Reply to Reviewers

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**TITLE: “INDICATOR BACTERIA OF FECAL CONTAMINATION IN SPRING AREA OF PLITVICE LAKES NATIONAL PARK”**

**Manuscript no. 2849-6723-1-RV**

Dear Editor,

We accepted almost all reviewers` suggestions which helped us to significantly improve our manuscript. Below are detailed answers to all comments (in italic).

We hope you will find this reviewed manuscript suitable for publication in Periodicum Biologorum.

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**Reviewer A**

**General comments:**

R: Data in Figs 5 - 8 are not clearly shown. It is almost impossible to decipher individual values, and there is no statistical data. Table with required information (mean value, SD, results of statistical analysis) should be much more convenient.

A: *We accept the comment and we changed figures, i.e. we showed results using Box and whiskers plot (so there are explanatory statistical values). Statistical analysis for this data is in the text, and we also added t-test to test the differences between source area and lake.*

R: In Table 2 and Figs 7-8 data for March are missing.

*A: The data for March are missing due to the fact that we did not carried out the analysis of fecal streptococcus and Pseudomonas aeruginosa bacteria in that month and the main reason are technical difficulties (we did not yet received the corresponding agar). We added the sentence: “The data for FS and Pseudomonas aeruginosa were not obtained in March due to some technical difficulties with agar base.”*

R: English language must be improved.

*A: We accept the comment and the whole text is reviewed by the professor of English language.*

**Specific comments:**

R: *Abstract*

Avoid the use of abbreviations without explanation (FC/FS).

*A: We accept the comment and reviewed sentence is: ”Standard microbiological methods were used to determine densities of total coliforms (TC), fecal coliforms (FC), fecal streptococcus (FS), Pseudomonas aeruginosa and Clostridium perfringens in water samples.”*

R: *Methods*

The authors did not indicate the number of replicates for each of the analysis. How long did it take from water sampling to bacteriological analysis and how were the samples treated during transport?

*A: We accept the comment and we explained this in a sentence: “Analytical procedures were tested for reproducibility by means of quadruplicates for random samples. As we determined low standard deviation (around 15%), analysis were mainly done on single samples.”*

*As for the question regarding transport, we added the sentence under the headline Sampling and identification: “Water samples were stored in the mobile fridge, and in two to three hours upon collecting they were processed in the laboratory of the Plitvice Lakes National Park.”*

R: How are the results presented: mean value, median, what about statistical parameters?

*A: We accept the comment and we presented results using mean values, medians, quartiles and outliers. One statistical method is also added. The sentence by which we explain statistical analysis in manuscript is: “All collected data were processed in the Excel program (MS Office) where tables and graphics were generated. Differences in TC, FC and FS numbers between the sampling months were tested by one-way ANOVA, followed by Fisher LSD post-hoc test. T-test was used for differences between source area and Prošćansko Lake in previously mentioned variables. All values were log (x+1) transformed prior to analysis. One-way ANOVA was done using Statistica 12 program (StatSoft).”*

R: *Study area*: According to reference 14, in village Plitvički Ljeskovac live 20 residents.

*A: We accept the correction and the corrected sentence is: “After the flow of both rivers through populated area of village Plitvički Ljeskovac with 20 residents (17)…”*

R: *Results and discussion*

Calcium ions are 2+, and not 3+. Furthermore, ions are present in solutions and do not precipitate as such. Precipitation occurs when poorly soluble salts are formed; therefore possible salts should be named.

*A: We accept the correction for calcium ions. It was lapsus calami. Considering the comment about the possible salts, we are not trying to go into the detail about the precipitation process itself because it is not so relevant for the context of this manuscript. Also, we did not measure all relevant chemical parameters to draw such conclusions. Furthermore, we are not presenting the results of calcium ions and the stated reference is mainly connected to the values of electrical conductivity meaning the connection of electrical conductivity and calcite precipitation. The conclusion about this connection is stated by the mentioned authors and we are not reassessing further information. However, we concluded that the sentences considering the conductivity and calcite precipitation are not so relevant for this manuscript because the chosen localities are in the area of very low calcite precipitation, and mentioned authors are mainly referring to the downstream area of lakes (from Upper to Lower lakes) in the cascading system.*

R: Speaking about the water temperature it seems more appropriate to speak about an increase from deeper layers to the surface (instead of temperature decrease). The temperature is more constant in depth while on surface seasonal effects are more prominent.

*A: We cannot accept this comment. It is known in limnology of freshwater ecosystems that the temperature in the lakes is described from the surface to the bottom and that in dimictic lakes like Prošćansko Lake the water temperature is decreasing towards the bottom except in winter when whole layers are isothermic. There is also a characteristic thermocline development at specific depth, and it would be unusual to describe this going from the bottom towards the surface.*

R: "Fecal streptococcus bacteria were increased in July and October at…" Increased compared to what?

*A: We accept the comment and the reviewed sentence is: “FC bacteria were also more numerous in source area than in Prošćansko Lake (t-test, t=3.66, p<0.001). Highest values were in July and October at the sampling site WRb (39 – 70 CFU 100 mL-1) (Figure 7, maximal values), while they were for instance not recorded at the sampling site BRs in July.”*

R: "Number of FS was not high in Prošćansko Lake and the highest recorded value in the period of study was 15 CFU 100 mL-1 at Ple10m." Again it is not clear what was used as a standard for comparison ("was not high"). The time (month) of the highest recorded value should be indicated.

*A: We accept the comment and the corrected sentence is: “The presence of FS was recorded at most of the sampling sites in Prošćansko Lake except PLd1m. The values of FS varied between 1 and 15 CFU 100 mL-1. The highest recorded value was 15 CFU 100 mL-1 at PLe10m in July 2012.”*

R: *References*

Refs. 4 and 22: Names of microorganisms should be written in italic.

*A: We accept the correction. However, due to adding of references, mentioned references are now numerated differently and it is written as following:*

6. MENA K D, GERBA C P 1989 Risk Assessment of *Pseudomonas aeruginosa* in Water**.** *Rev Environ Contam 201*: 71-115

31. WHITMAN R L, NEVERS M B, KORINEK G C, BYAPPANAHALLI M N 2004 Solar and Temporal Effects on *Escherichia coli* Concentration at a lake Michigan Swimming Beach. *Appl Environ Microbiol 70 (7)*: 4276-4285

R: References 14 and 26 should be amended.

*A: We accept the correction. Also, we added new reference to the Regulation of water quality and it is written as following:*

17. BURŠIĆ I 2013 Popis stanovništva, kućanstava i stanova 2011. Stanovništvo prema spolu i starosti. Statistička izvješća 1468. Državni zavod za statistiku Republike Hrvatske, Zagreb, p 682. [29.06.2015]

39. Regulation on Water Quality (NN 77/98)

40. Regulation on Water Quality Standards (NN 73/13 and 151/14)

**Reviewer B**

R: **General comments:**

I am convinced that sometime there is a thin line between scientific paper and research report but in the case of this paper I do not see it as original scientific paper. According to my opinion manuscript entitled: “Indicator Bacteria of Fecal Contamination in Spring Area of Plitvice Lakes National Park” has to be classified as research report similar to several others mentioned in the manuscript and quoted within the references pages.

*A: After corrections made in our manuscript we believe it is evident that our manuscript is scientific paper. Microbiological research received considerable attention in recent decades, with questions regarding national regulation and sources of pollutants, distribution and outcome of pollution being a subject of high quality papers (e.g. Anderson et al. 2005, Poté et al. 2009, Surbeck et al. 2010). There are still many open questions on persistence of fecal indicator bacteria in plankton, and not so many investigations. We added new references in our manuscript, done in countries but having comparable research scope (and those are original scientific papers). The reason for using research reports was the lack of published results in journals from previous microbiological investigations in Plitvice Lakes NP.*

*Having all that in mind, we believe that our manuscript falls in the scope of scientific papers.*

R: In the MATERIAL AND METHODS section there is much more information on sampling procedures and study area than about used physicochemical and microbiological methods. At the same time all applied procedures were standard and routine methods.

*A: We accept the comment about the lack of information on microbiological methods, so those were added. We think that physicochemical methods are described enough for this paper especially because the analysis were conducted with digital field instruments. For example, the same was done by Atkinson et al. (2007) and it is a standard in limnology.*

*More precisely, for the information on microbiological methods we added further sentences under the headline Sampling and identification:*

*“The water samples for microbiological analysis were collected into 500 mL sterile bottles under the water surface and with a plastic sampler Niskin type (Hydro-Bios, Germany) for water samples in the lake. Water samples were stored in the mobile fridge, and in two to three hours upon collecting they were processed in the laboratory of the Plitvice Lakes National Park.*

*All samples for microbiological analysis of FIB were processed using the filtration method according to standard methods (2) and by filtrating 100 mL of sample through the membrane filter with pores of 0,45 µm (Sartorius Stedim Biotech, Germany).*

*After the filtration, the filters were transferred to different agar bases: m - LES endo agar (Biolife, Italy) for TC and m - FC agar (Biolife, Italy) for FC. The incubation period for TC was 24 hours at 35°C. FC were incubated 24 hours at 44°C. For growth of FS we used Slanetz-Bartley agar (Biolife, Italy) and incubated plates 48 hours at 37°C. After the 48 hours we counted the characteristic colonies on membrane filter and then transferred the membrane filter on Bile aesculine agar (Biolife, Italy) with the incubation time of 2 hours at 44°C. After the membrane filtration of sample for Pseudomonas aeruginosa analysis we placed the filter on Pseudomonas agar base (Biolife, Italy) and incubated the plates 48 hours at 37°C. Afterwards we counted the fluorescent colonies as a part of the confirmation test by using UV lamp (Hach, Mini UV lamp, Germany) at λ=365 nm. Further identification of isolated Pseudomonas colonies was carried out through applying the biochemical API 20 NE test (bioMérieux, France). 1 mL of water sample used for determination of Clostridium perfringens were first inoculated into the sterile test tubes then inactivated at 80 °C for 5 minutes. After the inactivation, the SPS agar (Biolife, Italy) was poured into tubes and samples were incubated 24 hours at 37°C.”*

R: Also, research period is rather short for this type of ecological investigation.

*A: We cannot fully agree with this comment and the main reason is because we think that in one year of investigation and with inclusion of all seasons (winter, spring, summer and autumn meaning March 2012, May 2012, July 2012, October 2012 and January 2013) we collected enough data to present the seasonal number of fecal indicator bacteria.*

R: In REFERENCES section, research reports on similar topics, together with review papers and manuals describing routine methods are dominating over scientific papers. Recent papers on water contamination with fecal bacteria are totally missing.

*A: We accept the comment and we reviewed the REFERENCES section and added new references and deleted main portion of research reports, review papers and manuals. However, in some parts this was not an option due to the lack of recent scientific papers considering the investigated area of Plitvice Lakes National Park, as we mentioned previously.*

**References used in authors answers to Reviewer 2:**

Anderson K L, Whitlock J E, Harwood V J 2005 Persistence and differential survival of fecal indicator bacteria in subtropical waters and sediments". *Appl Environ Microbiol 71 (6)*: 3041-3048

POTÉ J, HALLER L, KOTTELAT R, SASTRE V, ARPAGAUS P, WILDI W 2009 Persistance and growth of faecal culturable bacterial indicators in water column and sediments of Vidy Bay, Lake Geneva, Switzerland. *J Environ Sci 21*: 62-69

Surbeck C Q, Jiang S C, Grant S B 2010 Ecological control of fecal indicator bacteria in an urban stream. *Environ Sci Technol 44 (2)*: 631-637

ATKINSON C A, JOLLEY D F, SIMPSON S L 2007 Effect of overlying water pH, dissolved oxygen, salinity and sediment disturbances on metal release and sequestration from metal contaminated marine sediments. *Chemosphere 69 (9)*: 1428–1437

***Additional changes:***

*We also changed the title (from “Indicator bacteria of fecal contamination in spring area of Plitvice Lakes National Park” to* ***“Distributional patterns of fecal indicator bacteria in spring area of Plitvice Lakes National Park”****) to make it more precise.*

*Abstract was also changed due to the changes made in the manuscript.*