Microbiology of subterranean waste sites

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Summary. Excavated repositories for radioactive waste in deep and shallow geological formations will be subject to microbial contamination; therefore, a number of groundwater environments have been examined in Europe for the presence and activity of microorganisms. Common soil bacterial isolates were found to predominate in the groundwaters. Their activity is curtailed by the oligotrophic conditions encountered. It still remains to be demonstrated whether waste and waste isolation materials such as cellulose and bitumen will provide an exogenous nutrient source for the microorganisms and whether microbial activity will compromise nuclear waste immobilisation. A further constraint to the microorganisms is the high pH near-field environment generated by cement/concrete barriers.
Key words. Groundwater microorganisms; nuclear waste; oligotrophy.

Introduction

Plans for the disposal of low-, intermediate- and high-level waste (LLW, ILW and HLW) in subterranean (geologic) environments is well advanced in most radioactive waste-producing countries. Almost all countries plan to dispose HLW in repositories in deep geological formations. LLW and ILW, on the other hand, may be subject to shallow land burial or to deep disposal. Since the objective of such disposal is the long-term protection of the environment and man, it is imperative to research all aspects which may compromise the safety aspects of the disposal options.

The potential role of microorganisms on the containment of radioactive wastes has been recognised[20, 27, 28] and research initiated by various national bodies.

Microbiology of selected sites in Europe

In Europe, various geological formations are being examined as potential host environments for repository construction (table 1). In some geological formations, underground laboratories have been specially constructed to assess their performance as disposal sites, e.g., in clay formation in Mol, Belgium. As a consequence the microbiology of existing boreholes, mines and specially constructed underground laboratories has been examined. Primarily, groundwater samples were obtained from various sites (table 1) and microorganisms looked for as well as their potential activity in natural groundwaters.

Table 1. Location and geology of European sites examined for microorganisms

Type	Location	Geology	Depth (m-bgl)
Boreholes	Harwell, UK	Oxford clay	165–331
	Altnabreac, UK	Granite	10–281
	Stripa, Sweden	Granite	340
Used/disused mines	Cumbria, UK	Anhydrite	ND
	Derbyshire, UK	Limestone	61
	Cornwall, UK	Granite	80–800
	Stripa, UK	Iron ore/granite	340
	Mol, Belgium	Boom clay	190–223
	Asse, FRG	Salt	750
	Konrad, FRG	Iron-ore	1100
	Grimsel, Switzerland	Granite	ND*
	Felsenau, Switzerland	Gypsum/anhydrite	ND*

ND, not determined/unknown; m-bgl, m below ground level; *, beneath mountains.

Normally groundwater sampling did not employ sterile techniques. The objective was not to differentiate between autochthonous and allochthonous microbial types. Ultimately it does not matter if the organisms of a repository groundwater are native or introduced. The importance lies in whether such microbial presence will affect the integrity of a repository. However, during groundwater sampling from a borehole in Altnabreac[5], we tested whether microorganisms present at a depth of between 259 and 281 m were surface soil contaminants or were being introduced through groundwater movement into the sampling depth enclosed by a double packer sampling system. A hypochlorite solution was introduced into the packer system and left to disinfect the region before sampling. In new infiltrating groundwater, no microorganisms were recovered, whereas significant populations were cultured prior to hypochlorite treatment. This implied that either natural groundwaters were microbial-free or the types of microorganisms present (novel types?) could not be cultivated using standard techniques employed. Thus the initial standing microorganisms were contaminants via the drilling process and the types present were those normally found in soils. These include autotrophic and heterotrophic types. Table 2 provides estimates on the aerobic heterotrophic bacterial populations in various mines.

The major heterotrophic bacteria detected were species of *Aeromonas, Flavobacterium, Pseudomonas, Alcaligenes, Micrococcus, Corynebacterium, Acinetobacter, Bacillus* and *Clostridium*. In addition, the use of selective and differential techniques revealed the presence of groups such as denitrifying bacteria, sulphate-reducing bacteria (SRB), methanogenic bacteria, iron precipitating bacteria and fungi. Autotrophic types detected included ammonium- and nitrite-oxidising (nitrifying)

Table 2. Bacterial populations in deep groundwater environments

Location	Bacterial count* CFU ml^{-1}
Harwell	$8.6 \times 10^3 - 3.5 \times 10^5$
Altnabreac, UK	9.4×10^5
Stripa, Sweden	$3 \times 10^1 - 1.3 \times 10^5$
Cornwall, UK	$2.5 \times 10^2 - 2.5 \times 10^4$
Mol, Belgium	1.2×10^3
Asse, FRG	ND
Konrad, FRG	ND $- 7.5 \times 10^2$
Grimsel, Switzerland	$9.5 \times 10^1 - 9 \times 10^4$
Felsenau, Switzerland	$1 \times 10^1 - 1.6 \times 10^4$

*Aerobic heterotrophs; ND, not detected.

bacteria and sulphur and iron-oxidising bacteria. Pasteurisation of natural samples showed that a variable proportion of organisms isolated were originally present as spores. A large number of genera demonstrated, however, are non-spore-formers and are either active or dormant in deep groundwaters recovered.

Unlike shallow aquifers, deep groundwaters are almost always limited in organic carbon. In deep repository environments, low organic carbon levels can be supplemented by contamination (soil, drilling fluids) and by backfill/buffer materials used in repository construction (e.g. clays, bitumen). Oligotrophic bacteria [12, 16] were isolated from various sites using media containing less than 10 mg l^{-1} organic carbon.

Microbiological techniques

Groundwater, particularly in deep geological formations, would be expected to contain or support only small numbers of microorganisms, or none at all. The experience of the present investigations in granitic and clay formations suggests that organisms detected, using cultural techniques generally developed for surface microorganisms, are contaminants from surface environments. Isolation techniques may, however, preclude novel microbial types.

Both direct and indirect methods exist for the detection of microorganisms in natural samples. Methodology includes epifluoresence [7, 11, 13] and other microscopy utilising light and electron beams; the detection of biological substances such as ATP [14], enzymes [3], nucleic acids, fatty acids, lipids and phospholipids [29], and cell wall and envelope components such as lipopolysaccharides, muramic acids and chitin. Cultural techniques are also available such as plate counting and the multiple tube dilution (Most Probable Number - MPN) technique [17, 30].

The presence of microorganisms in groundwaters does not constitute activity. Methods used to determine microbial activity include the examination of substrate utilisation and product formation. Examples include depletion of electron acceptors such as O_2, NO_3 and production of substances such as CO_2, CH_4, H_2S and volatile fatty acids. Perhaps the best and most sensitive way of detecting such microbial activity is to utilise stable and radioisotope labelled substrates. Radioisotopes commonly used are ^3H, ^{14}C and ^{35}S and stable isotopes include ^{13}C, ^{15}N and ^{18}O. Inhibitors are often employed to detect the microbial activity of specific groups (e.g. acetylene-inhibition technique for denitrifying bacteria [15]).

For a comprehensive review of methods available see Atlas [2], Fry [9], Costerton and Colwell [6] and references therein.

In the present examination of deep mine and borehole waters in relation to radionuclide waste disposal the following methods were employed: selective and differential solid (agar) and liquid media for viable microorganisms utilising spread plate and MPN techniques [26]; epifluorescence techniques utilising acridine orange; radioisotopic techniques with ^{14}C-bicarbonate incorporation as a measure of autotrophic metabolism and ^{35}S-sulphate uptake as an indication of productivity; and CO_2-production using gas chromatography.

Oligotrophy

In the granitic groundwaters of the Grimsel mine, autotrophic activity was demonstrated while heterotrophic activity was absent until groundwaters were supplemented with organic carbon sources. The majority of samples examined appeared to be organic carbon-limited and bacterial species detected could be oligotrophic types.

Oligotrophic bacteria represent a diverse group of microorganisms capable of growth at low levels of organic carbon. Mallory et al. [18], for example, isolated slow-growing bacteria from an estuarine environment, on a nutrient-limited medium. The oligotrophic bacteria isolated were placed into three broad groups; 1) gram-positive organisms including *Corynebacterium, Listeria, Nocardia, Staphylococcus* and *Planococcus*, 2) stalked or sheathed organisms including *Hyphomicrobium, Hyphomonas, Streptothrix, Sphaerotilus* and *Pediomicrobium* and 3) gram-negative rods including *Alcaligenes* and *Acinetobacter*. Some of the above genera were commonly detected in deep groundwaters examined. Clearly, oligotrophy is not limited to a single genus and may represent an enforced adaptation to very low levels of organic substances in natural environments.

Kuznetsov et al. [16] recognise four groups of oligotrophic bacteria. These are: a) isolated bacteria that do not grow on recultivation on either nutrient-poor or nutrient-rich media; b) bacteria that grow initially only on nutrient-poor media, but can be recultured on nutrient-rich media; c) bacteria isolated or cultivated only on specialised nutrient-poor media; and d) bacteria that do not grow on nutrient media. The latter has important implications to the detection and enumeration of microorganisms from novel environments using standard viable counting techniques.

Most natural habitats (soil, marine or freshwaters) unlike laboratory culture media are nutrient-limited and con-

Table 3. Chemical data for selected groundwaters examined for microorganisms and microbial activity

Groundwater	Depth (m)	pH	HCO_3	Cl^-	SO_4^{2-}	SiO_2	Na^+	K^+	Ca^{2+}	Mg^{2+}	TIN	PO_4^{3-}	Total Fe	TOC
			($mg l^{-1}$)											
Harwell, UK	165	8.75	31	30	61	0.8	18	3.5	35	3.1	22.4	0.05	0.05	ND
Altnabreac, UK	259–281	7.8	124	20	4	28	31	0.7	26	1.81	0.4	0.03	0.18	1.0
Mol, Belgium	190	12.7	7	2800	18	2	350	140	1700	0.05	8	0.5	0.1	29
Konrad, FRG	1100	6	5	132,000	820	6	64,400	300	12,600	2640	71	1.2	47	50
Asse, FRG	750	ND	203	307,700	25,980	2.8	84,450	140	31.4	95,060	69	0.1	4.84	1115
Stripa, Sweden	340	8.84	86	40	4.9	11.8	46	0.59	14	0.23	0.23	0.03	0.006	1.1

TIN, total inorganic nitrogen (NH_4^+, NO_2^-, NO_3^-); TOC, total organic carbon; ND, not determined/not detected.

tain carbon levels between 1 and 15 $mg \cdot l^{-1}$ [19]. A large oligotrophic region is the open sea with dissolved organic carbon concentration ranging from 0.3 to 1.2 mg $C \cdot l^{-1}$ [23].

Table 3 presents chemical data for some of the groundwater sites examined. There is wide variation in water chemistry from the highly saline waters of Asse with a relatively high organic carbon content, to the nutrient deficient granitic groundwaters of Stripa. Microbial success would reflect the differences in groundwater chemistry. At present one can only speculate on the possible microbial activity in deep groundwaters and compare conditions with marine systems. Similar to marine bacteria, those present in the subsurface environments would rely on nutrients and other physico-chemical factors present in the groundwater, and the majority would occur as spores or as vegetative cells with reduced metabolic activity [22]. Long residence times for deep groundwater would lead to extremely low throughput of novel nutrients. Microorganisms would have to rely on turnover of existing levels and this would be too slow to sustain a significant microbial population.

Examples of long turnover rates are seen in the oligotrophic environments of the deep sea where ultra low concentrations of organic carbon (0.05 $mg \cdot l^{-1}$), most of which is not readily degraded, occur. Turnover times of 3300 years have been estimated [21]. It is likely that in deep groundwaters which are almost exclusively anaerobic, turnover rates of organic material are even longer than those calculated for deep sea waters which are aerobic. Alexander [1] attributed persistence or lack of degradation of organic substances to a lack of oxygen in natural clay materials. Clay formations or clay backfill materials which may be used in waste isolation contain up to 10% organic material, a high proportion of which may be unavailable because it is of high molecular weight and non-degradable under anoxia. Another reason for non-biodegradability of organic material in clays is because clays such as montmorillonite reduce the activity of microbial enzymes such as proteases, amylases, cellulases, hemicellulases and phosphotases [1].

During the examination of relevant groundwaters in Europe it has been shown that although microorganisms are present, their activity depends on an exogenous input of organic carbon [4]. Microbial heterotrophic activity could only be detected after addition of, for example, yeast extract. In some cases, organic carbon additions also needed to be supplemented with nitrogen and phosphorus. It therefore appears that repository microorganisms would be nutrient-limited if activity depended on natural concentrations of nutrients in groundwater. There is a tendency for nutrients to accumulate on surfaces and it is envisaged that introduced or native microorganisms will be concentrated on rock surfaces as observed in other oligotrophic environments [8]. Rounding up of bacterial cells and miniaturisation are important survival strategies under nutrient limitation [25]. Microbial organic acid production may lead to the release of important elements such as phosphorus trapped in rock material. For a review of survival strategies see Roszak and Colwell [24]. There is an urgent need to determine whether materials used in repository construction would enhance or encourage significant activity.

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Evaluation of cement degradation induced by the metabolic products of two fungal strains

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Summary. During their metabolism, microorganisms can produce acids able to bring about the degradation of cement. Two acid-producing alkalophilic fungal strains have been isolated from soil, a strain of *Aspergillus niger* which produces gluconic and oxalic acid and of *Mycelia sterila* which produces gluconic and malic acid. After eleven months of contact the acids produced by *Aspergillus* dissolved portlandite with a low leaching of calcium, increased the cement porosity by 11.4%, and reduced the bending strength by 78%. The second strain is responsible for a significant dissolution of portlandite with a leaching of calcium of 4.2% of the initial content, an increase of the porosity of 11%, and a loss of the bending strength of 62%. Direct contact of mycelia with the cement surface is not necessary for effective dissolution to take place. Low pH and a high temperature favor the production of acids.
Key words. Radioactive waste; cement; degradation; microorganisms; organic acids.

Introduction

Waste repositories can be situated in geological formations where microorganisms capable of degrading complex organic materials are present[21]. As part of a program studying the long-term stability of the materials used for nuclear waste disposal, the biodegradability of cement (a coating matrix for the low and intermediate level waste) was determined.

Cements are mineral materials, primarily hydrated calcium silicate C–S–H and portlandite ($Ca(OH)_2$) for the ordinary Portland cement. Their biodegradation is due to the products of microbial metabolism and thus is an indirect effect of microbial growth. Examples of deterioration of stones or cement have often been described[20], mainly in sewer systems but also on building structures. In the sewer systems, degradation is due to the activity of autotrophic sulphur-oxidizing bacteria[14–16]. Hetero-