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EXPERIMENTAL ARTICLES

Distribution and Physiological State of Microorganisms in Petrochemical Oily Sludge

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Abstract—The occurrence, vertical distribution, and physiological state of microorganisms in a petrochemical oily sludge deposit were studied. The total number and the number of viable microbial cells at depths of 0.2 and 3 m were about 10^{10} and 10^8 cells/g dry wt sludge. Most microbial cells taken from the middle (1 m deep) and the bottom (3 m deep) sludge horizons showed a delayed colony-forming ability, which suggested that the cells occurred in a hypometabolic state. The relative number of microaerobic denitrifying microorganisms steeply increased with depth. The amount of microorganisms tolerant to 3, 5, and 10% NaCl and capable of growing at 7 and 40°C varied from 10^2 to 10^8 CFU/g dry wt sludge. Petrochemical oily sludge was found to maintain the growth of heterotrophs, among which the degraders of oily sludge and ten different individual polycyclic aromatic hydrocarbons were detected. The occurrence of highly adaptable microorganisms with an adequate metabolic potential in the petrochemical oily sludge deposit implies that its bioremediation is possible without introducing special microorganisms.

Key words: petrochemistry, by-products, oily sludge, microflora, bioremediation, physiological state, tolerance.

The petroleum industry is one of the most rapidly developing industries. The plants of this industry discharge a great deal of gaseous, liquid, and solid wastes [1, 2], including recalcitrant oily sludge, which is effluent treatment plant sludge and sludge separated from oil wastes by flotation or other processes. Petrochemical oily sludge may contain activated sludge from secondary settlement ponds and microorganisms from local waste treatment plants. The deposition of oil wastes is one of the most widespread methods of their disposal in many countries [3–6]. The processing of oily sludge is hindered by a high content of oil hydrocarbons (sometimes more than 50%) [7]. Oily sludge may also contain benzene, toluene, phenols, xylenes, styrene, acetophenone, acetonitrile, methylphenylcarbinol, methylethylketone, and other compounds [1, 6, 8], many of which are toxic and carcinogenic [9, 10]. The presence of ecologically hazardous polycyclic aromatic hydrocarbons (PAHs) in oily sludge [11] inevitably affects the composition and the metabolic activity of the sludge microflora.

The relevant data available in the literature [4, 5, 12, 13] primarily deal with the microbiological aspects of bioremediation of oily sludge–contaminated sites, whereas information concerning the microbiology of oily sludge deposits as specific anthropogenic ecosystems is scarce.

This prompted us to study the microbiological status of a petrochemical oily sludge deposit, namely, the distribution and physiological state of particular groups of microorganisms that inhabit sludge. The information obtained can be used for the development of a biotechnology for the detoxification and utilization of petrochemical wastes.

MATERIALS AND METHODS

The object for investigation was a petrochemical oily sludge deposit on the territory of the JSC Nizhnekamskneftekhim, Nizhnekamsk, Russia. Petrochemical oily sludge is a viscous substance with a severe odor of petroleum and its products. Depending on the deposit horizon, the content of organic substances in oily sludge varied from 384 to 607 g/kg dry wt sludge (hereafter, simply, g/kg). The content of the major fraction, malthenes, including monocyclic hydrocarbons and PAHs, reached 142 g/kg [6]. The concentration of the mineral forms of nitrogen (ammonia, nitrates, and nitrites) was 780–950, 8–13, and 1–2 mg/kg, respectively. The concentration of sulfates and total phosphorus was 3–12 and 1–2 mg/kg, respectively. Sulfides, including hydrogen sulfide, were absent. The redox potential Eh in the subsurface sludge horizon (0.2 m) was equal to –50 mV. Oxygen was absent. The pH of the aqueous extracts of oily sludge samples was 7.4–7.5. The elec-

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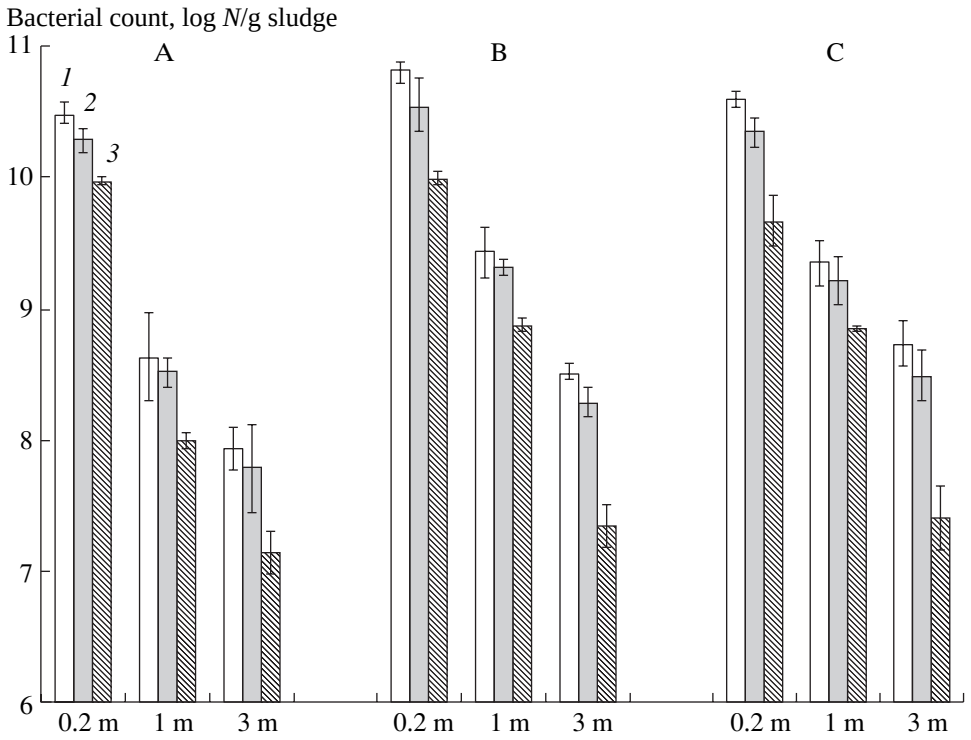

Fig. 1. The vertical distribution of (*1*) the total microbial cells, (*2*) viable microbial cells, and (*3*) aerobic heterotrophic microorganisms at three sites (A, B, and C) of the petrochemical oily sludge deposit.

tric conductivity of the extracts varied from 0.184 to 0.393 S/m.

Oily sludge samples were taken from three depths (0.2, 1, and 3 m) at three representative sites A, B, and C of the sludge deposit using a homemade sampler, which provided for aseptic sampling. Triplicate samples from each depth were immediately subjected to microbiological analysis.

Microbial count. Oily sludge samples were suspended in sterile tap water. To determine the total number of culturable aerobic heterotrophic microorganisms, the appropriate sludge suspension dilutions were plated onto nutrient agar. To evaluate the relative number of hypometabolic microbial forms by the method of Kozhevin [14], the incubation time of agar plates was extended to 8–11 days. The direct microscopic count of microbial cells was carried out as described in [15], using serial sludge suspension dilutions from 10^{-3} to 10^{-5}. The suspensions were stained with an acridine orange solution and examined under a LUMAM-I2 microscope equipped with an FS-1-4 light filter. The number of viable cells was determined microscopically as described by Kogure *et al.* [16]. Micromycetes and actinomycetes were enumerated using an acidified Czapek medium and starch–ammonia agar, respectively. Spore-forming bacteria were counted on wort agar using sludge suspensions that were preliminarily pasteurized at 90°C for 5 min [17]. Halotolerant microorganisms were counted on nutrient agar supplemented with 3, 5, or 10% NaCl. Thermo- and psychrotolerant microorganisms were enumerated on nutrient agar plates incubated at 40 and 7°C, respectively (in all other cases, the incubation temperature was 28°C). Denitrifying and sulfate-reducing bacteria were counted on Giltey and Postgate B media, respectively [17]. All the experiments were performed in five replicates. The number of microorganisms was referred to dry weight of oily sludge.

The number and the activity of oily sludge–degrading microorganisms were determined using an agar medium with oily sludge as the source of carbon and energy. Taking into account the phosphorus deficiency of oily sludge, it was suspended in 0.05 M phosphate buffer (pH 7.0). The amount of oily sludge in the agar medium was such that the total content of organic matter in the medium was about the same as in nutrient agar (this corresponded to a chemical oxygen demand of 40 g/l). Oily sludge with a moisture content of 60% comprised one-sixth of the volume of the agar medium. PAH-degrading microorganisms were recovered using a solid medium containing (g/l) $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.25; KH_2PO_4, 3.0; $Na_2HPO_4 \cdot 12H_2O$, 4.5; yeast extract, 0.05; agar, 20.0; and one of the following PAHs: naphthalene, phenathrene, anthracene, or fluorene. The pH of the medium was 7.0–7.2. Naphthalene crystals were placed on the lids of inverted petri dishes. The other PAHs were added to the medium at a concentration of 400 mg/l. The inoculated agar plates were incubated for 7–14 days. The most typical individual

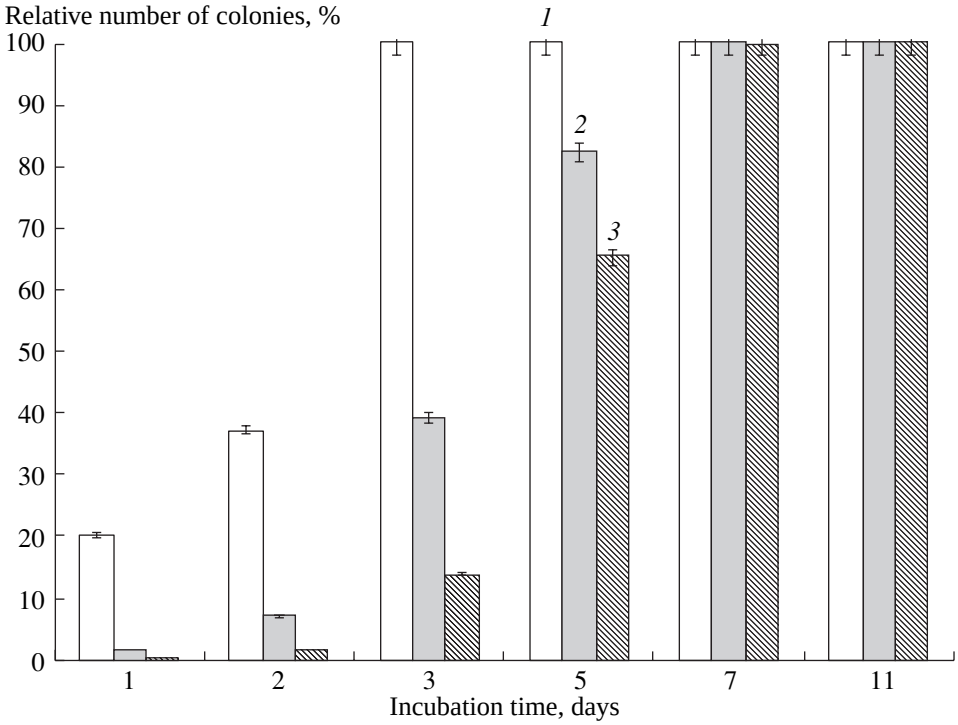

Fig. 2. The time dynamics of the relative number of colonies of aerobic heterotrophs grown from oily sludge samples taken at depths of (*1*) 0.2, (*2*) 1, and (*3*) 3 m. The maximum number of colonies grown from oily sludge samples collected from the particular horizon was taken as 100%.

colonies (the number of colonies on one plate was no more than 50) were used for the isolation of PAH-degrading microorganisms. PAH-degrading isolates were tested for purity by incubating them on nutrient agar, after which pure PAH degraders were grown on oily sludge agar. To estimate the degrading activity of the isolates, cells washed off from the oily sludge agar were suspended in a sterile phosphate buffer (pH 7.0) and inoculated into an oily sludge–containing liquid medium (25 ml in 250-ml cultivation flasks) to a density of 10^5 cells/ml. The flasks were incubated for 10 days on a shaker (100 rpm). The control flasks contained the sterile medium. The degrading activity of the isolates was estimated from the decrease (relative to the control) in the content of chloroform-extractable organic compounds and PAHs. The amount of chloroform-extractable organic compounds was determined gravimetrically and that of PAHs was determined by HPLC as described below.

Analytical methods. The culture liquid of PAH-degrading isolates was extracted with chloroform, and the solvent was removed in a vacuum rotary evaporator. The residue was weighed, and the amount of chloroform-extractable components of oily sludge was calculated relative to the control (the sterile medium). Then the residue was dissolved in acetonitrile, and undissolved material was removed by centrifugation at 14000 *g* for 3 min. The supernatant was analyzed for PAHs by HPLC using an LP series chromatograph equipped with a reversed-phase column (4.6×250 mm) packed with Ultracarb 5ODS, 5 μm (Phenomenex, United States). The column was eluted isocratically with an acetonitrile–water (70 : 30) mixture. The retention times of ten individual PAHs (acenaphthylene, naphthalene, biphenyl, fluorene, phenathrene, anthracene, fluoranthene, pyrene, benz[a]anthracene, and chrysene) determined at 254 nm with the aid of a UV-VIS detector were compared with those of the authentic samples of these PAHs purchased from Elsico (Russia).

Chemical oxygen demand (COD) was estimated by the standard method [18]. To evaluate the moisture content, samples of oily sludge were dried at 105°C. The results were statistically processed at significance level $P \le 0.05$.

RESULTS

The total number and the number of viable microbial cells (including aerobic heterotrophic cells culturable on nutrient agar) in the oily sludge deposit were unexpectedly large, especially in the subsurface sludge horizon (0.2 m), where the total bacterial count was about 10^{10} CFU/g dry wt sludge (at all the tested deposit sites A, B, and C). At depths of 1 and 3 m, this parameter decreased by 1–1.5 and 3 orders, respectively (Fig. 1). The percentage of viable cells was high and about the same in all three oily sludge horizons, whereas the percentage of aerobic heterotrophic micro-

The vertical distribution of some taxonomic and physiological groups of microorganisms at three sites (A, B, and C) of the petrochemical oily sludge deposit

Group of microorganisms	Depth, m	Site		
		A	B	C
Aerobic heterotrophs	0.2	$(9.2 \pm 0.9) \times 10^9$	$(9.6 \pm 1.2) \times 10^9$	$(4.6 \pm 2.5) \times 10^9$
	1	$(9.5 \pm 1.4) \times 10^7$	$(7.3 \pm 1.0) \times 10^8$	$(6.9 \pm 0.3) \times 10^8$
	3	$(1.4 \pm 0.6) \times 10^7$	$(2.2 \pm 0.9) \times 10^7$	$(2.5 \pm 1.9) \times 10^7$
Actinomycetes	0.2	$(5.0 \pm 4.3) \times 10^4$	$(9.6 \pm 2.5) \times 10^3$	$(2.8 \pm 1.2) \times 10^4$
	1	$(4.5 \pm 3.0) \times 10^4$	$(6.9 \pm 5.3) \times 10^3$	$(6.6 \pm 3.4) \times 10^3$
	3	$(2.6 \pm 1.0) \times 10^3$	$(1.5 \pm 0.3) \times 10^3$	$(9.1 \pm 4.1) \times 10^3$
Micromycetes	0.2	$(8.2 \pm 2.9) \times 10^4$	$(9.3 \pm 1.2) \times 10^3$	$(1.9 \pm 3.6) \times 10^4$
	1	$(7.1 \pm 1.1) \times 10^4$	$(7.7 \pm 3.2) \times 10^3$	$(5.6 \pm 3.5) \times 10^3$
	3	$(5.2 \pm 4.0) \times 10^3$	$(9.1 \pm 2.9) \times 10^3$	$(1.5 \pm 2.5) \times 10^3$
Spore-forming bacteria	0.2	$(1.1 \pm 3.4) \times 10^8$	$(8.7 \pm 3.2) \times 10^8$	$(8.0 \pm 1.6) \times 10^8$
	1	$(9.0 \pm 2.5) \times 10^6$	$(3.8 \pm 1.2) \times 10^7$	$(3.2 \pm 2.5) \times 10^7$
	3	$(3.0 \pm 1.9) \times 10^5$	$(9.1 \pm 1.7) \times 10^5$	$(6.0 \pm 2.4) \times 10^5$
Denitrifiers	0.2	$(1.4 \pm 0.5) \times 10^7$	$(2.5 \pm 0.5) \times 10^7$	$(1.4 \pm 0.5) \times 10^7$
	1	$(1.2 \pm 0.2) \times 10^7$	$(4.5 \pm 0.5) \times 10^7$	$(2.0 \pm 0.2) \times 10^7$
	3	$(9.5 \pm 0.8) \times 10^6$	$(7.0 \pm 1.0) \times 10^6$	$(8.2 \pm 0.8) \times 10^6$
Sulfate-reducers	0.2	$(9.5 \pm 0.5) \times 10^2$	$(4.5 \pm 0.5) \times 10^2$	$(1.5 \pm 1.0) \times 10^2$
	1	$(4.2 \pm 1.2) \times 10^2$	$(2.5 \pm 1.2) \times 10^2$	$(4.2 \pm 1.5) \times 10^2$
	3	$(9.5 \pm 0.1) \times 10^3$	$(1.5 \pm 0.5) \times 10^3$	$(5.5 \pm 0.1) \times 10^3$
Psychrotolerant (7°C) bacteria	0.2	$(5.0 \pm 0.6) \times 10^5$	$(1.9 \pm 0.6) \times 10^5$	$(2.5 \pm 1.3) \times 10^5$
	1	$(1.8 \pm 0.5) \times 10^6$	$(1.6 \pm 0.5) \times 10^6$	$(3.6 \pm 2.8) \times 10^6$
	3	$(2.5 \pm 1.3) \times 10^4$	$(3.1 \pm 1.5) \times 10^4$	$(9.5 \pm 1.3) \times 10^5$
Thermotolerant (40°C) bacteria	0.2	$(1.8 \pm 0.3) \times 10^8$	$(2.3 \pm 1.3) \times 10^8$	$(2.5 \pm 0.9) \times 10^7$
	1	$(1.9 \pm 0.5) \times 10^7$	$(6.5 \pm 5.1) \times 10^7$	$(3.6 \pm 0.8) \times 10^6$
	3	$(3.7 \pm 2.4) \times 10^5$	$(9.5 \pm 1.2) \times 10^4$	$(1.1 \pm 0.9) \times 10^5$
Osmotolerant bacteria	0.2	$(1.3 \pm 0.3) \times 10^9$	$(9.9 \pm 2.3) \times 10^8$	$(8.5 \pm 5.6) \times 10^8$
3% NaCl	1	$(1.3 \pm 0.5) \times 10^7$	$(8.9 \pm 0.8) \times 10^7$	$(5.6 \pm 2.5) \times 10^7$
	3	$(6.7 \pm 1.2) \times 10^5$	$(3.5 \pm 1.5) \times 10^6$	$(6.5 \pm 3.4) \times 10^6$
5% NaCl	0.2	$(4.6 \pm 0.5) \times 10^6$	$(6.8 \pm 4.2) \times 10^6$	$(7.6 \pm 6.1) \times 10^6$
	1	$(5.7 \pm 2.3) \times 10^5$	$(1.5 \pm 0.9) \times 10^5$	$(8.7 \pm 2.3) \times 10^4$
	3	$(6.7 \pm 1.6) \times 10^3$	$(1.8 \pm 0.6) \times 10^4$	$(4.0 \pm 1.2) \times 10^4$
10% NaCl	0.2	$(2.3 \pm 0.9) \times 10^5$	$(3.5 \pm 4.5) \times 10^5$	$(4.6 \pm 2.8) \times 10^5$
	1	$(1.4 \pm 0.2) \times 10^4$	$(1.5 \pm 0.8) \times 10^4$	$(2.7 \pm 0.4) \times 10^4$
	3	$(1.5 \pm 1.0) \times 10^3$	$(9.0 \pm 2.5) \times 10^3$	$(9.1 \pm 2.3) \times 10^2$
Oily sludge–degrading bacteria	0.2	$(1.7 \pm 0.7) \times 10^9$	$(8.2 \pm 1.8) \times 10^8$	$(9.6 \pm 0.5) \times 10^8$
	1	$(4.6 \pm 0.4) \times 10^7$	$(5.1 \pm 1.0) \times 10^7$	$(2.2 \pm 2.5) \times 10^7$
	3	$(3.4 \pm 1.6) \times 10^6$	$(1.8 \pm 0.7) \times 10^6$	$(7.5 \pm 5.9) \times 10^6$

organisms culturable on nutrient agar considerably decreased with depth (Fig. 1).

The physiological state of heterotrophic microorganisms can be estimated from the dynamics of the number of colonies grown on nutrient agar in the course of incubation. As can be seen from Fig. 2, the number of colonies grown on nutrient agar from samples taken from the subsurface sludge horizon did not increase any longer after 3 days of incubation. However, in the case of the sludge samples taken from depths of 1 and 3 m, the number of grown colonies tended to rise over an incubation period of 7 days (it should be noted that the

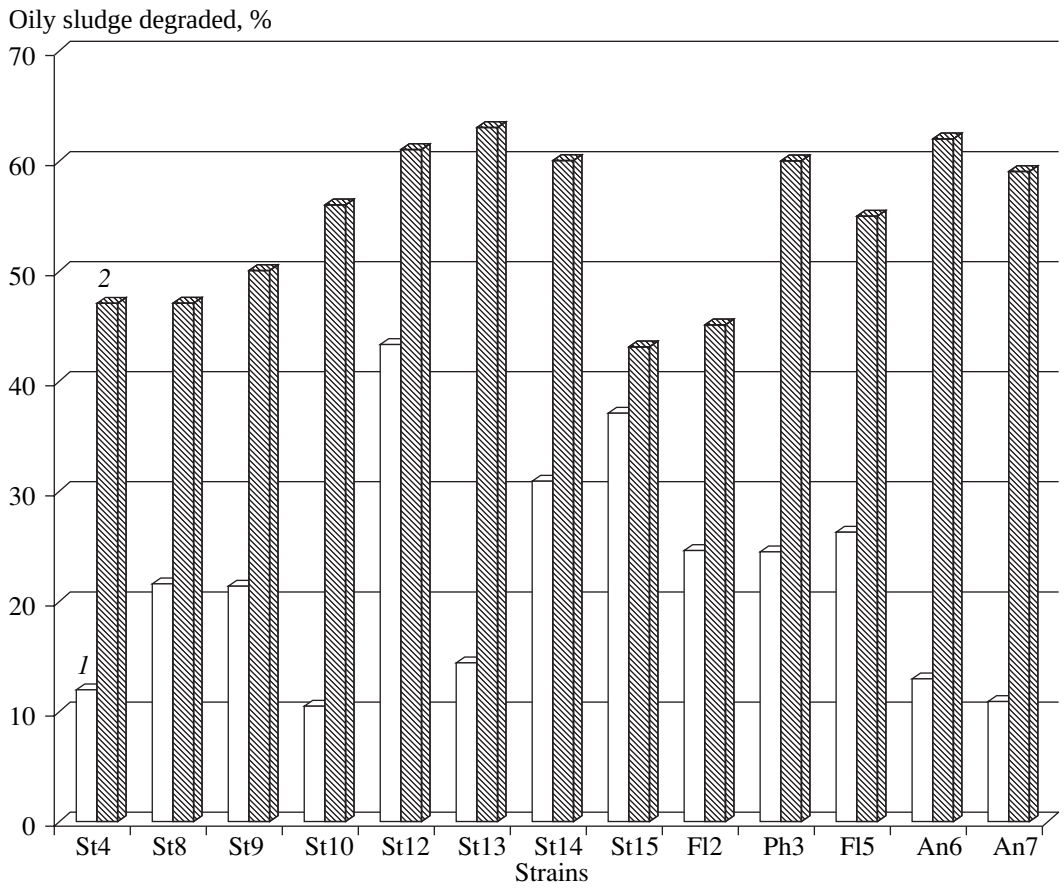

Fig. 3. The degradation of (*1*) the chloroform-extractable components of oily sludge and (*2*) the sum of ten major PAHs for the most active isolates.

data presented in Fig. 2 refer to site A, and the data for sites B and C are similar). This observation was taken into account when counting bacteria in oily sludge.

The microscopic test of Kogure *et al.* [16], which is able to distinguish viable and nonviable microbial cells, showed that incubation in the presence of an easily metabolizable substrate and nalidixic acid (an inhibitor of cell division) gave rise to anomalously large (i.e., actively growing) cells, whose number tended to increase in the course of 20-h incubation relative to the number of nongrowing (i.e., either resting or dead) cells. The percentage of viable cells estimated by this test was found to be about 95%.

Experimental data on the vertical distribution of various groups of microorganisms in the oily sludge deposit are presented in the table. It is evident that the populations of actinomycetes and micromycetes in the deposit were four to six orders smaller than that of heterotrophic bacteria and varied insignificantly with depth and sampling site (from $(5.6 \pm 3.5) \times 10^3$ to $(8.2 \pm 2.9) \times 10^4$ CFU/g sludge).

The number of spore-forming bacteria varied from $(1.1 \pm 0.4) \times 10^8$ in the subsurface horizon (0.2 m) to $(3.0 \pm 1.9) \times 10^5$ CFU/g in the bottom horizon (3 m).

The number of facultatively anaerobic bacteria capable of the dissimilatory reduction of nitrates was almost the same (about 10^7 cells/g) in all of the oily sludge horizons.

The amount of sulfate-reducing obligate anaerobes (the least abundant bacteria in the oily sludge deposit) in the bottom horizon $(9.5 \pm 0.1) \times 10^3$ cells/g was an order of magnitude greater than in the subsurface horizon.

Most of the heterotrophic bacteria had an optimal growth temperature of 28°C. The number of psychrotolerant bacteria capable of growing at 7°C was much smaller (in the case of the bottom horizon, by three orders) than the number of thermotolerant bacteria capable of growing at 40°C (table).

Oily sludge (more specifically, its aqueous phase) is, presumably, an osmotically active medium, since, in spite of its low mineral content (as is evident from its low electric conductivity equal to 0.4 S/m), the osmolarity of oily sludge must be significant due to the high content (up to 607 g/kg sludge) of organic substances. For this reason, we determined the content of osmotolerant heterotrophic bacteria using nutrient agar supplemented with NaCl as an osmoticum [19]. The number

of microorganisms tolerant to 3% NaCl at depths of 0.2, 1, and 3 m was found to be $(1.3 \pm 1.3) \times 10^9$, $(3.3 \pm 2.5) \times 10^7$, and $(2.7 \pm 1.2) \times 10^5$ CFU/g sludge, respectively. A similar tendency was observed for bacteria capable of growing at 5% NaCl (table). The number of bacteria tolerant to 10% NaCl varied from 10^2 to 10^5 CFU/g and was considerably smaller in deeper sludge horizons. These tendencies can be accounted for either by the limited osmotolerance of oily sludge–inhabiting microorganisms (like the limited osmotolerance of oil-degrading nocardioform bacteria [20]) or by a reduction in the general stress tolerance of microbial cells with depth (and, hence, the age of oily sludge). The latter is confirmed by the similar vertical dynamics of different groups of microorganisms in the oily sludge deposit.

The total metabolic potential of the sludge microflora was evaluated from its ability to utilize oily sludge as the sole source of carbon and energy. Experiments showed that the growth rate and morphology of colonies grown on nutrient agar and oily sludge agar considerably differed; however, the number of colonies grown on these two media differed insignificantly (table). The number of oily sludge–degrading bacteria tended to decrease with depth, being equal to $(1.4 \pm 1.0) \times 10^9$, $(3.3 \pm 1.3) \times 10^7$, and $(4.2 \pm 3.3) \times 10^6$ CFU/g sludge at depths of 0.2, 1, and 3 m, respectively.

The study of the ability of 24 isolates of PAH-degrading bacteria to utilize oily sludge as the source of organic nutrition showed that 13 of them utilized the chloroform-extractable substances of oily sludge by 10–40% and the sum of 10 PAHs (mentioned in the Materials and Methods section) by 40–60% in the course of 10-day incubation (Fig. 3).

DISCUSSION

The petrochemical oily sludge remediation strategy can be developed based on knowledge of the amount, physiological state, and metabolic activity of microorganisms inhabiting oily sludge. Bearing in mind that oily sludge had been deposited for many years, samples for analysis were taken at three representative sites at three different depths. The maximum depth of the deposit was found to be about 3 m.

Unexpectedly, all three studied horizons of the sludge deposit (0.2, 1, and 3 m) showed high contents of microbial cells, which, though, tended to decrease with depth. The percentage of viable microbial cells throughout the deposit was as great as 95%. Most microbial cells taken from the middle (1 m deep) and the bottom (3 m deep) layers of the oily sludge deposit displayed the colony-forming ability after a 5- to 7-day delay, suggesting that these cells occurred in a hypometabolic state. This state was reversible (at least in many microbial cells), as is evident from the fact that microbial strains isolated from slow-growing colonies showed normal growth rates after 2–3 transfers to fresh nutrient media capable of maintaining their growth.

In view of the above, the microbial communities of oily sludge very likely have evolved as follows: Some microorganisms could actively grow in the uppermost layer of oily sludge due to the high content of easily metabolizable sludge compounds, sufficient aeration, and the presence of atmospheric moisture. With the next discharge of oily sludge, the superficial layer with developed microflora turned out to be buried below the new layer of oily sludge. Ecological conditions in the buried layer (primarily, aeration, moisture, osmotic, and temperature conditions) became adversely altered. For this reason, the microbiological status of mature oily sludge (including the bottom layers of the deposit with an age of 35 to 40 years) is determined by the high capability of its microorganisms for long-term (over tens of years) survival under extreme conditions.

In view of the specific character of oily sludge as a habitat of microorganisms, of great interest are data on their salt tolerance, their ability to grow at temperatures within the interval of seasonal temperature variations, and the capability of heterotrophic bacteria for anaerobic respiration with nitrate as the terminal electron acceptor.

The mineral nitrogen of oily sludge is mainly found in the form of ammonia, indicating that nitrification processes are blocked at the first, aerobic, stage of the transformation of oily sludge soon after its deposition. Under these conditions, the potential activity of denitrifying bacteria cannot be fully implemented. Although the anoxic conditions of the buried oily sludge horizons are favorable for sulfate reduction, the high potential of sulfate reducers present in oily sludge cannot be implemented either, because of the low content of sulfates (3–12 mg/kg sludge) and unfavorable conditions for the growth of aerobes and anaerobic acido-/acetogenic bacteria responsible for the generation of easily metabolizable organic substrates. The latter speculation agrees with the finding that oily sludge contains C_5 through C_{16} acids, whereas lower homologues are absent [6].

The hypometabolic state of most microorganisms present in oily sludge is due to unfavorable physicochemical conditions (such as an anoxic and hydrophobic environment) and the absence of alternative electron acceptors.

The data obtained in this study show that most microorganisms inhabiting oily sludge over long periods of time (tens of years) remained viable and metabolically active in utilizing not only easily metabolizable substrates but also the recalcitrant PAH components of oily sludge.

It should be noted that the water-soluble components of petroleum wastes are quite easily oxidized under the conditions of the biological treatment of waste waters [1], whereas PAHs are a matter of great concern because of the recalcitrance, mutagenicity, and

carcinogenicity of many of them [11]. Most of the microbial degraders isolated by us showed a high capability for utilizing these hazardous compounds.

Thus, the occurrence of highly adaptable microorganisms with an adequate metabolic potential in oily sludge deposits implies that their bioremediation is possible without introducing special microorganisms.

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