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Investigation of the microbial diversity of an extremely acidic, metal-rich water body (Lake Robule, Bor, Serbia)

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Abstract: An investigation of the microbial diversity in the extremely acidic, metal-rich Lake Robule was performed using culture-dependant and culture-independent (T-RFLP) methods. In addition, the ability of the indigenous bacteria from the lake water to leach copper from a mineral concentrate was tested. T-RFLP analysis revealed that the dominant bacteria in the lake water samples were the obligate heterotroph *Acidiphilium cryptum* (≈50 % of the total bacteria) and the iron-oxidizing autotroph *Leptospirillum ferrooxidans* (≈40 %) The iron/sulfur-oxidizing autotroph *Acidithiobacillus ferrooxidans* was reported to be the most abundant bacteria in the Lake in an earlier study, but it was not detected in the present study using T-RFLP, although it was isolated on solid media and detected in enrichment (bioleaching) cultures. The presence of the two bacterial species detected by T-RFLP (*L. ferrooxidans* and *A. cryptum*) was also confirmed by cultivation on solid media. The presence and relative abundance of the bacteria inhabiting Lake Robule was explained by the physiological characteristics of the bacteria and the physico-chemical characteristics of the lake water.

Keywords: acidophiles; bioleaching; biomining; tailings; T-RFLP.

INTRODUCTION

The Copper Mine Bor has been in operation since 1903, and during that time millions of tons of mine tailings have been deposited in the close proximity of the town of Bor. Exposure of the tailings to air and water initiated the microbially accelerated oxidative dissolution of sulfide minerals, forming an acid mine drainage (AMD) containing elevated concentrations of metal cations and sulfates. It is known that acidophilic bacteria and archaea, some of which are directly involved in the process of sulfide mineral oxidation and thereby accelerate the

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production of AMD by a factor of up to 10^6, rapidly populate acidic environments.[1] This commonly leads to the formation of acidic lakes and ponds under deposits of tailings and in open pits.

Lake Robule is located at the foot of the overburden of the open pit named Visoki planir, which was created during the five-year period from 1975 until 1980. It is the largest overburden of the Copper Mine Bor (100 m in height) and is composed of 150 million tons of waste rocks and off-balanced ore.[2] By preventing water circulation, the Visoki planir overburden created Lake Robule. The lake also collects water draining the overburden after rainfall. The lake is 200 m in length and 150 m in width with a maximum depth of about 10 m. Although water is drained from the lake into the Bor River at a constant rate of about 500 m^3 per day,[3] the lake water level is constant.

Lake Robule has become an extreme environment due to the acidic solutions containing elevated concentrations of metal cations and sulfates continuously flowing into it from the surrounding overburden following rainfall. The input of AMD from the overburden has caused the water of the lake to become highly acidic, and deep red in color due to high concentration of ferric iron. Such an environment is a potential source of acidophilic bacteria that could be used in biohydrometallurgy, specifically for leaching of copper from concentrates, ores and tailings, referred to as bioleaching. Bioleaching technology enables economically feasible exploitation of low-grade ores and tailings, as it is significantly cheaper than the classical pyrometallurgical technology.[4] In addition, the environmental impacts of the bioleaching technology are generally much lower than those associated with the pyrometallurgical processing of copper-containing ores.[5]

It is estimated that tailings of the Copper Mine Bor still contain about one million tons of copper. According to its mineral composition and average contents of copper, the tailing deposits Visoki Planir, Cerovo and the Old Flotation are particularly suitable for microbial leaching. These dumps contain 200 million tons of tailings, with estimated content of 346 000 tons of copper.[6]

It is now accepted that compositions of microbial communities cannot be determined solely using culture-dependant methods[7] and that culture-independent approaches generally yield data that are more comprehensive. In order to better define the bacterial community inhabiting Lake Robule, terminal restriction enzyme fragment length polymorphism (T-RFLP) analysis was used in this study, in parallel with cultivation of acidophilic bacteria on solid media. T-RFLP enables the microbial diversity and relative abundance of microorganisms in samples to be estimated based on the different lengths of polymerase chain reaction (PCR)-amplified DNA (often 16S rRNA genes) that were digested with restriction endonucleases.

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The aims of this study were to investigate microbial diversity of the Lake Robule, to isolate and identify indigenous acidophilic bacteria, and to test the bioleaching potential of the microbial community in the lake water.

EXPERIMENTAL

Sampling and measurement of the physicochemical parameters of the lake water

Lake water samples were collected in 50 ml sterile plastic containers on July 26th, 2012. Water temperature, pH and conductivity were measured on site using a Hanna Instruments HI98311 mobile instrument. The redox potential of the water was measured using a combined Pt–Ag/AgCl electrode system.

Copper analysis

The total copper concentrations in the water samples were measured using a modified method described by Anwar *et al*.[8] Cu(II) was reduced to Cu(I) by adding 200 μL of 10 % hydroxylamine solution to 100 μL of water sample. The solution was mixed well and incubated for 5 min at room temperature. First, 1 mL of tartrate buffer (1 mL of 0.5 M HCl added to 100 mL of 0.5 M sodium tartrate and pH adjusted to 5.5) was added and the solution vortexed. Next, 500 μL of phosphate buffer (87.7 mL of 0.2 M NaH_2PO_4 with 13.3 mL of 0.2 M Na_2HPO_4) and 100 μL of 0.1 % bicinchoninic acid (Sigma Chemical, USA) diluted in tartrate buffer was added and the mixture vortexed. Finally, 0.8 mL distilled H_2O was added and the solution was mixed well. After 10 min at room temperature, the absorbance was measured at 562 nm (Cecil CE1011 spectrophotometer).

Iron analysis

The ferrozine assay was used to determine the concentrations of both soluble Fe(III) and Fe(II). The concentrations of Fe(II) were determined using the standard method described by Lovely and Phillips.[9] Then, the total iron concentration was determined by repeating the analysis following addition of hydroxylamine (to reduce the Fe(III) present to Fe(II)). The Fe(III) concentrations were obtained from the difference the obtained values.

Isolation and cultivation of acidophilic bacteria from the Lake Robule on selective solid media

Acidophilic bacteria from Lake Robule were cultivated on overlay solid media[10] that comprised a bottom layer of solid medium inoculated with an acidophilic heterotrophic bacterium *Acidiphilium cryptum* (strain SJH) and a top layer of the same medium inoculated with water from Lake Robule. *A. cryptum* SJH, a heterotrophic acidophilic bacterium, was added to a bottom layer of a plate in order to metabolize products of the hydrolysis of the agarose, which have an inhibitory effect on the growth of most acidophilic chemolithoautotorphs. Bacteria were cultivated on two types of overlay solid media: iFeo, medium, which contained ferrous sulfate as the sole energy source, and is suitable for the cultivation of iron oxidizers such as *Leptospirillum ferrooxidans*, while the FeSo medium that contains ferrous iron, tetrathionate and tryptone soya broth, supports the growth of iron- and sulfur-oxidizers, such as *Acidithiobacillus ferrooxidans*, and also some heterotrophic acidophiles.[11] The iFeo medium contained 1 basal salts of 50×concentrated solution (12.5 g L^{-1} $(NH_4)_2SO_4$, 5 g L^{-1} $MgSO_4 \cdot 7H_2O$), 0.1 % trace elements solution, 25 mM $FeSO_4$, while the composition of the FeSo medium was as follows: 1×basal salts of 50×concentrated solution, 0.1 % trace elements solution, 0.025 % tryptone soya broth, 5 mM $FeSO_4$, and 10 mM $K_2S_4O_6$, in final concen-

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trations. All media were gelled using a 0.5 % agarose solution. The inoculated plates were incubated for 30 days at 30 °C.

Extraction and analysis of DNA from the bacterial colonies

A small amount of biomass from several colonies displaying the same morphologies was suspended in 20 μL cell lysis solution (0.05 M NaOH, 0.25 % sodium dodecyl sulfate) and heated at 95 °C for 15 min in a PCR thermocycler. The crude cell lysates were allowed to cool and 180 μL of MilliQ:Tris buffer (0.01 mM Tris, pH 7.5) added. The Fe(III)-encrusted colonies were washed first in 100 mM oxalic acid, and then in sterile ultra-pure water, followed by the addition of the cell lysis solution addition. To identify the isolates, their 16S rRNA genes were amplified using PCR (described below) and the obtained products were digested with the restriction enzyme *Hae*III and the fragment lengths determined using T-RFLP (described below). Bacterial identities were determined by comparing the fragment lengths obtained with those in the databank of acidophilic bacteria maintained at Bangor University, UK.

PCR amplification of 16S rRNA genes

Bacterial 16S rRNA genes were amplified using 27F:
5′-AGAGTTTGATCMTGGCTCAG-3′ and
1387R: 5′-GGGCGGAGTGTACAAGGC-3′
primers. For PCR, a final volume of 25 μL, containing 12.5 μL master mix (Promega, USA), 10 pmol of each primer, 2.5 mM $MgCl_2$. 0.5 μL ultra-pure dimethylsulfoxide and 1 μL DNA template, and ultra-pure water, was used. The PCR reactions were realized in a Techne TC-312 thermocycler. Amplification was performed as follows: initial denaturation 95 °C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing 55 °C for 30 s and elongation at 72 °C for 90 s. Final extension was performed at 72 °C for 10 min. The PCR products were analyzed by gel electrophoresis on a 0.7 % agarose gel.

Isolation of DNA from Lake Robule

Approximately 400 mL of lake water was filtered through a 0.2 μm (pore size) sterile membrane filter. The filter was cut into segments and DNA was isolated using a MoBio Ultra Clean Soil DNA isolation kit following manufacturer's instructions. The isolated DNA was used as a template for the amplification of the 16S rRNA genes.

Terminal restriction fragment length polymorphism (T-RFLP) analysis of the amplified 16S rRNA genes

T-RFLP analysis was used to identify the isolated bacteria, and to study the diversity and relative abundance of the microorganisms present in the Lake water samples, as well as in samples following bioleaching of copper concentrate. In this case, PCR amplification was performed as described using a 27F primer labeled with Cy5 dye at the 5′ end (MWG Biotech, Germany) and unlabelled 1387R primer. The PCR products were digested using three different restriction endonucleases, *Hae*III, *Alu*I, and *Cfo*I in three separate reactions. The Reaction mixture consisted of 0.5 μL of enzyme, 1 μL of enzyme specific buffer, 1 μL of PCR product and 7.5 μL of ultra-pure water. The reaction mix was incubated at 37 °C for 1 h. Mixes containing 2 μL of digestion products and 28 μL of sample loading solution were analyzed using a Beckman Coulter CEQ 8000 capillary electrophoresis apparatus. The sample loading solution contained 0.5 μL 600b CEQ DNA size standard dissolved in 27.5 μL of formamide. The T-RFLP analysis for each restriction enzyme was performed in triplicate and the summarized results are presented.

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Bioleaching of copper concentrate

To evaluate the bioleaching potential of bacteria inhabiting Lake Robule, a concentrate containing 17 % of copper from the Copper Mine Majdanpek, Serbia, which contained chalcopyrite as the dominant copper sulfide mineral, was used as the test material. The basal salts solution (100 mL, pH 2.0) was transferred into 250 mL conical flasks (in triplicate) and 1 g of concentrate and 1 mL of water from Lake Robule were added. The cultures were incubated at 30 °C and shaken at 150 rpm. The concentrations of soluble iron and copper, pH, redox potentials (using a combined Pt–Ag/AgCl electrode) and the bacteria present in the cultures were determined after three weeks of incubation.

RESULTS

Physical and chemical properties of Lake Robule

The physical and chemical properties of Lake Robule measured on site are given in Table I. The Lake water is highly acidic and characterized by high conductivity due to the presence of elevated concentrations of dissolved ions. The highly positive redox potential of the Lake water is a consequence of the high concentration of Fe(III), which accounts for 99.7 % of the total iron present.

TABLE I. Physical and chemical properties of Lake Robule water

Sampling date	*t* °C	Color	pH	*Eh* mV	Conductivity mS cm^{-1}	$[Fe^{3+}]$ mg L^{-1}	$[Fe^{2+}]$ mg L^{-1}	$[Cu^{2+}]$ mg L^{-1}
26/07/12	26	Deep red	2.55	+850	10	614	1.68	73

T-RFLP analysis of the bacterial community of Lake Robule

Results of T-RFLP analysis of the PCR-amplified 16S rRNA genes sug-

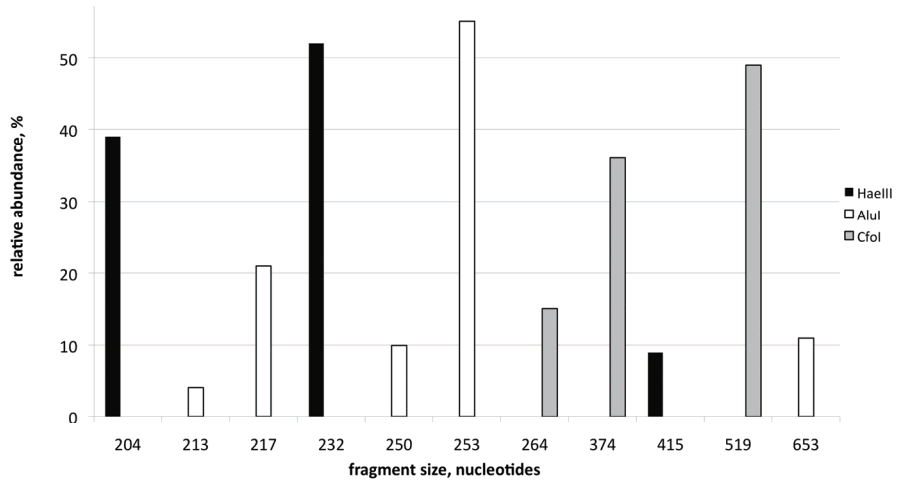

Fig. 1. Analysis of restriction fragments obtained from a sample of Lake Robule water. The length of T-RFs identified after digestion with three endonucleases (*x*-axis) and their relative abundance (*y*-axis).

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gested that the bacterial diversity in Lake Robule was very limited, as only three bacterial species were identified (Fig. 1). According to the T-RFLP profiles, the bacteria present in this extreme environment were *L. ferrooxidans*, *A. cryptum* and (more tentatively) *Acidisphaera rubrifaciens*. The presence of *L. ferrooxidans* and *A. cryptum* was confirmed by terminal restriction fragments (T-RFs) produced with all three restriction enzymes, but the presence of *Acd. rubrifaciens* was less certain as only one corresponding T-RF (*Alu*I digests) was detected (Table II). The terminal restriction fragments observed in T-RFLP profiles that could not be related to any fragment in the database are most likely pseudo T-RFs, PCR-related artifacts.[12] The approximate relative abundance of bacteria in the Lake water was calculated from the peak areas of each terminal restriction fragment as a percentage of total peak area. The most abundant bacteria were *A. cryptum* (50 %), followed by *L. ferrooxidans* (40 %), and *Acd. rubrifaciens* (1.3 %). The relative abundance of unidentified T-RFs (pseudo T-RFs) was 8.7 %.

TABLE II. Comparison between the observed T-RFs and those from the database

Enzyme	T-RF from database, nucleotide	Observed T-RF, nucleotide	Identified bacteria
HaeIII	204	204	*L. ferrooxidans*
AluI	217	217	
CfoI	374	374	
HaeIII	232	231	*A. cryptum*
AluI	255	253	
CfoI	519	519	
AluI	213	214	*Acd. rubrifaciens* (?)

Isolation of bacteria from Lake Robule

Three species of acidophilic bacteria were isolated from Lake Robule on overlay plates. Only very small Fe-encrusted colonies (identified as *L. ferrooxi-*

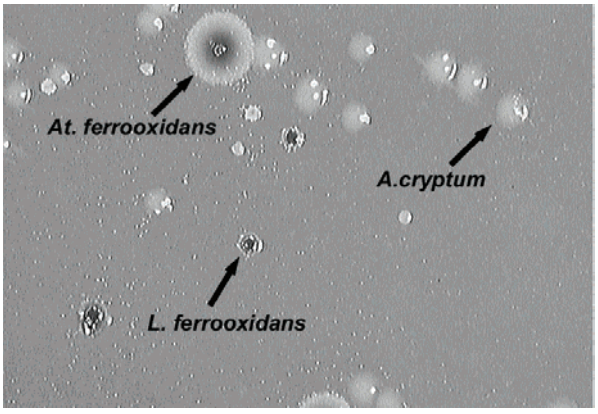

Fig. 2. Colonies of the three species of acidophilic bacteria on a FeSo overlay plate, inoculated with water from Lake Robule.

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dans) grew on the iFeo medium. In contrast, three colony variants were identified on the FeSo overlay media: very small Fe-encrusted colonies of *L. ferrooxidans*, larger Fe-encrusted colonies with translucent halos of *At. ferrooxidans* and round, non ferric-iron stained colonies of *A. cryptum*. The colony variants that grew on FeSo overlay plates are shown in Fig. 2. The most abundant colonies were colonies of *A. cryptum*, followed by those of *L. ferrooxidans* and the colonies of *At. ferrooxidans* were the least abundant.

Bioleaching test

After three weeks of the bioleaching experiment, the pH value of the solution was 2.20 and the redox potential was 820 mV. The concentration of the total iron was 815±1.633 mg L^{-1} and the concentration of the total copper was 808.97±5.735 mg L^{-1}. These concentrations of total iron and copper are the mean values of three measurements.

T-RFLP analysis was conducted using only *Hae*III digests, as this restriction endonuclease was able to produce different T-RFs for each of the bacterial species identified in the Lake water. Two bacterial species were identified: *L. ferrooxidans* and *At. ferrooxidans*. However, an additional (and relatively minor) T-RF, not found in the HaeIII digests of the amplified genes from Lake Robule, itself was observed. No acidophilic bacterium corresponding to this T-RF was present in the database. The relative abundances of the microorganisms present in the bioleach liquor are shown in Fig. 3.

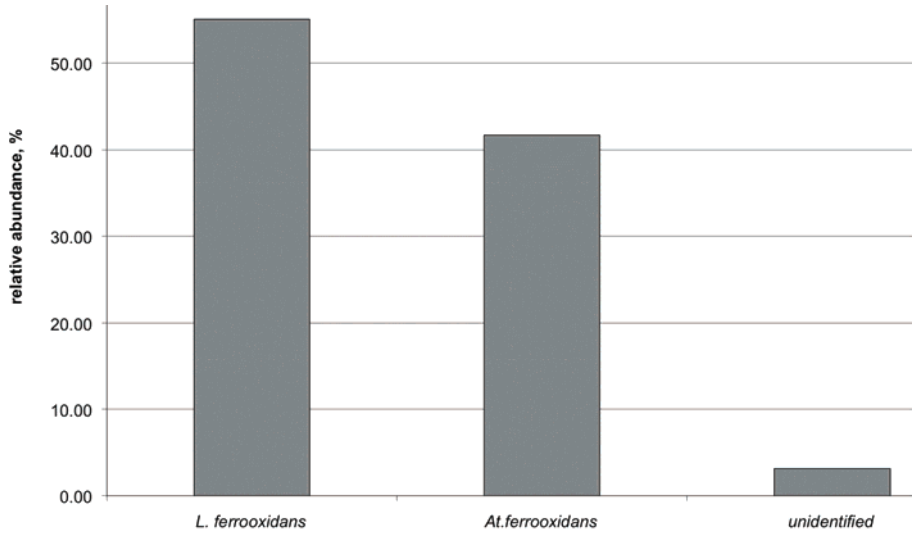

Fig. 3. Relative abundance of bacteria in solution after bioleaching determined by T-RFLP analysis.

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DISCUSSION

Lake Robule has been studied for over thirty years. Korać and Kamberović[13] reported that the pH of the lake was 2.97, and that it contained large concentrations of iron, 895 mg L^{-1}, sulfate, 4145 mg L^{-1}, and copper, 55.6 mg L^{-1}. Beškoski *et al.*[3] monitored the physical and chemical properties as well as the microbial diversity of the lake water between 1975 and 2008, and identified *At. ferrooxidans* as the most abundant bacterium in the lake. These authors reported that concentration of copper decreased between 1975, when it was 153 mg L^{-1}, and 2008, when it was 96 mg L^{-1}. The concentration of soluble iron as well as the pH and redox potential fluctuated during this time. The highest and lowest concentrations of iron were detected in 1988, 961 mg L^{-1} and in 1975, 562 mg L^{-1}, respectively. Redox potential of the water was highest in 1988, 527 mV, and lowest in 1975, 297 mV. The redox potential of the lake water was measured by using saturated calomel reference electrode (personal correspondence with the author). The lowest pH of the lake water was detected in 1975, 2.40, and the highest was in 1988, 2.81. Results obtained in the present study were concordant to the results obtained by these authors, with exception of the redox potential of the water (measured with Pt–Ag/AgCl electrode pair), which was higher than any value reported in previous studies. This could be explained by the dominance of Fe(III), which constitutes 99.7 % of total iron in the water.

T-RFLP analysis is a molecular fingerprinting technique that is widely used when studying microbial ecology. It does not require microorganisms to be isolated in order for them to be identified, as it is based on the analysis of genes amplified using environmental DNA as a template.[14] It is a rapid and reliable molecular method for the identification of microorganisms in environmental samples when the microbial diversity of the analyzed sample is low.[15]

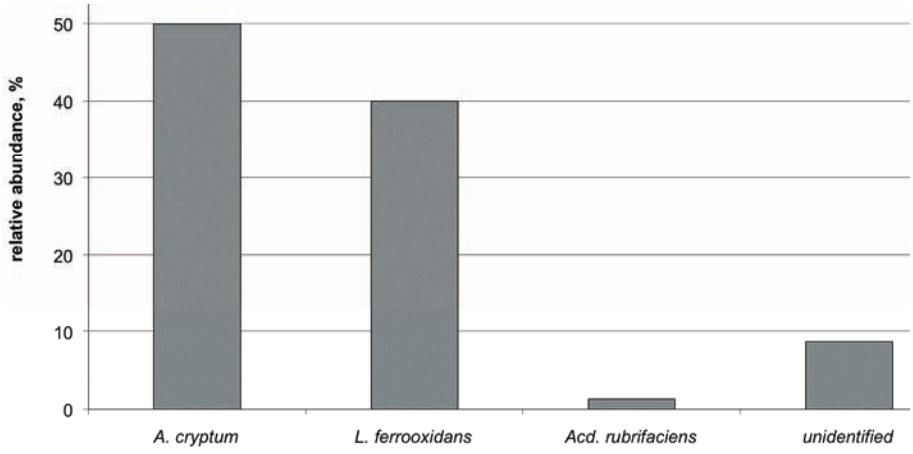

Fig. 4. Relative abundance of bacteria in Lake Robule determined by T-RFLP analysis.

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The T-RFLP profiles obtained with three different restriction enzymes confirmed with high confidence that the most abundant bacteria in Lake Robule are *A. cryptum* and *L. ferrooxidans* (Fig. 4). In addition, there is an indication that a bacterium related to *Acd. rubrifaciens* might be present in relatively low numbers, but since only one T-RF characteristic of this bacterium was observed following digestion with AluI, further analysis (*e.g.*, construction and analysis of a clone library) needs to be performed to elucidate this. Interestingly, *At. ferrooxidans*, previously reported as the dominant bacterial species in Lake Robule[3] was not been detected in the Lake water by T-RFLP analysis, although it was isolated on the solid medium (FeSo plates), along with *A. cryptum* and *L. ferrooxidans*. This indicates that while *At. ferrooxidans* is present in the lake, its relative abundance is low compared to those of both *L. ferrooxidans* and *A. cryptum*.

Earlier studies suggested that microbial communities in acidic environments were dominated by *At. ferrooxidans*, but this appears to have been an artifact of the methods, particularly in the enrichment culture and most probably the number counts.[7] Media for cultivation of acidophilic bacteria that have been widely used, and sometimes still are, such as 9K,[16] contain very high concentrations of Fe^{2+} (9 g L^{-1} in 9K) that favor the growth of *At. ferrooxidans*. Even if there is a very small number of *At. ferrooxidans* in a sample, it will be dominant after cultivation in 9K medium. Therefore, the results obtained in such studies are not surprising, as *At. ferrooxidans* thrives in environments with high concentrations of Fe^{2+} and a low redox potential.[17] In contrast, *L. ferroxidans* has a far higher affinity for ferrous ions and greater tolerance to ferric ions, and therefore tends to out-compete *At. ferroxidans* in high redox potential environments.[18]

Both direct plating of mine waters onto overlay media[19] and molecular methods such as T-RFLP and fluorescent *in situ* hybridization (FISH)[7] have revealed that the most abundant bacterium in iron-rich acidic environments is often *L. ferrooxidans*. The concentration of ferric iron in Lake Robule at the time of sampling was 614 mg L^{-1} (11 mM), and the redox potential was 850 mV, which are conditions that are far more conducive for the growth of *L. ferrooxidans* than for *At. ferrooxidans* (Table I). Reports on the composition of microbial community in the Lake published by Beškoski *et al.* (2009) that differ significantly from the results presented in this paper are, probably, the consequence of the methods that were used previously to cultivate bacteria from the Lake water. However, it is also possible that *At. ferrooxidans* was indeed more relatively abundant in the past when redox potentials were generally lower (and more variable) than more recently.

At the end of the bioleaching experiment, only *L. ferrooxidans* and *At. ferrooxidans* were detected in the mineral leachate (Fig. 3). At the start of the experiment, both the ratio of the Fe(III) to Fe(II) concentrations and the redox potential were low, but both increased during culture incubation. Initially, *At. ferrooxi-*

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dans would have outgrown *L. ferrooxidans*, since *At. ferrooxidans* has faster growth rate than *L. ferrooxidans* in low redox potential solutions. However, because the leptospirilli have a greater affinity for Fe(II) and are less sensitive to Fe(III), they would become dominant in the later stages of the bioleaching process.[18] These data indicate that *At. ferrooxidans* exist in the lake water, but the numbers of this bacterium in the lake are extremely low, and are undetectable by T-RFLP. The obligatory acidophilic heterotroph *A. cryptum*, the most abundant bacterium in the lake water as determined by T-RFLP analysis and isolation on the solid medium was not detected at the end of the bioleaching period since it is more sensitive to copper than both *At. ferrooxidans* and *L. ferrooxidans*, tolerating up to a maximum of about 10 mM of Cu (635 mg L^{-1}).[20] However, the concentration of copper determined in bioleaching solution was greater than this, *i.e.*, 808.97 mg L^{-1} .

The numbers of heterotrophic acidophiles in acidic, sulfide mineral-rich environments are often much lower than those of chemolithoautotrophic acidophiles, such as *L. ferrooxidans* and *At. ferrooxidans*. Heterotrophic acidophiles in these environments use metabolic products (lysates and exudates) of autotrophic acidophiles as growth substrates, as well as any extraneous organic carbon. In this mutualistic relationship, autotrophs produce growth substrate for heterotrophs, while heterotrophs, utilizing them, eliminate organic compounds (notably small molecular weight aliphatic acids) that are toxic to most acidophiles.[21] Since autotrophic acidophiles produce only small amounts of organic compounds, the numbers of heterotrophic acidophiles are often less than the number autotrophic acidophiles. However, if there is enough organic substrate, acidophilic heterotrophs can grow faster and can outnumber the autotrophs. One potential source of organic matter in the Lake is a municipal waste dump, which is in close proximity to the Lake, while other potential sources could be acidophilic algae. On the bottom of the Lake, green and filamentous biomass exists in the form of a microbial mat, indicating the presence of algae and fungi. Recent reports showed that in acidic environments exposed to sunlight, primary producers of organic matter are algae. Acidophilic algae excrete glycolic acid and sugars and sustain the growth of heterotrophic acidophilic bacteria, including *Acidiphilium* spp.[22] Production of oxygen by algae also helps in the growth of chemolithoautotrophic acidophiles. Since *Leptospirillum* spp. are very sensitive to the presence of organic compounds in the environment (particularly organic acids), it appears that heterotrophic acidophilic bacterium *A. cryptum* efficiently metabolizes organic compounds, facilitating the growth and activity of *L. ferrooxidans* within Lake Robule.

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CONCLUSIONS

The bacterial consortium populating Lake Robule is limited in its biodiversity, and is dominated by two bacterial species: *A. cryptum* and *L. ferrooxidans*. The most abundant microorganism in lake is the heterotrophic bacterium *A. cryptum*. This finding suggests that the lake water has a constant supply of organic matter. A possible source of organic matter could be the municipal waste dump that is very close to the lake. Another source of organic matter in the lake is probably acidophilic algae that populate a microbial mat at the bottom of the lake. *L. ferrooxidans* is an autotrophic iron oxidizer. This bacterium thrives in environments, such as that of Lake Robule, that have high concentrations of Fe^{3+} and very positive redox potentials. These conditions are less suitable for the growth of *At. ferrooxidans*, which was not detected by T-RFLP analysis, but was isolated directly from lake water on an overlay solid medium. *At. ferrooxidans* was also detected in the leach liquor from a test performed on the bioleaching of copper from a chalcopyrite concentrate using Lake Robule water as the inoculum. This bacterium prefers low redox potentials and high concentrations of Fe^{2+}, and it grew faster than *L. ferrooxidans* during the initial stages of the bioleaching process. This finding indicates that the lake water contains *At. ferrooxidans*, but in relatively small abundances. Cultivating bacteria from Lake Robule on media with high concentrations of ferrous ions could lead to the wrong conclusions concerning the microbial diversity of the lake. Moreover, this study showed that using only molecular, cultivation independent methods (such as T-RFLP) to evaluate the microbial diversity of environmental samples is not sufficient since *At. ferrooxidans* was not detected by this method. For the most accurate evaluation of microbial diversity under extremely acidic environments, the employment of both molecular- and cultivation-based methods is required.

Physical and chemical properties of the Lake display both seasonal and long-term variations. Consequently, the microbial community of the Lake Robule is also probably subject to variation, and the results presented in this paper are of lake water sampled during the summer months in the recent past. Future research should focus on tracking the changes in physical properties and chemistry of the Lake Robule, followed by an investigation of microbial diversity by combining molecular methods and plating on overlay solid media. This approach would give insight into changes in the microbial communities that populate Lake Robule over time and should explain correlations between changes in physical and chemical properties of the Lake water and the structure of bacterial consortium that inhabits this extreme environment.

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ИЗВОД

ИСТРАЖИВАЊЕ МИКРОБИОЛОШКОГ ДИВЕРЗИТЕТА ЕКСТРЕМНО КИСЕЛЕ ВЕШТАЧКЕ АКУМУЛАЦИЈЕ ВОДЕ СА ВИСОКИМ САДРЖАЈЕМ МЕТАЛА (ЈЕЗЕРО РОБУЛЕ, БОР, РЕПУБЛИКА СРБИЈА)

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Истраживање микробиолошког диверзитета екстремно киселе вештачке акумулације воде, језера Робуле код Бора, спроведено је култивацијом бактерија на селективним чврстим подлогама и применом молекуларне методе која не захтева изолацију и култивацију бактерија из животне средине (T-RFLP). Такође, испитивана је способност нативних бактерија из језера да врше лужење бакра из узорка минералног концентрата. T-RFLP анализом је утврђено да у води језера доминирају облигатно органохетеротрофна бактерија *Acidiphilium cryptum* (≈50 % од укупног броја бактерија) и облигатно аутотрофна бактерија која оксидује гвожђе *Leptospirillum ferrooxidans* (≈40 %). Према резултатима које су пре неколико година објавили други аутори, најзаступљенији микроорганизам у језеру је била аутотрофна бактерија која оксидује гвожђе и сумпор - *Acidithiobacillus ferrooxidans,* међутим присуство ове бактерије у води језера није потврђено T-RFLP анализом. Ова бактерија је изолована на селективној чврстој подлози и детектована T-RFLP методом у раствору након теста биолужења. Присуство две бактеријске врсте које су детектоване T-RFLP методом у води језера (*A. cryptum* и *L. ferrooxidans*) потврђено је култивацијом на селективним чврстим подлогама. Присуство и релативна заступљеност бактерија у језеру Робуле су објашњени у складу са физиолошким карактеристикама ових бактерија и физичко-хемијским особинама језерске воде.

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