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EXPERIMENTAL ARTICLES

Effect of Temperature on the Rate of Oxidation of Pyrrhotite-Rich Sulfide Ore Flotation Concentrate and the Structure of the Acidophilic Chemolithotrophic Microbial Community

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Abstract—Oxidation of flotation concentrate of a pyrrhotite-rich sulfide ore by acidophilic chemolithoautotrophic microbial communities at 35, 40, and 45°C was investigated. According to the physicochemical parameters of the liquid phase of the pulp, as well as the results of analysis of the solid residue after biooxidation and cyanidation, the community developed at 40°C exhibited the highest rate of oxidation. The degree of gold recovery at 35, 40, and 45°C was 89.34, 94.59, and 83.25%, respectively. At 40°C, the highest number of microbial cells (6.01×10^9 cells/mL) was observed. While temperature had very little effect on the species composition of microbial communities (except for the absence of *Leptospirillum ferriphilum* at 35°C), the shares of individual species in the communities varied with temperature. Relatively high numbers of *Sulfobacillus thermosulfidooxidans*, the organism oxidizing iron and elemental sulfur at higher rates than other acidophilic chemolithotrophic species, were observed at 40°C.

Keywords: flotation concentrate, biooxidation, temperature, pH and Eh of the medium, Fe^{3+}, Fe^{2+}, As_{total}, degree of oxidation of sulfide minerals, degree of gold recovery, microbial community, species composition

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Compared to roasting and autoclave leaching, biohydrometallurgy is the simplest, most efficient, and environmentally friendly method for processing gold-containing flotation concentrates of sulfide ores, which have been successfully used in industry for almost thirty years [1]. This technology is based on the oxidation of sulfide minerals by communities of acidophilic chemolithotrophic microorganisms, which utilize sulfide minerals, sulfur, or reduced sulfur compounds as energy substrate and CO_2 for constructive metabolism. Long duration of the process (6 to 10 days, depending on the concentrate composition) is the main shortcoming of the modern biohydrometallurgical technologies for gold recovery. Incomplete oxidation of elemental sulfur and antimony-containing sulfide minerals may also lead to gold loss and high NaCN expenditure during the cyanidation stage. Accumulation of elemental sulfur in the solid phase (up to 3–7%) results from high pyrrhotite content in some concentrates. Increased rates of elemental sulfur oxidation are therefore important for improvement of the biohydrometallurgical technology.

The species and strain diversity of acidophilic chemolithotrophic microorganisms (ACM) involved in the oxidation of sulfide minerals in biogeotechnological processes, apart from its theoretical importance, may also result in higher rate and efficiency of recovery of noble and nonferrous metals from sulfide ores and concentrates.

Our previous studies revealed some patterns of formation of ACM communities [2]. Modification of the technological conditions of biooxidation of sulfide ore flotation concentrates was shown to result in adaptive changes in the composition of microbial communities. For example, in the case of semicontinuous cultivation in a line of laboratory reactors oxidizing high-pyrrhotite pyrite–arsenopyrite gold–arsenic sulfide ore concentrate, an increase in the temperature from 40 to 50°C resulted in the shift of the dominant species from *Acidiferrobacter thiooxydans*, *Leptospirillum ferriphilum*, and *Ferroplasma acidiphilum* to *Sulfobacillus thermosulfidooxidans* strains HT-1 and HT-3 [3]. The composition of the mineral substrate may be affecting the composition of microbial communities [4]. A change in the composition of the microbial community during the stage of biooxidation was also observed when the concentrate was pretreated at 80°C with Fe^{3+}-containing culture liquid in order to oxidize pyrrhotite with formation of elemental sulfur [5]. *S. thermosulfi-*

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dooxidans HT-4 dominated in the resultant community. Changes in the species composition and ratios of the species were observed in microbial communities after shifting to oxidation of a different substrate [4].

The goal of the present work was to determine the effect of temperature on the rate of bacterio–chemical oxidation (BO) of high-pyrrhotite flotation concentrate and the degree of elemental sulfur oxidation, as well as the species composition of the microbial community in continuous culture.

MATERIALS AND METHODS

Subject of study. Pyrite–arsenopyrite flotation concentrate of gold–arsenic ore with high pyrrhotite content (30–35%) was the subject of investigation. The concentrate contained 32.4% Fe_{total}, 25.76% Fe_{sulf}, 8.45% As_{total}, 6.69% As_{sulf}, 23.5% S_{total}, 22.6% S_{sulf}, 0.7% S^0, 2.67% Sb_{total}, and 3.28% Ca_{total}. Sulfide minerals included pyrrhotite (30–35%), arsenopyrite (9–11%), pyrite (6–8%), and antimonite (2–3%). Other minerals were quartz (15–20%), mica (over 10%), magnetite (8–9%), and calcite (6–8%). Gold content was 58–64 g/t.

A community of acidophilic chemolithotrophic microorganisms with high rates of S^0 oxidation selected in the course of optimization of biooxidation of flotation concentrate in a line of three laboratory reactors under semicontinuous cultivation conditions [6] was used as inoculum. The dominant bacteria and archaea identified in this community were *Acidithiobacillus caldus* OP-1 and *F. acidiphilum* OP-2. Other microorganisms detected in the community were *Acidithiobacillus ferrooxidans* OP-3, *L. ferriphilum* OP-4, and *S. thermosulfidooxidans* OP-5.

Experimental conditions. Experiments were carried out in continuous culture in a line of four reactors (2 L working volume each) under agitation (450 rpm) and aeration (6.5–7.5 L/min) at temperature of 35 ± 1, 40 ± 1 or 45 ± 1°C and the 1 : 5 ratio of the pulp solid and liquid phase. Mass exchange was carried out by transferring 1600 mL of the pulp every 24 h at the process duration of 120 h and flow rate of 0.008 h^{-1}. The following parameters were monitored: microbial number, pH, Eh, and the concentrations of Fe^{3+}/Fe^{2+} and As_{total}.

Analytical methods. The values of pH and Eh were measured with a pH-150MA pH meter–millivoltmeter (Belarus); Eh was expressed relative to the normal hydrogen electrode. Fe^{3+} and Fe^{2+} concentrations in the pulp liquid phase were determined by trilonometric titration [7], As_{total} concentration was determined by iodometric titration [8].

The content of sulfide minerals in procession products of the gold-containing flotation concentrate (biocakes) was determined by fluorescent X-ray radiometric analysis after washing the solid phase with 5% HCl for 24 h at 30°C [9]. Gold content in the solid phase was determined by assay analysis. The degree of gold recovery was determined by sorption cyanidation of the residues after bacterial–chemical leaching. Cyanidation was carried out with 0.1% NaCN solution at pulp density 30% (wt/vol) and pH 10.2–10.5. Prior to cyanidation, the pulp was purged with air (25 L/h for 14 h). Sorption cyanidation was carried out with 8% sorbent (carbon Norit 3515) for 34 h at room temperature. Gold adsorption on the sorbent was 99–100%.

Investigation of microbial species composition. Microbiological techniques used to investigate the microbial communities included direct cell count under a phase contrast microscope (CX-41, Olympus, Japan) and inoculation of selective media with tenfold dilutions with subsequent incubation at different temperatures [10]. Pure cultures for molecular biological identification were obtained by repeated transfers of the highest growth-positive dilution of the culture liquid into selective media and incubation at a specified temperature.

DNA was isolated from microbial biomass using a modified alkaline hydrolysis method [11]. DNA concentration in the preparation was 30–50 μg/mL. RNA was present in the preparation in trace amounts (<1% according to the results of electrophoretic analysis). For polymerase chain reaction, cloning of the 16S rRNA gene PCR fragments, and sequencing of the clonal inserts, the universal primers (8-27f-1492r) [12] and (8fa-1492r) [13] were used for bacteria and archaea, respectively

In both cases, amplification mixture (50 μL) contained the following: 1× Bio*Taq* DNA polymerase buffer (17 mM $(NH_4)_2SO_4$; 67 mM Tris-HCl, pH 8.8; and 2 mM $MgCl_2$), 12.5 nmol of each dNTP, 50 ng template DNA, 5 pmol of each relevant primer, and 3 U Bio*Taq* DNA polymerase (Dialat, Russia). PCR was carried on a Gradient MasterCyckler (Eppendorf, Germany) as follows: the first cycle 94°C, 9 min; 55°C, 1 min; 72°C, 2 min; 30 cycles: 94°C, 1 min; 55°C, 1 min; 72°C, 2 min; final cycle: 72°C, 7 min. Analysis of PCR products was carried out in 2% low-melt agarose at 6 V/cm. Isolation of PCR products from agarose and their purification were carried out using the Wizard PCR Preps reagent kit (Promega, United States) according to the manufacturer's recommendations. Amplification products were cloned using the pGEM-T System reagent kit (Promega, United States) according to the manufacturer's recommendations.

Sequencing was carried out according to Sanger et al. [14] using the Big Dye Terminator v. 3.1 reagent kit (Applied Biosystems, United States) and an ABI PRIZM 3730 automatic sequencer (Applied Biosys-

Table 1. Physicochemical parameters of the pulp liquid phase in the reactors during the oxidation of flotation concentrate at different temperatures

Temperature, °C	Reactor no.	pH	Eh, mV	Fe^{3+}, g/L	Fe^{2+}, g/L	Fe_{total}, g/L	As_{total}, g/L
35	1	1.65	573	0.5	11.1	11.6	1.82
	2	1.57	620	7.8	21.5	29.3	3.10
	3	1.54	627	11.0	23.5	34.5	4.83
	4	1.56	660	28.0	11.3	39.3	6.20
40	1	1.65	579	0.7	12.6	13.3	1.85
	2	1.38	625	4.8	20.5	25.3	3.15
	3	1.32	640	15.4	23.3	38.7	4.71
	4	1.26	742	36.9	1.3	38.2	6.39
45	1	1.67	585	0.7	9.2	9.9	1.35
	2	1.39	605	1.5	15.5	17.0	1.83
	3	1.39	615	2.5	16.3	18.8	2.30
	4	1.27	633	5.7	15.4	21.1	4.67

tems, United States) according to attached recommendations. Both external and internal primers were used for sequencing; reading was carried out in two directions. Initial analysis of the similarity between the sequences was carried out using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast). The sequences were aligned with the relevant sequences of most closely related bacteria and archaea using the CLUSTALW software package [15]. The sequences were edited using the BioEdit software package (http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html).

The obtained 16S rRNA gene sequences were deposited to GenBank under accession nos. KF356023–KF356026.

RESULTS AND DISCUSSION

Physicochemical parameters of oxidation of flotation concentrate at different temperatures. At 35 and 40°C the rates of sulfide mineral oxidation in reactors 1, 2, and 3 were the same, as can be seen from the concentrations of total iron (11.6, 29.3, 34.5 and 13.3, 25.3, 38.7 g/L, respectively) and total arsenic (1.82, 3.10, 4.83 and 1.85, 3.15, 4.71 g/L, respectively) in the culture liquid (Table 1). In reactor 4, however, the highest rate of Fe^{2+} oxidation was observed by the end of oxidation at 40°C: Fe^{2+} concentration in the culture liquid was 1.3 g/L, 11.3 and 15.4 g/L at 35 and 45°C, respectively. In reactor 4, the highest Eh (742 mV) was observed at 40°C, compared to 660 and 633 mV at 35 and 45°C, respectively. Oxidation of reduced sulfur compounds in reactor 4 was more active at 40°C than at 35°C, with pH decreasing from 1.65 in reactor 1 to 1.26 in reactor 4 at 40°C and from 1.65 to 1.56, at 35°C, respectively. The rate of oxidation of reduced sulfur compounds at 45°C was also high, with pH decreasing from 1.67 in reactor 1 to 1.27 in reactor 4, although the oxidation rates for sulfide minerals and ferrous iron were lower (the lowest concentration of total iron in the culture liquid of 21.1 g/L).

Arsenopyrite oxidation in reactor 4 was more active at 40°C than at 45°C, as may be seen from the total arsenic concentrations (6.39 and 4.67 g/L, respectively). At 35°C the rate of arsenopyrite oxidation was

Table 2. Elemental sulfur content in solid residue after biooxidation of flotation concentrate by microbial communities at different temperatures

Temperature, °C	Reactor no.	S_{elem}
35	1	1.30
	2	1.39
	3	1.58
	4	2.54
40	1	0.79
	2	1.04
	3	1.88
	4	2.24
45	1	0.97
	2	1.06
	3	1.66
	4	2.01

Table 3. Gold content in the products of cyanidation of solid residue from four reactors after biooxidation of flotation concentrate by microbial communities at different temperatures

Temperature, °C	Products	Au content, g/t	Au recovery, %	NaCN consumption, kg/t
35	Tar	55.50	89.34	53.6
	Cyanidation tailings	6.62	10.66	
	Initial biocake	62.12	100.00	
40	Tar	55.21	94.59	52.8
	Cyanidation tailings	3.16	5.41	
	Initial biocake	58.37	100.00	
45	Tar	52.94	83.25	42.8
	Cyanidation tailings	10.65	16.75	
	Initial biocake	63.59	100.00	

the same as at 40°C, and the concentration of residual arsenic in reactor 4 was 6.20 g/L.

Data on the content of elemental sulfur in the solid residue after biooxidation in all reactors at different temperatures are presented in Table 2. Since the microbial community used as inoculum has been previously selected for high rates of elemental sulfur oxidation [6], sulfur content in reactor 4 was low at all temperatures: 2.01, 2.24, and 2.54% at 45, 40, and 35°C, respectively.

The results of cyanidation of the solid residue after biooxidation of the flotation concentrate obtained at the exit from reactor 4 are presented in Table 3. The highest rate of gold recovery (94.59%) was observed after BO at 40°C, resulting in significantly lower gold losses in cyanidation tailings (5.41 g/t).

Physicochemical parameters of the pulp liquid phase and analysis of the solid phase showed that biooxidation of sulfide ore flotation concentrate was more active at 40°C.

Analysis of microbial communities. Our results on the rates of oxidation of pyrrhotite-rich flotation concentrate at different temperatures were confirmed by the data on the numbers and composition of microbial communities developing in the reactors and on the ratio of iron- and sulfur-oxidizing microorganisms.

Direct cell counts and incubation at different temperatures of selective media inoculated with tenfold terminal dilutions were found to supplement each other. Thus, direct count was suitable for rapid assessment of the changes in community structure, since the cells of different morphotypes were present: rods of different size (genera *Acidithiobacillus, Acidiferrobacter*, and *Sulfobacillus*), vibrios or spiral-shaped cells (genus *Leptospirillum*), and disk-shaped archaeal cells (genus *Ferroplasma*). This method, however, did not make it possible to differentiate between living and dead cells, or between *Acidithiobacillus* species. Since *A. ferrooxidans, A. thiooxidans, Acidiferrobacter*, and *Sulfobacillus* are capable of oxidation of ferrous iron and elemental sulfur, the ratio of iron- and sulfur-oxidizing microorganisms in the communities, as well as the isolation of pure cultures, were carried out by inoculating selective media with Fe^{2+} or S^0 with tenfold terminal dilutions with subsequent incubation at different temperatures. This method reveals only the living cells.

Microbial community oxidizing flotation concentrate at 35°C. Abundance of the members of the genera *Acidithiobacillus, Sulfobacillus*, and *Ferroplasma* during biooxidation in the lie of laboratory reactors at 35°C is shown on the figure (a). At this temperature, bacteria of the genus *Acidithiobacillus* and archaea of the genus *Ferroplasma*, oxidizers of ferrous iron and pyrite, played the major role. The numbers of sulfobacilli were 10–100 times lower.

Microbial community oxidizing flotation concentrate at 40°C. At 40°C, bacteria of the genus *Acidithiobacillus* and archaea of the genus *Ferroplasma* prevailed in the community (figure b). The numbers of archaea were stable in all reactors. No sulfobacilli were present in reactors 1 and 2. They were present in reactor 3, while their number increased considerably in

reactor 4 (4.2×10^8 cells/mL), in which leptospirilla were also detected (0.3×10^8 cells/mL).

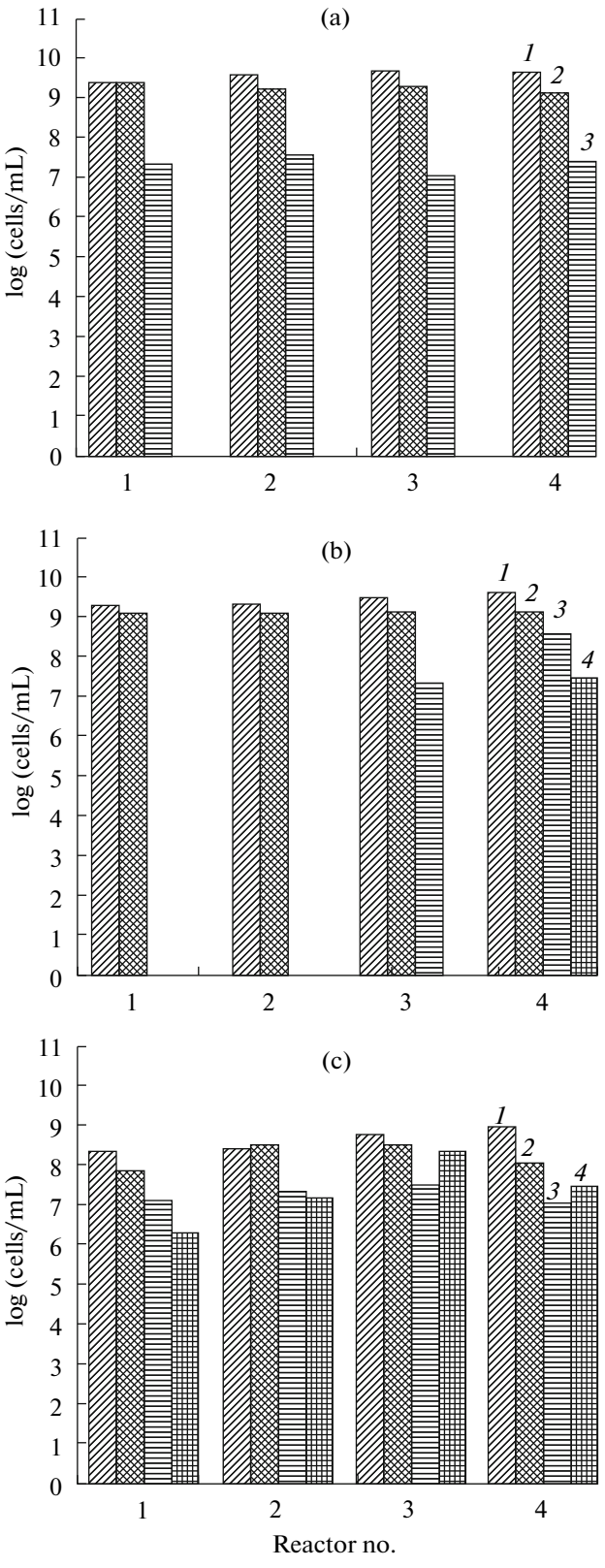
Microbial community oxidizing flotation concentrate at 45°C. Similar to other temperature regimes, during biooxidation at 45°C the cells of acidithiobacilli predominated in the community, although their numbers were considerably less than at other temperatures (8.4×10^8 cells/mL in reactor 4) (figure c). Archaea made up the second most numerous group, while the numbers of sulfobacilli and leptospirilla did not exceed 0.28×10^8 cells/mL.

Composition of the microbial community developed in three laboratory reactors in the course of pyrite concentrate oxidation at 45°C was described in [16]. Abundance of the mixotrophic archaeon *Ferroplasma* was found to be higher in the third reactor than in the previous three, which was attributed to higher concentration of organic compounds in the medium. Similar results were reported for high-temperature communities in the installations of a BIOX® factory [17]. According to our data, the number of mesophilic archaea *Ferroplasma* in the reactors at 45°C was insignificant.

Ratio of iron- and sulfur-oxidizing microorganisms in the communities in the course of oxidation of flotation concentrate at different temperatures. The ratios of sulfur- and iron-oxidizing bacteria in the communities are presented in Table 4. During the oxidation of flotation concentrate at 35°C, the number of sulfur-oxidizers was 7 times higher than that of iron-oxidizers. Pyrrhotite oxidation at this temperature probably resulted in the formation of sulfur, which was actively consumed by microorganisms at the expense of the oxidation of sulfide minerals. In reactors 2 and 3 oxidation of both sulfur and iron occurred, with a certain prevalence of the prokaryotes oxidizing sulfur-containing substrates. Sulfur-oxidizing microorganisms predominated significantly in reactor 4.

The results presented in Table 4 demonstrate that the highest rates of biooxidation of the pyrrhotite-rich flotation concentrate occurred at 40°C. At this temperature, the organisms oxidizing ferrous iron predominated in reactor 1. This finding correlated with the data on Fe^{2+}/Fe^{3+} concentrations, indicating active oxidation of pyrrhotite and pyrite, as well as of the produced ferrous iron. Predominance of sulfur-oxidizers in the community was found in reactor 2. The highest rate of elemental sulfur oxidation was observed in reactor 3, as was evident from the ratio of sulfur- and iron-oxidizing microorganisms. In reactor 4, sulfur-oxidizers were more numerous than iron-oxidizers, although to a lesser degree than in reactor 3.

The oxidation of flotation concentrate at 45°C resulted in a considerably higher abundance of sulfur-



Number of microorganisms in reactors at different temperatures during the oxidation of flotation concentrate: 35 (a), 40 (b), and 45°C (c); *Acidithiobacillus* sp. (*1*), *Ferroplasma* sp. (*2*), *Sulfobacillus* sp. (*3*), and *Leptospirillum* sp. (*4*).

Table 4. Ratios of sulfur- and iron-oxidizing microorganisms in reactors at different temperatures of flotation concentrate oxidation

Temperature, °C	Reactor no.	Ratio of S- and Fe-oxidizing microorganisms
35	1	7.07
	2	1.41
	3	1.45
	4	5.12
40	1	0.3
	2	1.69
	3	7.62
	4	3.15
45	1	16.8
	2	12.3
	3	4.5
	4	0.6

oxidizers, compared to iron-oxidizers, probably due to the presence of *A. caldus*, which has an optimum for growth and elemental sulfur oxidation at this temperature. Oxidation of sulfide minerals with formation of ferrous iron occurred at a low rate. Only in reactor 4 the number of iron-oxidizers exceeded that of sulfur-oxidizers.

The data on total microbial numbers in the communities and on the abundance of their individual groups in reactor 4 during the oxidation of flotation concentrate at different temperatures are presented in Table 5. The highest cell number (60.1×10^8 cells/mL) was observed at 40°C. Under these conditions, the number of sulfobacilli was an order of magnitude higher than in the communities incubated at 35 or 45°C. Sulfobacilli are able to oxidize sulfide minerals, iron, and various sulfur species at higher rates than members of other groups of acidophilic chemolithotrophic microorganisms [18]. Their high abundance in the community at 40°C therefore resulted in higher oxidation rates of flotation concentrate. The lowest cell number (9.88×10^8 cells/mL) was found in the reactors operating at 45°C; the numbers of *Acidithiobacillus* species were relatively high. A decrease in the operation temperature to 35°C resulted in the loss of iron-oxidizing leptospirilla.

Identification of the dominant microorganisms in the communities oxidizing flotation concentrate at 40°C. Strain OL12-3 oxidizing elemental sulfur and not oxidizing iron was isolated from the pulp of reactor 4 operating at 40°C by tenfold terminal dilutions. Analysis of the nucleotide sequence of its 16S rRNA gene made it possible to identify it as an *A. caldus* strain (99% similarity to *A. caldus* SM-1). Two bacterial strains (OL12-1 and OL12-2) and one archaeal strain (OL12-4) were also isolated from the pulp of reactor 4. Analysis of the 16S rRNA gene sequences made it possible to identify strain OL12-1 as *S. thermosulfidooxidans* (99% similarity to *S. thermosulfidooxidans* DSM 9293^T), strain OL12-2 as *L. ferriphilum* (99% similarity to *L. ferriphilum* ATCC 49881^T), and strain OL12-4 as *F. acidiphilum* (99.5% similarity to *F. acidiphilum* Y^T).

Our results show that the species composition of microbial communities carrying out biooxidation of flotation concentrate at the studied temperatures did not change significantly. Only the loss of the moderately thermophilic *L. ferriphilum* at 35°C should be noted. The ratio of the species in the communities varied significantly, which affected the rates of the substrate oxidation. Higher rate of biooxidation of flotation concentrate at 40°C was shown.

Table 5. Total numbers of microorganisms and abundance of different microbial groups in four reactors at different temperatures (cells/mL × 10^8/relative abundance, %)

Temperature, °C	Total number, cells/mL × 10^8	*Acidithiobacillus* sp.	*Ferroplasma* sp.	*Sulfobacillus* sp.	*Leptospirillum* sp.
35	53.64	40.40/75.32	13.0/24.23	0.24/0.45	0
40	60.10	41.60/69.22	14.0/23.29	4.20/6.99	0.30/0.50
45	9.88	8.40/85.02	1.10/11.13	0.10/1.01	0.28/2.83

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