Microbiology of formation waters from the deep repository of liquid radioactive wastes Severnyi

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Received 21 November 2002; received in revised form 28 October 2003; accepted 6 February 2004
First published online 17 April 2004

Abstract

The presence, diversity, and geochemical activity of microorganisms in the Severnyi repository of liquid radioactive wastes were studied. Cultivable anaerobic denitrifiers, fermenters, sulfate-reducers, and methanogens were found in water samples from a depth of 162–405 m below sea level. Subsurface microorganisms produced methane from [2-14C]acetate and [14C]CO2, formed hydrogen sulfide from Na235SO4, and reduced nitrate to dinitrogen in medium with acetate. The cell numbers of all studied groups of microorganisms and rates of anaerobic processes were higher in the zone of dispersion of radioactive wastes. Microbial communities present in the repository were able to utilise a wide range of organic and inorganic compounds and components of waste (acetate, nitrate, and sulfate) both aerobically and anaerobically. Bacterial production of gases may result in a local increase of the pressure in the repository and consequent discharge of wastes onto the surface. Microorganisms can indirectly decrease the mobility of radionuclides due to consumption of oxygen and production of sulfide, which favours deposition of metals. These results show the necessity of long-term microbiological and radiochemical monitoring of the repository.

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Keywords: Subsurface; Liquid radioactive waste repository; Microbial gas production; Sulfate reduction; Methanogenesis; Gas chromatography–mass spectrometry

1. Introduction

Disposal of radioactive wastes in deep geological formations requires knowledge about the host rock environment, including possible effect of microorganisms on the future of the repository. The role of microorganisms in radioactive waste disposal was extensively studied in the course of performance of national programs in Great Britain, Sweden, USA, and Canada [1–3]. In these countries a conception of multibarrier storage of nuclear wastes has been accepted. This conception involves disposal of nuclear waste in titanium or copper containers surrounded by compacted clay-based buffer and backfill materials, in a vault 500–1000 m deep in granitic rock. Investigations using cultivation and isolation techniques have established that subsurface microorganisms are physiologically and phylogenetically diverse [4–6]. Pure cultures of aerobic heterotrophs and anaerobic acetogenic, methanogenic, and sulfate-
iron-reducing bacteria were isolated from deep granitic aquifers and subsurface Cretaceous rock [7–11].

Of particular interest is microbial waste degradation in low-level radioactive waste disposal sites, since this waste can contain significant amounts of degradable organic materials [12,13]. The use of geological formations as the final destination for liquid radioactive waste (LRW) is based on the fact that they are located outside of the sphere of human activity and are not involved in rapid circulation of living material. One such site is the Severnyi disposal site at the Mining and Chemical Complex (MCC) in Eastern Siberia (Zheleznogorsk, Russia). The deep Severnyi repository has been receiving waste since 1967, with approximately 2.8×10^6 m^3 of waste with a total activity of about 100 millions Ci disposed of to date [14]. Liquid radioactive wastes injected into subsurface horizons of the repository contain fission products of uranium, sodium salts (nitrate, acetate, carbonate, and sulfate), and ions of several metals. The toxicity of the LRW is due to both transplutonium elements and non-radioactive components of wastes (nitrate and acetate).

The disposal of wastes into deep horizons is accompanied by physicochemical and radiochemical processes and changes in pH and Eh, especially in the zone of dispersion (mixing) of wastes with subsurface waters. The above-mentioned processes can influence radionuclides and other components of wastes. A range of geophysical, geochemical, and radiochemical methods for the control of underground waters from injection and observation wells have been applied, but microbiological investigations of the repository were started only in 1998 [15,16].

The aquifer complexes of the upthrown block of the repository contact the Yenisei River Valley to the west. Thus, rigorous monitoring is necessary in this area to ensure the safety of the disposal operation and prevent environmental pollution.

The aim of the present work was to study the physicochemical characteristics of the fluids and the abundance, biodiversity, and geochemical activity of microorganisms of the Severnyi repository intended for storage of liquid radioactive wastes. The capacity of microorganisms inhabiting the repository to produce gases from the components of wastes (acetate, nitrate, and sulfate) was also studied.

2. Materials and methods

2.1. Site characteristics and sampling method

The object of investigation was groundwater of the Severnyi repository of liquid radioactive wastes at the Mining and Chemical Complex (Zheleznogorsk, Russia). The complete characteristic of the repository was given earlier [14]. Water samples were studied in the summer periods of 1998–2001 at increasing number of wells.

The reservoir horizons are composed of weakly cemented sand–clay rocks and are separated by relatively impermeable clay strata. Horizons I and II are located at depths from 370 to 465 and 180 to 280 m below sea level, respectively. Hydrologically, the region of the Severnyi disposal site can be regarded as a small artesian basin located in the downthrust block and open to the Chulym artesian basin on the north. The groundwater typically moves from south to north at velocities of 5–6 m year⁻¹. The reservoir formations contain water with a low salt content (0.3–0.6 g l⁻¹) and pH in a range of 7.5–9.3 (horizon I) and 7.0–8.4 (horizon II).

The injection of intermediate-level wastes into horizon I of the Severnyi site was started in 1967. The wastes contain sodium salts (nitrates, acetates, carbonates, and sulfates), silica gel, and ions of several metals. The total salt content is up to 240 g l⁻¹, with a specific activity not exceeding 10⁻² Ci l⁻¹. The injection of low-level wastes into horizon II was started in 1968. These wastes contain salts (up to 20 g l⁻¹) and detergents. The radionuclide composition is generally similar to that of intermediate-level wastes (10⁻⁸–10⁻⁶ Ci l⁻¹). Wastes are injected into subsurface horizons through injection wells, which are arranged in linear fashion (H-1–H-11, AH-16, and AH-18); there are also observation-injection wells, observation wells, and relief wells (Fig. 1). The relief wells were drilled at the site to relieve the reservoir pressure and have been used to pump out subsurface waters simultaneously with waste injection. It should be noted that a part of subsurface waters from the relief wells is used as a source of industrial water.

Samples of groundwater were collected directly from wellhead of relief wells and observation wells located on the periphery or in the zone of penetration of wastes. Samples were collected into sterile 5-l bottles and analysed in the laboratory within 4–6 h for bacterial numbers, biodiversity, and rates of microbial processes. The pH, temperature, and sulfide concentration of formation waters were measured at the time of sampling. Samples were stored at 6 °C until all chemical analyses were performed.

2.2. Enumeration of bacteria

A technique for cultivation of strictly anaerobic microorganisms [17] was applied for the cultivation and enumeration of microorganisms. Anaerobic bacteria were cultivated in tubes with pure argon in the gas phase; an exception was the medium with H₂/CO₂ for methanogens. The numbers of cultivable anaerobic bacteria were estimated by serial decimal dilutions of water samples in tubes with various enrichment media designed to promote growth of specific functional groups. The highest dilution in which growth occurred was assumed to approximate the inverse log of the population size, as
determined by the most probable number (MPN) procedure [18]. Enrichment media were degassed by boiling under a steam of O₂-free Ar (for denitrifiers and fermentative bacteria) or prereduced (for sulfate-reducing bacteria and methanogens) and were adjusted to pH 7.0–7.2. Anaerobic organotrophic bacteria with a fermentative type of metabolism were determined by measuring the increase in H₂ in medium with peptone (4 g l⁻¹) and glucose (10 g l⁻¹) [19]. Sulfate-reducing bacteria were analysed by measuring the increase in H₂S in medium B containing sodium lactate (4 g l⁻¹) and reduced with Na₂S·9H₂O (0.2 g l⁻¹) [19]. Denitrifying bacteria were determined by measuring the increase in N₂ in media with sodium acetate (2 g l⁻¹) or sucrose (5 g l⁻¹) as a source of carbon and energy and sodium nitrate (0.85 g l⁻¹) as an acceptor of electrons [20]. Methanogens were determined by measuring the increase in CH₄ in media [21] with acetate (2.2 g l⁻¹) or H₂/CO₂ (80:20% v/v), supplemented with microelements and yeast extract (1 g l⁻¹) [22,23].

Aerobic bacteria were cultivated in Hungate tubes with air as the gas phase. Viable aerobic heterotrophs were also enumerated by serial decimal dilutions of water samples in medium composed of bacto-tryptone (5.0 g l⁻¹), yeast extract (2.5 g l⁻¹), and distilled water (1 l, pH 7.0). All media were inoculated with samples of formation water using syringes and were incubated at room temperature (i.e., 24°C) for 30 days. Samples from all tubes were examined by phase-contrast microscopy.

2.3. Analytical methods

Molecular hydrogen and nitrogen were analysed with a model 3700 gas chromatograph ("Chromtograph", Fig. 1. Location of wells at the Severnyi disposal site. Injection (H-1–H-10), relief (horizon I: P-1–P-6, P-11, and P-12; horizon II: P-7–P-10), observation-injection (horizon I: A/H 10, A/H 12, A/H 14, and A/H 18) and observation wells (horizon I: A-5, A-19, A-22–A-27, A-32, A-37, A-45, P-1, P-3, P-11, P-12, P-19, C-26, C-35, C-36, and C-38; horizon II: A-11, A-18, A-36, A-42, A-44, A-46, A-47, A-50, A-52, A-56–A-63, A-65–A-67, C-15: C-20, C-45, P-2, P-5–P-7, P-13–P-15, and P-20).
Moscow, Russia). A column (2 m × 2 mm i.d.) packed with the 5A molecular sieve, mesh 60/80, was used for analyses. The carrier gas was argon. The thermal conductivity detector and injector were kept at 70 and 40 °C, respectively. Methane was determined with the same chromatograph equipped with a flame ionisation detector and a SOVKOL prepacked column (1 m × 3 mm i.d.). The carrier gas was argon. The detector and injector were kept at 150 and 140 °C, respectively. The standard gas mixtures of H₂, N₂, and CH₄ produced by Balashikha Oxygen Plant (Balashikha, Russia) were used for calibration of gas chromatograph.

Hydrogen sulfide was determined with dimethyl-p-phenylenediamine by a modified colorimetric method of Pachmair [24]. pH values of subsurface waters were measured using an OP-212/1 digital laboratory pH meter (Radelkis, Hungary). Iron, magnesium, calcium, and sodium in groundwater were analysed on AASIN atomic absorption spectrophotometer (Carl Zeiss, Jena, Germany). Nitrites were analysed on an Ecotest-01 device (‘‘NPP Ekoniks’’, Moscow, Russia) fitted with an ELIT-21 (NPP ‘‘Ekoniks’’, Moscow, Russia) ion-selective electrode. Nitrites were determined colorimetrically with sulfanilic acid and α-naphthylamine [25]. Other components of groundwater were analysed by routine methods [26].

Concentration of lower fatty acids was determined in samples fixed with saturated KOH (2 ml/50 ml of sample). In the laboratory, 0.98 ml of each sample was acidified with 0.02 ml of 25% HCl and analysed on a model 3700 chromatograph (“Chromtograph”, Moscow, Russia) equipped with a flame ionisation detector. The column (2 m × 3 mm i.d.) was packed with Porapak Q (100/120 mesh). The temperature of injector was 180 °C. A mixture of N₂ (95%) and CO₂ (5%) was used as the carrier gas. The standard solutions of lower fatty acids produced by Chemical department of the Lomonosov Moscow State University (Moscow, Russia) were used for calibration of the chromatograph.

2.4. Radioisotope methods

The rates of sulfate reduction and methanogenesis were determined in formation waters by radioisotope methods using labelled Na₂³⁵SO₄, ¹⁴CH₃-COONa, and NaH¹⁴CO₃ [27–29]. About 50 ml sterile serum bottles were completely filled with samples of water without any gas phase and closed with butyl rubber stoppers and aluminium caps. Four replicate bottles were used for measuring each process in each sample. One of the bottles was supplemented with 1 ml of 40% NaOH and served as control. About 200 μl of a radioisotope solution was added to each bottle with a syringe; the surplus of the solution from the bottles was leaked through the second needle inserted into the flask. The incubation proceeded for 24 h at 24 °C. In the case of methano-

2.5. Gas chromatography–mass spectrometry analysis of microbial community composition in the groundwater

Water sample (1 l) from the observation well D-1 of horizon II was passed through a 0.22-µm membrane filter. To a half of the filter with biomass, 200 μl of a 5.4 N solution of anhydrous HCl in methanol was added and the mixture was heated at 70 °C for 2 h. The methyl esters of fatty acids and aldehyde derivatives obtained were extracted twice with 100 μl of hexane. The extract was dried and silylated in 20 μl of N,O-bis(trimethylsilyl)trifluoroacetamide for 15 min at 65 °C. About 1 μl portions of the reaction mixture were analysed with a model HP-5985B gas chromatography-mass spectrometry (GC–MS) system (Hewlett–Packard, Palo Alto, USA) equipped with a fused-quartz capillary column (25 m by 0.25 mm) containing an Ultra-1 non-polar methylsilicone phase. The temperature program was run from 150 (2-min isotherm) to 250 °C at 5 °C min⁻¹ and then from 250 to 300 °C at 10 °C min⁻¹. Data processing was carried out with an HP-1000 computer by using the standard programs of the GC–MS system (Hewlett–Packard, Palo Alto, USA).
Concentrations of fatty acids, hydroxy fatty acids, fatty aldehydes, sterols, and methanolysate of the biomass lipid fraction were used to determine the population size of the individual community members. The calculation was based on the information, stored in a data bank, about the chemical composition of the probable members of the community. An algorithm for rapid assessment of the genus or species composition from the GC–MS data for total biomass has been developed for quantitative analysis of the community under study [34–36].

3. Results

3.1. Physicochemical and radiochemical characteristics of groundwater

In the period of 1998–2001, about 50 samples of subsurface waters of the Severnyi disposal site from horizons I and II were studied. Investigation of the chemical composition of groundwater revealed that the content of main compounds slightly varied in both horizons (Table 1). Typical waters of both horizons had pH in a range of 7.5–8.5. Eh values were in a range from 210 to 310 mV (data not presented). The content of calcium varied from 0.8 to 64.0 mg l\(^{-1}\), the content of carbonate-ion was from 128.1 to 347.7 mg l\(^{-1}\), nitrate did not exceed 4.09 mg l\(^{-1}\), sulfate did not exceed 6.9 mg l\(^{-1}\); total salinity was 0.23–0.49 g l\(^{-1}\) (wells: C-35, A-38, A-39, D-1, and D-2). In the waters, phosphate-ion and hydrogen sulfide were absent; the content of \(\text{NH}_4^+\) was less than 2.7 mg l\(^{-1}\) (data not presented). Iron was present in several samples in quantities from 6.5 to 29.5 mg l\(^{-1}\); in waters from well D-4, its quantity reached 237.5 mg l\(^{-1}\). The temperature of the studied groundwater samples was 12–14 °C, but in the near-bottom zone of injection wells it was higher. We did not study highly radioactive underground waters.

The groundwater from observation wells closely related to the injection wells had another chemical composition due to its mixing with wastes. The appearance of waste in the area of the observation and relief wells was detected by a rise in the total salt level and an increase in the content of nitrate and sulfate ions. In horizon I, these changes could be seen in waters from wells A-22, A-25, A-26, and A-32. In these samples, the concentration of sulfate varied from 78.6 to 133.3 mg l\(^{-1}\), and nitrate reached 102.9 mg l\(^{-1}\). In horizon II, wastes were dispersing preferentially in the direction of wells A-56, A-57, A-39, and P-10. For example, in waters of well A-57, the content of nitrate reached 347.7 mg l\(^{-1}\), that of sulfate, 31.4 mg l\(^{-1}\), and total salinity, 0.76 g l\(^{-1}\). Lower fatty acids (acetate, propionate, and butyrate) were not found in the studied water samples. Despite the significant changes in the chemical composition of the waters from the above-mentioned wells, we did not register in these samples tritium, \(^{14}\)C, \(^{90}\)Sr, and \(\alpha\) radiation or \(\gamma\) activity during the period of investigation (since 1998 till June 2002). Thus, the groundwater studied varied in its composition depending on the hydrodynamic connection with injection wells, was generally neutral or slightly alkaline, fresh, and oligotrophic.

Table 1

| Well number | Year of analysis | Depth of sampling, m | Ph | \(\text{SO}_4^{2-}\), mg l\(^{-1}\) | \(\text{Fe}_{\text{total}}\), mg l\(^{-1}\) | \(\text{Mg}^{2+}\), mg l\(^{-1}\) | \(\text{Ca}^{2+}\), mg l\(^{-1}\) | \(\text{NO}_3^-\), mg l\(^{-1}\) | HCO\(_3^-\) | \(\sum(\text{Na}^+ + \text{K}^+)\), mg l\(^{-1}\) | Total dissolved solids, g l\(^{-1}\) |
|-------------|-----------------|---------------------|----|-----------------|-----------------|-----------------|-----------------|-----------------|-------------|-----------------|-----------------|-----------------|
| Horizon I   |                 |                     |    |                 |                 |                 |                 |                 |              |                 |                 |                 |
| C-35        | 2000            | 335                 | 9.25 | 5.4              | Nd              | 0.3             | 0.8             | 1.2             | 270.8        | 80.0           | 0.37            |                     |
| A-19        | 2000            | 405                 | 8.65 | 56.8            | Nd              | 8.3             | 17.0            | 0               | 201.3        | 86.6           | 0.39            |                     |
| A-22        | 2000            | 400                 | 8.25 | 125.4           | Nd              | 7.6             | 38.0            | 0               | 191.5        | 76.9           | 0.47            |                     |
| A-25        | 1999            | 405                 | 8.13 | 78.6            | 0               | 7.0             | 25.0            | 1.44            | 276.0        | 78.8           | 0.46            |                     |
| A-26        | 2000            | 403                 | 8.09 | 133.3           | Nd              | 10.9            | 49.2            | 0               | 228.1        | 56.9           | 0.52            |                     |
| A-32        | 2000            | 337                 | 8.55 | 80.4            | Nd              | 7.6             | 17.0            | 102.9           | 268.4        | 67.6           | 0.57            |                     |

| Horizon II  |                 |                     |    |                 |                 |                 |                 |                 |              |                 |                 |                 |
| A-38        | 1998            | 180                 | 7.9 | <4.0            | 6.50           | 13.10           | 23.0            | 4.09            | 158.6        | 25.70          | 0.23            |                     |
| A-39        | 1998            | 168                 | 7.8 | <4.0            | 6.65           | 14.25           | 31.0            | 1.87            | 335.5        | 64.20          | 0.45            |                     |
| D-1         | 1998            | 189                 | 9.1 | <4.0            | 16.30          | 9.50            | 17.5            | 1.25            | 305.0        | 71.30          | 0.42            |                     |
| D-2         | 1998            | 198                 | 10.05 | 6.9             | Nd             | 7.3             | 16.0            | 1.9             | 242.2        | 86.1           | 0.37            |                     |
| D-4         | 1998            | 162                 | 7.9 | <4.0            | 29.50          | 23.00           | 45.3            | 2.0             | 347.7        | 18.70          | 0.47            |                     |
| P-15        | 1998            | 163                 | 7.5 | <4.0            | 23.00          | 15.30           | 64.0            | 0.35            | 311.1        | 19.00          | 0.43            |                     |
| A-56        | 2000            | 190                 | 8.2 | 31.4            | Nd             | 26.4            | 52.4            | 337.9           | 128.1        | 160.0          | 0.76            |                     |
| P-10        | 2000            | 230                 | 8.13 | 2.5             | Nd             | 18.6            | 9.4             | 35.3            | 332.5        | 62.7           | 0.47            |                     |

*No data.
3.2. Distribution of microorganisms and rates of anaerobic microbial processes in the groundwater

Formation fluids of both horizons of the Severnyi repository were used for the enumeration of microorganisms of some physiological groups that could, theoretically, reside in this habitat. Aerobic heterotrophic bacteria and anaerobic microorganisms of various physiological groups were observed in the groundwater. The lowest number of microorganisms was observed in wells located beyond the zone of waste penetration (horizon I, wells A-5, II-19; horizon II, well D-1, 1999) (Table 2). In this zone, the number of cultivable aerobic heterotrophs, anaerobic denitrifiers, fermenters, and sulfate-reducers did not exceed hundreds of cells ml\(^{-1}\). The number of lithotrophic and aceticlastic methanogens was very low (several cells ml\(^{-1}\)).

In wells located in the zone of waste dispersion (as established by the presence of nitrate and sulfate in the waters), the numbers of cultivable microorganisms were highly variable. The number of aerobic heterotrophs reached 10\(^6\) cells ml\(^{-1}\). Denitrifying bacteria able to grow on acetate and nitrate were present practically in all samples (10\(^2\)–10\(^4\) cells ml\(^{-1}\)). Sulfate-reducing bacteria and methanogens, usually involved in the last stage of organic matter degradation in various ecosystems, were observed in subsurface horizons; however, they were not numerous, which could testify to the low rate of modern microbial processes in this ecosystem. The number of sulfate-reducing bacteria growing on lactate was mainly in the range from 10 to 10\(^3\) cells ml\(^{-1}\); the highest value (10\(^4\) cells ml\(^{-1}\)) was observed in groundwater of well D-4 in year 2001. The number of cultivable fermentative bacteria did not exceed 10\(^3\) cells ml\(^{-1}\). In most water samples, autotrophic methanogens (0–10\(^2\) cells ml\(^{-1}\)) predominated over aceticlastic methanogens (0–several cells ml\(^{-1}\)). Thus, the microflora of the groundwater had a high catabolic potential and was able to grow autotrophically or heterotrophically, mineralising efficiently various organic substrates in the processes of aerobic oxidation and anaerobic fermentation, denitrification, sulfate reduction, and methanogenesis.

In 2001, for estimation of geochemical activity of microorganisms in subsurface horizons, we measured the rates of sulfate reduction and methanogenesis. Radioisotope investigations revealed that sulfate reduction was the major anaerobic process, having a rate in a range from 0.014 to 12.19 l S\(^2\) g\(^{-1}\) day\(^{-1}\). Autotrophic and acetotrophic methane formation was registered in two wells (A-26 and D-4) of 10 studied; the total rate of methanogenesis did not exceed 0.1093 l CH\(_4\) g\(^{-1}\) day\(^{-1}\) (Table 3). The highest rates of methanogenesis and sulfate reduction were observed in waters of wells A-26 and D-4.

### Table 2

| Well number | Year of analysis | Depth of sampling, m | Aerobic heterotrophs | Denitrifiers | Fermenters | Sulfate-reducers | Methanogens |
|-------------|-----------------|----------------------|----------------------|-------------|------------|----------------|-------------|
|             | Horizon I       |                      |                      | Tryptone + glucose + yeast extract | Acetate + NO\(_3\) | Sucrose | Lactate + SO\(_4\) | H\(_2\) + CO\(_2\) | Acetate |
| A-5         | 2001            | 307                  | 10\(^2\)             | Single cells | 10         | 0              | 0           |
| A-15        | 2000            | 360                  | 10\(^4\)             | 10          | 10         | 0              | 0           |
| A-19        | 2000            | 405                  | 10\(^3\)             | 10          | 10         | 0              | 0           |
| A-22        | 2001            | 400                  | 10\(^5\)             | 10          | 10         | 0              | 0           |
| A-26        | 1999            | 380                  | 10\(^3\)             | 10          | 10         | 0              | 0           |
|             |                  |                      | 10\(^3\)             | 10          | 10         | 0              | 0           |
| A-32        | 2000            | 337                  | 10\(^3\)             | 10          | 10         | 0              | 0           |
| II-19       | 2000            | 423                  | 10\(^3\)             | 10          | 10         | 0              | 0           |
|             | 2001            | 423                  | 10\(^3\)             | 10          | 10         | 0              | 0           |
| C-35        | 2001            | 355                  | 10\(^3\)             | 10          | 10         | 0              | 0           |
|             | Horizon II      |                      |                      | Tryptone + glucose + yeast extract | Acetate + NO\(_3\) | Sucrose | Lactate + SO\(_4\) | H\(_2\) + CO\(_2\) | Acetate |
| D-1         | 1999            | 162                  | 10\(^3\)             | Single cells | 0          | 0              | 0           |
|             | 2000            | 162                  | 10\(^3\)             | 10          | 10         | 0              | 0           |
| D-4         | 1998            | 171                  | 10\(^3\)             | Single cells | 10         | 0              | 0           |
|             | 2000            | 170                  | 10\(^3\)             | 10          | 10         | 0              | 0           |
| A-39        | 2000            | 182                  | 10\(^3\)             | 10          | 10         | 0              | 0           |
| P-10        | 2000            | 230                  | 10\(^3\)             | 10          | 10         | 0              | 0           |
|             | 2001            | 230                  | 10\(^3\)             | 10          | 10         | 0              | 0           |

Nd, no data.

*Absence of the growth in a liquid medium inoculated with 1 ml of the formation fluid.

*Presence of bacteria.
(Horizon I) and D-4 (Horizon II), located in the zone of the radioactive waste influence.

3.3. Production of gases by microorganisms of the Severnyi repository

Active enrichment cultures of fermentative, sulfate-reducing, denitrifying, and methanogenic bacteria were isolated from both horizons studied. Growth of nitrate-reducing enrichment cultures in medium with acetate and nitrate was accompanied by a considerable decrease in the concentration of nitrate, from 620 to 15–98 mg l\(^{-1}\) (Fig. 2). Among the products of nitrate reduction, we detected nitrite and molecular nitrogen. Microorganisms able to produce other gases (methane, carbon dioxide, and hydrogen sulfide) from the components of waste were also isolated from subsurface horizons. Sulfate-reducing bacteria from wells P-10, C-35, D-4, and A-26 produced about 400 mg hydrogen sulfide per l l on medium with lactate + sulfate and about 200 mg l\(^{-1}\) on medium with acetate + sulfate. The most active enrichment cultures of aceticlastic methanogens from wells A-5, A-26, and A-32 produced methane and CO\(_2\) from acetate, increasing the pressure in vessels to 3–4 bars. Carbon dioxide was produced in all aforementioned processes, but usually it was in the form of bicarbonate. The data obtained show that due to the activity of subsurface microorganisms the quantity of acetate, nitrate, and sulfate in wastes may decrease. On the other hand, biogenic production of gases may result in a local increase of the pressure in the repository and discharge of wastes onto the surface.

3.4. Microbial community composition of the groundwater of horizon II as determined by GC–MS analysis of the total biomass lipid components

Data on the species composition of the microbial community of the formation water taken from observation well D-1 are summarised in Table 4. GC–MS analysis on an HP-5985B gas chromatograph–mass spectrometer performed after methanalysis of the lipid fraction of the total biomass showed the presence of 57 compounds belonging to fatty acids, hydroxy acids, and fatty aldehydes. Using these data on the chemical composition of total biomass and relying on biomarkers and analysis of obtained profiles, we unravelled generic or even species composition of the community.

The composition of microorganisms of the water sample included eucaryotic and procaryotic microorganisms. Proaroytic microorganisms were represented mainly by Gram-positive actinobacteria of the genera Rhodococcus, Corynebacterium, and Pseudonocardia. Some actinobacteria could not be classified into any of the known genera. Spore-forming microorganisms belonged to the genera Bacillus and Clostridium. The majority of the Gram-negative bacteria were representatives of the genus Sphingomonas often observed in subsurface horizons [37].

![Fig. 2. Utilisation of nitrate by enrichment cultures of denitrifying bacteria grown on acetate over 30 days.](https://academic.oup.com/femsec/article-abstract/49/1/97/529314)
formation waters studied; however, we did not find ac-
croorganisms. Nitrate and sulfate were found in the
and ions of several metals, which can be used by mi-
(such as acetate) and electron acceptors: nitrate, sulfate,
injected into these horizons contain organic compounds
nant anion was bicarbonate. Liquid radioactive wastes
substances, nitrogen and phosphorous. The predomi-
native ecosystems limited by the content of organic
zons of the Severnyi repository (located beyond the zone
characteristics shows that the original subsurface hori-
sory
for storage of liquid radioactive waste has been only
scantily investigated. We performed microbiological and
physicochemical studies of the Severnyi repository used
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and Chemical Complex as a preliminary step to eluci-
date the potential microbial influence on the compo-
nents of wastes.

4. Discussion

The microbiology of deep aquifers has received much
attention [38,39]. Compared with surface waters, how-
ever, ground waters have been studied to a lesser extent,
probably due to the great difficulty and expensiveness of
obtaining uncontaminated, representative samples. In
particular, the microbiology of deep aquifers used for
storage of liquid radioactive waste has been only
scantily investigated. We performed microbiological and
physicochemical studies of the Severnyi repository used
for storage of liquid radioactive wastes at the Mining
and Chemical Complex as a preliminary step to eluci-
date the potential microbial influence on the compo-
nents of wastes.

4.1. The groundwater environment at the Severnyi repos-
itory

Our monitoring of component levels and geophysical
characteristics shows that the original subsurface hori-
zons of the Severnyi repository (located beyond the zone
of waste dispersion) are oligotrophic slightly alkaline
freshwater ecosystems limited by the content of organic
substances, nitrogen and phosphorous. The predomi-
nant anion was bicarbonate. Liquid radioactive wastes
injected into these horizons contain organic compounds
(such as acetate) and electron acceptors: nitrate, sulfate,
and ions of several metals, which can be used by mi-
croorganisms. Nitrate and sulfate were found in the
formation waters studied; however, we did not find ac-
etate or other lower fatty acids. We did not reveal tri-
utium, $^{14}C$, $^{90}Sr$, $\alpha$ or $\gamma$ radiation. Upon the injection of
wastes to subsurface horizons, a rise in the temperature
was observed, which is due to energy release during
radioactive decay [14,40]. Thus, the physicochemical
conditions (carbon and energy sources, pH, and tem-
perature) of the subsurface horizons containing low-
and intermediate-level wastes were favourable for the
growth of microorganisms.

4.2. The numbers of cultivable microbial communities and their
activity at the repository

This study revealed the existence of active microbial
communities at all depth investigated, from 162 to 405
m below sea level. Subsurface waters contained meso-
philic microorganisms of various physiological groups.
In wells located beyond the zone of radioactive waste
penetration, the number of cultivable aerobic hetero-
traphs, anaerobic denitrifiers, fermenters, and sulfate-
reducers did not exceed hundreds of cells ml$^{-1}$. The
number of lithotrophic and acetotrophic methanogens
was still lower (several cells ml$^{-1}$). In the zone of waste
dispersion (as was established by the presence of nitrate
and sulfate in the waters), the number of cultivable
microorganisms, especially aerobic heterotrophs ($10^6$
cells ml$^{-1}$), anaerobic denitrifiers ($10^4$ cells ml$^{-1}$),
and sulfate-reducers bacteria ($10^4$ cells ml$^{-1}$) increased.

In our experiments, one physical parameter was not
similar to that of subsurface: temperature. The tempera-
ture at both of the studied horizons was 12–14 ºC,
despite their different depth. Injection of intermediate-
level and low-level wastes in horizons I and II, respec-
tively, lead to uncontrolled change of the temperature,
especially in the zone of waste dispersion [40]. We
incubated subsurface microorganisms at room tempera-
ture, assuming that an increase in the temperature by
8–10 ºC is unlikely to result in any significant underes-
timation of the bacterial numbers and activity. For ex-
ample, a pure culture of the sulfate-reducing bacterium
Desulfovibrio aespoeensis, isolated from deep ground
water from the Aspö hard rock, grows in the wide range
of temperature from 4 to 35 ºC and have an optimum of
growth at 25–30 ºC, despite the fact that the tempera-
ture of their habitat is 9–20 ºC [8]. Methanogens and
homoacetogens isolated from this environment from
waters with a temperature 9 ºC are also able to grow at
20 ºC and higher temperatures [7,9].

Sulfate reduction was the predominant microbial
process (from 0.014 to 0.93 µg S$^{2-}-1^{-1}$ day$^{-1}$) in the
Severnyi repository; methanogenesis was not registered in
eight of the 10 water samples studied. The injection of
liquid radioactive waste into subsurface horizons led to
activation of microbial processes, especially in waters
from wells A-26 (Horizon I) and D-4 (Horizon II),
which are characterised by higher rates of sulfate re-

Table 4
Genus and species composition of microbial community of water from
dev VA as measured by GC–MS whole cell lipid components analysis

| No. | Microorganism                  | Number of cells, 10$^3$ ml$^{-1}$ | Content, % |
|-----|-------------------------------|-----------------------------------|------------|
| 1.  | Rhodococcus sp.               | 2.2                               | 0.55       |
| 2.  | Cellulomonas sp.              | 15.5                              | 3.78       |
| 3.  | Clostridium sp.               | 5.5                               | 1.33       |
| 4.  | Nocardia sp.                  | 4.8                               | 1.17       |
| 5.  | Unknown actinobacteria        | 68.9                              | 16.77      |
| 6.  | Clostridium propionicum       | 6.2                               | 1.52       |
| 7.  | Pseudonocardia sp.            | 48.4                              | 11.80      |
| 8.  | Mycobacterium chelonae        | 22.3                              | 5.43       |
| 9.  | Sphingomonas capsulata        | 5.0                               | 1.22       |
| 10. | Rhodococcus equi              | 74.0                              | 18.03      |
| 11. | Mycobacterium sp.             | 1.6                               | 0.39       |
| 12. | Corynebacterium sp.           | 41.3                              | 10.07      |
| 13. | Micromonospora/Actinomadura   | 10.0                              | 2.44       |
| 14. | Fungi                         | 72.5                              | 17.66      |
| 15. | Bacillus subtilis             | 2.7                               | 0.66       |
| 16. | Agrobacterium radiobacter     | 0.3                               | 0.07       |
| 17. | Rhodococcus terrae            | 28.1                              | 6.85       |
| 18. | Acetobacter diazotrophicus    | 1.0                               | 0.24       |
| 19. | Eucaryotes                    | 0.1                               | 0.03       |
| Total|                               |                                   | 100.0      |
duction (up to 12.19 µg S²⁻¹ day⁻¹) and methane formation (up to 0.109 µg CH₄ 1⁻¹ day⁻¹), by a higher content of sulfate and higher numbers of sulfate-reducing bacteria and methanogens. The rates of anaerobic processes in the repository were close to respective values determined for lakes and formation waters of petroleum reservoirs (Mykhpskoe, Talinskoe, Romashkiniskoe, and Daqing), which are fresh-water environments with low content of sulfate and utilizable organic compounds [41–43]. In groundwater from the deep Aspó hard rock [7], the rates of methane formation from acetate (from 0 to 12.46 µM CH₄ h⁻¹) and from NaH¹⁴CO₃ with hydrogen as energy source (from 0 to 1.47 µM of CH₄ h⁻¹) were higher than in the repository. The rate of methane formation from NaH¹⁴CO₃ in the experiments of Kotelnikova and Pedersen [7] may have been overestimated due to the addition of exogenous hydrogen and the long period of incubation (4–10 days). Earlier methanogenesis in Lake Mendota sediments was shown to be increased by H₂ additions [41]. Penetration of wastes stimulated the activity of the sulfate-reducing population and of other anaerobic bacteria in subsurface horizons. The scale of these processes is limited by the penetration of sulfate and organic compounds with wastes. Thus, our data show that the injection of waste stimulated methane and hydrogen sulfide formation in the Severnyi repository.

4.3. Gas production by enrichment cultures isolated from the repository

The microbial communities inhabiting the formation waters were able to produce gases (N₂, CH₄, CO₂, and H₂S) on media with acetate and various electron acceptors: sulfate, nitrate, and carbonate, which are usually present in wastes [14]. The microbial production of gases should be taken into account when evaluating the ecological safety of repositories of liquid radioactive wastes. We did not measure the rate of denitrification in the repository but obtained active enrichment cultures of denitrifying bacteria. The geochemical activity of nitrate-reducing bacteria have a positive aspect (cleaning wastes from nitrate); however, microbial production of gases, such as dinitrogen and methane, may have a negative effect: a local pressure increase in the repository may lead to a discharge of wastes onto the surface. It is necessary to control the pressure in injection and observation wells of the repository.

The arrival of acetate and sulfate with wastes stimulated the activity of sulfate-reducers and other anaerobic bacteria in subsurface horizons. The production of sulfide by sulfate-reducing bacteria may initiate corrosion of metal equipment of the repository and favours the deposition of metals and radionuclides. The scale of these processes is restricted by the penetration of sulfate and organic compounds with wastes.

4.4. Biodiversity of the groundwater of horizon II

We also studied the bacterial diversity in the repository by GC–MS techniques. The applied method made it possible to detect the presence of strains whose cell number is more than 10⁶ cells ml⁻¹ sample. The GC–MS results correlated with those obtained by cultural and radioisotopic methods. Thus, we could not detect sulfate-reducing bacteria or methanogenic archea, whose number and activity were very low. We did not find biomarkers of representatives of the genera Acinetobacter, Comamonas, and Aeromonas, isolated from the repository earlier in enrichment or pure cultures [15,16]. In formation waters, we revealed eucaryotic and procaryotic microorganisms. Among procaryotes Gram-positive actinobacteria of the genera Rhodococcus, Corynebacterium, and Pseudonocardia, spore-forming representatives of the genera Bacillus and Clostridium, and Gram-negative bacteria of the genus Sphingomonas dominated. The application of the GC–MS method may be useful for long-term monitoring of changes of predominant representatives of the microbial community in the repository. It can be anticipated that the penetration of wastes will lead to eutrophication of this oligotrophic subsurface ecosystem and change the predominant microbial populations.

4.5. The possible effect of microorganisms on the mobility of radionuclides

Another important aspect of the burial of liquid wastes is the microbial transformation of their radioactive components, which remained out of the scope of our investigation yet. Various microorganisms are known to be capable of reducing metals in dissimilatory processes with energy conservation [44,45]. Microbial utilisation of Fe(3+), Mn(4+), and U(6+) as electron acceptors may influence their migration in aquatic ecosystems and in subsurface waters. Dissimilatory reduction of U(6+), Se(6+), Cr(6+), Hg(2+), Tc(7+), V(5+), and Au(3+) is a potential mechanism for removing and concentrating these metals from contaminated environments or waste streams. Although microorganisms able to reduce of each of these metals are available in pure culture, the information on microorganisms catalysing metal reduction in subsurface environments is scarce [46–48]. The biogeochemical metal cycles have a strong impact on many other elements, including carbon, sulfur, phosphorous and trace metals [43].

The obtained data show that radionuclide components of waste migrate with a lower rate than other components of wastes dissolved in the waters (nitrate, sulfate, and acetate). This phenomenon was explained by the transport of radionuclides in sorbed form with fine suspended particles of pelite and clay, which move with a lower rate than dissolved compounds [14]. The
role of bacteria as biocolloids in the transport of actinides ($^{232}$Th, $^{238}$U, $^{237}$Np, $^{239}$Pu, and $^{243}$Am) from a deep radioactive waste repository (New Mexico) was studied [49]. It was shown that the amount of actinide associated with suspended cell fraction was very low ($10^{-12}$–$10^{-21}$ mol cell$^{-1}$).

It cannot be excluded that the separation of radioactive and non-radioactive components of wastes, observed in the repositories, is due not only to chemical and colloid processes, but also to the effect of metal-reducing and sulfate-reducing bacteria and their metabolites on radionuclides and other metals. Apart from the microbial groups studied (denitrifiers, fermenters, sulfate-reducers, and methanogens), iron-reducing and sulfur-reducing bacteria usually also occur in subsurface environments [50]. As it was shown for petroleum reservoirs, microbiological processes proceed most actively in the near-bottom zone of injection wells, and further in the stratum their activity becomes lower [42,43]. A similar phenomenon can take place in deep repositories of LRW, where metals and radionuclides may be reduced by microorganisms with the formation of insoluble compounds and thus be concentrated in the near-bottom zone of injection wells. The data presented show the necessity of microbiological and radiochemical monitoring of the Severnyi repository.

5. Conclusions

The injection of liquid radioactive wastes into deep repositories has made it possible for significant part of the wastes to be isolated from the sphere of human habitation. Subsurface horizons of the repository Severnyi contain small but metabolically diverse microbial population, representing various physiological groups of microorganisms: aerobic heterotrophs, anaerobic fermenters, denitrifiers, sulfate-reducers, and methanogens. Sulfate reduction is the major anaerobic process in subsurface horizons. The number and geochemical activity of microorganisms is higher in the zones of waste penetration. The microbial communities are able to decrease the quantity of pollutants (acetate, sulfate, nitrate) in waste, producing thereby gases ($\text{N}_2$, $\text{CH}_4$, $\text{CO}_2$, and $\text{H}_2\text{S}$); however, biogenic gas production may result in a local increase of the pressure in the repository and consequent discharge of wastes onto the surface. Thus, there is an urgent need for monitoring of the abundance, biodiversity, and geochemical activity of microorganisms inhabiting the Severnyi repository of LRW.

Acknowledgements

We thank Valeriy S. Ivoilov, Artem S. Davidov, and Alexandre A. Grigoriyan (Institute of Microbiology, Russian Academy of Sciences, Moscow, Russia), for their help in the estimation of geochemical activity of microorganisms.

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