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LETTER TO THE EDITOR

# Reply on the Commentary on paper "Investigation of microbial diversity of an extremely acidic metal-rich water body Lake Robule (Bor, Serbia) published in *Journal of the Serbian Chemical Society*, Volume 79, Issue 6, Pages: 729–741

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This is a reply to the Commentary (hereafter: **Commentary**) by V. Beškoski and M. Vrvić on the article entitled "Investigation of the microbial diversity of an extremely acidic, metal-rich water body (Lake Robule, Bor, Serbia)" by Srđan Stanković, Ivana Morić, Aleksandar Pavić, Branka Vasiljević, D. Barrie Johnson and Vladica Cvetković, published in the *Journal of the Serbian Chemical Society*, Volume 79, Issue 6, Pages: 729–741.

We welcome the **Commentary** and thank the authors for showing interest in our research. In this reply, we only address the most important remarks of the **Commentary**, and provide additional arguments for the conclusions originally presented in the paper. We, therefore, put the strongest emphasis on the comments related to the major scientific results of our paper, leaving aside all technical remarks of the Commentary as well as those comments focused on improving the editorial handling of the *Journal of the Serbian Chemical Society*.

The aim of our work was to study the microbial diversity of Lake Robule using the molecular fingerprinting method (Terminal Restriction Fragment Length Polymorphism – T-RFLP) and cultivation of bacteria on selective solid media. In the last twenty years or so, new molecular methods were introduced into microbiology, allowing more comprehensive detection of bacterial diversity in environmental samples in comparison to traditional cultivation-based approaches.[1] It is estimated that traditional cultivation based methods can isolate only 0.1 to 1 % of total bacterial species present in the analyzed samples.[2]

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At the time of sampling (July 2012), the most abundant bacteria in the analyzed samples of the water collected at several locations from Lake Robule were *Acidiphilium cryptum* and *Leptospirillum ferrooxidans,* with a relatively smaller number of *Acidithiobacillus ferrooxidans.* We clearly showed that the diversity and relative abundance of bacteria grown on selective solid media reflects the relative abundance and diversity of bacteria determined by T-RFLP analysis. Of course, both methods have their drawbacks and for this reason, we combined these two approaches.

The authors of the **Commentary** ignore strong evidence presented in the paper and suggest that microscopic examination of the samples would be enough to prove that our findings were wrong. We performed microscopic examination of the samples and saw numerous small spirilli and larger bacilli. It is all documented in microscopic images that can be presented on the Editor's request.

The authors of the **Commentary** based their conclusion that "some methodological and/or conceptual errors may have been made in this paper" on the fact that *At. ferrooxidans* was not detected in the T-RFLP profile, but it was detected on the selective solid media. In order to remove confusion and to solve this dispute, we need to explain in more detail some basic concepts of the T-RFLP analysis.

The T-RFLP analysis of the bacterial community structure is a technique based on Polymerase Chain Reaction (PCR) amplification of the bacterial 16S rRNA genes, enzymatic digestions of the amplified genes, and detection of the size of each of the individual resulting terminal restriction fragments.[3] PCR reaction in T-RFLP analysis requires the use of a primer labeled with a fluorescent dye at its 5′ end. The primers used for 16S rRNA gene amplification (fluorescently labeled 27F and unlabeled 1387R) are designed to bind to a highly conserved region in bacterial DNA. These primers are regularly used to amplify successfully 16S rRNA genes of all known species of acidophilic bacteria, and many other bacterial species. After PCR amplification, the amplified fragments were digested by endonucleases, producing restriction fragments of different lengths. Capillary electrophoresis instrument (DNA sequencer) detects restriction fragments labeled with a fluorescent dye; the so-called terminal restriction fragments (T-RFs), and determines their length. The most abundant T-RFs produce the most intensive fluorescent signals, identified as peaks in the electropherogram, while T-RFs with low abundance produce low intensity peaks, which are undetectable, or masked, by more intensive signals. It is not uncommon to isolate bacterial species on solid media, but not to detect it by T-RFLP analysis since, at least theoretically, a single bacterial cell is enough to produce a colony on selective solid media. On the other hand, T-RFLP analysis can reveal the presence of bacteria that were unable to grow on selective solid media. The best approach to estimate microbial diversity of environmental samples is to use both molecular-



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and cultivation-based methods.[4–7] The T-RFLP analysis was performed in the laboratory of Bangor Acidophile Research Team (BART), Bangor University, UK under supervision of Professor D. Barrie Johnson, one of the most influential authors in this area of microbiology. Professor Johnson kindly provided BART's comprehensive database of the T-RFs of acidophilic bacteria, since this information is not available in public T-RF databases.

Chunbo *et al*. (2010) reported *A. cryptum* as the dominant bacteria in an extremely acidic open pit lake in China. The physicochemical properties of the lake at the time of sampling were very similar to those of Lake Robule.[8] The physical and chemical conditions in both lakes at the time of sampling were nearly optimal for the growth of *A. cryptum*.[10] This fact could be one of the reasons for the dominance of this bacterial species in the analyzed water samples.

Again, it must be stressed that our results reflect only the structure of the bacterial community at the moment of sampling. Summer of 2012 was one of the hottest and driest summers ever recorded in Serbia, and it certainly affected the structure of the bacterial community of the Lake. The physical and chemical properties of Lake Robule express significant seasonal variations.[11] In the conclusion of the paper, we suggested tracking seasonal changes in physicochemical properties and microbial diversity of the Lake in order to provide a more detailed insight into the dynamics of the bacterial population of the Lake.

In conclusion, we truly hope that this reply clarifies the major scientific results of our paper on the microbial diversity in Lake Robule.

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И З В О Д

## ОДГОВОР НА КОМЕНТАР НАУЧНОГ РАДА "INVESTIGATION OF MICROBIAL DIVERSITY OF AN EXTREMELY ACIDIC METAL-RICH WATER BODY LAKE ROBULE (BOR, SERBIA)" ОБЈАВЉЕНОГ У *JOURNAL OF THE SERBIAN CHEMICAL SOCIETY* (2014), *VOLUME* 79, *ISSUE* 6, PP. 729–741

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Колеге В. Бешкоски и М. Врвић са Хемијског факултета Универзитета у Београду су у свом коментару изразили сумњу у резултате објављене у студији "*Investigation of the microbial diversity of an extremely acidic metal-rich water body lake Robule (Bor, Serbia)*" објављеној у *Journal of the Serbian Chemical Society* (2014), *Volume* 79, *Issue* 6, pp. 729–741. Аутори коментара су изнели тврдњу да су објављени резултати последица методолошких или концептуалних грешака у научно–истраживачком раду описаном у овој публикацији. Као одговор на ове тврдње, изнели смо додатне доказе који поткрепљују резултате



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изнете у овом научном раду, као и додатна појашњења примењених метода идентификације микроорганизама, која ће отклонити недоумице у вези са валидношћу приказаних резултата.

(Примљено 5. новембра, прихваћено 11. новембра 2014)

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