Botanica SERBICA 41 (1): (2017) 47-54

Original Scientific Paper

Evaluation of microbial diversity of the microbial mat from the extremely acidic Lake Robule (Bor, Serbia)

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ABSTRACT: Extremely acidic environments are frequently formed in areas impacted by mining activities, and Lake Robule is such an ecosystem. Although an extreme environment, Lake Robule is inhabited by acidophilic microorganisms. We investigated biodiversity of the macroscopic structure known as a microbial mat formed on the lake bottom in shallow waters. Microbial mats are common in acidic environments, but their composition can differ significantly from site to site. Microbial diversity of the mat from Lake Robule was investigated using both cultivation-dependent and metagenomic approaches. The results showed the mat to be mostly inhabited by heterotrophic acidophilic bacteria. When compared to the microbial community of Lake Robule's surface water, the microbial mat proved to be a more complex community. A biogeochemical model of the mat of Lake Robule is proposed on the basis of our results and available published data.

Keywords: Lake Robule, acidic environment, microbial mat, acidophiles, metagenomic analysis, T-RFLP

Received: 28 July 2016 Revision accepted: 12 December 2016

UDC: 579(497.11 jezero Robule)
DOI: 10.5281/zenodo.453792

INTRODUCTION

Acidic waters are extreme environments that are usually formed in mining-impacted areas. Although typically low in overall microbial diversity, these rare environments harbour metabolically active, acidophilic microorganisms well adapted to such harsh habitats. When exposed to sunlight or when they are rich in reduced inorganic chemicals (predominantly ferrous iron), acidic waters can be quite complex ecosystems, and generally inhabited not just by prokaryotic (bacteria and archaea), but also by eukaryotic (algae, protozoa, fungi) microorganisms (Rowe *et al.* 2007; Chunbo *et al.* 2010). Besides planktonic forms, acidophilic microorganisms under such conditions are able to form macroscopic structures known as microbial streamers and mats, mainly consisting of bacteria and an extracellular matrix (Johnson 2009).

Dugan *et al.*(1970) studied microbial streamers in the acid mine drainage (AMD) formed in a coal mine, and these authors were the first to conclude that the filamentous structures in question are inhabited by bacteria embedded in an extracellular matrix under physicochemical conditions (e.g., with higher pH) that are different in comparison with the surrounding water. Similarly, the microbial community of long filamentous streamers from the Tinto River (Spain), a naturally occurring extremely acidic river 100 km long, proved to be unusual for an acidic environment. While its acidic waters were inhabited by *Leptospirillum ferrooxidans*, *Acidithiobacillus ferrooxidans* and *Acidiphilium cryptum*, acidophilic bacteria typical of such habitats (Gonzalez-

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Toril *et al.* 2003), culture-independent analysis of the streamers revealed the presence of species closely related to heterotrophic neutrophilic bacteria belonging to the genera *Bacillus*, *Sphingomonas* and *Pseudomonas* (Lopez-Archilla *et al.* 2004). These results also suggest that physicochemical properties inside microbial streamers differ significantly from the surrounding environment. Kimura *et al.* (2011) identified *Ferrovum myxofaciens* as the dominant bacterium in streamers collected from the underground pyrite mine Cae Coch in North Wales (UK). These chemolithoautotrophic iron-oxidisers produce extraordinarily large amounts of extracellular polymers and rapidly form "streamers" under *in vitro* conditions (Kay *et al.* 2013). The microbial community of streamers located in the channel that drains acidic, metal-rich water from an abandoned underground copper mine was dominated by the autotrophic iron-oxidisers *Acidithiobacillus ferrivorans* and *Ferrovum myxofaciens* (Kay *et al.* 2013). Analysis of the microbial mat formed in the channel that drains acidic water from an abandoned copper mine in Spain showed the presence of acidophilic microalgae at its surface, while the deeper thicker stratified layer was mostly inhabited by heterotrophic acidophilic bacteria, including *Acidobacterium capsulatum*, *Acidiphilium* sp. and some not yet cultured bacteria related to heterotrophic acidophiles (Rowe *et al.* 2007). Anaerobic sulphate-reducing bacteria were also identified, suggesting that the microbial mat beneath the algal layer was anoxic, although the surrounding water was rich in oxygen. Growth of these heterotrophic acidophilic facultative anaerobes in the mat was probably supported by organic molecules produced by acidophilic algae and their ability to survive in anoxic environments is a result of using Fe^{3+} ions instead of oxygen (Coupland & Johnson 2008). Clearly, microbial populations of the streamers and mats in extremely acidic environments can differ significantly.

Lake Robule (Fig. 1a) is an extremely acidic, metal-rich artificial accumulation of water formed in the late 1970s as a consequence of intensive mining activities at an open pit of the Copper Mine Bor in eastern Serbia. Formation of an overburden prevented surface water flow, so a lake was created. The length of Lake Robule is 450 metres, and it has a maximum width of 150 metres. Exposure of sulphide minerals, predominantly pyrite, in the overburden to oxygen and water initiated chemical and biological processes leading to continuous formation of AMD waters characterised by low pH and extremely high concentrations of metal cations (such as Fe^{2+}, Fe^{3+}, Cu^{2+}, Mn^{2+}, etc.), thus constantly providing an influx of acidic water to Lake Robule. More details of the process of AMD generation are presented elsewhere (Dimitrijevic 2013; Vera *et al.* 2013).

While microbial diversity of Lake Robule's surface water has been investigated (Stankovic *et al.* 2014), the thick green-coloured mat visible at the bottom of the lake in shallow waters has not yet been studied. The aim of the present work was therefore to assess microbial diversity of the mat formed in Lake Robule employing both culture-dependent and culture-independent (terminal restriction fragment restriction polymorphism – T-RFLP) approaches to obtain integrated information that would provide better insight into the ecology of this extreme environment.

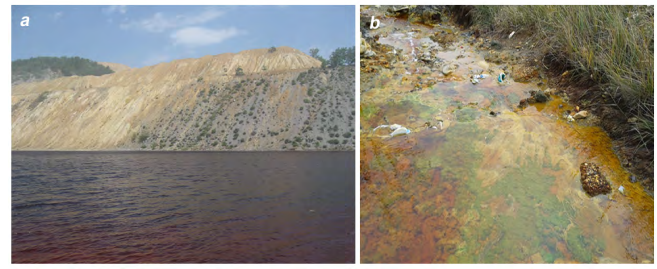

Figure 1. Lake Robule, located near the town of Bor, eastern Serbia. a) Deep red-coloured water of Lake Robule formed at the foot of the open pit's overburden in the Copper Mine Bor; b) Shallow water of the lake where the microbial mat sample was collected.

MATERIALS AND METHODS

Sampling. A microbial mat sample was collected on June 13th 2013 from the bottom of Lake Robule (Fig. 1b) in shallow water near the pipe that drains water from the lake. This was done using a 50-ml sterile plastic container. The collected sample was then transported in a mobile refrigerator to the laboratory, where it was kept at +4°C until analysis. Thickness of the collected mat sample was approximately 10 cm. The top layer of the microbial mat was thin and green in colour, while the underlying thicker layer was brown-coloured. The sample was processed within the next 24 hours. Temperature of the lake's water and its pH value were measured on-site using a portable HI 98312 device (Hanna Instruments, USA).

Isolation of acidophilic bacteria. Acidophilic bacteria from the microbial mat sample were isolated and cultivated using overlay solid media particularly developed for their isolation (Johnson 1995). In short, these solid media consist of a bottom layer always inoculated with an acidophilic heterotrophic bacterium such as *Acidiphilium cryptum* SJH, and a top solid layer of the same composition. Media were solidified using 0.5% agarose, since it is more stable in acidic conditions than agar. Inoculation of the bottom layer with *A. cryptum* SJH ensured scavenging of hydrolysis products of agarose and other organic molecules that inhibit growth of most acidophilic chemolithoautotrophs.

Composition of solid overlay media can vary, and acidophilic bacteria from the mat sample were isolated using three different overlay solid media. The first of them, iFeo medium, contained ferrous sulphate as the

sole energy source, and it was suitable for cultivation of iron oxidisers like *Leptospirillum ferrooxidans* and *Acidithiobacillus ferrooxidans*. The second medium, FeYeo medium, contained ferrous iron and yeast extract, which support the growth of iron-oxidisers (mostly *Acidithiobacillus ferrooxidans*) and some heterotrophic acidophiles (such as *Ferrimicrobium* sp.). The third medium, Yeo medium with yeast extract, was suitable for isolation of moderately acidophilic heterotrophic bacteria. Composition of the media was as follows: iFeo medium contained 1× basal salts [50× basal salts: 12.5 g/L $(NH_4)_2SO_4$, 5 g/L $MgSO_4 \cdot 7H_2O$], 0.1% trace elements solution and 25 mM $FeSO_4$; FeYeo medium had the same composition as iFeo, with 0.02% yeast extract in addition; Yeo medium contained 1× basal salts, 0.1% trace elements solution, 100 µM $FeSO_4$ and 0.05% yeast extract. The final pH value of iFeo and FeYeo media was 2.5, whereas the final pH value of Yeo medium was 4. Inoculated plates were incubated for 30 days at 30°C. Isolated bacteria were identified on the basis of phenotypic characteristics (i.e., colour, morphology, size) of their colonies formed on selective solid media (Johnson *et al.* 2005).

Optical microscopy. Bacterial colonies grown on overlay solid media in Petri dishes and the microbial mat were observed under a Leica DM 1000 microscope (Leica, Germany) using up to 50× magnification, and digital pictures were taken.

Isolation of total DNA from the mat sample. Total DNA was isolated from 1 g of the mat sample using the MoBio Ultra Clean Soil DNA Isolation Kit (MoBio Laboratories, USA) following the manufacturer's instructions. Isolated metagenomic DNA was used as a template for amplification of 16S rRNA genes.

PCR amplification of 16S rRNA genes. Amplification of 16S rRNA genes for T-RFLP analysis was performed using the KAPA2G Fast PCR Kit (Kapa Biosystems, USA). Primers used in the PCR reaction were: 27F primer labelled with 6-FAM fluorescent dye at the 5' end (5'-6-FAM–AGAGTTTGATCMTGGCTCAG-3'; Invitrogen, USA); and unlabelled 1492R primer (5'-CGGCTACCTTGTTACGACTT-3'; Invitrogen, USA). The reaction mixture contained: 1× KAPA2G Buffer A; each dNTP in a final concentration of 0.2 mM; each primer in a final concentration of 0.5 µM; 0.5 U of KAPA2G DNA polymerase; 2 µL of DNA template; and distilled water. The PCR reaction was performed in a 2720 Thermal Cycler (Applied Biosystems, USA) under the following conditions: 3 min of initial denaturation at 95°C, followed by 35 cycles of denaturation for 15 s at 95°C, annealing for 15 s at 60°C and final elongation at 72°C for 30 s. Amplification was done in triplicate, and PCR products (approx. 1500 bp) were analysed on 1% agarose gel. Products of the replicates were pooled, purified (QIAquick Gel Extraction Kit; Qiagen, USA) and used as such for further analysis.

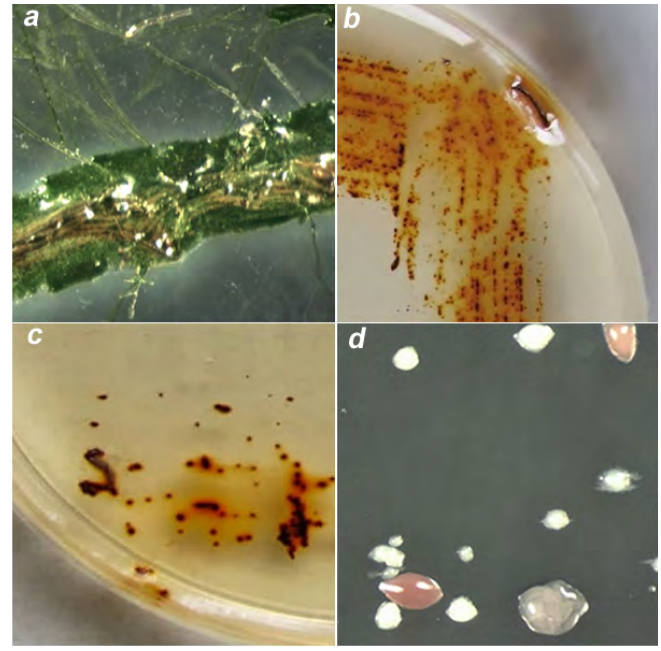

Figure 2. Mat sample from Lake Robule under a microscope.
a) Microscopic image of filamentous algae on the surface of the microbial mat; b) Small iron-encrusted bacterial colonies of the iron-oxidising obligatorily aerobic acidophilic bacterium *Leptospirillum ferrooxidans* grown on iFeo plates when the mat sample was inoculated; c) Iron-oxidising bacterial colonies grown on FeYeo plates, most likely the acidophilic bacterium *Acidithiobacillus ferrooxidans*, a facultative anaerobe; d) Colonies of heterotrophic acidophiles grown on Yeo overlay solid medium. The pinkish colonies are most likely *Acidiphilium cryptum*, while the white, non-transparent colonies are probably colonies of the heterotrophic acidophilic bacterium *Acidocella aromatica*.

Terminal restriction fragment length polymorphism (T-RFLP) analysis. PCR products were cut, in separate reactions, with three restriction endonucleases: *Alu*I, *Bsu*RI (*Hae*III) and *Msp*I (*Hpa*II) (Fermentas, Lithuania). Each digestion reaction was performed in a 10-µL volume and contained 1 µL of enzyme-specific buffer, 0.5 µL of the restriction enzyme, 7.5 µL of distilled water and 1 µL of DNK. The reaction mixtures were incubated for 1 h at 37°C, and endonucleases were denaturated according the manufacturer's protocol. All restrictions were performed in triplicate. One microlitre of each digestion reaction mixture was added to 18 µL of formamide (Hi-Di Formamide; Applied Biosystems, USA) containing 1 µL of an internal size standard (GeneScan500Lys; Applied Biosystems, USA). Mixtures were denatured for 3 min at 95°C (Thermomixer comfort, Eppendorf, Germany), immediately chilled, and kept on ice until electrophoresis on 3130 Genetic Analyzer (Applied Biosystems, USA)

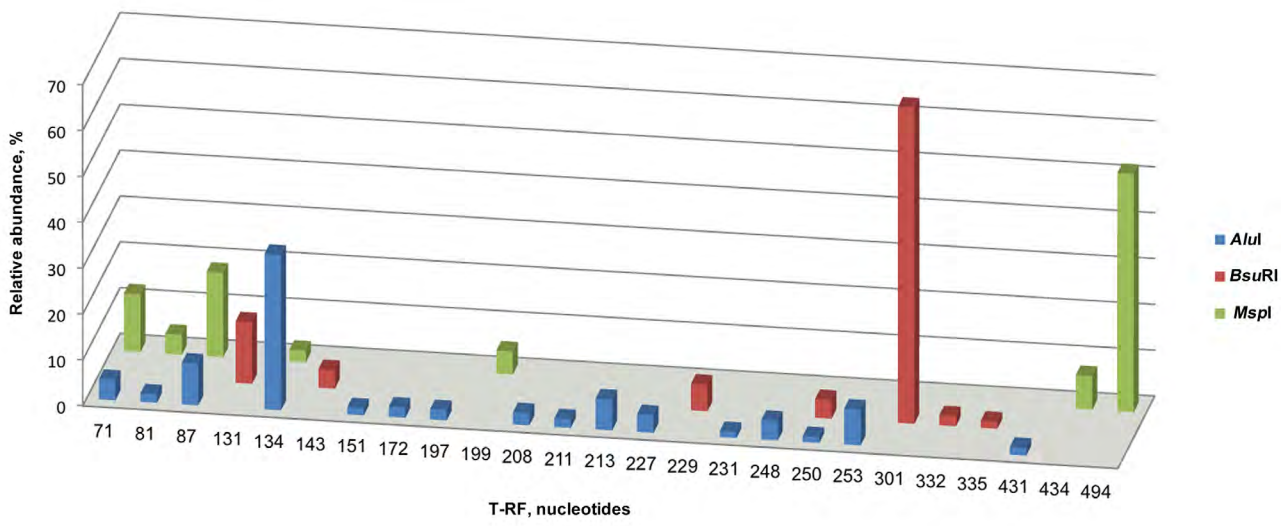

Figure 3. Graphic representation of the results of T-RFLP analysis of the microbial mat sample.

operating in fragment analysis mode. Terminal restriction fragment (T-RF) calling was performed in GeneMapper Software (Applied Biosystems, USA). For each sample, peaks over a threshold of 50 fluorescence units were used, while T-RFs shorter than 30 bp were excluded from the analysis to avoid detection of primers. Obtained T-RFs were compared with data in a T-RF length database specific for acidophilic microorganisms (provided by Professor D. Barrie Johnson, head of the Bangor Acidophile Research Team, Bangor University, UK) and identified. Relative abundance of each T-RF was calculated as the percentage of total peak area.

RESULTS

At the time when the mat sample was collected (June of 2013), temperature of the water in Lake Robule was 21°C, while its pH was 2.53. Microscopic examination of the microbial mat sample confirmed that the green colour of the superficial layer of the mat is due to the presence of filamentous algae (Fig. 2a). Inoculation of iFeo media with the microbial mat sample resulted in the appearance of numerous iron-encrusted colonies, mostly small in size (Fig. 2b). On FeYeo media, on the other hand, fewer iron-encrusted colonies were isolated, but they were larger in size. In addition, small white colonies of heterotrophic bacteria also developed on FeYeo plates (Fig. 2c). On Yeo media, microbial mat inoculation resulted in growth of white and pink colonies of heterotrophic bacteria (Fig. 2d). According to Johnson *et al.* (2005) larger iron-encrusted colonies were identified as *Acidithiobacillus ferrooxidans* species, and smaller iron-encrusted colonies as *Leptospirillum ferrooxidans*. Colonies of the heterotrophic acidophilic bacterial species *Acidiphilium cryptum* could also be easily identified under the microscope on the basis of their white to pinkish or sometimes intensive pink appearance. Such pigmentation of members of the genus *Acidiphilium* is a result of the presence of bacteriochlorophyll A in their cells (Johnson 2009). Opaque white colonies (Fig. 2d) were most likely colonies of the heterotrophic acidophilic species *Acidocella aromatica* (personal communication with Prof. D. B. Johnson).

Results of bacterial diversity analysis of the microbial mat sample by the T-RFLP method are presented in Fig. 3 and summarised in Table 1. Digestion of amplified 16S rRNA genes from metagenomic DNA with three endonucleases generated a total of 29 terminal fragments. The most discriminatory results were obtained in the case of *Alu*I digestion, since it produced as many as 16 T-RFs. The most abundant T-RF detected with *Alu*I was 134 bp long (with relative abundance of 33.9%). Search of a database with T-RFs of acidophilic microorganisms revealed that a fragment of this size could represent the acidophilic heterotrophic bacteria *Acidocella aromatica* and/or *Ferromicrobium acidophilum*. The presence of isolates closely related to the heterotrophic bacteria *Acidisphaera rubrifaciens* and *Acidiphilium cryptum* was indicated by the appearance of T-RFs with lengths of 213 bp (6.7%) and 253 bp (7.8%), respectively. Occurrence of the genera *Acidocella* and *Acidiphilium* was additionally supported by the appearance of T-RFs with lengths of 208 bp and 431 bp, respectively, although the relative abundance of these fragments was low. Other T-RFs with low relative abundance (each below 5%) indicated the presence of *Acidobacterium* sp. (151 bp); *Sulfobacillus thermotolerans* (172 bp); the anaerobic sulphate-reducing bacterium *Desulfobacca acetoxidans* (211 bp);

Table 1. Assignment of terminal fragments and their relative abundance.

***Alu*I**			***Bsu*RI**(*Hae*III)		
T-RF[a]	Identification	Relative abundance[b]	T-RF[a]	Identification	Relative abundance[b]
71	/	4.8	131	*Sulfobacillus* sp.	13.4
81	*Sulfobacillus* sp.	1.8	143	*Sulfobacillus acidophilus*	4.1
87	/	9.1	229	*Acidocella aromatica*	5.8
134	*Acidocella aromatica* / *Ferrimicrobium acidophilum*	33.9	250	*Alicyclobacillus* sp.	4.1
151	*Acidobacterium* sp.	1.6	301	Chloroplast 16S rRNA gene	68.9
172	*Sulfobacillus thermotolerans*	2.2	332	/	2.2
197	Chloroplast 16S rRNA gene	2.2	335	/	1.5
208	*Acidocella* sp.	2.7	***Msp*I**(*Hpa*II)		
211	*Desulfobacca acetoxidans*	1.9	T-RF[a]	Identification	Relative abundance[b]
213	*Sulfobacillus* sp. / *Acidisphaera rubrifaciens*	6.7			
227	/	16.7	71	/	12.6
231	*Acidithiobacillus* sp. / *Alicyclobacillus* sp.	1.3	81	*Leptospirillum ferrooxidans*	4.3
248	*Leptospirillum* sp.	4.3	87	/	18.4
250	*Leptospirillum* sp.	1.3	199	*Sulfobacillus thermotolerans*	5.0
253	*Acidiphilium cryptum*	7.8	434	/	7.3
431	*Acidiphilium* sp.	1.7	494	*Acidithiobacillus ferrooxidans*	51.9

[a] Terminal restriction fragment size is expressed in base pairs (bp).
[b] Relative abundance is expressed as percentage.

Acidithiobacillus sp. and/or *Alicyclobacillus* sp. (231 bp); and members of the genus *Leptospirillum* (T-RFs with lengths of 248 bp and 250 bp). The terminal fragment with a length of 197 bp that corresponded to the 16S rRNA gene from chloroplasts suggested the presence of algae in the microbial mat sample. Fragments with lengths of 71 bp, 87 bp, and 227 bp were unidentified.

Digestion with *Bsu*RI (*Hae*III) produced seven T-RFs (Fig. 3; Table 1). With a length of 301 bp, the most abundant T-RF (68.9%) corresponded to the chloroplast 16S rRNA gene. The second most abundant T-RF (13.4%) was a 131-bp-long fragment referable to *Sulfobacillus* sp., whereas the third most abundant one (5.8%) was a terminal fragment 229 bp long, which indicated the presence of the heterotrophic acidophile *Acidocella aromatica*. The presence of members of the genus *Sulfobacillus* was additionally supported by the presence of a less abundant T-RF with a length of 143 bp, which corresponded to *Sulfobacillus acidophilus*. The terminal fragment with a length of 250 bp most likely belonged to *Alicyclobacillus* sp., while two fragments (332 bp and 335 bp) were not identified.

Restriction with *Msp*I (*Hpa*II) produced the least number of fragments, only six T-RFs (Fig. 3; Table 1), with the most abundant T-RF (51.9% of relative abundance) having a length of 494 bp, indicating the presence of *Acidithiobacillus ferrooxidans*. Two other T-RFs were also identified – fragments with a length of 81 bp belonged to *L. ferrooxidans* (4.3%), while the 199-bp-long T-RF corresponded to *Sulfobacillus thermotolerans* (5%). Terminal fragments with lengths of 71 bp, 87 bp and 434 bp and relative abundance of 12.6%, 18.4% and 7.3%, respectively, were not identified.

DISCUSSION

In a recent study that combined both the cultivation and the metagenomic approaches to assess microbial diversity of the surface water of Lake Robule, it was shown that the lake's water is inhabited by a simple bacterial consortium. It was composed mostly of the iron-oxidising autotrophic obligate aerobe *Leptospirillum ferrooxidans* and the heterotrophic acidophilic facultative anaerobe *Acidiphilium cryptum*, with a relatively small number of the chemolithoautotrophic obligate aerobe *Acidithiobacillus ferrooxidans* (Stankovic *et al.* 2014). However, in the present study diversity of the microbial mat sample has proven to be more complex. The culture-

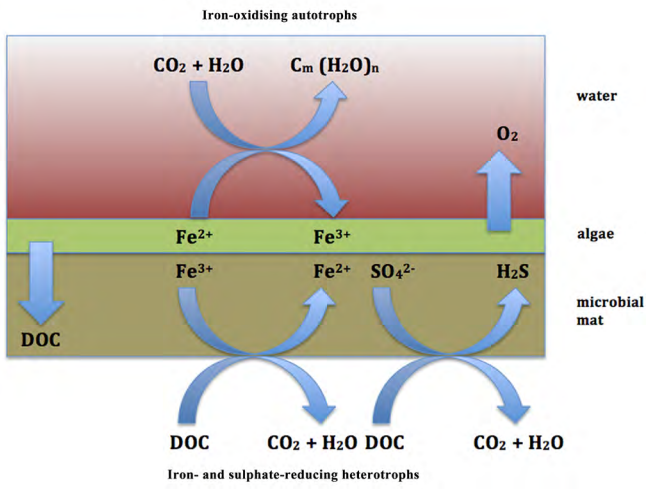

Figure 4. Proposed biogeochemical model of the microbial mat in Lake Robule.
Organic molecules released by algae are oxidised by heterotrophic acidophiles and sulphate-reducing bacteria. In the anoxic environment of the mat, oxidation of organic molecules is followed by reduction of Fe^{3+} to Fe^{2+} and SO_4^{2-} to H_2S. In the oxygen-rich shallow water of the lake, iron-oxidising bacteria oxidise Fe^{2+} and produce carbohydrates during chemosynthesis.
DOC – dissolved organic carbon, Cm(H2O)n – carbohydrates.

independent approach revealed that the microbial mat is more diverse than surface water and is mostly inhabited by heterotrophic acidophiles. In T-RFLP analysis of 16S rRNA genes, a total of 29 terminal fragments were generated, four of which were referable to autotrophic iron-oxidisers, two were referable to algae and eight were not identified, while 15 were T-RFs referable to heterotrophic acidophilic microorganisms. By comparing them to a database of TRFs specific for acidophilic microorganisms, we were able to identify ones belonging to the heterotrophic acidophilic genera *Acidiphilium* and *Acidocella*, mixotrophic *Sulfobacillus* sp. and chemolithoautotrophic iron-oxidisers belonging to the genera *Acidithiobacillus* and *Leptospirillum*. There were a few fragments that suggested the possible presence of other heterotrophic acidophiles (of the genera *Acidobacterium*, *Acidisphaera*, *Alicyclobacillus* and *Ferrimicrobium*), as well as that of sulphate-reducing bacteria (*Desulfobacca acetoxidans*) in the mat. It should be noted that a few signals were biased (Table 1), i.e., they could be assigned to two species belonging to completely different phyla, thus making identification harder. Still, the obtained results indirectly indicate greater community complexity.

The cultivation approach confirmed the presence of *Acidithiobacillus ferrooxidans*, *Leptospirillum ferrooxidans*, *Acidiphilium cryptum* and *Acidocella aromatica*, but not that of the genus *Sulfobacillus,* whose colonies can be easily detected on the basis of their morphology (resembling a "fried egg") (Johnson *et al.* 2005; Watling *et al.* 2008). In T-RFLP analysis, fragments corresponding to *Sulfobacillus* sp. were present in all three digestions, indicating the high probability of its presence. Members of this genus are moderate thermophiles, although some species like *Sulfobacillus thermotolerans* are able to survive in a wide temperature range (Bogdanova *et al.* 2006), so these bacteria could exist in an environment like Lake Robule. But for now whether or not this species is present will remain unresolved.

In acidic environments exposed to sunlight, the primary producers of organic carbon are chemolithoautotrophic bacteria and acidophilic algae (Rowe *et al.* 2007). Nancucheo & Johnson (2010, 2012) proved that exudates and lysates of acidophilic algae can sustain the growth of populations of acidophilic heterotrophic bacteria using the cell-free liquor in which the acidophilic algae were cultivated to support growth of *Acidiphilium cryptum* and *Acidocella aromatica*. Algae growing on the top of the mat produce organic molecules able to sustain populations of heterotrophic acidophiles and, possibly, sulphate-reducing bacteria in the deeper, anoxic layers of the mat. The presence of algae in the mat sample from Lake Robule was also confirmed in T-RFLP analysis, since two terminal fragments from two endonuclease digestions corresponded to 16S rRNA genes of algal chloroplasts.

The presence of unidentified terminal fragments is always expected in a T-RFLP analysis, since like any other method it has its own inherent pitfalls. Some of the methodological drawbacks are related to formation of pseudo-terminal restriction fragments or incomplete digestions. Formation of single-stranded pseudo-T-RFs can be a result of incomplete amplification during PCR (Egert & Friedrich 2003), while incomplete digestions occur for various reasons, including template impurity and complexity, as well as peculiarities of the PCR reaction itself. Moreover, incomplete databases with T-RF sizes make identification of some of the obtained terminal fragments impossible. We used the best database available so far, but many microorganisms are not part of it since they have not yet been cultivated.

The obligately aerobic chemolithoautotroph *Leptospirillum* sp. was detected by both cultivation-based and molecular methods. These bacteria probably originated from water of the lake, since they are abundant at its oxygen-rich surface and are very sensitive to traces of organic molecules in the environment (Baker & Banfield 2003). Similarly, presence of *Acidithiobacillus ferrooxidans* was also detected in the microbial mat sample by both culture-dependent and culture-independent approach, as well as in the lake water (Stankovic *et al.* 2014). Members of the genus *Acidithiobacillus* are facultative anaerobes generally less sensitive to organic molecules than leptospirilli, so it is possible that these bacteria also exist in the microbial mat. The validity of this finding is supported

by results of the study of Kay *et al*. (2013), who identified *Acidithiobacillus ferrivorans* as one of the dominant species in the microbial community of streamers in the acidic, metal-rich water of an abandoned underground copper mine.

Our results suggest that although they most likely share some common members, microbial communities of the surface water and the microbial mat at the bottom of Lake Robule are separate microenvironments. The lake's microbial mat is stratified and inhabited by acidophilic algae, as well as by acidophilic heterotrophic and autotrophic bacteria. In the light of our findings and published data on similar extreme environments, we here propose a biogeochemical model of the microbial mat in Lake Robule (Fig. 4). In this model, the upper layer is inhabited by producers of organic molecules (microalgae that make the environment at least locally oxygenated) and iron-oxidising chemolithoautotrophic bacteria (*Leptospirillum ferrooxidans* and *Acidithiobacillus ferrooxidans*). Deeper layers are inhabited by facultatively and obligately anaerobic heterotrophic acidophiles that metabolise organic compounds produced by the upper-layer microorganisms, thus removing "toxic" compounds from the chemolithoautotrophs. In return, they (e.g., *Acidiphilium cryptum*) reduce Fe^{3+} to Fe^{2+} and continuously supply upper-layer iron-oxidisers with Fe^{2+} ions. Under anoxic conditions in lower strata of the mat, heterotrophic acidophiles (e.g., *Desulfobacca acetoxidans*) reduce SO_4^{2-} ions.

CONCLUSION

Based on the obtained results, it can be concluded that a dual methodological approach is appropriate when analysing extreme habitats in biodiversity studies. We have confirmed a suspected difference in diversity of the mat and water samples from the acidic, metal-rich Lake Robule, i.e., showed that the microbial mat represents a microenvironment within the lake with a distinct microbial population and probably distinct physicochemical properties. Better understanding of the biodiversity and ecological and physiological challenges that indigenous organisms encounter in harsh environments provides insights into the mechanisms governing adaptation of an organism to extreme habitats and sets the basis for our comprehension of biogeochemical cycles.

Ackowledgements — The authors are grateful to Professor D. Barrie Johnson (Bangor University, UK), who kindly provided T-RF database. This study was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Project No. 173048). VC was supported by Ministry of Education, Science and Technological Development of the Republic of Serbia (Project No. 176016).

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REZIME

Diverzitet mikrobijalnog tepiha sa dna ekstremno kiselog jezera Robule (Bor, Srbija)

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Ekstremno kisela staništa se obično formiraju u oblastima intenzivnih rudarskih aktivnosti. Jedan takav ekološki sistem je i jezero Robule koje je, uprkos ekstremnim uslovima koji u njemu vladaju, nastanjeno acidofilnim mikroorganizmima. U ovom radu je opisan mikrobiološki diverzitet makroskopske strukture koja se formira na dnu kiselih vodenih staništa, poznata pod nazivom mikrobijalni tepih. Iako je prisustvo mikrobijalnih tepiha uobičajena pojava u kiselim staništa, struktura ove zajednice može značajno da se razlikuje od jedne do druge ekstremno kisele sredine. Diverzitet mikrobijalnog tepiha formiranog na dnu jezera Robule je ispitivan klasičnim mikrobiološkim metodama, ali i savremenim metodama zasnovanim na metagenomici. Pokazano je da u mikrobijalnom tepihu dominiraju heterotrofne acidofilne bakterije. Utvrđeno je, takođe, da tepih karakteriše kompleksnija mikrobiološka zajednica u odnosu na mikrobiološku zajednicu površinske vode jezera Robule. Na osnovu dobijenih rezultata, ali i postojećih literaturnih podataka, predložili smo biogeohemijski model mikrobijalnog tepiha jezera Robule.

Ključne reči: jezero Robule, kisela staništa, mikrobijalni tepih, acidofili, metagenomska analiza, T-RFLP