Continuous Bioleaching of Arsenic-Containing Copper-Zinc Concentrate and Shift of Microbial Population under Various Conditions

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Abstract: The goal of this work was to study the bioleaching of arsenic-containing polymetallic concentrate that contained 6.2% Cu, 7.3% Zn and 1.7% As, depending on different temperatures and in the presence of CO2 and molasses in the medium, as well as the difference in the composition of microbial population formed under various conditions. A mixed population of moderately thermostable and thermotolerant acidophilic microorganisms formed during the continuous bioleaching of copper concentrate was used as an inoculum. The experiments were carried out in a continuous mode in laboratory scale reactors, with a temperature range of 40 °C to 60 °C. To assess the effect of CO2 and molasses on metal leaching and microbial population composition, the experiments were carried out in three reactors: CO2 (~0.01 L/min) was supplied into the first reactor; 0.02% molasses were added to the pulp of the second reactor; and no additional carbon sources were supplied into the control reactor. The highest copper recovery (27%) was achieved at 50 °C in the experiment with molasses, while the highest zinc recovery (82.1%) was reached at 45 °C in the control experiment. Additional carbon sources affected the extraction of non-ferrous metals only at 60 °C and increased the extraction of copper and zinc by 12.6% and 24.2%, respectively. Both the temperature and carbon source used affected the microbial population composition. The main microbial genera revealed in the populations by next generation sequencing (NGS) were bacteria of the genera Sulfobacillus and Acidithiobacillus, as well as archaea of the genera Ferroplasma, Acidiplasma, and Cuniculiplasma. At low temperatures (40 and 45 °C), Acidithiobacillus, Sulfobacillus, and Ferroplasma predominated, while at temperatures 50–55 °C, the decrease in relative abundance of these genera occurred, and the predominance of Acidiplasma archaea was observed. The usage of both CO2 and molasses led to the increase in Sulfobacillus and Acidiplasma in relative abundance.

Keywords: bioleaching; acidophiles; tennantite; chalcopyrite; sphalerite; polymetallic concentrates; microbial population analysis

1. Introduction

Currently, the extraction of non-ferrous metals, in particular copper, from sulfide ores is carried out mainly using pyrometallurgical technologies. To obtain high grade concentrates appropriate for further pyrometallurgical processing, ores are subjected to flotation to concentrate copper and/or other valuable metals (zinc, lead, nickel), and the resulting concentrates are then processed by roasting, smelting and converting. High-grade concentrates obtained from sulfide ores usually contain chalcopyrite (CuFeS2), the most widespread copper-bearing sulfide mineral, and do not contain large amounts of...
impurities, and therefore can be efficiently processed using pyrometallurgy approaches without high environmental risks [1–3]. At the same time, the composition of some sulfide copper-zinc ores either does not allow the production of concentrates conditioned for pyrometallurgical processing, or forces the processing of ores at an additional cost. For example, concentrates with a high content of arsenic, obtained from sulfide ores containing arsenic minerals, including tennantite (Cu$_{12}$As$_4$S$_{13}$) and enargite (Cu$_3$AsS$_4$), require the application of special gas purification systems to avoid the emission of highly toxic volatile As$_2$O$_3$ and As$_4$O$_6$, which makes their processing less cost-effective [4,5]. Reducing arsenic content in the concentrate by removing arsenic-containing sulfides in the process of flotation lead to a decrease in the content of copper, i.e., target component. In addition, some polymetallic ores with a high content of arsenic cannot be effectively processed due to the fine-grained intergrowth of copper and zinc minerals with pyrite [6]. Technologies based on pressure oxidation (POX) [4,7,8] and sulfide alkaline leaching [4,9–14] may be used to remove arsenic from copper concentrates. However, POX requires comparatively high capital and operating expenditure and is usually used in practice for gold-bearing sulfide concentrates treatment [15]. Sulfide alkaline leaching with Na$_2$S solutions is successfully applied to remove arsenic from minerals such as enargite and convert it to a liquid phase to obtain concentrate without arsenic, which may be processed using pyrometallurgical approaches [4,9–14]. Thus, this process makes it possible to extract undesirable arsenic from the concentrate, although it does not allow the removal of other impurities, including zinc.

Therefore, the development and commercialization of novel approaches for processing polymetallic copper-containing ores with different impurities impeding copper extraction (i.e., zinc, arsenic, antimony) are promising. These methods include different hydrometallurgical technologies, for example, biohydrometallurgy.

Currently, biorecovery technologies are widely used in the non-ferrous metallurgy for the processing of copper ores: about 5% of the global Cu production is provided by heap bioleaching [16,17]. Heap biooxidation can also be used to deliberate gold in pyrite-arsenopyrite ore, which is unsuitable for obtaining a standard concentrate [18]. Tank (reactor) leaching is not economically viable for the processing of low-grade non-ferrous metal ores; therefore, this technology is widely used for gold-bearing concentrate treatment. However, reactor bioleaching can also be successfully used for the processing of sulfide concentrates containing Cu and Zn [19] as well as other non-ferrous metals [20,21]. Thus, biohydrometallurgical technologies may be successfully used to extract copper from different ores and concentrates, including those containing an excess of zinc [19].

Sulfide minerals biooxidation both in natural ecosystems and in industry (in heaps and bioleach reactors) is carried out by the communities of acidophilic sulfur and iron oxidizers belonging to different phyla of bacteria and archaea [17,22,23]. The composition of the microbial community is affected by different environmental factors, including the mineral composition of oxidized ores and concentrates and physicochemical parameters of the biooxidation process. Since sulfide mineral biooxidation is exothermic, bioleaching processes in industrial-scale heaps and reactors are accompanied by a temperature increase in the bioleach reactor pulp, which leads to the dominance of moderately thermophilic and thermotolerant microorganisms in microbial populations [24–26]. It is known that representatives of the bacterial genera Acidithiobacillus and Sulfobacillus and the archaea Acidiplasma and Ferroplasma predominate in such microbial communities [27–31]. For technological process performance, it is important that acidophilic microorganisms, which are used for the biohydrometallurgical processing of sulfide ores and concentrates, have different physiological properties and allow adaptation to a wide range of conditions. This makes it possible to develop novel, more efficient processes for the treatment of various sulfide ores and concentrates, which requires preliminary laboratory testing to determine optimal conditions.

The purpose of this work was to study the process of reactor bioleaching of arsenic-containing copper-zinc concentrate by a mixed population of acidophilic moderate thermophiles and thermotolerant microorganisms in a continuous mode at various temper-
atures (40–60 °C) and in the presence of various additional carbon sources (CO₂ and molasses) to assess the effect of different conditions on metal and arsenic bioleaching and on microbial populations formed during long-term experiments.

It is well-known that the chalcopyrite bioleaching rate is significantly affected by the temperature [32–34], while the data on the effect of temperature on the bioleaching of arsenic-bearing minerals (enargite, tennantite) [34,35] are controversial. Therefore, a study of the dependence of copper bioleaching from the concentrate containing chalcopyrite and tennantite on temperature is of interest for bioleaching optimization. At the same time, our previous works have shown that different carbon sources may affect biooxidation pyrite-arsenopyrite concentrates at an elevated temperature [36,37]. Therefore, it was important to determine the influence of carbon sources on copper concentrate bioleaching, since it may also increase copper and zinc bioleaching rates.

A study of the shift of the microbial population composition formed in bioleach reactors during long-term experiments caused by different conditions will contribute towards an understanding of the importance of different groups of microorganisms in the bioleaching of various sulfide concentrates.

2. Materials and Methods

2.1. Flotation Concentrate

Concentrate was provided by Ural Mining Metallurgical Company (Verknaya Pyshma, Russia). A mixture of copper-zinc ores from deposits located in the Ural Region (Russia) was used to obtain the flotation concentrate. The main element of content in the concentrate was determined by using a phase analysis method [38], as shown in Table 1. Chalcopyrite (CuFeS₂), tennantite (Cu₁₂As₄S₁₃), sphalerite (ZnS) and pyrite (FeS₂) were the main minerals of the concentrate (Table S1) according to an XRD analysis performed using a DRON-2 diffractometer (Burevestnik, St. Petersburg, Russia).

| Element | Content, % |
|---------|------------|
| As      | 1.7        |
| Fe      | 24.4       |
| Cu      | 6.2        |
| Zn      | 7.3        |
| S_total | 35.1       |
| S sulfate | 2.6       |
| S⁰      | 0.6        |
| S sulfide | 31.9      |

A mixed population of moderately thermophilic and thermotolerant acidophilic microorganisms with a predominance of the bacterial species Acidithiobacillus caldus and Sulfobacillus benefaciens and archaea Ferroplasma acidiphilum and Cuniculiplasma divulgatum, and containing minor species, bacteria Leptospirillum ferriphilum and archaea Acidiplasma sp., was used as an inoculum; its composition was determined using the sequencing of 16S rRNA gene V3–V4 regions [31]. As this population was formed during a long-term biooxidation of similar concentrate, its biomass was used in the present study without preliminary adaptation. Cells of the mixed population were separated from the liquid phase of the pulp [31] by centrifugation in 500-mL tubes (9500 rpm, 15 min) using Sigma 4–15 centrifuge (Sigma, Osterode am Harz, Germany), resuspended in a mineral medium, and inoculated in the reactors in such a way that the initial total cell number in the pulp was about 1 × 10⁸ cells/mL.

2.2. Experimental Setup and Biooxidation

Concentrate bioleaching was performed in continuous mode in a single-stage laboratory reactors with a working volume of 1 L under the following conditions: stirring rate—500 rpm, aeration rate—5 L/min, temperature at different modes was 40, 45, 50, 55, and 60 °C, the pulp density (solid to liquid ratio, S: L) was 1: 10 (100 g of the concentrate per 1000 mL of liquid medium), the residence time was 10 days. At each temperature, biooxidation was performed in continuous mode for at least 25 days. Required temperature in was maintained using TW-2.03 circulating water baths (Elmi, Riga, Latvia) and U-shaped
titanium heat exchangers; RW20 overhead stirrers (IKA, Staufen, Germany) were used for stirring.

We used liquid mineral nutrient medium, which was applied in our previous work [36,37], containing the following salts (g/L): (NH$_4$)$_2$SO$_4$–0.750, KCl—0.050, MgSO$_4$ × 7H$_2$O—0.125, K$_2$HPO$_4$–0.125, distilled water–1.0 L. The pH of the pulp was adjusted by adding either concentrated sulfuric acid or calcium carbonate to the medium. Initial pH of the medium was 1.5.

Experiments with additional carbon sources was performed as described in the work [37]. Control experiment was performed without additional carbon sources and the sole carbon source for microorganisms was CO$_2$ supplied with air. To assess effects of carbon dioxide and organic nutrients on bioleaching, CO$_2$ was fed into the pulp of the first reactor (approximately 0.01 L/min, i.e., approximately five times greater than carbon dioxide supplied with air in the control) and molasses (KDF, Moscow, Russia) (final concentration in the pulp was of 0.02% wt/vol by dry weight) was added to the pulp of the second one. The molasses was added in the form of 20% (wt/vol by dry weight) solution, which was sterilized using 0.22 µm membrane filter (Merck, Darmstadt, Germany).

The amounts of supply of carbon sources were based on the results of previous works, as it was shown that in these amounts both carbon dioxide and molasses affected biooxidation of pyrite-arsenopyrite concentrations [36,37].

2.3. Sampling and Analysis

The pH and redox potential (Eh) levels were measured using pH-150MI pH meter (Izmeritel’naya Tekhnika, Moscow, Russia). Ferrous and ferric iron and arsenic concentration were determined by trilonometric and iodometric titration, respectively [39,40]. Copper, zinc, and arsenic concentrations in reactor pulp were determined using a Perkin Elmer 3100 flame atomic absorption spectrometer (Perkin Elmer, Waltham, MA, USA). The degrees extraction of copper, zinc, and arsenic from concentrate were calculated by the concentration of Cu, Zn, and As ions in liquid phase.

The solid residues of biooxidation were separated from the liquid phase, dried, and analyzed to determine element content. Pulp samples to obtain solid residues were collected over several days when steady state conditions were reached. Averaged sample of the solid residue obtained in each experiment was used for the further analysis. Determination of the content of iron, arsenic, and sulfur was carried out using phase analysis methods [38].

2.4. Microbial Population Analysis

The composition of microbial populations that formed during the experiments under different conditions was performed by the metabarcoding of 16S rRNA gene fragment using the MiSeq system (Illumina, San Diego, CA, USA) as described in the works [36,37]. Samples for the analysis of microbial populations were collected twice at the end of each experiment and averaged results are shown.

To perform the analysis, biomass from the liquid phase of the pulp was collected using an Allegra X-22 centrifuge (Beckman Coulter, Brea, CA, USA). To separate the biomass from the pulp sample, the solid phase was first separated by centrifugation at 1000 rpm, then the biomass was precipitated from the supernatant by centrifugation at 9500 rpm (9299 g). The biomass preparation, DNA isolation, and library preparation based on the V3–V4 region of the 16S rRNA gene, with amplicon preparation and sequencing using the MiSeq system (Illumina, San Diego, CA, USA) was being performed as described previously in the articles [36,37,41]. Amplicons were obtained with the forward primer (5′-CAAGCAGAAGACGGCATACGATGTAGTCTCGAGGAGATGTCTTCTCTTCTGAT CTXXXXXXXAAGXXXXXXXXZCCTgyGJGDBGCSACAG-3′) consisting of “5′ Illumina Linker Sequence”, “Index 1”, “Heterogeneity Spacer” and the Pro-mod-341F primer sequence, respectively, as well as the reverse primer (5′-AATGATACGGCGACCACACCAGATCTACACT CTTCCTACAGCCAGCTCTTCCGATCTXXXXXXXAAGXXXXXXXXZZZZGACTACNVGGGTMT CTAATCC-3′) consisting of “3′ Illumina Linker Sequence”, “Index 2”, “Heterogeneity
“Spacer” and the Pro-mod-805R primer sequence, respectively. Sequencing was performed with the MiSeq System (Illumina, San Diego, CA, USA). Subsequent sequence processing and analysis were also performed in QIIME ver. 1.9.1 [42], USEARCH version 6.1544 [43], and the Silva 123 version reference base of 16S rRNA reads [44]. On average, 10,144 fragments were analyzed for each sample.

Samples from each experiment were collected in duplicate and analyzed separately. The average results obtained in each experiment are presented in the article.

2.5. Data Processing

MS 15.0.459.1506 Excel 2013 software (Microsoft, Redmond, WA, USA) was used to process the results obtained. The average values of the parameters are presented.

3. Results

3.1. Concentrate Bioleaching

Data obtained in experiments on the bioleaching of arsenic-containing polymetallic concentrate are shown in Tables 2, 3, S2 and S3 as well as in Figure 1.

![Figure 1. As, Cu, and Zn extraction (%) during biooxidation.](image-url)
Table 2. Parameters (pH, Eh, ferric and ferrous ion concentration) of the liquid phase of the pulp at the end of the experiment (steady state conditions).

| T, °C | Carbon Source | pH   | Eh, mV   | Concentration, g/L | Fe³⁺ | Fe²⁺ |
|-------|---------------|------|----------|---------------------|------|------|
| 40    | CO₂           | 1.08 ± 0.03 | 776 ± 12 | 16.32 ± 0.50 | 0.04 ± 0.06 |
|       | Molasses      | 1.04 ± 0.02 | 778 ± 9 | 15.51 ± 0.55 | 0   |
|       | Aeration (control) | 1.05 ± 0.01 | 773 ± 9 | 15.18 ± 0.54 | 0   |
| 45    | CO₂           | 1.04 ± 0.03 | 802 ± 29 | 18.70 ± 0.76 | 0.03 ± 0.06 |
|       | Molasses      | 1.08 ± 0.04 | 804 ± 26 | 15.76 ± 0.29 | 0   |
|       | Aeration (control) | 1.05 ± 0.05 | 792 ± 35 | 15.79 ± 0.27 | 0.06 ± 0.08 |
| 50    | CO₂           | 1.04 ± 0.04 | 805 ± 10 | 17.56 ± 0.67 | 0.06 ± 0.08 |
|       | Molasses      | 1.02 ± 0.07 | 800 ± 4 | 14.64 ± 0.13 | 0.14 ± 0.00 |
|       | Aeration (control) | 0.97 ± 0.08 | 802 ± 8 | 16.13 ± 0.51 | 0.03 ± 0.06 |
| 55    | CO₂           | 1.26 ± 0.03 | 832 ± 5 | 9.59 ± 0.54 | 0.35 ± 0.08 |
|       | Molasses      | 1.37 ± 0.04 | 804 ± 4 | 4.97 ± 0.18 | 1.54 ± 0.11 |
|       | Aeration (control) | 1.91 ± 0.22 | 760 ± 28 | 1.12 ± 0.57 | 3.22 ± 0.00 |
| 60    | CO₂           | 2.34 ± 0.07 | 580 ± 10 | 0.06 ± 0.02 | 1.10 ± 0.46 |
|       | Molasses      | 2.52 ± 0.07 | 573 ± 9 | 0.09 ± 0.09 | 0.28 ± 0.04 |
|       | Aeration (control) | 2.52 ± 0.04 | 572 ± 5 | 0.11 ± 0.06 | 0.66 ± 0.18 |

Table 3. Parameters (As, Cu, and Zn concentrations) of the liquid phase of the pulp at the end of the experiment (steady state conditions).

| T, °C | Carbon Source | As  | Cu²⁺ | Zn²⁺ |
|-------|---------------|-----|------|------|
| 40    | CO₂           | 0.42 ± 0.03 | 1.21 ± 0.05 | 5.65 ± 0.26 |
|       | Molasses      | 0.41 ± 0.01 | 1.25 ± 0.02 | 5.83 ± 0.12 |
|       | Aeration (control) | 0.42 ± 0.00 | 1.10 ± 0.04 | 5.80 ± 0.02 |
| 45    | CO₂           | 0.46 ± 0.02 | 1.52 ± 0.11 | 5.74 ± 0.12 |
|       | Molasses      | 0.44 ± 0.02 | 1.62 ± 0.08 | 5.76 ± 0.14 |
|       | Aeration (control) | 0.43 ± 0.03 | 1.43 ± 0.08 | 5.99 ± 0.13 |
| 50    | CO₂           | 0.48 ± 0.01 | 1.58 ± 0.09 | 5.19 ± 0.37 |
|       | Molasses      | 0.44 ± 0.03 | 1.67 ± 0.07 | 4.94 ± 0.26 |
|       | Aeration (control) | 0.46 ± 0.01 | 1.48 ± 0.35 | 4.97 ± 1.23 |
| 55    | CO₂           | 0.34 ± 0.04 | 1.38 ± 0.06 | 5.14 ± 0.28 |
|       | Molasses      | 0.24 ± 0.03 | 1.25 ± 0.06 | 4.89 ± 0.36 |
|       | Aeration (control) | 0.09 ± 0.13 | 1.35 ± 0.04 | 5.15 ± 0.11 |
| 60    | CO₂           | 0.01 ± 0   | 1.25 ± 0.17 | 4.55 ± 0.39 |
|       | Molasses      | 0.01 ± 0   | 0.61 ± 0.02 | 3.00 ± 0.13 |
|       | Aeration (control) | 0.01 ± 0   | 0.64 ± 0.07 | 3.30 ± 0.27 |

Parameters of the liquid phase did not differ noticeably under different temperatures in the range of 40 to 50 °C, while at 55 and 60 °C, significant differences between the reactors were observed (Table 2). At temperatures of 40, 45, and 50 °C, the pH values were comparable in all three reactors (1.04–1.08 and 0.97–1.04, respectively). At 55 °C, the lowest pH value was in the reactor with a CO₂ supply (1.26), and the highest pH value was observed in the control reactor (1.91). At 60 °C, the pH values were comparable in the control reactor and in the reactor with molasses (2.52 and 2.52, respectively), while a lower pH value was noted in the reactor with CO₂ (2.34).

The Eh values of the medium at temperatures of 40–50 °C were greater than 770 mV and were comparable in all reactor variants (773–805 mV). At 55 °C, in the reactors with additional carbon sources, the redox potential was greater than 770 mV, while a relatively low potential value was observed in the control reactor (804–832 and 760 mV, respectively).
At 60 °C, the Eh values were below 600 mV in all three reactors (572–580 mV), indicating a low intensity of biooxidation.

The Fe³⁺/Fe²⁺ concentration ratio in the liquid phase of the reactors corresponded to the observed values of the redox potential (Table 2). At temperatures of 40–50 °C, Fe³⁺ ion concentration was considerably greater than that of the Fe²⁺ ions in all reactors (14.64–18.70 and 0–0.14 g/L, respectively). At 55 °C, the Fe³⁺/Fe²⁺ ratio was high in the reactor with a carbon dioxide supply (9.59 and 0.35 g/L, respectively), while the Fe³⁺ concentration in the control reactor was lower than the Fe²⁺ concentrations (1.12 and 3.22 g/L, respectively).

At 55 °C, in the reactor with a molasses supply, the Fe³⁺/Fe²⁺ concentration ratio (4.97 and 1.54 g/L, respectively) was lower than in a reactor with a carbon dioxide supply. In all variants of the experiment at 60 °C, the predominance of ferrous ions over ferric ions was observed (0.28–1.10 and 0.06–0.11 g/L, respectively). Thus, at temperatures of 40 to 50 °C, the Fe³⁺ ion concentrations were higher than at temperatures of 55 and 60 °C, which suggests a greater activity regarding the biooxidation of the iron-containing minerals, chalcopyrite and pyrite, at lower temperatures. At the same time, at 40–50 °C, the biooxidation of iron-containing minerals was affected by the presence of an additional carbon source to a lesser extent than at 55 °C.

At 55 °C, both in the reactors with a carbon dioxide and molasses supply, the Eh values were comparable to those observed at 40–50 °C, while in the control reactor at 55 °C, it was significantly lower than at lower temperatures. This suggested an inhibition in the microbial oxidizing activity at 55 °C in the absence of an additional carbon source. At 60 °C, the low Eh values, as well as the ferric ion concentrations, demonstrated sharp a decrease in microbial activity. Despite the high iron content in the concentrate, which was used in the experiment, the iron ion concentrations in the liquid phase at 60 °C were low. This may be explained by the fact that sulfide mineral leaching (tennantite, chalcopyrite, and pyrite) depends on the microbial oxidation of ferrous iron and sulfur compounds and does not occur in the absence of microbial activity, even at a comparatively high temperature [34,44–47].

The concentrations of arsenic, copper and zinc in the liquid phase and, accordingly, their recovery, differed more significantly at higher temperatures (55 and 60 °C) (Tables 3 and S2, Figure 1). At temperatures below 55 °C, the concentration of arsenic in the medium was 0.41–0.48 g/L, which corresponds to a 24.1–28.4% recovery. At 55 °C, the highest content of arsenic in the solution was in the reactor with a CO₂ supply and amounted to 0.34 g/L (the recovery was 20.0%), while the lowest concentration was determined in the control reactor, 0.09 g/L (the recovery was 5.4%). At 60 °C, the lowest concentrations of arsenic in the medium, 0.01 g/L were observed in all three reactors (the extraction reached 0.6–0.7%). Thus, at lower temperatures, a higher degree of extraction of arsenic was observed, however, the activity of the process did not depend on the presence of additional carbon sources. At 55 and 60 °C, less active leaching was observed, while at 55 °C the supply of CO₂ and, to a lesser extent, addition of molasses into the reactor pulp increased the degree of extraction of arsenic into the solution. These data corresponded to those on iron ion concentrations. Low concentrations of both iron and arsenic in a liquid phase may be explained by the inhibition of microbial oxidative activity at 60 °C that was crucial for the decrease in tennantite leaching rate [34], and in turn led to the decrease in arsenic concentration.

Both at 40 and at 55 °C in all three reactors, the copper content in the medium was 1.10–1.38 g/L (the extraction level was 18.7–22.2%). The copper concentrations at 45–50 °C were comparable in the three reactors, differing in the presence of additional carbon sources, amounting to 1.43–1.67 g/L, which corresponded to a copper recovery of 23.0–27.0%. At 60 °C, the copper concentration in the reactor with a carbon dioxide supply was 1.25 g/L (the recovery was 20.1%), which was significantly higher than in the reactor with molasses (0.61 g/L, recovery, 9.9%) and in the control reactor (0.64 g/L, recovery 10.3%).

Thus, the degree of copper leaching increased at 60 °C, when an additional carbon dioxide was supplied to the reactor. Probably, the supply of CO₂ led to an increase in the
activity of auto- and mixotrophic organisms, which increased the activity of the bioleaching. At 55–60 °C, lower degrees of arsenic leaching compared to lower temperatures were observed in all the reactors, and the degree of copper extraction did not depend severely on temperature in the reactor with a CO₂ supply, while in the other reactors (control and with molasses supply), copper extraction was noticeably lower than in the corresponding reactors at lower temperatures. This can be explained by the fact that the extraction of these elements was mainly due to biological oxidation that was less active at higher temperatures, in which the leaching was more dependent on the presence of additional carbon sources that affected the activity of both auto- and mixotrophic microorganisms (fixing CO₂), and heterotrophic microorganisms (consuming molasses) and on the lower pH value of the pulp that resulted in a higher solubility of oxide minerals containing arsenic. Therefore, it can be assumed that at lower temperatures copper leaching was more dependent on the oxidation of tennantite, while at higher temperature, it was determined mainly by the oxidation of chalcopyrite. Previously, it was shown that the bioleaching of chalcopyrite significantly depended on temperature and accelerates with its increase [44–46], while tennantite bioleaching depends on a temperature at a lesser extent [34]. At the same time, the extraction of copper at all temperatures and conditions was comparatively low, which indicates the resistance of copper minerals to the bioleaching process.

At 40–45 °C, the zinc content in the solution in all experiments was comparable and amounted to 5.65–5.99 g/L, which corresponds to 77.4–82.1% extraction. At 50–55 °C, zinc concentrations in the liquid phase were slightly lower and amounted to 4.89–5.19 g/L (that corresponded to the recovery of 67.0–71.1%). At 60 °C, the difference between the zinc concentrations in the control reactor (3.3 g/L, recovery 45.3%) and the reactor with molasses (3.0 g/L, recovery 41.2%) was insignificant, and the highest concentration and, respectively, the Zn recovery was observed in the reactor with a CO₂ supply (4.55 g/L, recovery was 62.3%). Thus, the degree of zinc extraction significantly dependent on temperature, and the effect of a carbon dioxide supply was observed only at the highest temperature, at 60 °C. It is known that sphalerite is easily subjected to oxidation, including chemical oxidation, which probably explains the regularity obtained [46].

The chemical composition of the solid residues of biooxidation is presented in Table S3. The content of arsenic in the residues in almost all variants of the experiment was higher than in the original raw materials (1.80–3.10 and 1.7%, respectively). This may be explained by the fact that arsenic extraction was comparatively low, and the decrease in the mass of biooxidation residue, compared to the initial concentrate, led to the accumulation of the element in the residues. At 40–50 °C, the content of copper in the bioleaching residue was higher than in the concentrate (9.10–11.95 and 6.2%, respectively), but at 60 °C, the Cu content, on the contrary, was lower in the biooxidation residues (5.5–6.1%). The Zn content in the residues was lower than in the concentrate (2.05–5.20 and 7.3%, respectively). The data obtained indicate that sphalerite was comparatively easily bioleached, while the copper minerals, mostly tennantite, were more refractory to the bioleaching.

3.2. Microbial Population Analysis

The analysis of the composition of microbial populations made it possible to evaluate the effect of temperature and additional carbon sources on the activity of certain physiological groups of microorganisms (Figure 2).

No data were obtained for all three reactors at 60 °C and the control reactor at 55 °C due to the low cell number (less than 10⁶ cell/mL), meaning it was not possible to obtain a sufficient amount of biomass from the pulp sample collected.

At 40–45 °C, in the experiments with a CO₂ supply, the proportion of *Sulfobacillus* increased in comparison to the control experiment (from 8.50 and 2.50% to 29.50 and 6.00%). At temperatures of 40–50 °C, the proportion of *Acidithiobacillus* (probably *A. caldus*) was the highest in the control reactors (36.0–77.5%) compared to those in the experiments with CO₂ (21.0–43.5%) and molasses (1.5–65.5%). The proportion of *Leptospirillum* was insignificant in all variants of the experiment (0.03–0.70%). The proportion of archaea
increased at temperatures of 40–50 °C, both in the reactors with a CO₂ supply and in the reactors with the addition of molasses, while at 40 °C the supply of carbon dioxide caused a greater increase in the proportion of archaea (from 14.10 to 27.5%) than the addition of molasses (to 21.0%), while at 45–50 °C, on the contrary, the addition of molasses increased the proportion of archaea to a greater extent (from 53.50–63.8 to 56.97–98.20%) than the addition of CO₂ (up to 55.98–79.75%). The data obtained indicate that, in the absence of additional carbon sources, autotrophic *A. caldus* might play the most important role in the process of biooxidation. The supply of carbon dioxide led to an increase in the number of mixotrophic *Sulfobacillus* and to an increase in the number of exometabolites of the autotrophic bacterium, which could be used by heterotrophic archaea. Molasses were also used by heterotrophic archaea, which led to an increase in their numbers in the population.

**Figure 2.** Analysis of microbial populations performing bioleaching under different conditions (proportion of the 16S rRNA gene fragment).

### 4. Discussion

The results obtained in the present work demonstrated that stirred tank reactor bioleaching under different conditions allowed the partial extraction of copper and zinc from the concentrate studied. It was shown that both the temperature and presence of additional carbon sources affected both copper and zinc extraction, as well as the microbial population composition.

In all experiments, zinc extraction was significantly higher than the copper recovery. This can be explained by the fact that the sphalerite contained in the concentrate is more easily leached than the copper minerals present in the concentrate (chalcopyrite and tennantite) [46–50].

The copper extraction was affected by both temperature and additional carbon source presence. Thus, at 45 and 50 °C, Cu extraction was the highest, but in the range of 40 to 55 °C, the recovery did not depend on carbon source. At 60 °C, both copper and zinc extractions were affected by CO₂ supply and was about 2 and 1.5 times higher than in the control experiment and in the experiment with molasses. This suggests that at 60 °C, the carbon shortage caused by a high temperature affected bioleaching activity, while a CO₂ supply could eliminate the effect of temperature increase. Both the temperature and pH level affect CO₂ solubility, and in turn, the carbon availability to autotrophic and mixotrophic microorganisms, and the pH decrease and the temperature increase lead to the decrease in CO₂ solubility. However, in our experiments, at the highest temperature (60 °C), the pH level was higher (about 2.3–2.5), than at a lower temperature. Therefore,
in this case, the pH level could not lead to the decrease in CO₂ solubility compared to other experiments at 40–55 °C as in these cases, the pH levels were lower (in the range 0.97–1.91). Thus, temperature was the main factor that effected CO₂ availability under the experimental conditions used.

The results on the effect of the CO₂ supply at a high temperature obtained in the present work partially corresponded to those obtained in our previous work on the biooxidation of pyrite-arsenopyrite concentrates [36,37]. However, in the case of pyrite-arsenopyrite concentrates, the effect of CO₂ and molasses supply on biooxidation was observed at temperatures 40–50 °C, while in the present work, a significant effect was observed only at 60 °C.

Furthermore, the temperature effect on copper bioleaching observed in the present work corresponded to the results of our previous works. It is well-known that the bioleaching of chalcopyrite significantly depended on temperature and accelerates with temperature increase [34,45,46]. At the same time, tennantite bioleaching depends on temperature to a lesser extent, and its bioleaching was mainly affected by microbial activity [34]. In the works [49–51], we have studied the bioleaching of copper concentrates with different a chalcopyrite and tennantite ratio. It was shown that the copper leaching rate of the concentrate with higher chalcopyrite content in the range of 40 to 60 °C increased, while copper extraction from the concentrate with higher tennantite content was the highest at 55 °C, and decreased at 60 °C. In both cases, arsenic leaching was higher in the range of 40 to 50 °C and inhibited at higher temperatures. In the work [31], in contrary, copper and zinc recovery was the highest at 40 and 45 °C under autotrophic conditions, at 50 °C in the presence of yeast extract and molasses in the medium. Arsenic extraction was at a maximum at 45 °C in all variants of the experiments.

An analysis of microbial populations suggest that, in the absence of additional carbon sources, autotrophic A. caldus might play the most important role in the process of biooxidation. The supply of carbon dioxide led to an increase in the number of mixotrophic Sulfobacillus. The addition of molasses into the pulp led to an increase in their numbers in the population. In all cases, a temperature exceeding 45 °C resulted in the predominance of Acidiplasma archaea. Thus, at high temperature and in the presence of additional carbon sources, the proportion of mixo- and heterotrophic iron oxidizing acidophiles increased, which should enhance the bioleaching rate. At the same time, in some experiments, the predominance of sulfur-oxidizing autotrophs, At. caldus, was observed. Despite these bacteria not being able to oxidize ferrous iron, they may play a key role in the oxidation of some sulfide minerals, producing exometabolites which may be consumed by mixo- and heterotrophs [52], and oxidizing sulfur-compounds, which are intermediates of sulfide mineral oxidation [53,54]. Thus, A. caldus may play an important role in concentrate bioleaching.

Thus, it was shown that metal leaching from the concentrate and microbial population composition depended on different factors, but copper extraction was comparatively low in all experiments. Despite this, the results obtained may be used in further studies to improve bioleaching efficiency of the studied concentrate and similar ones. For example, different approaches, including sulfide alkaline leaching, may be used to increase the copper bioleaching rate [55], while patterns revealed in the present work may be used to optimize process conditions.

5. Conclusions

In the present work, reactor bioleaching of an arsenic-containing polymetallic concentrate containing 6.2% Cu, 7.3% Zn, and 1.7% As, at temperatures 40–60 °C and in the presence of an additional carbon source (CO₂ and molasses) in the medium, as well as changes in the composition of the microbial population under different conditions, were studied. It was shown that temperature had a weak effect on the extent of leaching of both copper and zinc, and additional carbon sources affected extraction of non-ferrous metals only at 60 °C. At temperatures of 40–55 °C in all reactors, the extent of copper extraction was 18.7–27.0%, while zinc extraction was 67.0–82.1%. At 60 °C, the supply of carbon
dioxide increased the copper and zinc recovery by about two and 1.5 times in comparison to other variants, respectively. Thus, by using carbon dioxide as an additional source, we could avoid a significant decrease in the degree of Cu and Zn bioleaching when the pulp temperature rises to 60 °C. This suggests that a carbon dioxide supply may be used on an industrial scale to prevent the inhibition of bioleaching caused by the temperature rise caused by exothermic reactions of mineral oxidation, and decrease costs for reactor cooling.

Both the temperature and carbon source used affected the microbial population composition. The main microbial genera revealed in the populations were bacteria Acidithiobacillus and Sulfobacillus as well as archaea Ferroplasma, Acidiplasma, Cuniculiplasma. At low temperatures (40 and 45 °C), Acidithiobacillus, Sulfobacillus, and Ferroplasma were predominant, while an increase in the temperature up to 50–55 °C resulted in the decrease in relative abundance of these genera, and predominance of Acidithiobacillus archaea. The usage of both CO2 and molasses led to the increase in Sulfobacillus and Acidiplasma in relative abundance.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/min12050592/s1, Table S1: Mineral content in the concentrate (based on XRD data); Table S2: As, Cu, and Zn extraction (%) during biooxidation; Table S3: Chemical composition of bioleaching residues.

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