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EXPERIMENTAL ARTICLES

Microbiological Processes in the Severnyi Deep Disposal Site for Liquid Radioactive Wastes

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Abstract—Local monitoring of physicochemical, radiochemical, and microbiological parameters was performed in the deep horizons of the Severnyi site used for disposal of liquid radioactive waste (LRW). Analysis of the chemical and radiochemical composition of the wastes and formation fluid revealed that the boundary for migration of radionuclides lagged behind that for nonradioactive waste components (sodium nitrate) and tritium. The physicochemical and radiochemical conditions in deep horizons did not prevent microbial growth. The numbers of microorganisms (aerobic organotrophs, denitrifying, fermentative, sulfate-reducing, and methanogenic) were low, as were the rates of sulfate reduction and methanogenesis; they increased in the waste dispersion zone. The microorganisms from deep horizons were able to produce gases (CH_4, CO_2, N_2, and H_2S) from possible waste components. Denitrifying bacteria belonged to different *Pseudomonas* species and reduced nitrate to dinitrogen under the conditions of pH, salinity, temperature, and radioactivity found in the disposal site. These results suggest the need for control of microbiological processes in deep disposal site for liquid RW.

Key words: deep disposal site for liquid radioactive waste, sulfate reduction, methanogenesis, 16S rRNA, denitrifying bacteria.

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During the last few decades, deep horizons have been used frequently for disposal of liquid wastes of the chemical, food, metallurgical, and oil-refining industries. Radioactive wastes (RW) of nuclear power plants and the waste of the industries of the nuclear fuel cycle present an especially challenging problem [1, 2]. Liquid radioactive wastes arrive at a water-bearing layer through injection wells, displace formation water from the pore space, and interact with the components of the geological environment. While physicochemical and radiochemical investigation have been carried out throughout the operating period of the disposal sites, the microbiological and biogeochemical processes in these environments remain poorly studied.

Organic matter and mineral components arriving with the waste may act as carbon and energy sources for microorganisms and affect the activity and composition of the subsurface microbial communities [3–6]. Microbial metabolites (for example, sulfide) may interact chemically with the radioactive waste components and affect their migration.

In Russia, the first microbiological investigation of deep disposal sites for liquid RW have been carried out on the Severnyi site of the Chemical Mining Combine (Krasnoyarsk krai, Russia) [7, 8]. In these works, attention was paid mainly to the role of microorganisms in the transformation of nonradioactive waste components. A diverse microbial community was found in the subsurface horizons, including aerobic organotrophs together with anaerobes (fermentative, denitrifying, sulfate-reducing, and methanogenic microorganisms), which are able to produce gases from the possible waste components (acetate, nitrate, and sulfate). The real rate of gas production by formation water microorganisms was shown to be low, not endangering the site [9, 10].

Low rates of sulfate reduction and methanogenesis were revealed in deep disposal site for liquid radioactive wastes of the Siberian Chemical Combine (Seversk, Tomsk oblast) [11]. The microorganisms isolated from the deep horizons were able to participate in sorption and transformation of uranium and actinides [12].

An objective assessment of the safety of disposal of liquid RW is presently impossible without consideration of the microbiological processes, the specific conditions of liquid waste localization, and the partic-

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ulars of their chemical and radiochemical composition.

The goal of the present work was to investigate the dynamics of the radiochemical composition, occurrence, and geochemical activity of microorganisms in the waters of the Severnyi deep disposal site for liquid radioactive waste and to determine the diversity of denitrifying microorganisms, their adaptation to the environment, and their role in the transformation of waste components.

MATERIALS AND METHODS

Characterization of the deep horizons of the Severnyi disposal site has been provided earlier [1, 2, 8]. Monitoring of the physicochemical, radiochemical, and microbiological characteristics of formation water of the Severnyi disposal site for low-level liquid radioactive wastes of the Mining and Chemical Combine (MCC) (Zheleznogorsk, Russia) was carried out from 1998 to 2006. The disposal site was investigated yearly in summer (June to July), while the number of observation and relief wells increased.

The subsurface horizons used for liquid waste disposal at the Severnyi site are composed of weakly camented sand-clay rocks isolated from the upper and lower horizons by relatively impermeable clay layers [1, 2]. Horizons I and II are located at depths of 370–465 and 180–280 m below sea level, respectively. The subsurface waters are weakly mineralized (0.3 g/l) and move from the south to the north (5–6 m per year). Injection of intermediate-level radioactive wastes into horizon I commenced in 1967. The wastes contained sodium salts (nitrates, acetates, and sulfates), silicic acid, and some metal ions. The total salt content was as high as 240 g/l, and the specific activity did not exceed 10^{-2} Ci/l. The wastes contained strontium, cesium, ruthenium, cerium, and other short-lived radionuclides. Injection of low-level radioactive wastes (LLW) into horizon II commenced in 1968. These wastes contained various salts (up to 10 g/l), sodium nitrate and sulfate, condensates, drainage and desorbing solutions, surfactants, extragents, and diluents. The radionuclide composition was similar to that of the technological waste (10^{-8}–10^{-6} Ci/l). The volume of radioactive wastes injected into horizon II before 1992 was about 2×10^6 m^3 [1].

Liquid wastes are injected into the subsurface horizons via a row of injection wells. Relief wells were drilled in order to decrease the formation pressure and are used for extraction of groundwater simultaneously with waste injection. The relief wells of horizon I (P-1–P-6, P-11, and P-12) and horizon II (P-7–P-10) are located 1 and 1.5 km, respectively, from the relevant injection wells in the direction opposite to the natural direction of groundwater movement. The observation wells are located between the rows of injection and relief wells and are used for monitoring of conditions in horizons I and II. The locations of the wells on the Severnyi site have been depicted earlier [1, 2, 8].

Sampling. Groundwater samples were collected directly at the wellhead of the observation wells located at the periphery and inside the waste migration zone, as well as from relief wells. During extraction of groundwater with external pumps, the fluid was collected in sterile 1-l bottles. The bottles were hermetically sealed and transported to the laboratory within 6–20 h for determination of microbial numbers and rates of biogenic processes. Prior to chemical analyses, the samples were stored at 6°C.

Growth media and enumeration of bacteria. Microorganisms of major physiological groups were enumerated by tenfold serial dilutions in liquid media in two repetitions. The most probable numbers were calculated using McCrady tables. The media used for microbiological monitoring have been described earlier [8]. The numbers of aerobic organotrophs were determined on TYEG medium containing bacto tryptone (5.0 g/l), yeast extract (2.5 g/l), and glucose (1.0 g/l), with pH about 7.0. Growth of sulfate-reducing bacteria was assayed as an increase in sulfide concentrations in Postgate B medium with sodium lactate supplemented with 100 mg/l $Na_2S \times 9H_2O$ [13]. The growth of denitrifying bacteria was determined as dinitrogen formation in Adkins mineral medium [14] supplemented with sodium acetate (2.0 g/l) as the source of carbon and energy and sodium nitrate (1.0 g/l) as the electron acceptor. Bacteria with fermentative metabolism were assayed by hydrogen formation in the terminal dilutions on the medium with peptone (4.0 g/l) and glucose (10.0 g/l) [15]. Methanogens were determined by methane production in terminal dilutions in media with acetate (2 g/l) or $H_2 + CO_2$ [16]. Trace elements according to [17] were added.

Hungate tubes were used for enumeration of aerobic and anaerobic bacteria. Groundwater was injected with syringes. For anaerobic bacteria, except for methanogens growing on $H_2 + CO_2$ (4 : 1 vol/vol), oxygen-free argon was used as the gas phase. Air was the gas phase for aerobic bacteria. The tubes were incubated at 18–20°C until visible growth occurred. To confirm the absence of growth, the tubes were incubated for 2 months. The cultures were examined under an Olympus phase microscope.

Analytical techniques. Molecular hydrogen and molecular nitrogen were determined by gas chromatography [8]. Sulfide was determined by the colorimetric reaction with dimethyl-*p*-phenylenediamine in the modification cited in [18]. The pH value was measured on an OP-211/1 digital laboratory pH meter. The content of iron, magnesium, calcium, and sodium in groundwater was determined using an AAS1N atomic absorption spectrometer. Nitrates were analyzed with an Ecotest-01 device equipped with an ELIT-21 ion-selective electrode. Nitrites were determined colorimetrically with sulfanilic acid and α-naphthylamine. The concentrations of other com-

Table 1. Chemical composition of formation water and the rates of sulfate reduction and methanogenesis in fluids from horizons I and II of the Severnyi deep disposal sites for liquid radioactive waste

Well no.	Year of analysis	Concentration, mg/l							Sulfate reduction rate, μg S^{2-} l^{-1} day^{-1}	Methanogenesis rate, μg CH_4 l^{-1} day^{-1}	
		Na^+	K^+	Cl^-	NO_3^-	SO_4^{2-}	HCO_3^-	Acetate		from HCO_3^-	from acetate
Horizon I											
A-5	2004	83.1	3.0	11.2	0	45.2	277.5	3.1	0.171	0.006	0.020
A-19	2004	78.4	3.4	9.9	0	30.8	330.0	4.3	0.090	0	0.190
A-22	2004	67.4	3.4	18.6	3.3	84.4	195.2	6.6	0.380	0	0.020
A-26	2004	60.3	4.1	20.9	0	79.8	236.9	6.5	0.026	0	0.030
P-19	2004	65.2	4.0	18.9	0	65.0	231.8	5.4	0.028	0	0.010
P-20	2006	54.0	3.5	5.3	1.4	7.5	243.3	0.7	0.007	0	0.003
C-35	2004	102.9	2.4	5.8	0	8.9	255.6	6.5	0.092	0	0.020
R-4	2004	61.5	4.7	26.6	0	69.0	237.9	4.4	0.050	0	0.020
R-6	2004	67.2	3.4	19.6	0.1	65.5	231.8	3.7	0	0	0.050
Horizon II											
A-38	2004	75.3	2.9	1.2	0	8.5	253.1	3.9	0.060	0	0.010
A-39	2004	76.0	2.8	1.9	0	6.4	349.3	10.7	0.052	0.618	0.020
D-2	2004	73.4	3.2	2.5	0	11.8	378.2	8.6	0.122	0.035	0.020
	2006	49.0	2.0	2.2	1.3	5.9	385.2	0.9	0.002	0.860	0
D-4	2001	33.0	6.7	3.3	0.2	7.5	170.0	0*	0.760	0.110	0.001
	2004	79.1	3.0	3.4	0	12.0	348.9	11.1	0.011	0	0.001
R-10	2004	45.6	3.1	1.4	0	5.1	433.1	11.7	0.022	0.018	0.130

* Methanogenesis rates in the absence of acetate were calculated subject to the labeled acetate introduced into the sample.

ponents of the groundwater were determined by traditional methods [19]. Volatile fatty acids were analyzed in the samples of groundwater fixed with KOH [8].

Radioisotope techniques. The rates of sulfate reduction and methanogenesis were determined by radioisotope techniques [21, 22], using labeled $Na_2^{35}SO_4$, $NaH^{14}CO_3$ and $^{14}CH_3$–COONa with specific activities of 1.9, 3.8, and 1.3 MBq/ml, respectively. The labeled substrates (0.2 ml) were added to 40 ml of groundwater, incubated for 24 h at room temperature, fixed with 1 ml of the saturated KOH solution, and analyzed as described earlier [8]. All radioisotope measurements were carried out on a Rack Beta scintillation counter (LKB, Finland). Radiochemical analysis of tritium, ^{14}C, and ^{90}Sr was carried out using an LS-6500 automatic scintillation counter (Beckman). Alpha radiation and gamma activity of the water samples were analyzed using low-background alpha-radiometer and gamma-spectrometer [23–25].

Molecular cloning and sequencing of 16S rRNA genes from denitrifying enrichment. The enrichment culture of denitrifying bacteria maintained for 2 years in Adkins medium with sodium acetate and sodium nitrate was used to isolate the total DNA as described previously [26]. DNA isolation, amplification with the primers 8-27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3'), cloning of the fragments into the pGEM-T plasmid vector, transformation of *E. coli* Z85 by recombinant plasmids, selection of the colonies with required inserts, plasmid purification, and sequencing of 16S rRNA gene fragments were carried out as described previously [26]. The sequences of 16S rRNA genes were analyzed using the NCBI GenBank Blast software package.

RESULTS AND DISCUSSION

Physicochemical and radiochemical characterization of the groundwater. From 1998 to 2006, a total of 55 and 50 water samples of horizons I and II, respectively, have been analyzed. The physicochemical characteristics of the 1998–2001 samples have been reported earlier [7, 8]. Most of the water samples were similar in composition to the original groundwater. In the waters of both horizons, the concentrations of the main macrocomponents varied within a narrow range (Table 1). Carbonate concentration varied from 170 to 433.1 mg/l, the concentrations of nitrate and sulfate did not exceed 8.9 mg/l (wellC-35). Total salinity was 0.23–0.49 g/l (wells C-35, A-38, A-39, D-1, and

Table 2. Chemical composition of groundwater samples from the wells of horizon II located at different distances from the injection contour

Parameters, components, mg-eq/dm^3	Wells, distance from the injection contour, sampling year								
	A-36 (930 m)			A-38 (420 m)			An-34 (390 m)		
	1969*	2005	2007	1967*	2005	2007	1967*	2005	2007
pH	7.7	7.6	7.7	8.0	8.0	7.8	7.6	7.6	7.7
$\Sigma Na^+ + K^+$	1.6	1.6	1.5	2.2	2.8	2.5	2.3	2.1	2.4
$\Sigma Ca^{2+} + Mg^{2+}$	4.8	4.8	4.6	3.4	3.0	3.3	3.2	3.1	3.6
Σ cations	6.4	6.4	6.1	5.6	5.8	5.8	5.5	6.2	6.0
HCO_3^-	6.4	6.5	6.7	5.6	5.7	5.5	5.6	7.0	6.4
SO_4^{2-}	0.1	0.12	0.09	0.09	0.2	0.13	0.04	0.02	0.06
NO_3^-	≤0.002	≤0.002	≤0.002	≤0.002	0.016	≤0.002	≤0.002	≤0.002	≤0.002
Σ anions	6.5	6.5	6.7	5.7	5.9	5.6	5.64	7.0	6.4
Σcat. /Σ an.	0.98	0.98	0.98	0.96	0.98	0.98	0.98	0.89	0.94

* Data obtained at site development.

D-2), while pH varied from 7.6 to 8.0 (not all results are presented in Tables 1 and 2). Phosphates were not present, and the NH_4^+ concentration did not exceed 2.7 mg/l. In several samples, iron was found (6.5–29.5 mg/l); in the water from well D-4, the iron concentration was as high as 237.5 mg/l. The temperature of the groundwater was 12–14°C.

Due to mixing with the waste, the waters from the observation wells located close to injection wells had a different chemical composition. The presence of the waste manifested itself in increased levels of nitrate, sulfate, and total salinity (Tables 1 and 2). During 2003–2006, similar changes were observed in the fluids from horizon I (wells A-5, A-19, A-22, A-26, A-32, P-19, etc.)

Changes in the chemical composition of formation fluid resulting from the presence of the waste were established by comparison of our data and those obtained in 1964–1969 during the site development (kindly provided by the MCC personnel). The results of investigation of the chemical and radiochemical composition of the samples from individual wells located 45–930 m from the injection contour are presented below (Table 2; Figs. 1a, 1b).

It can be seen from Tables 1 and 2 that Na^+, K^+, Ca^{2+}, and Mg^{2+} were the major cations, while HCO_3^- and SO_4^{2-} were the major anions. The content of nitrate and sulfate ions in the samples from the observation wells was low in both 1967 and 2005. Thus, in 1969–2007, no significant changes occurred in the composition of groundwater from the wells remote from the injection contour.

In the zone of wells located 200–300 m from the injection contour, temporary changes in the composition of groundwater were observed, especially noticeably in the concentration of nitrate ions. For example, in well P-20, waste filtration occurred in 1972, with nitrate concentration increasing from ≤0.002 (in 1966) to 38.9 mg-eq/dm^3. Subsequently, the chemical composition of groundwater returned to the initial level. In 2003, NO_3^- content decreased to 0.6 mg-eq/dm^3, while in 2006 it was 0.002 mg-eq/dm^3, similar to nitrate concentration in the initial groundwater (Fig. 1a).

The well A-56 exemplifies the dynamics of the changes in groundwater composition in wells located 40–50 m from the injection contour (Fig. 1b). From 1967 to 2004, the concentration of $Na^+ + K^+$ increased from 1.8 to 17 mg-eq/dm^3 and the concentration of NO_3^- increased from 0.002 to 5.1 mg-eq/dm^3 (Fig. 1a, 1b). In 2004–2007, $Na^+ + K^+$ and NO_3^- concentrations reached 18 and 16 mg-eq/dm^3, respectively, which is comparable to their concentrations in diluted waste.

Acetate was occasionally found in the fluids from both horizons (Table 1), as well as propionate and butyrate; their maximum concentrations were 22, 52, and 6 mg/l, respectively.

The results of radiochemical analysis of the samples collected from wells A-36, An-34, and A-56 in 2004 and 2007, as well as the values of the interference level (IL), are presented in Table 3. The radiochemical characteristics of the samples from other wells did not differ significantly from those listed in the

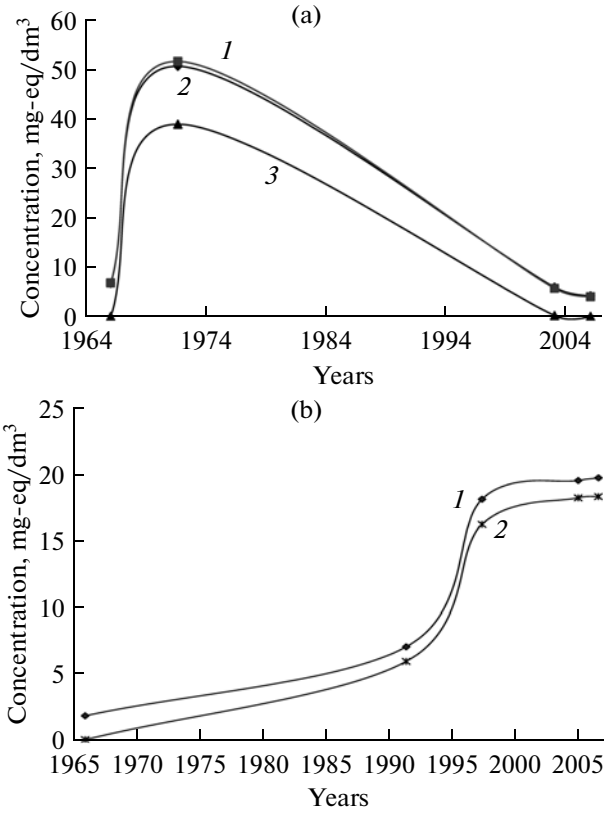

Fig. 1. Dynamics of concentrations of Σ cations (*1*), Σ anions (*2*), and NO_3^- (*3*) in groundwater from well P-20 (a) and concentrations of NO_3^- (*1*) and $\Sigma Na^+ + K^+$ (*2*) in formation fluid from well A-56 (b).

table. Both induced and natural radionuclides were present in the samples. The levels of induced radionuclides (^{90}Sr, ^{137}Cs and ^{241}Am, ^{60}Co, ^{95}Zr, ^{95}Nb, ^{106}Ru, and ^{144}Ce) in wells A-36 and An-34 were below the sensitivity of our equipment, not exceeding 1 Bq/dm^3; they were within the standards of the interference level [25]. The levels of total β-activity increased closer to the injection contour. For example, they did not exceed 1.5 Bq/dm^3 for the samples from wells A-36 and An-34, while in well A-56 they reached 170 Bq/dm^3. While the latter sample contained ^{60}Co, ^{90}Sr, and ^{137}Cs, a tenfold excess was observed only for ^{90}Sr. The concentrations of other induced radionuclides were below the interference level.

Natural radionuclides were detected in all samples, even in those from wells localed 900 m from the injection contour. In some samples, the ^{40}K content exceeded the IL for drinking water (Table 3).

The results of chemical and radiochemical analysis of injected waste and groundwater confirm the conclusion of [1, 2] that the borders of distribution of radionuclides lagged considerably behind the front of the movement of nonradioactive components of wastes (sodium nitrate) and tritium. Radionuclides do not migrate significant distances from the injection contour. The tentative radiation exposure calculated based on these data did not exceed 0.06 rad/h.

Thus, the subsurface horizons of the Severnyi disposal site are a freshwater ecosystem limited by organic substrates, nitrogen, and phosphorus. Bicarbonate was the dominant anion. Liquid waste injected into these horizons contained small amounts of organic compounds and electron acceptors (nitrate and sulfate), which may be utilized by microorganisms.

Distribution of microorganisms and rates of anaerobic processes in groundwater. The substrates and mineral components (nitrate and sulfate) arriving with the waste may change the ratio of electron donors and acceptors available to microorganisms. The theoretically possible microbial processes in contaminated horizons include aerobic biodegradation of organic matter using the oxygen dissolved in injected wastes and (in the absence of oxygen) anaerobic processes, denitrification, sulfate reduction, methanogenesis, etc. Thus, the numbers of aerobic organotrophs, denitrifiers, fermentative microorganisms, sulfate reducers, and methanogens were determined in groundwater.

During 1998–2006, the number of microorganisms in the zone remote from the injection contour was low (wells P-3, P-6, P-10, and D-1) (Table 4). The numbers of aerobic organotrophic, denitrifying, and fermentative bacteria were 10^4–10^5 cells/ml; the number of sulfate-reducing bacteria did not exceed hundreds of cells per ml, while the number of methanogens was still lower (several cells per ml).

In the zone of waste dispersion (determined by the presence of nitrate and sulfate in the water), microbial numbers varied in the course of investigation, reaching 10^8 cells/ml (aerobic organotrophs and anaerobic fermentative bacteria). In most of the samples, the number of H_2-utilizing methanogens was low (from several cells to tens of cells per ml), while in the zone of waste penetration it increased to 10^2–10^3 cells/ml (Table 4). Low levels of aceticlastic methanogens, which have been previously only occasionally detected, were found in waste-contaminated formation fluids. Denitrifying bacteria growing on acetate and nitrate were found in practically every sample. The dynamics of their numbers exhibited a direct correlation to the dynamics of nitrate concentration in the samples of formation fluid collected at the same time.

In the zone of well P-20, waste arrival in 2004 resulted in increased numbers of aerobic organotrophic, denitrifying, and sulfate-reducing bacteria up to 10^8, 10^5, and 10^3 cells/ml, respectively (Fig. 2a). Due to consumption of the growth substrates by 2006, their numbers decreased to background values. In the vicinity of well A-56, waste filtration was observed in 2004–2007, resulting in stable high numbers of the microorganisms of all the groups investigated (Fig. 2b). These results demonstrate the effect of waste penetration on microbial numbers in deep horizons.

Table 3. Radionuclide content in groundwater from wells A-36, An-34, and A-56

Nuclides	Concentration, Bq/dm^3 in samples from wells						IL [25], Bq/dm^3
	A-36		An-34		A-56		
	2004	2007	2004	2007	2004	2007	
Sb	<1.5	<1.5	<1.5	<1.5	170	170	–
^{90}Sr	<0.5	<0.5	<0.5	<0.5	43	51	5.0
^{137}Cs	<0.12	<0.11	<0.15	<0.14	0.286	0.312	1.1 + 1
^{60}Co	0	0	0	0	0.693	0.723	4.1 + 1
^{144}Ce	<0.14	<0.14	<0.12	<0.12	<0.17	<0.17	2.7 + 1
^{241}Am	<0.17	<0.17	<0.17	<0.17	<0.25	<0.25	6.9 – 1
^{40}K	<3.7	<3.3	<1.4	<1.4	3.42	3.27	2.2 + 1
^{226}Ra	<1.3	<1.3	<0.32	<0.32	0.72	0.72	5.0 – 1
^{232}Th	<0.92	<0.92	<0.41	<0.41	<0.88	<0.88	6.0 – 1
^{235}U	<0.28	<0.28	<0.16	<0.16	<0.087	<0.087	3.0

Table 4. Microbial numbers (cells/ml) in groundwater of horizons I and II of the Severnyi RW disposal sites in 1998–2006

Well	Number of samples	Aerobic organotrophs	Denitrifying	Fermentative	Sulfate-reducing	Methanogens	
		Tryptone + glucose + yeast extract	Acetate + NO_3^-	Peptone + glucose	Lactate + SO_4^{2-}	$H_2 + CO_2$	Acetate
Horizon I							
A-5	7	10^2–10^6	0–10^6	Single-10^6	0–10^4	0–10^2	0–10
A-19	6	10^3–10^6	10–10^5	10–10^8	Single-10^3	0–10^3	0-Single
A-22	7	10^5–10^6	10^3–10^5	10–10^8	Single-10^3	0–10	0-Single
A-26	8	10^5–10^6	10^2–10^7	Single-10^8	10–10^3	0–10^3	0–10
A-32	3	Single-10^8	Single-10^3	Single-10^4	Single	Single-10^2	0-Single
P-19	5	10–10^6	Single-10^4	0–10^2	10–10^2	0–10	0
C-35	7	10^4–10^7	10–10^6	Single-10^7	10^2–10^3	0–10^3	0–10
R-3	2	10^3	10^3	10^4	0–10	0	0
R-6	3	10^4–10^5	10^2	10–10^5	Single-10^2	0–10	0
Horizon II							
D-1	6	Single-10^3	Single-10^5	Single-10^5	0–10^2	0–10	0–10
D-2	5	10^2–10^5	10^2–10^5	Single-10^5	10–10^3	0–10^3	0-Single
D-4	7	10–10^8	10^3–10^5	Single-10^8	Single-10^4	0–10	0-Single
A-36	1	Н.д.@	10	10^2	10	0	0
A-38	7	10^2–10^8	Single-10^6	10–10^7	Single-10^2	0–10^2	0–10
A-39	7	10^4–10^8	10^2–10^5	10–10^7	Single-10^3	0–10	0-Single
An-34	1	10^6	0	10^7	Single	Single	0
R-10	5	10^5–10^6	Single-10^4	0–10^8	0–10^3	0–10^2	0-Single
P-20	3	10^5–10^8	10^2–10^6	Single-10^6	10–10^3	0–10^2	0-10

Note: “0” indicates no cells detected in 1-ml inoculum.

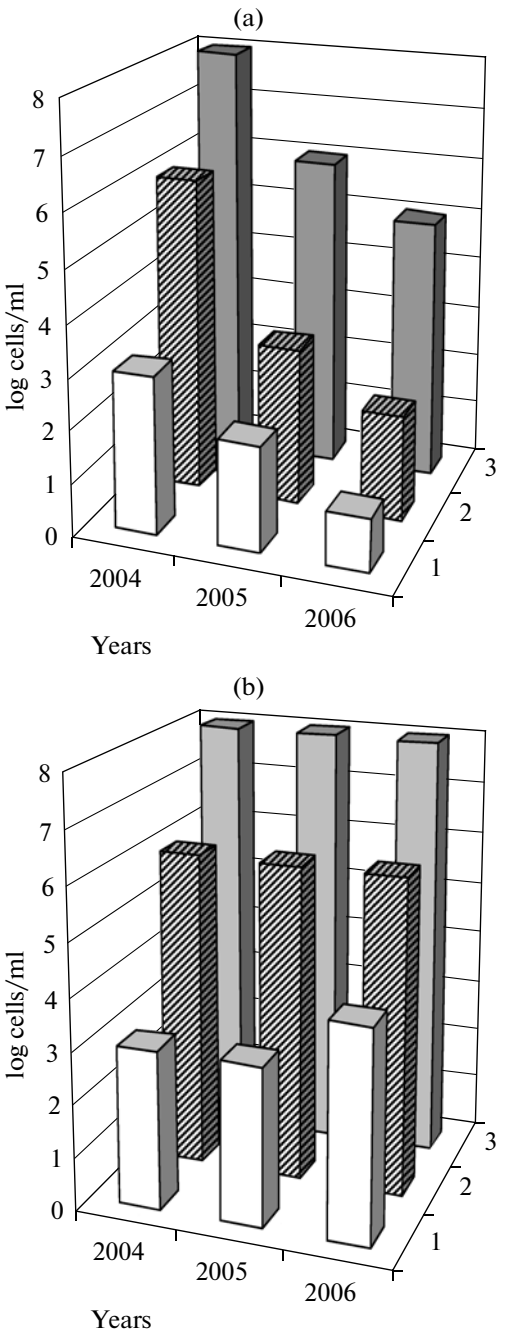

Fig. 2. Microbial numbers in groundwater from wells P-20 (a) and A-56 (b) located 200-300 and 45-60 m from the injection zone, respectively. Sulfate reducers (1), denitrifiers (2), and aerobic organotrophs (3).

Rates of sulfate reduction and methanogenesis were determined by radioisotope techniques in 29 and 37 samples of groundwater from horizons I and II, respectively. The rates of both processes were low. Sulfate reduction rate was the highest in the sample from well A-26 (12.19 μg S^{2-} l^{-1} day^{-1} in 2001) [8]. It was from 0.1 to 0.76 μg SS^{2-} l^{-1} day^{-1} in seven samples (wells A-5, A-22, R-4, D-2, and D-4) and did not exceed 0.1 μg SS^{2-} l^{-1} day^{-1} in other samples (Table 1; some data are not presented). Methane formation from labeled bicarbonate was detected only in ten samples. In five of them (wells A-26, A-39, D-2, and D-4), it was from 0.11 to 0.86 μg CH_4 l^{-1} day^{-1}, while in the five other samples it did not exceed 0.1 μg CH_4 l^{-1} day^{-1}. The rate of methanogenesis from 2-^{14}C-acetate in the samples from wells A-19 and R-10 was 0.19 and 0.13 μg CH_4 l^{-1} day^{-1}, respectively; in the remaining 35 samples, it was below 0.05 μg CH_4 l^{-1} day^{-1}.

Addition of possible waste components (acetate, sulfate, and nitrate) and molecular hydrogen to the samples of groundwater promoted sulfate reduction, methanogenesis (data not shown), and denitrification (Fig. 3). Dinitrogen was not produced in the investigated samples. Supplementing formation water with sodium nitrate and organic substrates or H_2 resulted in production of gases, mostly N_2. At 4 g/l $NaNO_3$ and 8 g/l CH_3COONa, dinitrogen prevailed in the gas, while at higher concentrations of nitrate and acetate, CO_2 was detected as well. Denitrification occurred at concentrations of nitrate and acetate up to 10 and 20 g/l, respectively, i.e., at the concentration found in almost undiluted waste.

Denitrifying Bacteria from Deep Horizons

This work focused mainly on denitrifying bacteria. Enrichment cultures producing N_2 on medium with acetate and nitrate were obtained from deep disposal sites. One of the cultures was investigated by molecular techniques (isolation of total microbial DNA and construction of the 16S rRNA gene library). Phylogenetic analysis of 42 sequences revealed predominance of 16S rRNA genes of the genus *Pseudomonas* (class *Gammaproteobacteria*), belonging to eight phylotypes (37 clones). *Pseudomonas stutzeri* was the closest relative for 22 clones (99–100% similarity). This bacterium is known to reduce nitrates to N_2. For four sequences, the similarity to *P. stutzeri* 16S rRNA gene sequence was lower (98% similarity for two clones and 94 and 92% similarity for the other two). Moreover, 11 sequences were closely related to 16S rRNA genes of other *Pseudomonas* species: *P. alcaligenes* (six clones, 100% similarity), *P. putida* (three clones, 99% similarity, and one clone, 98% similarity), and *P. mendocina* (one clone, 98% similarity). Two phylotypes belonged to the classes *Alphaproteobacteria* (three clones with 99% similarity to *Agrobacterium tumefaciens*) and *Actinobacteria* (two clones, 94% similarity to *Tessarococcus* sp.).

Pure cultures of *P. stutzeri* (strains A-26 and A-38) and *Acinetobacter calcoaceticus* (strain A-39) were isolated from the enrichments and their physiological and biochemical characteristics were investigated. Strain A-39 reduced nitrate only to nitrite, while the *P. stutzeri* strains produced molecular nitrogen under anaerobic conditions and used a broader spectrum of substrates for aerobic growth compared to strain A-39.

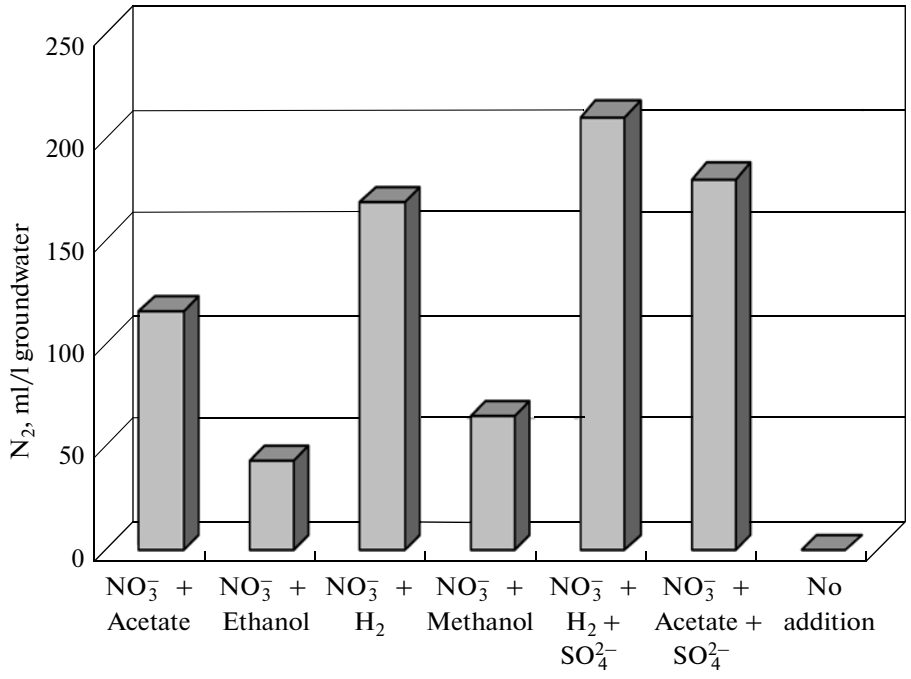

Fig. 3. Nitrogen formation in groundwater from well A-38 enriched with $NaNO_3$ (0.85 g/l) and different electron donors (sodium acetate, 2 g/l; ethanol, 5 ml/l; methanol, 5 ml/l; or hydrogen, 2 ml/50 ml water) during 20 days of incubation at 18°C.

All strains grew within the temperature range from 6 to 43°C, with the optimum being at ~28°C, which is higher than the temperature of the subsurface horizons (12–14°C).

From the ecological point of view, enrichments are more appropriate objects of investigation than pure cultures. The effect of the stratal conditions (temperature, nitrate concentration, pH, and ionizing radiation) on microbial numbers in enrichments and biogenic gas production was investigated. The growth optimum for denitrifying enrichments was observed at 2 g/l $NaNO_3$ [9], temperatures from 20 to 40°C, pH 7–8, and radiation exposure up to 10 kGy (Figs. 4a, 4b).

Effect of ionizing radiation on growth of enrichments of aerobic organotrophs and anaerobic fermentative bacteria obtained from horizon II was investigated. An absorbed dose of up to 1 kGy had little effect on the number of aerobic bacteria, while no viable cells were detected at an absorbed dose of 10 kGy. Fermentative bacteria remained viable at the maximum absorbed dose used (12 kGy). At 7 kGy, their number was half that in the initial culture.

Thus, the radiation exposure, temperature, pH, and nitrate content in horizon II do not prevent microbial growth. The rates of biogenic gas formation depend on the rate of arrival of nitrate and organic matter with the waste. Among the gases, nitrogen is predominant, with lower concentrations of CO_2. Denitrification may result in decreased toxicity of the

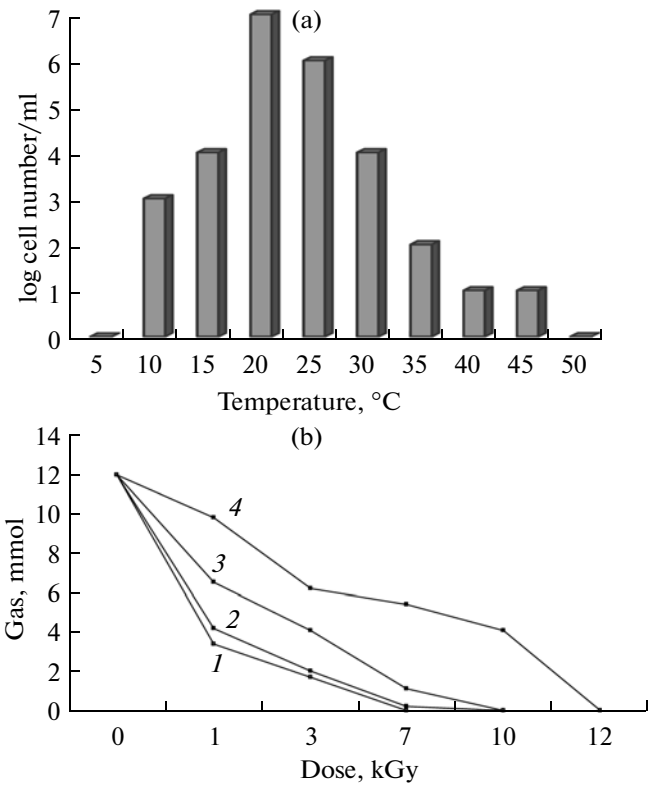

Fig. 4. Effect of temperature (a) and ionizing radiation (b) on bacterial numbers in enrichment cultures of denitrifying microorganisms and on nitrogen production (Adkins medium with 2 g/l $NaNO_3$ and 4 g/l CH_3COONa). Radiation exposure of 0.5 (*1*), 1 (*2*), 5 (*3*) and 8.7 mGy/s (*4*).

waste caused by the presence of NO_3^- ions due to their reduction to environmentally safe molecular nitrogen.

Our results demonstrate that the physicochemical and radiochemical conditions of the Severnyi disposal site for liquid radioactive wastes do not prevent microbial growth. Induced radionuclides present in the waste were shown to migrate no more than 200 m from the injection zone. A diverse microbial community was detected in the subterranean horizons. In formation waters, both the numbers of microorganisms and the rates of sulfate reduction and methanogenesis were low and increased in the zone of waste dispersion. The microorganisms isolated from the subsurface horizons produced gases (methane, H_2S, CO_2, and nitrogen) from the possible waste components (nitrate, acetate, and sulfate). A direct correlation was found between the number of denitrifying bacteria and nitrate concentration in formation fluids. In the presence of organic compounds, denitrifying bacteria isolated from deep horizons reduced nitrate to molecular nitrogen at the values of pH, temperature, salinity, and radioactivity found in the disposal site for low-level waste.

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