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Microbial degradation of bitumen

M. Wolf and R. Bachofen

Institute of Plant Biology, University of Zürich, Zollikerstr. 107, CH-8008 Zürich (Switzerland)

Summary. Bitumen is commonly employed as a matrix for the long-term storage of low and intermediate level radioactive waste. As bitumen can be degraded by microbial activity, it is of great significance to determine the rates at which it may occur in nuclear waste repositories.
Experiments have been carried out under optimal culture conditions using bitumen with a highly increased surface area. The potential of different microbial consortia to degrade bitumen has been examined. The investigations showed clearly that bitumen-degrading organisms are ubiquitous. In general the organisms formed biofilms on the accessible substrate surface area. Under oxic culture conditions a bitumen degradation rate of 20–50 g bitumen $\cdot m^{-2} \cdot y^{-1}$ leading to a CO_2 liberation of 15–40 l was observed. Anoxic conditions yielded a 100 times smaller degradation rate of 0.2–0.6 g bitumen $\cdot m^{-2} \cdot y^{-1}$ and a CO_2 production of 0.15–0.45 l.
Based on linear extrapolation the experimentally determined degradation rates would lead to a 25–70% deterioration of the bitumen matrix under oxic and 0.3–0.8% under anoxic conditions within 1000 years.
Key words. Bitumen; microbial activity; biofilm; degradation; gas production.

Introduction

Bitumen has been used by man since early times and prehistorical findings document various applications such as glue or mastic as surface protection material or for tightening. The presence of asphalt mines has been documented for ancient middle eastern civilizations and the material has been used e.g. to isolate water pipes for the gardens in Ninive or for proof coating the ark of Noah. Historical details are given by Hellmuth[18] and Poll[20]. Todays' use of bitumen is mainly in the construction industry, for road pavements, for protection of roofs and pipes and in the automobile industry[13]. In most recent times bitumen is also used for the disposal of radioactive wastes, particularly for the solidification of low- and intermediate level radioactive wastes. This technology is documented in various publications[12,13,22].
Bitumen and bitumen-like substances e.g. tar, pitch or asphalt, may last for long time periods[18], as seen in remains in the caves of Lascaux in France (around 15 000 years)[12] or as components of crude oil (10^8 years)[26]. However, under certain conditions rapid degradation has also been observed. As early as 1935 microbial degradation of bitumen was described[20] and the phenomenon was investigated later in more detail[6,15,16,21,24,33,39]. To determine the long-term stability of the bitumen matrix used for the solidification of the waste radionuclides it is of great importance to quantify the microbial degradation and to investigate the environmental conditions which support microbial activity on bitumen as the substrate.
The mechanical, physical and chemical properties of the type of bitumen used in nuclear waste disposal, e.g. leaching, radiolysis and thermolysis, aging and change in consistency have been investigated in detail[4,10,13,17,19,22,23,27,31,32,34,38]. However, a possible influence of microorganisms on the stability of the bitumen matrix has been considered only in recent years although as described earlier microbial degradation of bitumen has been known for a long time[11,13,40]. No clear quantitative data were available which would allow an estimation of the long-term stability of bitumen at the sites of the waste disposal. In the present paper a quantification of the degradation rate of the water-insoluble and recalcitrant substrate bitumen is presented and growth on bitumen characterized. Since knowledge of the environmental conditions in a waste repository is minimal and since these conditions may change during the storage period, the experiments were carried out under optimal conditions. At the beginning, after closure of the repositories, the conditions will be oxic turning later to anoxic. Degradation studies therefore have been carried out for both conditions.

Materials and methods

Bitumen

Depending on the production procedure bitumen is of different composition and presents variable properties. The exact chemical composition of bitumen is unknown[25]. It usually contains various hydrocarbons, linear and branched alkanes, substituted aromatic compounds, etc. It also comprises sulfur compounds such as mercaptans, sulfides and thiophenes, oxygen containing substances such as phenols, naphtenacids and heterocyclic nitrogen compounds. The elemental composition can be given roughly as follows: carbon 80–88%, hydrogen 8–11%, oxygen 1–15%, sulfur 1–8%, nitrogen 1%[13,25,40]. Tables 1a and 1b show the composition of the bitumen (Ebano B15, Esso) used in this study. The properties of bitumen are determined more by its structure than by its chemical composition. In general bitumen are defined as colloidal systems containing basically asphaltenes and oil resins[25].

Bitumen suspension

Since the microbial degradation rate of water-insoluble substrates is dependent on the accessible surface, a large and defined surface area of the substrate material was achieved by freezing a weighed amount of bitumen in liquid nitrogen followed by grinding in a mortar and sieving out particles greater than 1 mm in diameter. Then 10% (w/w) of bentonite (Tixoton, Süd-Chemie AG) was added and the mixture suspended in the culture medium of the composition given in table 2.

Enrichment and isolation of bitumen degrading organisms

According to ZoBell and Molecke[40] only a few, but widely distributed species mainly present in soil are able to grow with bitumen as the carbon and energy source. Soil from a local garden as well as from the asphalt mines in Travers (NE, Switzerland) therefore were used as inocula. After gentle homogenization the soil material was suspended in culture medium, coarse particles removed by sedimentation and the slurry added to the culture system. Enrichment was achieved either in Erlenmeyer flasks (250 ml, shaking speed 160 rpm) or in percolation systems according to Audus[2] where culture medium was flowing through a mixture of powdered bitumen and soil (1:1). All culture systems were protected from light.

Table 1a. Elemental composition of the organic part of the bitumen used (Ebano B 15, Esso), data by Elektrowatt[12]. No values are given for oxygen.

Element	Amount %
C	85.2
H	10.2
S	2.8
N	1.8
P, metals, etc.	Residues

Table 1b. Elemental composition of the inorganic part of the bitumen used (Ebano B 15, Esso). Other elements were only present in traces.

Element	Amount (mg/g bitumen)
Al	0.27
Ca	0.93
Cu	0.05
Fe	0.9
Mg	0.14
Na	0.67
Ni	0.78
P	0.64
Pb	0.26
Zn	0.53

Table 2. Composition of the culture media for bitumen degrading microorganisms.

Macroelements	Aerobic (mM)	Anaerobic (mM)
$(NH_4)_2SO_4$	5	–
NH_4Cl	–	5
$MgSO_4 \cdot 7\ H_2O$	1	0.1
$MgCl_2$	–	0.5
$CaCl_2 \cdot 2\ H_2O$	0.5	1
NaCl	5	1
KCl	2.5	–
KNO_3	1	–
KH_2PO_4	10	30
$FeCl_2$	–	0.1
{FeS}	–	0.123 g/l
Trace elements	**Aerobic (μM)**	**Anaerobic (μM)**
$CuSO_4 \cdot 5\ H_2O$	0.03	–
$CuCl_2 \cdot 6\ H_2O$	–	0.25
$Fe_2(SO_4)_3 \cdot 5\ H_2O$	10	–
$Na_2MoO_4 \cdot 2\ H_2O$	0.2	0.5
$Co(NO_3)_2 \cdot 6\ H_2O$	0.8	–
$CoSO_4 \cdot 7\ H_2O$	–	0.25
$ZnSO_4 \cdot 7\ H_2O$	0.5	–
$ZnCl_2$	–	0.5
$MnSO_4 \cdot H_2O$	0.45	–
$MnCl_2 \cdot 4\ H_2O$	–	1
H_3BO_3	0.25	0.25
$NiCl_2 \cdot 6\ H_2O$	0.1	0.25
V_2O_5	0.01	–
Alternative electron acceptors if added		**Anaerobic (mM)**
$NaNO_3$		5
$FeCl_3$		5
Na_2SO_4		5
pH adjusted to	7.0	7.2

Aerobic cultivation system

The oxygen requirement for the complete oxidation of bitumen is high. To ensure sufficient oxygen supply to growing cultures, rate determinations were done in 2-l fermentors, holding 1.5 l culture medium, aerated with 0.5 l air/min and stirred at 500 rpm (temperature 30 °C). The microbial activity was determined by measuring the CO_2 concentration of the gas leaving the fermentor system by a Beckman IR analyzer. The CO_2 of the inflowing air was adsorbed in a column containing soda lime and the water vapor removed before IR analysis by a column filled with silica-gel. The gas flow rate was controlled by valves and a rotameter. Further controlled parameters in the fermentor cultures concerned temperature and pH. Since organic substrates may be carried into the cultivation system with the inoculation, CO_2 production in a

control fermentor lacking the substrate bitumen was run in parallel.

Anaerobic cultivation system

Anaerobic degradation is expected to be slow. It was measured in long-time experiments in 250-ml vessels sealed with butyl-rubber membranes holding a gas phase of helium. Degradation was tested in the absence and in the presence of the electron acceptors NO_3^-, SO_4^{-2} and Fe^{+3}. The microbial activity was determined by measuring the gases CO_2 and CH_4 (gas chromatography) and the dissolved organic carbon (IR-spectroscopy).

Analytical methods

1. Determination of particle size and particle surface: Volume and size distribution of the suspended particles were determined with a Malvern 2600 particle counter in the range of 11–1100 µm for the bitumen- and 2–190 µm for the bentonite-particles in 32 channels. The surface of the particles was calculated for each size class.
2. Multi-element analysis: Bitumen put into concentrated nitric acid, enclosed in a pressure bomb, was heated to 150 °C for 30 h. Remaining particles were removed from the sample by filtration (0.45 µm pore size). The filtrate was analyzed ICP-spectrometrically to determine the concentration of the elements.
3. Gaschromatographic determination of CO_2 and CH_4: Gas analysis was carried out with a Shimadzu R-1A gas chromatograph with both TCD (for CO_2) and an FID (for methane) detector. Methane was analyzed at 200 °C with nitrogen as carrier gas (50 ml/min) on a MS-5A 80/100 mesh column (Supelco), carbon dioxide with a temperature gradient program (70 °C 4 min, 70–200 °C 5 min, 200 °C 3 min) with helium as carrier gas (70 ml/min) on a Carbosieve-S 120/140 mesh column (Supelco).
4. Determination of dissolved organic carbon: Complete oxidation to CO_2 and H_2O was achieved at 95 °C in a DOC/TOC analyzer (Procon). Liberated CO_2 was detected by IR analysis.
5. CO_2 concentration in gas flux: The continuous production of CO_2 by a fermentor culture was monitored by an IR gas analyzer (Beckman 215A or 865).
6. Dosimetry: Quantification of the radiation dose was achieved by an alanine dosimeter enclosed in the fermentor. Measurements were done at the Paul Scherrer Institute in Würenlingen.
7. Epifluorescence microscopy: Active cells were stained by carboxy-fluorescein-diacetate (CFA, Molecular probes) as described by Brunner et al.[5].
8. Scanning electron microscopy: Samples were fixed in 4% glutaraldehyde, washed several times and freeze-dried, then covered with a gold-palladium alloy. Pictures were taken in a Cambridge S-4 SEM (20 kV) on Ilford Pan-F films.

Results and discussion

Properties of the bitumen suspension

The bitumen-bentonite suspension shows a particle size distribution with about 30% of the particles in the size range below 100 µm diameter, 55% below 200, and 90% below 400 µm. This size distribution was constant over a period of 18 months when the suspension was kept aerobically and unstirred in a flask. Also cultivation in a fermentor (500 rpm) for several weeks did not change the particle size distribution significantly. Furthermore, variation in pH (between 3 and 10.5) or temperature (between 5 and 35 °C) had no influence on the size distribution. In suspensions kept anaerobically the particle size distribution in most of the samples did not change during a time period of about 4 months. Then, a clear shift in particle size towards larger particles was observed, possibly due to the presence of surface active substances excreted by the organisms present. Surface active substances are known to be produced in the presence of hydrophobic substrates [7–9, 14]. The same effect could be observed after adding large amounts of organic substances together with the inoculum.

From the size distribution obtained, the surface of the suspended bitumen particles was calculated as the basis for activity determinations. With the method used 1 g of bitumen was suspended with a surface area of 350 cm^2 assuming that the particles are spheres and do not have an inner surface. However, SEM studies showed that this assumption does not hold and the real surface must exceed the calculated one. Not included in the calculations are also the much smaller bentonite particles (98% below 60 µm) which are probably coated with bitumen.

Interaction of the microorganisms with the substrate

Observation of bitumen particles after one week of cultivation in a fermentor gives clear evidence for microbial growth on the bitumen surface [5, 36]. As seen in figure 1, biofilms of bacilli type or filamentous organisms can be observed with SEM microscopy as well as in microscopical investigation after staining with carboxyfluorescein-diacetate [5].

CO_2 production as a measure of microbial activity

Quantification of the microbial activity of a consortium growing on an undefined substrate system as described is probably best obtained by measuring the rate of the carbon dioxide production. A time course (figs 2 and 3) shows in a first phase rapid growth. After a period of a few days, growth ceases and probably after build-up of the biofilm on the accessible surface area the CO_2 production rate remains approximately constant or declines slightly. The decline in rate immediately after the growth phase is due to the degradation of the better degradable substances in a first stage and the slower decomposition of the remaining solid material. A further slight decrease in microbial activity is caused by technical reasons since small amounts of bitumen material is lost at the liquid-

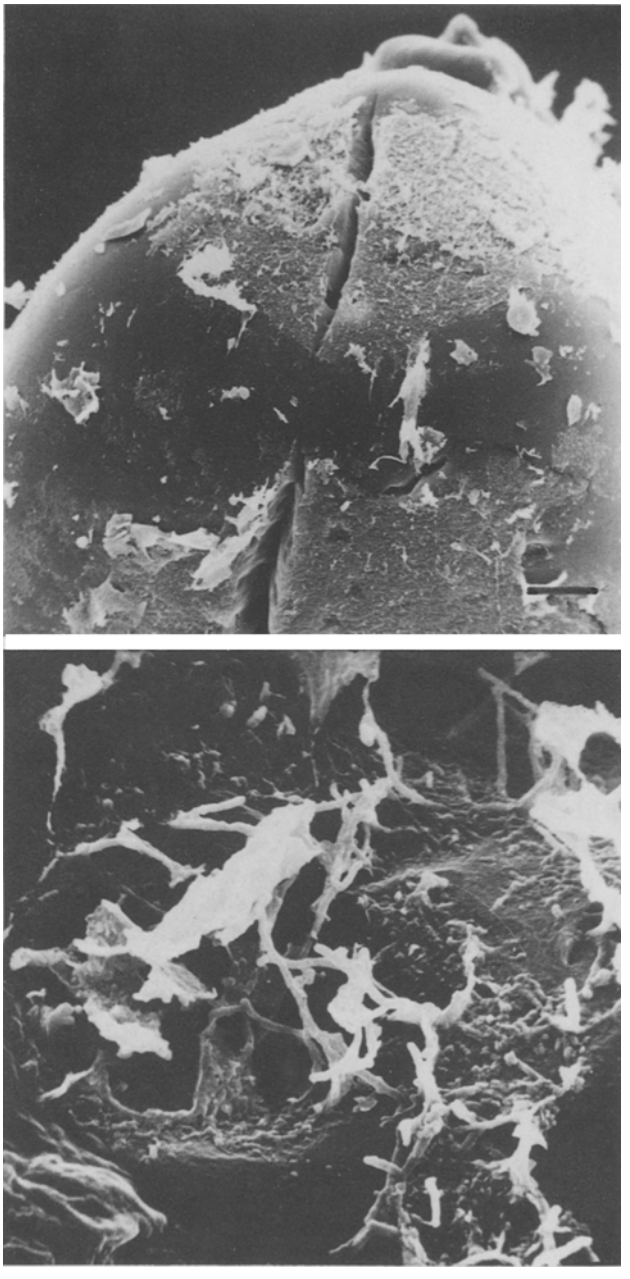

Figure 1. Bitumen particle coated with microorganisms after cultivation of 1 week in a bioreactor. a) bar = 20μm; b) bar = 2μm.

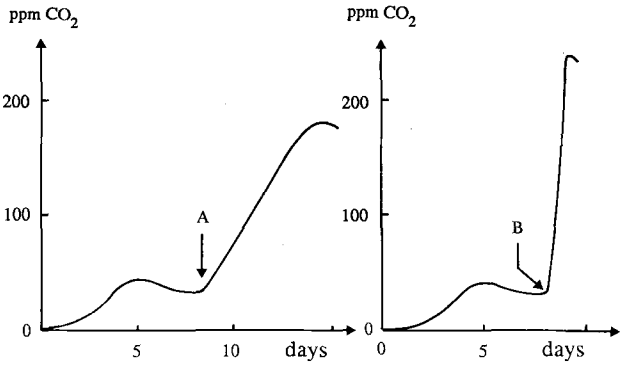

Figure 2. Microbial activity with bitumen and crude oil as the substrate (measured as concentration of released CO_2 in exit gas). (A) Addition of 25 g freshly prepared bitumen suspension. (B) Addition of 20 ml crude oil (Shell). Cultivation in 2-l bioreactor. Culture conditions: Temperature = 30 °C, pH = 7.0, stirring speed = 500 rpm, gas flow rate = 500 ml/min.

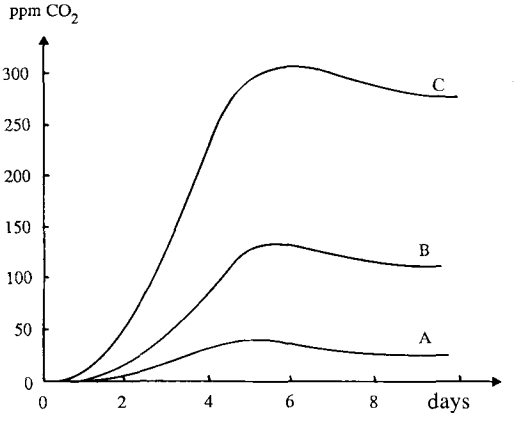

Figure 3. Microbial activity as a function of the suspended bitumen surface area (measured as concentration of released CO_2 in exit gas). Cultivation in 2-l bioreactor. Culture conditions: Temperature = 30 °C, pH = 7.0, stirring speed = 500 rpm, gas flow rate = 500 ml/min. Amount of bitumen added: (A) 5 g, = 0.175 m^2; (B) 25 g, = 0.875 m^2; (C) 50 g, = 1.75 m^2.

gas interphase of the glass vessel as well as precipitated at the mixing propeller. Since the medium contains all essential elements in excess, the growth-limiting factor is the carbon- and energy source bitumen. The fact that the biofilms formed on the surface of the bitumen particles have much higher metabolic capabilities is demonstrated when crude oil is added instead of new bitumen suspension to a bacterial culture in its equilibrium stage (fig. 2). The immediate response of the bitumen-adapted culture to the addition of oil points to the fact that the enzyme inventory necessary for oil degradation is present in the bitumen-adapted cells. As seen in fig. 3 the rate of CO_2 production is strongly proportional to the surface area of the suspended bitumen substrate. Experiments kept over periods of up to 4 months did not show a decline of the degradation rate suggesting that the substrate as well as the microbial population did not change during this period, that is, there is no obvious sequential degradation of the various bitumen components in the substrate offered during this time. Different inocula, e.g. garden soil, soil from an asphalt mine, or bitumen-adapted material from a percolation system, showed different adaptation times but the final activity reached a more or less similar value[36]. Furthermore, when a not-inoculated bitumen suspension was kept under non-sterile conditions a similar CO_2 liberation was observed demonstrating the development of a new bitumen-adapted microflora. Such inocula were later used for all experiments, since by this procedure no material not originally present in the bitumen-bentonite suspension was introduced into the system. A

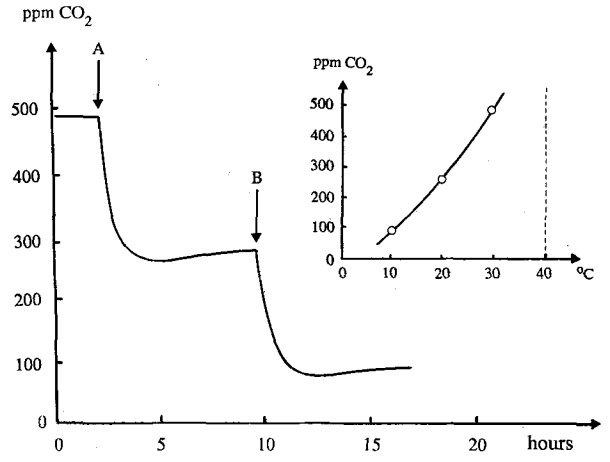

Figure 4. Influence of temperature on microbial activity with bitumen as the substrate. Cultivation in 2-l bioreactor. Culture conditions: Temperature = 30 °C, pH = 7.0, stirring speed = 500 rpm, gas flow rate = 500 ml/min. Time course of changes in temperature during the phase of constant microbial activity: (A) from 30° to 20 °C; (B) from 20° to 10 °C.
Insert: Activity (measured as concentration of CO_2 released in exit gas) as a function of temperature.

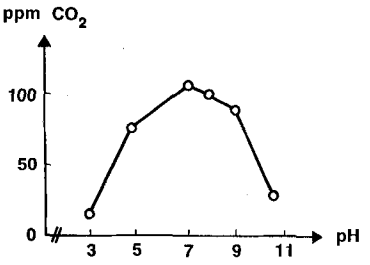

Figure 5. Influence of pH on microbial activity with bitumen as the substrate. Cultivation in 2-l bioreactor. Culture conditions: Temperature = 30 °C, pH = 7.0, stirring speed = 500 rpm, gas flow rate = 500 ml/min.

taxonomic analysis of the species present in this mixed culture showed a dominance of *Pseudomonas aeruginosa* together with not-identified species of the genus *Alkaligenes* and *Streptomyces*. *P. aeruginosa* had previously been found using bitumen as carbon source by Westsik et al.[35] and Barletta et al.[3].

Influence of temperature, pH and radiation on bitumen degradation

The goal of these experiments was to establish the influence of environmental conditions relevant in a waste repository on the activity of the microorganisms present. The results must be taken as example for the microbial population enriched under the conditions given; microorganisms adapted to other conditions, e.g. exposed to another stress factor might show a different behavior. Figure 4 shows the microbial activity measured at the temperatures 10, 20 and 30 °C. In general an increase of the temperature by 10 °C resulted in an increase of metabolic activity by a factor of two. Bitumen suspensions were only stable up to about 35–40 °C; at higher temperatures the suspended particles tend to conglomerate leading to a reduced surface area of the substrate and consequently to a smaller microbial activity. Figure 5 demonstrates the change in activity in the pH range from 3 to 10.5. The pH optimum is rather broad between 7 and 9 with a maximum at pH 7 as known for hydrocarbon degrading microorganisms [1, 39]. However, microbial degradation of hydrocarbons and bitumen was also found at a quite alkaline pH. Roffey et al.[30], for instance, demonstrated bitumen degradation at a pH above 11. Microorganisms in a waste depository are subjected to a constant radiation. In figure 6 an experiment is shown in which a culture is exposed to a dosis of 50 000 rad during 3 h. The initial radiation results in a drastic emission of CO_2. Afterwards the microbial activity decreases followed by a recovery within one week leading to the original metabolic activity. Since the radiation in a repository is much lower than the experimental radiation used[12], it is assumed that radiation in a repository will have no limiting effect on the microbes, but instead could lead to adaption of resistant cells. An increased rate of mutation might furthermore create new metabolic properties of the cells and probably faster adaptation to new environments.

Quantification of the aerobic degradation of bitumen

These calculations are based on the CO_2 production under optimal culture conditions for growth and metabolism of a mixed culture with bitumen as the only carbon source. Under the experimental conditions a maximal degradation rate of 20–50 g for bitumen/$m^2 \cdot y$ is observed resulting in 15–40 l of released CO_2. The reproducibility of the degradation rate is within ± 20%, probably due to the difficulty in making reproducible bitumen suspensions. It is still unknown whether bitumen is completely degraded to water and CO_2 or whether incomplete oxidized organic products are excreted into the medium or remain undegraded. Since the size distri-

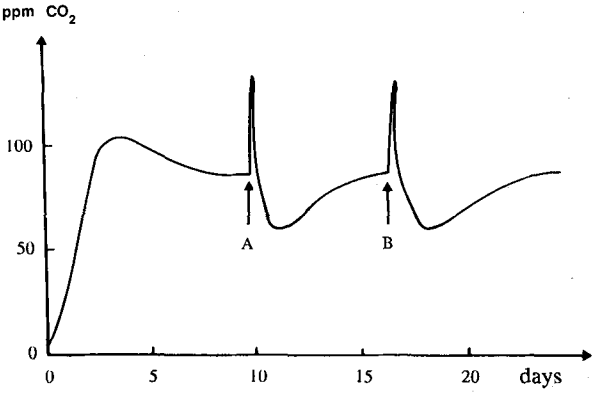

Figure 6. Effect of radiation on microbial activity with bitumen as the substrate. Cultivation in 2-l bioreactor. Culture conditions: Temperature = 30 °C, pH = 7.0, stirring speed = 500 rpm, gas flow rate = 500 ml/min. Radiation was achieved with an electron beam of 30 kV during 3 h yielding a radiation dose of 50 000 rad. Radiation at times indicated with an arrow.

Table 3. Anaerobic degradation of bitumen in the absence and presence of different electron acceptors. Cultivation in 250-ml flasks. Culture conditions: Temperature = 35 °C, pH = 7.2, shaking speed = 160 rpm, gas phase = helium. Each flask contained 24 g of bitumen, equal to 0.8 m^2 surface area. Cultivation time = 2 months. Parameters measured in the gas phase: (a) CO_2; (b) CH_4.
Symbols: + gas quantified; (+) traces of gas detected, not quantified; – gas not detected.

Inoculum	Electron acceptor —	NO_3^-	SO_4^{2-}	Fe^{3+}	
–	–	–	–	–	a)
	–	–	–	–	b)
Garden soil (I)	–	+	+	+	a)
	+	–	–	+	b)
Sewage sludge (II)	–	+	+	+	a)
	–	–	–	–	b)
Bitumen-adapted culture (III)	(+)	+	+	+	a)
	+	–	+	+	b)
Mixture of I + II + III	(+)	+	+	+	a)
	–	+	+	+	b)

bution of the particles remains constant under aerobic conditions at least no surface active substances seem to be excreted. The calculation is based on the assumption that under aerobic conditions 40–70% of the substrate-carbon is assimilated into biomass.

Microbial metabolism under anaerobic conditions

When molecular oxygen is absent organic compounds may be degraded by fermentation or by anaerobic respiration in the presence of electron acceptors such as nitrate, sulfate, certain metals such as iron or manganese, or carbon dioxide. We investigated anaerobic degradation in the presence of NO_3^-, SO_4^{-2} and Fe^{3+}. As inocula for the experiments, garden soil as a non-adapted inoculum, bacterial cultures adapted to carbohydrates and to bitumen, and a mixture of all three cultures were used. Microbial activity was determined by measuring the production of the two gases CO_2 and CH_4. As seen in table 3 all suspensions when inoculated yielded either one of the two gases or both. It is concluded that anaerobic bitumen degraders must also be omnipresent. In contrast to experiments under oxic conditions, non-inoculated cultures gave no gas production at all. Possibly the slow growth under these conditions prevented a detectable activity of the cells present. CO_2 was liberated with all the various electron acceptors tested, while methane was observed mainly in the absence of electron acceptors, or in their presence preferably with bitumen-adapted cells. Depending on the number of cells in the various inocula up to 50 ml of CO_2 were detected after 2 months with a suspended bitumen surface area of 0.8 m^2. Maximal amounts of methane found were in the order of 1 ml per m^2 after 2 months. Measurement of dissolved organic carbon showed variable amounts of soluble organic carbon compounds to be present, but usually diminishing during cultivation, caused by adsorption at the large surface area of the suspended particles. It was not possible to quantify excretion products by this method.

When the degradation rate for bitumen is calculated for anaerobic conditions based on a mean value of 0.25 l CO_2 production per year and m^2, a degradation of approximately 0.2–0.6 g of bitumen is obtained, a degradation rate about 100-fold smaller than under aerobic conditions.

Interpretation and extrapolation of the experimentally determined degradation rates

Various microbial activities must be expected in depositories either from autochthonic species already present in the rock or in the ground water, or from microorganisms brought into the caverns by man, by the waste or the backfill material. Since bitumen-degrading microorganisms seem to be ubiquitous, their presence in such depositories must be assumed. The environmental conditions in such places are not well known, but factors like temperature, water potential or radiation will hardly limit microbial activities. The most likely limiting factor may be a high pH in the presence of concrete or cement. However, Roffey et al.[30] reported degradation of bitumen up to a pH of 12. Oxygen will probably be present only during the first 5 years[37] if no further supply is assumed e.g. through ground water. As demonstrated by the experiments the microbial activity is proportional to the substrate surface area accessible. Increased surface area, e.g. by the formation of pores or cracks as the consequence of gas formation or swelling of waste material, would accelerate any microbial activity. Furthermore, excreted detergent-like products may also lead to enlarged surface areas. Originally a standard container filled with an average bitumen-waste mixture of 60:40 will have a bitumen surface of 1.4 m^2. Under aerobic conditions a decomposition of 30–70 g per container and year are expected, leading to 700–2000 m^3 of carbon dioxide per year for the whole repository, and 7–20 m^3 under anoxic conditions, respectively. At a pH of 10–13 most of the CO_2 would be absorbed leading to hydrogencarbonate in the water phase, a process decreasing the initially high pH to a more physiological range.

If it is assumed that the rate observed under laboratory conditions remains constant over the complete time the radionuclides should be stored safely, anaerobic degradation would use up 0.3–0.8% of the bitumen originally present within 1000 years, while under aerobic conditions 25–70% would be metabolized. It can be questioned whether it is possible to extrapolate the rate of bitumen degradation linearly over such a long period of time. While an increase in surface by pores and cracks and partial solubilization by detergents would augment the rate of metabolism of the bitumen, the increasing proportion of slowly degredable material in the bitumen would reduce the rate. Changes in external factors may also alter the degradation rate in an unpredictable manner. Other experts give figures of, e.g., 3.3% of bitumen degraded in 1000 years[3] for solid bitumen blocks in mineral medium or embedded in soil respectively and under oxic

conditions. While the data of Roffey et al.[30] for anaerobic conditions are similar to the ones presented here, the values obtained by Roffey et al. under aerobic conditions differ by a factor of 10 from the data presented. Since in the experiments of Roffey et al.[30] oxygen supply must have been limiting by using closed and unstirred vessels the lower values obtained probably represent semianaerobic conditions rather than aerobic ones.

Conclusion

The experiments described clearly demonstrate that bitumen is degraded under aerobic as well as under anaerobic conditions although with different rates. The absence of molecular oxygen is of great importance for the long-term stability of the bitumen matrix used. Continuous supply of oxygen to the repository could result in a rapid deterioration of the bitumen matrix and lead to the liberation of radionuclides.

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