Taylor & Fletcher 1998).

To conserve the *in situ* conditions, and especially indicators of activity such as mRNA, the time between sampling and cooling to cryogenic temperatures should be as short as possible (Rochelle et al. 1994; Wilson et al. 1999). Rapid freezing is often deleterious to viability due to the formation of intracellular ice and membrane damage (Karlsson & Toner 1996). To maintain maximum viability, samples frozen rapidly should also be thawed rapidly (Karlsson & Toner 1996). Studies with microorganisms have shown that each cell type has different requirements for optimal cryopreservation and that the physiological state at the moment of cryopreservation affects survival (Karlsson & Toner 1996; Taylor & Fletcher 1998). Active cells are more sensitive to freezing than inactive cells (MacLeod & Calcott 1976). Since it is likely that the most active microorganisms in the subsurface will be those involved in biodegradation and because it is unlikely that the viability of all cells will be maintained during storage, biological redox and degradation assays and culturing studies cannot be made reliably after cryopreservation (or other methods of conservation; Table 1). Such assays should be performed on fresh samples.

For the conservation of chemical redox parameters, both sampling and storage should be made under anaerobic conditions as many indicators of anaerobic

Table 1. Influence of different storage conditions on the ability to suspend activity and maintain the in situ state of chemicals, DNA and RNA levels and viability.

Storage condition	Suspending activity	Maintaining *in situ* state of		
		Chemicals	DNA + RNA	Viability
Cold storage (0–8 °C)	$-^{1,4,5}$	$v^{1,5,11}$	$-^{1,4,5}$	$v^{1,4,5}$
Freezing (−20–80 °C)	$-^{1,5,10}$	$v^{1,5,11}$	$-^{3}$	$v^{1.5}$
Liquid nitrogen (−160–196 °C)	$+^{6,8}$	$+^{11}$	$+^{6-8}$	$v^{6,8}$
Freeze drying	$+^{6,8}$	$v^{2,9}$	$+^{6-8}$	$v^{6,8}$

−, no; +, yes; *v*, variable. Compiled from: 1. Zelles et al. 1991; 2. Lewis 1992; 3. Leonard et al. 1993; 4. Haldeman et al. 1994; 5. Haldeman et al. 1995; 6. Karlsson & Toner 1996; 7. Smith & Austin 1997; 8. Taylor & Fletcher 1998; 9. Bordas & Bourg 1998; 10. Rivkina et al., 2000; and 11. general ESB-knowledge.

conditions (sulphide, iron(II) and other microbially reduced metals) can react rapidly with oxygen (Heron et al. 1994). Storage in liquid nitrogen achieves anaerobic conditions.

Concluding remarks

Fundamental research is providing insight in the natural attenuation of many compounds in a wide variety of subsurface geochemical settings. However, current knowledge of natural attenuation of many compounds is insufficient for the development and implementation of general tools for monitoring intrinsic bioremediation. Readily accessible subsurface specimen banks for long-term storage of samples will help to elucidate subsurface natural attenuation, provide a more deterministic view of the potential, activities and capacity of natural attenuation and facilitate translation from fundamental research to monitoring tools.

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