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SUPPLEMENTARY MATERIAL TO

Commentary on the article titled "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 (available online 27 June 2013)

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ADDENDUM

Page 731, paragraph 2. "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 location or the site from which the samples were taken is not specified (the former cementation plant; at the foot of Visoki planir; at the centre of the lake; at the exit point; from the lake bottom; in the vicinity of communal wastewater, *etc*.). The authors should provide the GPS coordinates of the sampling sites. Authors also say "water samples", without specifying how many different water samples were actually taken on this day.

Page 731, paragraph 2. "The redox potential of the water was measured using a combined Pt–Ag/AgCl electrode." In the experimental section, it is not clear whether the oxidation/reduction potential (ORP) was measured in the field or not, so this data should be included. Furthermore, the authors do not specify the temperature conditions under which the samples were later transported to the laboratory. Finally, there are no data concerning the timeframe after sampling, and within which the microbiological analyses were commenced/finished.

Page 731. The authors do not mention whether microscopy methods were used to aid in the identification of the microorganisms (MOs). It is presumed they

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did use microscopy, as this is such a fundamental method for identification of MOs.

Page 731. The authors also do not specify whether biochemical characterization of any of the isolated MOs grown on solid media was conducted, or, indeed, any other standard microbiological characterization.

Page 731. MOs were isolated on a solid medium, but they were not counted, so the authors do not mention the number of MO per mL (or at least the order of magnitude) obtained by the methods of classical microbiology. Later on, they specify the prevalence of the MOs in percentages by T-RFLP methods. Unfortunately, their presence was confirmed only by this method (and this involved making a comparison with a database that is available only to the authors, but is not publically available to the scientific community).

Page 732, paragraph 2. "PCR products were analyzed by gel electrophoresis on 0.7 % agarose gel." The authors studied the 16S rRNA gene of colonies grown on a solid medium. However, they do not specify whether the identity of these MOs was confirmed by the sequencing of this gene, which is a standard method of identifying microorganisms, including environmental isolates, and is the best confirmation that the isolated MO is really the one that is being presumed.

Page 732, paragraph 3. "Approximately 400 mL of lake water was filtered through a 0.2 μm (pore size) sterile membrane filter." The authors should specify what type of membrane was used.

Page 734, paragraph 1. "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 the total peak area." The authors give the approximate relative presence based on the T-RFLP results, without the specification of any statistics. Data on the use of statistics should be included.

Page 734, paragraph 1. "The most abundant colonies were colonies of *A. cryptum*, followed by those of *L. ferrooxidans*; colonies of *At. ferrooxidans* were the least abundant." The authors must state clearly whether there were also other types of colonies on the solid medium. The authors should provide complete colony descriptions for all three species growing on their solid medium at a specified temperature, under a specified atmosphere and for a specified time in order to allow other researchers to replicate the methods of the study. The authors provide results in a descriptive manner, without specific numbers and the study would be enhanced if actual numbers were provided.

Page 735, paragraph 2. "After three weeks of bioleaching experiment, the pH value of the solution was 2.20 and redox potential was +820 mV. The concentration of total iron was (815±1.633) mg L^{-1} and the concentration of the total copper was (808.97±5.735) mg L^{-1}. Concentrations of total iron and copper are mean values of three measurements." and page 8, paragraph 1. "The concen-

tration of copper determined in bioleaching solution was greater than this, 808.97 mg L^{-1}." Such over-precision and mixing of the number of decimal places is seen throughout the **Paper.**

Page 736, paragraph 1. "The redox potential of the lake water was measured by a calomel electrode (personal correspondence with the author)." In the personal correspondence with the authors of the cited paper, it has been stated that the ORP was determined using a setup consisting of a calomel reference electrode and a platinum electrode, which the authors of the **Paper** wrongly quote, stating only that the reference electrode was used for the measurement. This does unfortunately cast doubt on knowledge of the authors' of the **Paper** of analytical chemistry. We would also like to mention that authors of the **Paper** were not granted authorization for publishing the personal correspondence.

Page 737, paragraph 1. The authors mention the presence of *Acidisphaera rubrifaciens* based on T-RFLP and confirmed the negligible presence of *At. ferrooxidans* regardless of biogeochemical indicators which show otherwise. Discussion about these extremely interesting results is missing and some discussion on this point should have been included.

Page 737, paragraph 1. "Interestingly, *At. ferrooxidans*, previously reported as the dominant bacterial species in Lake Robule, was not detected in the lake water by T-RFLP analysis, though was isolated on solid medium (FeSo plates), along with *A. cryptum* and *L. ferrooxidans*." The authors should have explained this.

Page 737, paragraph 2. "Earlier studies suggested that microbial communities in acidic environments were dominated by *At. ferrooxidans*, but it appears this was an artefact of the methods, particularly enrichment culture and most probably number (MPN) counts. Media for cultivation of acidophilic bacteria that have been widely used, and sometimes still are, such 9K, contain very high concentrations of Fe^{2+} iron (9 g L^{-1} in 9K) and favour the growth of *At. ferrooxidans*." The authors suggest that 9K is a selective media that favours the growth of *At. ferrooxidans.* Of course, this cannot be disputed, since in the cited investigation, it was of interest to determine, as accurately as possible, the count of *At. ferrooxidans* in the environment which was very heavily burdened with iron. We chose to do so using the MPN technique, which is a very common and well-accepted standard technique for estimating bacterial numbers in liquid samples (http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ /ucm109656.htm). In fact, the medium, 9K reflects, in an excellent manner, the natural conditions under which *At. ferrooxidans* live, it is a widely-accepted and the most suitable medium with which to obtain MPN counts of this MO from these types of water samples. The authors should note that the use of any medium, including their solid medium, is selective, as is the choice of the incubation atmosphere, time and temperature. For any given sample (*e.g.*, food,

water, soil, biological tissue, *etc*.), the choice of medium, atmosphere and time/ /temperature combination affects the ability of individual microorganisms contained within that sample to form colonies (and thus be noticed and "counted" under the chosen conditions). Thus, in any given sample, the "total bacterial population" or "total bacterial count" only ever encompasses those bacteria able to thrive under the given conditions on the given medium, while those which cannot do so are not included. Therefore, to selectively count a given microorganism in any given sample, the medium, atmosphere, time and temperature are all adjusted to suit the organism of interest, and to allow/promote its growth. The authors should have explained that it is not possible to compare the estimates of bacterial percentage prevalences obtained in the **Paper** with bacterial MPN counts obtained in the cited study.

Page 737. We note that polymerase chain reaction (PCR) is also selective and that it will multiply only those fragments for which primers have been added. It is only with repetition using different primer sets that the probability of finding all 16S rRNA present in a PCR-tube is increased.

Page 737, paragraph 3. "Reports on the composition of the microbial community in the lake published by Beškoski *et al*. (2009) that differ significantly from the results presented in this paper are..." In the quoted paper of Beškoski *et al*. (2009), the microbial community was not studied and is not mentioned, so this appears to be a misinterpretation of results obtained by us, and therefore, compares things that cannot be compared at all. Neither the total community nor the biodiversity were analysed in our study, but instead, *At. ferrooxidans* and other thionic bacteria were the main targeted MOs. The authors of the **Paper** speculate about a historical bacterial population, which is difficult to justify, as, to the best of our knowledge, no published record of the microbial community in this lake exists, and there is no evidence of its change, or lack thereof, over time.

Page 738, paragraph 1. "The obligatory acidophilic heterotroph *A. cryptum*, the most abundant bacterium in the lake water as determined by T-RFLP analysis and isolation on solid medium". Since the solid medium was prepared with the addition of *A. cryptum*, the issue of contamination cannot be excluded. The authors should have provided information about the steps they took to prevent such contamination, and their proof that the solid medium could not have been contaminated with supplemented *A. cryptum*.

Page 738, paragraph 2. "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." and *page 9, paragraph 1*. "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 municipal waste dump that is very close to the lake. Another source of organic matter in the lake is probably acido-

philic algae that populate the microbial mat at the bottom of the lake." The authors state that the organic substance from the municipal waste dump (or the acidophilic algae) is the reason for the presence of organic substance which stimulates the growth of *A. cryptum* and *Acidisphaera rubrifaciens*. However, they have not confirmed the presence and the quantity of the organic substance using basic analysis (*COD*, *BOD*, *TOC*, *etc*.).

Page 739, paragraph 1. "The most abundant microorganism in the lake is heterotrophic bacterium *A. cryptum*." The authors reach this conclusion based on one point-like sampling and the use of the solid medium on which this MO was used anyway to remove organic compounds. One (or even several) water sample(s) taken on one day would not seem to be representative of the entire lake. The authors should have replaced the word "lake" in this sentence with "the water samples".

Page 739, paragraph 1. "Those 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 overlay solid medium." This result, which indicates that this MO was present in sufficient numbers that its colonies appeared on the solid medium, but it was not present in sufficient numbers to enable isolation of metagenomic DNA, is very interesting and it is surprising that the authors did not attempt to explore it further. This is an important scientific point which must be developed, as it has wider scientific repercussions than just for the current **Paper**.

Page 739, paragraph 2. "Physical and chemical properties of the lake display both seasonal and long-term variations." This cannot be concluded for Lake Robule from the results of the authors of the **Paper**, since the results of the **Paper** are based on one sampling time. Nor can the results regarding microorganisms be compared directly with the results of others, as explained above.

Page 740, paragraph 1. "Такође, испитивана је способност нативних бактерија из језера да врше луженье бакра из узорка минералног концентрата." The term "indigenous bacteria" is translated into non-standard terminology by the authors as "нативне бактерије".

Page 733, Table I. No data are available about the concentration of dissolved O_2, or the content of organic substance.

Pages 733 and 736, Figures 1 and 2. Figure 2 has been produced based on the data taken from Figure 1. The authors should have specified what fragment of T-RF (*HaeIII, AluI* or *CfoI*) was used for this quantification and provide statistics.

Page 734, Figure 2. The quality of the black and white photograph is not satisfactory, so that readers cannot see what the authors refer to in the **Paper**.