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Abstract: Relating the treated wastewater quality and its impact on organismic biosensors (Prussian carp, Carassius gibelio and earthworm, Eisenia fetida) was the main objective of the study. The impact on health status of fish living downstream, microbiological contamination and antimicrobial resistance, fish tissue structure, blood biochemistry, oxidative stress, genotoxic effects, as well as multixenobiotic resistance mechanism (MXR) was assessed. Treated wastewater discharged from the WWTP modified the environmental parameters and xenobiotic concentrations of the receiving surface waters. Potential bacterial pathogens from fish and respective waters were found in relatively low numbers, although they comprised aeromonads with a zoonotic potential. High resistance profiles were determined towards the tested antimicrobial compounds, mostly sulfamethoxazole and erytrhomycin. Histopathology primarily revealed gill lamellar fusion and reduction of interlamellar spaces of effluent fish. A significant increase in plasma values of urea, total proteins, albumins and triglycerides and a significant decrease in the activity of plasma superoxide dismutase were noted in carp from the effluent-receiving canal. Micronucleus test did not reveal significant differences between the examined groups, but a higher frequency of erythrocyte nuclear abnormalities was found in fish sampled from the effluent-receiving canal. Earthworms indicated to the presence of MXR inhibitors in water and sludge samples, thus proving as a sensitive sentinel organism for environmental pollutants. The integrative approach of this study could serve as a guiding principle in conducting evaluations of the aquatic habitat health in complex bio-monitoring studies.

Response to Reviewers: The response is attached as a separate file.

Dear Editors,

According to the request of Dr. Mark Hanson for the role of the Associate Editor for this paper,

I am forwarding a revised manuscript (**STOTEN-D-15-01596R1**) titled IMPACT OF TREATED WASTEWATER ON ORGANISMIC BIOSENSORS AT VARIOUS LEVELS OF BIOLOGICAL ORGANISATION by Topic Popovic et al.

We request that this manuscript be considered for publication in Science of the Total Environment as an original, full paper. This manuscript has not been published and is not under consideration for publication in any other journal. All authors concur with the submission.

The manuscript is a result of a comprehensive study which integrated various disciplines and research fields. The authors decided to present it as a whole rather than splitting it in two or more papers, for a better understanding of the discussed subject. We believe we have presented a potential guiding principle for conducting evaluations of the aquatic habitat health in complex bio-monitoring studies.

I will be serving as the corresponding author for the manuscript. We hope that it is found to be interesting and appropriate for publication in Science of the Total Environment. Should you have any questions, please do not hesitate to contact me.

Yours truly, Natalija Topic Popovic, DVM, PhD

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August 7, 2015.

Dear Dr. Hanson,

Thank you for your letter and for the comments of the reviewers that we received on July 19th. We are very thankful for their assistance in improving our paper. We considered all the comments and have made all the necessary corrections in the manuscript (**STOTEN-D-15-01596R1**) titled: Impact of treated wastewater on organismic biosensors at various levels of biological organization by N. Topic Popovic et al.

Below we address each comment of the reviewers:

***Reviewer # 1**:*
The authors have addressed many of raised concerns and improved the manuscript. However, the following additional improvements/explanations are still necessary before the manuscript could be recommended for publication.

Main comments:
1. Page 20, last 6 lines and page 21, first 2 lines; also page 31, first 2 lines: I am sorry but I don't understand how and why you conducted PCA and what the findings are. It seems that you conducted PCA as a dimension reduction method. However, you do not report how you reduced the dimension of the dataset and in addition to that you discuss the results in terms of associations. This maybe just the semantics and if so I would appreciate if you could explain your approach and provide a reference where a similar approach was applied. Meanwhile, here is my understanding of the purpose and the process of PCA. In PCA you only consider the independent variables (i.e., predictors) but not the outcome (which in your case is presumably the location: A (reference), B or C). Because of that we do not test for associations between the variables and the outcome using PCA. In PCA, generally, the top principal components (PCs) explaining most of the total variation are identified and described. For example, you may say that plasma values for D, E and F loaded on the first PC, while variables G, H, I and J loaded on the second PC. Then you try to find some commonality between variables loading on the same PC (for example variables D, E and F in PC 1 may all be mediators of stress and so that would explain why they vary together). Once you identified the top PCs you can

calculate the principal component scores (PCS) for each PC and tests for an association between the PSC and the outcome of interest. A practical problem with this approach may be the interpretation of the whole PC (i.e., combined variables that comprise the PC). An alternative approach could be to pick one variable from each PC (as its representative) and test its association with the outcome of interest. Using the example from before maybe you picked D from PC 1 and H from PC 2 (because they had the lowest p-values from the univariable analysis of plasma values association with the location (i.e., the outcome)). Finally, you would run a multivariable model with D and H as predictors of the outcome. Your outcome has 3 levels (locations A (the reference), B and C) and so the approach to use would be the multinomial logistic regression. Again, this is based on my understanding of PCA; please feel free to explain the rationale of your approach if you believe that it is valid.

Response:

- Thank you for this clarification. We have taken a whole different approach and completely rewritten all sections relating to the PCA as they were indeed confusing, and created a new biplot for plasma parameters. We hope that the new text, as inserted in the respective paragraphs, will answer your concerns.
- Methods: "Principal component analysis (PCA) was applied to describe the main sources of variation and relationship among the selected blood biochemistry parameters. The results are presented graphically by a biplot."
- Results: "The PCA of eight blood biochemistry parameters from carp captured at three locations produced three significant components. First two principal components (PCs), as shown in Figure 5a, explain 76.86 % of variation in our dataset. PC1 accounted for 43.48 % of variation and loaded mainly on stress variables (CHOL, TRIG, TP, ALB, GLU), PC2 accounted for 33.38 % of variation and loaded mainly on liver impairment parameters, such as ALP and ALT. Axes PC1 and PC3 (Figure 5b) explain 54.47 % of variation, where PC1 (42.26 % of variation) loaded mainly on stress variables, and PC3 which accounted for 12.21 % of variation, loaded mainly of renal impairment parameters, (CRE, URE, TP and ALT)."
- Discussion: "The PCA allowed discrimination of the most relevant plasma mediators determining physiological responses of fish under assay. In the both PC1 and PC2, PC1 and PC3 axes (Figure 5), the first component was biologically consistent with parameters related to stress, and a clear effect was found for ALB and TP as well as for CHOL and GLU from fish originating from the effluent-receiving canal (B-5), which tend to group together and appear to have similar characteristics. There was a clear separation between the effluent fish and the unaffected stream fish, where stress impacted the liver impairment. Similar findings relating to stress-based tissue impairment were documented by Li et al. (2011). Renal impairment affected by stress reached downstream from the effluent, in the County canal (C-9), as evident from the CRE and URE parameters." The rest of the paragraph discusses the reasons for alteration of particular hematological and biochemical parameters.
- We have also introduced a new reference: Li Z-H, Velisek J, Grabic R, Li P, Kolarova J, Randak T (2011): Use of hematological and plasma biochemical

parameters to assess the chronic effects of a fungicide propiconazole on a freshwater teleost. Chemosphere, 83, 572-578.

2. In the context of the previous comment please check Figure 6 caption: what does "highly associated parameters" mean? Which test of association was applied?

Response:

- We have rewritten the caption and renamed the figure into Figure 5. The text now reads: "PCA correlation loading biplots for carp plasma parameters. Abbreviations: (A_metabolite: fish from the unaffected stream, B_metabolite: fish from the effluent-receiving canal, C_metabolite: fish from the County canal)."

3. Page 4, statements of hypotheses: I like that you included a statement of hypotheses. However, please confirm whether these two hypotheses encompass all the various evaluations and tests you conducted. More importantly, the two hypotheses are not at all integrated with the rest of the manuscript. Please tell the reader in the Materials and Methods which work was conducted to test each of the two hypotheses.

Response:

- Yes, the two hypotheses are each rather comprehensive and do encompass all the tests performed. Since we already have the text explaining the tests conducted to test the hypotheses in the Introduction, we have adapted it to clarify which work was done to test one or the other by inserting their respective designative numbers in the parentheses (1) (2) next to the methodologies applied. The text now reads: "To test our hypotheses, we conducted a series of tests to measure the impact on health status of fish living downstream, microbiological contamination, fish tissue structure, blood biochemistry, oxidative stress, genotoxic effects and multixenobiotic resistance mechanism (MXR): (i) general fish health examination, necropsy and histopathology were performed (hypothesis 1); (ii) rapid phenotypic tests and matrix assisted laser induced desorption ionization connected to the time of flight mass spectrometry (MALDI-TOF MS) were conducted on multiple samples of water, fish, and sludge (hypotheses 1, 2); (iii) fish blood biochemistry parameters and oxidative stress parameters were determined (1); (iv) erythrocytic nuclear abnormalities and micronuclei were enumerated and assessed (1); (v) cellular efflux mechanism mediated by ATP binding cassette (ABC) transporters that bind and actively remove toxic substrates from cells was analyzed post exposure to raw and treated water and sludge (1); (vi) physical-chemical characteristics and heavy metal contents were determined in water and sludge samples (1); (vii) antibiotic concentrations were measured in water and sediment (2)."

4. Page 22: "The goal of this study was to test the hypotheses that 1) the WWTP effluent will induce biological effects on organismic biosensors; 2) resistant bacteria and potential fish and human pathogens will be identified from water and sludge, and antibiotics will be retrieved from sediment." It is a bit awkward to see the text from

introduction repeated in the discussion per verbatim. A better approach would be to formulate your findings as "…we have confirmed our hypotheses that…."

Response:

- Thank you for this suggestion. However, in order to avoid repetition, since in the same section we have a similar sentence, this one we deleted and left the one that reads: "The results corroborate the hypotheses, indicating that antibiotic effects, bacterial pathogens, and the changes of biological responses related to the WWTP reach far downstream from the WWTP."

5. Page 22: "Such a combined approach was considered due to possible misinterpretations resulting from the isolated analyses of each aspect and therefore inherently implies a broader ecological and, ultimately, human health insight." This statement is overly vague. You must demonstrate, using examples from your results, how different conclusions would be made if different results are interpreted in isolation compared to if they are interpreted jointly.

Response:

- We have added the text in which we tried, by citing relevant works which used a suite of biological indices for measuring stress responses, to elaborate the benefits of such an approach. The sections that follow link the results and biological responses with water quality, heavy metal and drug concentrations, tissue contamination with potential pathogens, zoonoses and drug resistance, oxidative (immune) sensitivity, structural and functional tissue alterations related to stress. The added text reads: "Although most environmental studies take into account only a limited amount of biological responses, parameters which individually show no distinctive response to a stressor can, when used in conjunction with other parameters, be useful indicators of the extent of the environmental impact (Bernet et al., 2000). This impact can be demonstrated as either direct chemical or physical damage to cells or tissues, modification of physiological and biochemical reactions, increased infection pressure by facultative pathogens or reduced resistance to infection (Schmidt-Posthaus et al., 2001), thus their determination at the whole organism level can detect sublethal and chronic effects on organisms and present integrative responses to complex mixtures of pollutants."

6. Page 22: "The results corroborate the hypotheses, indicating that antibiotic effects, bacterial pathogens, and the changes of biological responses related to the WWTP reach far downstream from the WWTP." This is good and may be used to address comment #4 above. Next, for each described result (or group of results) link the results back to your hypotheses (this will also assure integration of hypotheses into your Discussion). Also, how do you define "far downstream"? Was the effect "far downstream" demonstrated for all types of evaluated samples?

Response:

- As explained in the response to the comment 4 above, this sentence was kept instead of the other one.

- We have linked the findings with the hypotheses. In section 4.2. it reads: "We have thus confirmed the hypothesis that resistant bacteria and potential fish and human pathogens will be identified from water and sludge"; in section 4.3. "As hypothesized, the inflow of high concentrations of SMX, also found in the sediment, must have contributed to significantly higher resistance of environmental aeromonads towards this drug"; in section 4.4. "Structural and functional alterations found in the carp gill tissues, particularly in the effluent-receiving canal (B-5), relate extensively to the secondary lamellae, indicating to environmental pollution, as presumed in the first hypothesis of this work."; in section 4.5. "The hypothesis that the WWTP effluent will induce biological effects on organismic biosensors is further discussed in this section and the following one."
- The expression "far downstream" we used for the sampling sites after the treated wastewater leaving the WWTP and the effluent canal, all the way to the remote Drava river. Those sampling sites are: 7: canal entering the County canal, 8: County canal, 9: County canal downstream before the Drava river. We used it to avoid repetitions and to shorten the text. The antibiotic effects, bacterial pathogens, and the changes of biological responses indeed reached further than just in the effluent waters, as we discuss throughout the paper.

7. Page 33: "The lack of correction for multiple testing, due to the preliminary nature of the study, is one of its limitations." This limitation should be moved to the end of the Discussion section. The only other limitations stated were in terms of the low number of fish and sampling over just 2 days. In addition to these limitations indicate other study limitations. Specifically, you evaluated only one WWTP; more WWTPs may very well not be logistically feasible but still based on just one WWTP it remains unknown if the results are generalizable to other WWTPs or if they are unique to this particular WWTP. If I understand this correctly, the number of samples tested per location, except for fish (8) and water for analytical chemistry (3), was only one per location and once in spring of 2014. Therefore, the within location variability in a point in time and over time could not be evaluated.

Response:

- We introduced a section 5. Limitations. It reads: "The study was conducted once in spring of 2014, with limited number of fish, sampling days, and samples per location. The relatively low fish sample size per sampling site is a consequence of reduced accessibility to the posts appropriate for fishing and fish avoidance of the bait. The lack of correction for multiple testing, due to the preliminary nature of the study, is another of its limitations. The tested hypotheses relate to the particular WWTP under assay."

Minor comments:

1. Page 7: spell out EC and EEC for readers outside of Europe.

Response:

- The phrase now reads: “This work has been carried out in accordance with the European Council Directive 86/609/European Economic Community for animal experiments”

2. Page 8, line 4: what do you mean with "Specimens were randomly sampled…"? Did you really sample fish at random or was it a convenience sample of all fish you were able to catch?

Response:

- It was a sample of the fish that took bait and the fish that we could net. We rephrased the sentence. Since in the previous paragraph we detail the ways of capture, here we state: “Specimens were transported live to the laboratory and within few hours sacrificed by overdose of tricaine methane-sulfonate….”

3. Page 16, line 3: based on your results section you used Spearman and not Pearson correlation, correct?

Response:

- Yes. That was a typo. We corrected it.

4. Page 19: Table 7 is described before Table 5 and 6. Please correct.

Response:

- We have moved the description in the respective text, as well as changed the title of the section 3.3. which now reads: “Physical and chemical properties of water; heavy metals; pharmaceuticals in water and sediment”

5. Page 20: please indicate the strength of correlation for each pair. With very large datasets two variables may be significantly correlated although the strength of correlation may be low; with just 24 fish in total I don't expect this problem in your results but still you should indicate the strength of correlation for each pair.

Response:

- The strengths of correlation ranged from moderate to very strong. The text now reads as follows: “The Spearman Rank Order Correlation demonstrated that pairs of variables with p values below 0.050 and positive correlation coefficients (r_s), such as URE/ALT (0.5), URE/TRIG (1.0), URE/CHOL (0.5), CRE/SOD (1.0), TP/TRIG (0.56), TP/CHOL (0.74), ALB/TRIG (0.73), ALB/CHOL (0.68), ALT/TRIG (0.86), ALT/CHOL (0.67), ALT/SOD (0.68) ALP/TRIG (0.59), TRIG/CHOL (0.87), TRIG/SOD (0.56) from the fish captured in the County canal, C-9, tend to increase together.

6. Page 20, line 7 from the bottom: replace "correlated" with "compared".

Response:

- Replaced.

7. Page 25, line 3: why is this a "valid first step"; how do you define validity? Generally something is valid if it is not biased. Your expression may be correct and I just ask that you confirm it.

Response:

- We referred to validity as to a clinical term for diagnosis. The API 20E is a tool for initial identification of non-fastidious Gram-negative rods. In that sense it is a valid first step in preliminary bacterial identification, as we stated.

8. Page 26, lines 7 and 16: please show in brackets the number of positives (resistant) out of the number tested for your reported percentages 50% and 11%.

Response:

- We inserted the number of fish aeromonads that were resistant to SMX, and also the number of *Aeromonas* spp. isolated from water and sludge resistant to OTC and erythromycin. These are 18 and 3 isolates, respectively.

9. Page 33: "The transferability of presented approach in assessing the impact of the WWTP effluent on biosensors and biological responses will be the basis of future investigations." I am sorry, but I don't understand what you mean here. Please provide details (e.g., which biosensors and which biological responses will be tested in future investigations to test the transferability).

Response:

- Here we meant that in the future studies of other WWTPs we will try to assess the treated wastewater quality and its impact on biota (fish and earthworms) in the same manner as we presented in this work. We found that, aside water, sludge and sediment testing, a combination of methods we applied to assess the health status of fish, microbiological contamination and antimicrobial resistance, fish tissue structure, blood biochemistry, oxidative stress, genotoxic effects and earthworm MXR mechanisms, are well chosen to establish links between environmental contamination and its effects on biota. However, we decided to remove the sentence from the section as it is probably redundant.

10. Table 4: How many isolates were tested per location and per sample type (water, sludge, fish)? From the table, it seems that in most locations as little as 4 (or even 3 in location 5) isolates were tested. Which location is 7/B-7? Should that be 5/B-5 instead?

Response:

- The table presents the number of samples per sampling location for a) fish for necropsy, microbiological, blood plasma, genotoxicity, histopathology assessment (8 specimens per location, at three locations, a total of 24 specimens); b) water for physico-chemical, heavy metal, and microbiological analyses (one sample per location, a total of 9 samples); c) sludge for physico-chemical, heavy metal, and microbiological analyses (one sample per location, a total of two samples of

sludge); d) water for analytical chemistry (3 samples per two locations, a total of 6 samples of water); e) sediment for analytical chemistry (3 samples per location, a total of 3 samples of sediment). Samples of water d) were taken at three time-points (morning 8:30, mid-day 14:00, evening 20:30 hrs) as a one-grab sample, while all other samples were taken in the early morning as a one-grab sample

- The location 7/B-7 is indeed the location 5/B-5. That was a typo. Thank you for pointing it out.

11. In several places you write "Total metal concentration of heavy metals…". Could you just write "Total concentration of heavy metals"?

Response:

- Of course. Thank you for noticing.

Reviewer #2:

This manuscript presents a first attempt at a multi-biosensor approach to assessing the potential impact of WWTP on the receiving aquatic and terrestrial environment. This manuscript is much improved from the original submission and I would like to commend the authors' efforts to improving this manuscript. I believe this manuscript makes a contribution to our understanding of how WWTP effluent can modify the receiving environment and alter biological parameters of organisms found in those environments. It is my recommendation that this manuscript be accepted pending revisions. Below there are a number of comments which need to be addressed.

Title: Check the spelling of "organization".

Response:

- We have set the spelling for English US. Thank you for pointing it out.

Abstract: Accurately represents the results presented in the manuscript however it is lengthy and should be abbreviated.

Response:

- We deleted the sentences “Water, sediment, sludge and fish were sampled from sites related to a Croatian wastewater treatment plant (WWTP) processing municipal, hospital and sugar plant wastewaters. Bioindicators chosen as links between the environmental contamination and its effects on biota were Prussian carp (*Carassius gibelio*) and earthworms (*Eisenia fetida*), demonstrating the toxicant-induced changes in their biological systems.” The first sentence now reads: “Relating the treated wastewater quality and its impact on organismic biosensors (Prussian carp, *Carassius gibelio* and earthworm, *Eisenia fetida*) was the main objective of the study.”

Introduction:
Pg 4 Ln 36: The sugar plant is a "significant contributor to the wastewater…". What is the total or percent volume which it contributes to the effluent?

Response:

- According to the data for the year 2014, the overall communal and technical water volume was 1,970.187 m^3, while the sugar plant contributed with approx. 250.000 m^3.

Ln 5 ln 9: Define MXR here. It is defined in the abstract but not in the manuscript.

Response:

- This part of the sentence now reads: "…..genotoxic effects and multixenobiotic resistance mechanism (MXR): ……."

Throughout the manuscript: the format for the references are inconsistent and needs to be corrected to meet the format criteria of the journal.

Response:

- We have corrected the format of the references and believe that they are now fully meeting the journal criteria. These corrections are highlighted in red.

Methods:
Pg 6 len 36: The authors separate out biological treatment from the rest of the treatment process. However normally the biological treatment plant is considered to be part of the treatment process as a whole. Please clarify.

Response:

- The final treated effluent is discharged into the water canal. This canal further downstream receives additional communal treated water from a biological treatment plant serving a small suburb of 3.600 inhabitants, and widens to enter a County canal. The "biological treatment plant" refers to a man-made two-pond lagoon with reed beds, providing settlement, nitrogen removal and further biological improvement. It is mentioned because it impacts the water quality, and is thus chosen as one of the sampling points. For clarification, the sampling site 6 is now throughout the text described as "6: canal after the biological treatment plant (reed beds);"

Pg 7 ln46: Define EC and EEC.

Response:

- The phrase now reads: "This work has been carried out in accordance with the European Council Directive 86/609/European Economic Community for animal experiments"

Pg 8 ln 4: Please clarify if both males and females carp samples were pooled and if they are mature or immature fish. Age of fish would provide an indication of residence or time of exposure to the effluent. For example, younger fish may not migrate as much so are then more resident or older fish would have longer exposure period to the effluent resulting in greater bioaccumulation or resistance depending on the parameter.

Response:

- Both male and female fish were fished out and pooled due to a small sample size. We did not establish their age, but according to the average weights and lengths, they were between one and two years of age, which is the age when they sexually mature. The largest specimens were captured in the County canal. The County canal corresponds with the river, and fish have more manoeuvre and feeding possibilities than in the other two shallower water bodies. The fish did not vary as much in weight as in length.

Pg 16 ln 27: "Data presented as mean + SD using Daniel's XL Toolbox open-source add-in for Microsolf…" I am uncertain if this is necessary. Please check with the editorial team.

Response:

- It probably isn't necessary; the relevant part is that the data are presented as mean ± SD. We deleted the rest.

Results
Tables: The tables require formatting as they are difficult to follow as they are spaced out. Perhaps formatting them into landscape would make them easier to follow.

Response:

- They are formatted into landscape, but the spacing was double throughout. In this submission we have reduced the spacing, and they are now easier to follow.

Pg 19 ln 51": Typo - Remove ")" of the end of the sentence"…decrease with distance from the WWTP."

Response:

- We did. Thanks for noticing.

Pg 20: Change the order of the tables. Physio-chemical properties are listed as Tables 5 and 6, however the list of pharmaceuticals in Table 7 is presented first. Change the order so that the table order coincides with the order in which the data is presented. I recommend presenting the physio-chemical data first (routine) then the pharmaceutical data.

Response:

- We have changed the order in the text, as well as the title of the section 3.3. It now reads: "Physical and chemical properties of water; heavy metals; pharmaceuticals in water and sediment"

Ln 16: What are the detection limits for each of the plasma parameters measured? If they are non-detects or close to detection limit it is important to have that information for follow-up studies.

Response:

- The Olympus AU 640 biochemistry analyzer is a very sensitive, fully automated apparatus, and will show any value above 0. The values for ALP were measured for a number of plasma samples, but are not presented in the descriptive statistics, as they are not complete. The values for GGT, GSJ-Px and PON 1 were not expressed at all, even after four repeated procedures, presumably due to haemolysis of the plasma and changed colorimetric properties. Their target values and acceptable ranges for fish species are not yet established.

Ln 48: PCA data presented in Figure 6 listed should be Figure 5. It is also important to enumerate the breakdown of the percent total variability each PC contributes (i.e. PC1 = X%; PC2 = Y%; PC3 = Z%). The data as presented it is difficult to see the relationships among the parameters and the principal components in 3 dimensions. Suggest separating the PCA figure into to biplot figures with PC1 and PC2 and then P1 and PC 3. It will be easier to see those relationships and interpret the results.

Response:

- We have changed Figure 6 into Figure 5 and *vice versa*.
- We have replaced the text with the new one and enumerated the total variability percents. The text in the Results section now reads: "The PCA of eight blood biochemistry parameters from carp captured at three locations produced three significant components. First two principal components (PCs), as shown in Figure 5a, explain 76.86 % of variation in our dataset. PC1 accounted for 43.48 % of variation and loaded mainly on stress variables (CHOL, TRIG, TP, ALB, GLU), PC2 accounted for 33.38 % of variation and loaded mainly on liver impairment parameters, such as ALP and ALT. Axes PC1 and PC3 (Figure 5b) explain 54.47 % of variation, where PC1 (42.26 % of variation) loaded mainly on stress variables, and PC3 which accounted for 12.21 % of variation, loaded mainly of renal impairment parameters, (CRE, URE, TP and ALT)."
- The PCA results are now presented as biplots in Figure 5. The Figure 5. caption reads: "PCA correlation loading biplots for carp plasma parameters. Abbreviations: (A_metabolite: fish from the unaffected stream, B_metabolite: fish from the effluent-receiving canal, C_metabolite: fish from the County canal)."

Pg 21 Ln 48: Figure 5 should be Figure 6: again order of presentation.

Response:

- Indeed.

Discussion

Pg 23 ln:" The sugar plant wastewaters mostly "excelled" the raw…". Do you mean that the sugar plant wastewater out-performed and was of a higher quality than the raw municipal water? If so then this is the proper terminology.

Response:

- It was the wrong wording choice. These values were higher, not of a higher quality. We have changed the verb into "surpassed…… in values": "The sugar plant wastewaters (3) mostly surpassed the raw municipal wastewaters inflowing the WWTP (2) in values of many parameters such as suspended solids, COD, COD-Mn, BODn, nitrate, nitrite, whilst they matched in ammonium and total nitrogen concentrations."

Pg 25 ln4: "Although API 20E showed a limited sensitivity in species distinguishing…" should read as follows" Although API 20E showed a limited sensitivity in distinguishing species…."

Response:

- Changed.

We have re-read the manuscript, corrected inconsistencies, and improved the syntax. Sentences with the changes, and corresponding references, are highlighted in yellow and red, respectively. The omitted parts of text are not shown. The authors wish to thank the reviewers for their valuable suggestions. We hope that the manuscript is, after this revision, to be found ready for publishing in Science of the Total Environment.

Yours truly,
Natalija Topic Popovic, DVM, PhD

Graphical Abstract

The authors opted not to present the results in the Graphical Abstract.

Highlights

- Bacteria retrieved from fish and water have a zoonotic potential and might pose a health risk
- High antimicrobial resistance profiles were determined; half of aeromonads were resistant to SMX
- The sediment SMX and total antibiotic concentrations decreased with the distance from the WWTP
- Histological, haematological, plasma biochemical parameters of fish significantly differed in effluent and downstream waters
- *Eisenia fetida* is an optimal sentinel organism for environmental pollutants (MXR activity)

Impact of treated wastewater on organismic biosensors at various levels of biological organization

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ABSTRACT

Relating the treated wastewater quality and its impact on organismic biosensors (Prussian carp, *Carassius gibelio* and earthworm, *Eisenia fetida*) was the main objective of the study. The impact on health status of fish living downstream, microbiological contamination and antimicrobial resistance, fish tissue structure, blood biochemistry, oxidative stress, genotoxic effects, as well as multixenobiotic resistance mechanism (MXR) was assessed. Treated wastewater discharged from the WWTP modified the environmental parameters and xenobiotic concentrations of the receiving surface waters. Potential bacterial pathogens from fish and respective waters were found in relatively low numbers, although they comprised aeromonads with a zoonotic potential. High resistance profiles were determined towards the tested antimicrobial compounds, mostly sulfamethoxazole and erytrhomycin. Histopathology primarily revealed gill lamellar fusion and reduction of interlamellar spaces of effluent fish. A significant increase in plasma values of urea, total proteins, albumins and triglycerides and a significant decrease in the activity of plasma superoxide dismutase were noted in carp from the effluent-receiving canal. Micronucleus test did not reveal significant differences between the examined groups, but a higher frequency of erythrocyte nuclear abnormalities was found in fish sampled from the effluent-receiving canal. Earthworms indicated to the presence of MXR inhibitors in water and sludge samples, thus proving as a sensitive sentinel organism for environmental pollutants. The integrative approach of this study could serve as a guiding principle in conducting evaluations of the aquatic habitat health in complex bio-monitoring studies.

Keywords: WWTP; Effluent; Bacteria; Fish; Genotoxicity; Earthworm; MXR

1. Introduction

Safe drinking water and proper sanitation are indispensable factors for sustaining life (Naidoo and Olaniran, 2014), while treated wastewater discharged to a body of water modifies its environmental parameters, both qualitative and quantitative. The discharge of effluent from domestic and industrial sources has detrimental effects on the aquatic ecosystem as this outfall can deposit large amount of organic matter, nutrients and pollutants leading to eutrophication, oxygen deficits and accumulation of pollutants into receiving waterways (Bhatia and Goyal, 2013). Urban wastewater treatment plants (WWTP) were originally designed to reduce the biological oxygen demand, total suspended solids and nitrogen and phosphorus pollution, while the removal of pathogenic microorganisms has received less attention (Lucas et al., 2014). Although the primary and secondary treatments are able to remove up to 99 % of fecal indicator bacteria (Servais et al., 2007, Lucas et al., 2014), the quality required to use treated wastewaters might be insufficient to achieve the level required for recreational activities in the receiving water bodies.

Environmental change can increase the vulnerability of aquatic species to toxic chemicals by challenging an organism's capacity to respond or to repair toxic injury or by modifying animal behavior like migration or predation (Couillard et al., 2008). Also, xenobiotics may affect the capacity of aquatic species to adapt to environmental challenges that come with stressors, such as pathogens. Fish are very susceptible to environmental variations, and their physiological status can serve as an early indicator of the specific ecosystem's health (Kaur and Dua, 2014).

A sampling strategy was developed to retrieve representative water, sediment, sludge, and fish samples related to a Croatian WWTP processing municipal, hospital and sugar plant wastewaters. It is a mechanical and chemical-biological facility with activated sludge, encompassing primary and secondary treatments of influents, treating mainly municipal wastewater deriving from a small city of 20,000 residents. Frequently, hospital wastewater is pretreated, but on this location it is connected directly to a municipal sewer and treated at the municipal WWTP. Treatment of such wastewater at the source has advantages of avoiding dilution due to mixing with the urban sewage and avoiding losses into the environment caused by sewer leakage and overflows. The sugar plant, operating at the time of this investigation, is a significant contributor to the wastewater to be treated at the WWTP. Sugarcane industry is among those industries with the largest water demands and, in addition, is an important source of non-toxic organic pollution (Ingaramo et al., 2009).

There is a gap in fundamental understanding of the specific contribution of the WWTP effluent in observed changes in organisms residing in/exposed to effluent- receiving waters and sludge. Also, the effectiveness of the WWTP in eliminating bacteria and pollutants which are not organic (particularly antibiotics), needs to be elucidated. Consequently, the objective of this wide-scale work was to test the hypotheses that 1) the WWTP effluent will induce biological effects on organismic biosensors; 2) resistant bacteria and potential fish and human pathogens will be identified from water and sludge, and antibiotics will be retrieved from sediment. To test our hypotheses, we conducted a series of tests to measure the impact on health status of fish living downstream, microbiological contamination, fish tissue structure, blood biochemistry, oxidative stress, genotoxic effects and multixenobiotic resistance mechanism (MXR): (i) general fish health examination, necropsy and histopathology were performed (hypothesis 1); (ii) rapid

phenotypic tests and matrix assisted laser induced desorption ionization connected to the time of flight mass spectrometry (MALDI-TOF MS) were conducted on multiple samples of water, fish, and sludge (hypotheses 1, 2); (iii) fish blood biochemistry parameters and oxidative stress parameters were determined (1); (iv) erythrocytic nuclear abnormalities and micronuclei were enumerated and assessed (1); (v) cellular efflux mechanism mediated by ATP binding cassette (ABC) transporters that bind and actively remove toxic substrates from cells was analyzed post exposure to raw and treated water and sludge (1); (vi) physical-chemical characteristics and heavy metal contents were determined in water and sludge samples (1); (vii) antibiotic concentrations were measured in water and sediment (2).

The impact of effluent on fish and earthworms as toxicity biosensors was specifically addressed, especially in the view of active influence of all contributors to the wastewater volume (municipal, sugar industrial, hospital) as it is frequently omitted from investigation of performance of wastewater treatment plants. Such an approach encompasses an integrated overview of the impact of treated wastewater on key environmental and organismal parameters.

2. Materials and methods

2.1. WWTP and description of the sampling sites

The study was carried out in spring 2014, and the samplings of fish, water, sludge and sediment were conducted during the April 23 and 24 (Table 1). The samplings were carried out during the treatment process of a municipal WWTP serving about 20,000 inhabitants, also receiving

hospital and sugar plant wastewaters. Treatment includes primary and secondary processes, including settling tanks, grit chambers, activated sludge biological process, aeration tanks, secondary tanks for removing the biomass and other suspended particles. The resultant final treated effluent is discharged into the water canal. This canal further downstream receives additional communal treated water from a biological treatment plant (reed beds) serving a small suburb, widens to enter a County canal (agricultural landuse), which eventually ends up in a Drava river. Therefore, sampling sites for water and sludge are defined as follows: 1: unaffected stream, not related to any industrial nor agricultural waters, considered as a reference site; 2: inflow of raw municipal wastewaters to the WWTP; 3: inflow of sugar plant wastewaters to the WWTP; 4: treated wastewater leaving the WWTP; 5: canal receiving the effluent; 6: canal after the biological treatment plant (reed beds); 7: canal entering the County canal; 8: County canal; 9: County canal downstream before the Drava river; 10: WWTP active sludge; 11: sludge from the depot (Figure 1). Water and sludge were collected in sterile glass and polypropylene flasks, refrigerated transported to the lab and immediately analyzed.

2.2. Physical and chemical properties and heavy metals analyses

Physico-chemical properties of water were analyzed according to the international standards as follows: determination of electrical conductivity, pH, suspended solids, dissolved oxygen, permanganate index (COD-Mn), chemical oxygen demand (COD), biochemical oxygen demand after n days (BODn) by dilution and seeding with allylthiourea, ammonium and phosphorus with spectrometric method, nitrite, total nitrogen by persulfate digestion method, total phosphorus by ascorbic acid method, nitrate by colorimetry, and cadmium reduction (ISO 7888:1985, ISO

10523:2008, ISO 872:2005, ISO 5813:1983, ISO 8467:1993, ISO 15705:2002, ISO 5815:1989, ISO 7150-1:1984, ISO 6878:2004, ISO 6777:1984, SM 4500-NO3-E, respectively).

Total concentrations of heavy metals (Cd, Cr, Pb, Hg Ni, Zn, Cu, Fe) were analyzed according to the international standards as follows: determination of total cadmium, chromium, and lead by atomic absorption spectrometry-graphite furnace technique (ISO 15586:2003, ISO 15586:2004, ISO 15586:2006); determination of mercury by atomic absorption spectrometry (ISO 12846:2012); determination of nickel, zinc, and copper by inductively coupled plasma optical emission spectrometry (ISO 11885:2007, ISO 11885:2011, ISO 11885:2012); determination of iron by spectrometric method using 1,10-phenanthroline (ISO 6332:1988).

2.3. Animals and tissue processing

This work has been carried out in accordance with the European Council Directive 86/609/European Economic Community for animal experiments, and fish were manipulated by the competent authorized persons (licensed veterinarians) in accordance with the provisions of national legislation. Fish were caught by nets and angling at three locations: A-1: unaffected stream, B-5: effluent-receiving canal, entering the County canal, C-9: County canal downstream before the Drava river (corresponding with water sampling sites 1, 5, 9, respectively) (Table 1). A total of 24 Prussian carp (*Carassius gibelio*) of both sexes were subjected to examination, 8 from each site: A-1 (mean weight 155.20 ± 16.99 g, mean length 77.00 ± 27.01 mm), B-5 (mean weight 144.00 ± 8.74 g, mean length 55.60 ± 6.43 mm), C-9 (mean weight 259.07 ± 49.13 g, mean length 398.00 ± 213.45 mm).

Specimens were transported live to the laboratory and within few hours sacrificed by overdose of tricaine methane-sulfonate (MS-222, Sigma, St. Louis, Missouri, USA). Blood withdrawal and necropsy were performed immediately and tissues (gills, spleen, liver) were fixed in 4 % neutral buffered formalin, and after graded ethanol-xylene dehydration were embedded in paraplast. Sagital and transverse sections of 3-5 μm thickness were stained with hematoxylin/eosin (H&E). Microphotographs were taken with a digital camera DP 70 Olympus® connected to an Olympus® BX51 binocular microscope, and transferred to Microsoft® AnalySIS Soft Imaging System for interpretation.

The *Eisenia fetida* earthworms (Oligochaeta, Lumbricidae) were obtained from an earthworm farm (Donja Bistra, Croatia). Before experiment, adult clitellate specimens (180-260 mg) were stored in Petri dishes on moist filter paper for 24h (in the dark at 20 ± 1 °C) to empty the gut contents.

2.4. Pharmaceuticals in water and sediment

Water was sampled at the inflow of raw wastewaters to the WWTP (site 1) and outlet of the treated wastewater leaving the WWTP (site 4), at three time-points (morning, mid-day, evening). Sediment was collected from three locations: treated water leaving the WWTP (4), canal receiving the effluent (5), County canal before the river (9) (Table 1).

2.4.1. Reagents and sample preparation

All chemicals were of high purity grade. Suprapur formic acid was from Merck (Darmstadt, Germany). Water, acetone and methanol (all from J.T.Baker, Deventer, Netherlands) were of LC

MS grade. Ammonium formate was from Fischer Scientific (New Jersey, USA). Analytical standards of sulfa drugs (sulfachloropyridazine, sulfadimethoxine, sulfamethazine, sulfamethizole and sulfametoxazole) were from Supelco (Sigma Aldrich , Steinheim, Germany). Ciprofloxacin, erythromycin and trimethoprim were purchased from Restek (Bellafonte, USA), and azithromycin was from Dr. Ehrenstorfer (Germany). Polycarbonate bottles containing 250 mL of water and sediments were stored for 24 h at 4°C until analysis. Prior analyses of water samples were filtrated on 0,2µ PTFE filter. Ultrapure laboratory water samples were always processed in parallel with the environmental water samples.

2.4.2. Methods and analytical procedure

The direct injection method was used for the determination of pharmaceuticals in water. Calibration curve was obtained by direct injecting, in triplicate standard solutions at 7 concentration levels. Correlation coefficients >0.999 were used as linearity acceptance criterion. By testing blank samples spiked at three concentration levels, accuracy and precision were calculated, and for each concentration were evaluated in quintuplicate. Acceptance criteria was for accuracy = recoveries (between 70 % and 110 %) and for repeatability precision lower than 20 %. The method reported by Darwano et al. (2014) was used for sediment samples. Pharmaceuticals were determined by ultra high performance liquid chromatography quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS).

Analyses were performed on 1290 UHPLC system, Agilent Technologies, USA (G4226A autosampler, G4220B binary pump and G1316C thermostated column). The separation of analytes was done using the RP column ACQUITY UPLC, HSS T3 (150 mm x 2.1 mm, 1.8

μm). The gradient was from 100 % water to 100 % organic in 20 min run. The injection volume was 100 µL. The temperature of the column chamber was set to 50°C. In positive ESI (ESI(+)), the mobile phases were composed of solvent A (5 mM ammonium formate/formic acid , and B (100 % MeOH). Gradient elution with a flow rate of 0.4 mL/min was used. The analytes were detected using a 6550 i-Funnel Q-TOF-LC/MS (Agilent Technologies, USA) in 4 GHz detector rate, with a 40,000 resolving power and <2 ppm accuracy. QTOF mass spectrometer (Agilent 6550) operated in positive (ESI+) ion mode. Ions were generated using a dual AJS ESI (Agilent Jet Stream) ion source. Operation conditions were as follows: sheath gas temperature 375 °C, gas temperature 125°C, heat gas 12 L N2/min, drying gas 15 L N2/min, capillary voltages 3500 V, fragmentor 400V, and nebulizer 35 psig. The resolution power for ESI+ was 52296 at 922.009798 m/z and 21801 at 118,086255 m/z. Correction during measuring for any possible drift in the mass axis was done automatically with lock 2 mass ion software. Analyses were performed using MS and MS/MS mode with fixed collision energy and in mass range of 50–1,000 m/z. Fata were further processed with Agilent MassHunter Workstation software (Quantitative Analysis version B.07.00/Build 7.0.457.0 for QTOF, Agilent Technologies, USA).

2.5.Microbiological analyses

Samples were streaked on media for fastidious organisms, general, selective isolation and standard plate count media (all Oxoid Ltd, England, UK). Total viable counts and methods for detection and enumeration of bacteria were as follows: Enumeration of culturable micro-organisms - Colony count by inoculation in a nutrient agar culture medium (ISO 6222:1999); Detection and enumeration of *Escherichia coli* and coliform bacteria – Part 1: Membrane

filtration method (ISO 9308-1:2000/Corr.1:2008); Detection and enumeration of intestinal enterococci – Part 2: Membrane filtration method (ISO 7899-2:2000); Detection and enumeration of *Pseudomonas aeruginosa* by membrane filtration (ISO 16266:2008); Microbiology of food and animal feeding stuffs – Horizontal method for detection of *Salmonella* spp. (ISO 6579:2002/Corr. 1:2004); Microbiology of food and animal feeding stuffs – Horizontal method for detection and enumeration of *Listeria monocytogenes* – Part 1: Detection method (ISO 11290-1:1996); Microbiology of food and animal feeding stuff – Horizontal method for detection and enumeration of *Listeria monocytogenes* – Part 2: Enumeration method (ISO 11290-1:1996/A1:2004; Detection and enumeration of the spores of sulfite-reducing anaerobes (clostridia) - Part 2: Method by membrane filtration (ISO 6461-2:1986); Microbiology - General guidance for enumeration of yeasts and moulds - Colony count technique at 25 degrees C (ISO 21527-1:2008, ISO 21527-2:2008).

Samples of fish gills, kidney and liver were inoculated onto Tryptone Soya Agar, MacConkey Agar (Oxoid) and Blood Agar (Certifikat doo, Croatia). The plates were incubated at 22°C for 48-72 h. Representative colonies were isolated and reincubated on fresh media. Pure colonies were Gram-stained and subjected to morphological, physiological and biochemical tests. The taxonomic position of the isolates was determined by API 20E panels (bioMerieux, Marcy l'Etoile, France) and Bruker Biotyper MALDI-TOF MS (Bruker Daltonics, Billerica, MA). The API 20E tests were performed according to the manufacturer's instructions with a few alterations in order to adapt the system to the bacteria of freshwater fish as described in Topić Popović et al. (2007).

Antimicrobial susceptibility of the isolated strains was determined with Kirby-Bauer disk diffusion method on Mueller Hinton agar (all Oxoid). The following antimicrobials with

respective concentrations (μg) were used in the test: oxytetracycline (OTC_{30}), amoxicillin (AMC_{30}), oxolinic acid (OA_2), erythromycin (E_{15}), sulfamethoxazole (SMX_{50}), florfenicol (FFC_{30}), norfloxacin (NOR_{10}), flumequine (UB_{30}). The inoculum was prepared in sterile 5 mL of 0.85 % Suspension medium (bioMerieux, Marcy l'Etoile, France) and turbidity was adjusted to 0.5 MacFarland's standard. The diameter of the zones of inhibition was read and expressed by referring to the manufacturer's standard table and reported as susceptible (S), intermediate (I) or resistant (R).

2.5.1. Bruker Biotyper MALDI-TOF

Bacterial isolates (one loopful of each bacterial culture) were smeared to a 24-spot steel plate (Bruker Daltonics) in the form of the direct colony technique in two replicates, and were allowed to visibly dry at 22°C. A saturated solution of α-cyano-4-hydroxycinnamic acid in 50 % acetonitrile and 2.5 % trifluoroacetic acid (MALDI matrix, 2 μL) was added to the bacterial colony and dried. The ions generated with a 337-nm nitrogen laser were captured in the positive linear mode between 2 to 20 kDa of mass range. The linear mode of accelerating voltage (20 kV) extracted the positive ions. Two hundred laser shots in five different regions of every well yielded a spectrum comprising the sum of the respective ions. The MALDI Biotyper automation control and Bruker Biotyper 2.0 software (Bruker Daltonics) were used to analyze the captured spectra. For each 24-spot plate, a standard (bacterial test standard; Bruker Daltonics) was included to calibrate the instrument and validate the run. The criteria for identification were as follows: a score of 2.300 to 3.000 indicated highly probable species level identification, a score of 2.000 to 2.299 indicated secure genus identification with probable species identification, a

score 1.700 to 1.999 indicated probable identification to the genus level, and a score of <1.700 was considered to be unreliable. The data obtained with the two replicates were added to the calculation in order to minimize any random effect. The fingerprints for every isolate were identified as presence or absence of peaks and matched with the reference strain in the database.

2.6. Blood biochemistry, oxidative stress, and genotoxic analyses

Blood was withdrawn from all sampled fish by caudal artery and vein puncture, immediately after capture. Tubes were coated with lithium heparin as anticoagulant and centrifuged at 12,000*g* for 90 seconds. Plasma was separated from the corpuscular fraction and frozen at -80ºC until analysis. Heparinized microhaematocrit capillaries were centrifuged at 12,000*g* for 120 seconds within 20 minutes from collection. Haematocrit (Hct) was determined as the volume percentage of red blood cells in blood (Wedemeyer and Yasutake, 1977).

In blood plasma, concentrations of glucose (GLU), urea (URE), creatinine (CRE), cholesterol (CHOL), triglyceride (TRIG), total proteins (TP), albumin (ALB) and activity of alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT) were determined by Beckman Coulter commercial kits (Olympus Life and Material Science Europe, Ireland) on the Olympus AU 640 biochemistry analyzer (Olympus, Japan). The activity of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were determined by Randox commercial kits on the Olympus AU 640 biochemistry analyzer. Paraoxonase activity (PON 1) in serum was assayed by modified method of hydrolysis of paraoxon described by Charlton-Menys et al. (2006) on the Olympus AU 640. Enzyme activity was presented in U/L (1 μmol p-nitrophenol formed/min/L).

For the genotoxicity assessment (micronucleus – MN and erythrocyte nuclear abnormalities – ENA assays), three blood smears per fish were allowed to air dry, then were fixed in 95 % methanol for 3 min and stained with May Grünwald/Giemsa stains. One thousand erythrocytes per slide were scored on randomized and coded slides at 1000x magnification. Nuclear anomalies such as nuclear buds, blebbed, lobed, notched, vacuolated nuclei and bi-nuclei were considered together, while micronuclei were recorded separately and represented as n/1000 (‰).

2.7. MXR activity analyses

2.7.1. Filter paper contact test

In order to determine the presence of inhibitors of the MXR activity in water samples, the filter paper contact toxicity method was applied (OECD, 1984) based on the accumulation of the model fluorescent substrate in the whole body of the earthworm (Bošnjak et al., 2014). *E. fetida* specimens were exposed to three concentrations of raw and treated wastewater and sludge as undiluted, diluted 100x and diluted 10,000x. Exposure was performed as follows: the total volume of 1.2 mL per tested concentration was added to filter paper cut-out discs (80 to 85 g/m^2, approximately 0.2 mm thick, medium grade), which were placed in Petri dishes, changed and moistened with sample to be tested every day during the experiment. Five earthworms per one Petri dish were used. Positive and negative controls were run in parallel with MK571 sodium salt and distilled water. After the pre-treatment (24h), 10 µM fluorescent substrate Rhodamine 123 (Sigma-Aldrich, CAS No. 62669-70-9; R123) was added on filter paper for additional 24 h.

After the end of the exposure, earthworms were weighed and rinsed with distilled water, and then used for measurement of efflux pump activity. All Petri dishes were kept in the dark at (20 ± 1 °C). Experiments were run 3 times in duplicate (total of 270 specimens) and behavioral and morphological changes were recorded during every stage of experiment.

2.7.2. MXR inhibitory potential: determination of inhibitors of MXR mechanism

Accumulation of R123 was measured in the whole earthworm body 24 hours after the exposure. Earthworms exposed were weighed and then homogenized (for 30 sec) with Ultra-Turrax T18 homogenizer (IKA, Germany) in 1 mL distilled water and centrifuged for 10 min at 9,000*g*. After centrifugation, the supernatant was transferred to black 96-well microplates (Nunc, Thermo Fisher Scientific, Denmark). The amount of R123 in the supernatant was determined in triplicate using Tecan Infinite M200 plate reader (Tecan Trading AG, Männedorf, Switzerland) excitation at 490 nm, emission at 544 nm.

2.8. Statistical analysis

Statistical analyses were performed by SigmaStat and SigmaPlot Statistical Software ver. 11.0 (Jandel Corp., San Rafael, California). Analyses and correlations between blood parameters were conducted by *t*-test, Mann-Whitney Rank Sum Test and Spearman correlation coefficients. The significant differences were regarded at $p<0.05$. Differences in MN and ENA frequencies between groups were assessed using Kruskal-Wallis ANOVA on Ranks since the data were not normally distributed. The amount of the accumulated R123 in the whole earthworm body was

calculated in relation to its mass and shown as fluorescent units per mg of earthworm tissue (FU mg^{-1}). To determine differences between treatment groups and controls we used the unpaired *t*-test. The probability level for significance was $p<0.05$. Data are presented as mean ± SD. Principal component analysis (PCA) was applied to describe the main sources of variation and relationship among the selected blood biochemistry parameters. The results are presented graphically by a biplot. The software used was SPSS 13.0 for Windows. The dendrograms were created by MALDI Biotyper 3.0 (Bruker Daltonics, Bremen, Germany) with following settings: distance measure was set at *correlation* and linkage at *complete*. Spearman's rank correlation as column distance measure was applied with pair-wise complete-linkage as the clustering method. Distance values were relative and normalized to a maximum value of 1000.

3. Results

3.1. Fish health and histopathology

The external gross signs and necropsy findings from fish captured in the three representative water bodies did not indicate to any manifest disease. However, fish from the unaffected stream (A-1) demonstrated a low parasitic infestation of gills with *Dactylogyrus* spp., while those from the County canal (C-9) carried a medium load, mainly *Dactylogyrus* spp. on gills and *Thelohanellus* spp. on opercula, skin and fins. Fish from the effluent-receiving canal (B-5) had no parasitic infestations. Histopathological findings of the Prussian carp gill tissues varied in fish sampled from the effluent-receiving canal (site 5), entering the County canal (site 7) and County

Canal (site 8) (Figure 2). Lamellar fusion was the most remarkable microscopic lesion (Fig. 2(a)), where the leading lateral edges of non-fused lamellae were turned towards the neighboring ones often possessing epithelial extensions, bridging the inter-lamellar space. Interlamellar spaces were reduced, with sparse mucus and undifferentiated cells. Epithelial hypertrophy and hyperplasia were also noted in several specimens, causing fusion of two or more lamellae. Fig. 2(b) demonstrates mild dilation of blood sinuses and enlargement of marginal blood channels of secondary lamellae.

3.2. Bacterial identification and antimicrobial susceptibility

In Table 2, total viable counts and concentration of bacteria across all sample collection sites are presented. Colony counts at both incubation temperatures retrieved the highest load from the active sludge (10) and sludge from the depot (11), as well as from the inflows of municipal (2) and sugar plant wastewaters to the WWTP (3). Interestingly, fecal coliforms and yeasts were even more abundant in the unaffected stream (1) than in the canals downstream from the effluent (5-9), while *E. coli* was retrievable from all the sampling sites. Gram-negative bacteria were recovered from all tissues under examination. The majority of isolates were retrieved from gills (61.22 %) and spleen (18.38 %), while less from liver (10.20 %) and kidney (10.20 %). Only 61.22 % of total isolates both identification systems (API 20E and MALDI-TOF MS) identified to the same genus, while most of the readings indicated to aeromonads (48.98 % MALDI-TOF MS; 71.43 % API 20E), particularly dominant in the canal C-9, as presented in Table 3. Of all aeromonads isolated from fish tissues, 50 % were resistant to sulfamethoxazole, which proved to

be the antimicrobial exerting the majority of resistance in all fish bacterial isolates, except amoxicillin, for which resistance was partly expected (Table 4).

From water and sludge, mainly Gram-negative bacteria were retrieved, whereas Gram-positives and Gram-variables (4.54 %) comprised *Bacillus simplex* and *Lysinibacillus fusiformis.* Overall, the most frequently identified genus from water and sludge, irrespective of the sampling site, was *Aeromonas* (38.64 % MALDI-TOF MS; 50.00 % API 20E). Of all the isolates, only 15.91 % matched completely in both API 20E and MALDI-TOF MS readings, while both panels identified 52.27 % of all isolates identically to the genus. For Gram-negative rods, the conventional method resulted in significantly fewer species identifications than with MALDI-TOF MS (11.36 % versus 81.82 %). With API 20E, the mean time to identification was 48 hours, whereas MALDI-TOF MS needed less than 10 minutes per bacterial isolate. MALDI-TOF MS spectra obtained from aeromonads isolated from water and fish from all locations are presented in Figure 3, showing characteristic fingerprints, with the highest intensity peaks ranging from 4,000 to 10,000 Da. Aeromonads retrieved from all samples were also separated by hierarchical clustering (Figure 4) which differentiated related *Aeromonas* species and clustered the closely related ones.

In Table 4, antimicrobial susceptibility of the isolated strains (fish, water, sludge) from all locations are presented as percentages of susceptible (S), intermediate (I) or resistant (R) strains. Except towards amoxicillin, most of the strains, even from the unaffected stream (1), showed resistance towards sulfamethoxazole and erythromycin. Resistance towards tested antimicrobials was most pronounced in the active sludge isolates (10, 11).

3.3. Physical and chemical properties of water; heavy metals; pharmaceuticals in water and sediment

Physico-chemical properties of water and total concentrations of heavy metals from water and sludge are presented in Tables 5 and 6, respectively. Of all pharmaceuticals entering the WWTP (2), antibiotics on average comprised 13.62 %, while at the outlet of treated wastewater (4) their portion increased to 27.45 %. The mean concentration of antibiotics at the inflow of raw wastewaters (2) was 15,367.45 ng/L, while at the outlet (4) it was 7,715.39 ng/L, of which sulfa drugs comprised 0.049 % (76.05 ng/L) and 0.839 % (64.76 ng/L), respectively (Table 7). The overall concentration of antibiotics entering the WWTP (2) increased five-fold from morning towards the evening time-points, but it remained constant over time at the outlet. By contrast, the concentration of sulfa drugs, sulfamethoxazole included, entering the WWTP decreased to half by the evening time-points, but also remained constant over time at the outlet (Table 7). The average SMX concentration in both locations was comparable, while erythromycin concentration (inflow at 19.57 ng/L) was not detectable at the outlet at any time. Erythromycin was also not detectable in any of the sediment tested, while the sediment SMX concentration decreased with the distance from the WWTP. Total pharmaceuticals in sediment also decreased, as well as total antibiotics, although not in the same proportion.

3.4. Fish plasma biochemistry, oxidative stress (SOD), MN, and ENA

A general comparison of the plasma values of GLU, URE, CRE, CHOL, TRIG, TP, ALB, ALT, ALP and SOD of Prussian carp are presented in Table 8. in the form of descriptive statistics. The values of ALP, GGT, GSH-Px and PON 1 were not measurable in most of the tested samples, probably due to haemolysis, and are therefore not presented. TRIG from fish captured in the effluent-receiving canal (B-5) failed the Normality test, as well as the URE, CRE, ALT and CHOL from fish captured in the County canal downstream. The Spearman Rank Order Correlation demonstrated that pairs of variables with p values below 0.050 and positive correlation coefficients (r_s), such as URE/ALT (0.5), URE/TRIG (1.0), URE/CHOL (0.5), CRE/SOD (1.0), TP/TRIG (0.56), TP/CHOL (0.74), ALB/TRIG (0.73), ALB/CHOL (0.68), ALT/TRIG (0.86), ALT/CHOL (0.67), ALT/SOD (0.68) ALP/TRIG (0.59), TRIG/CHOL (0.87), TRIG/SOD (0.56) from the fish captured in the County canal, C-9, tend to increase together. A significant increase in values of URE, TP, ALB and TRIG was noted in the fish from the effluent-receiving canal (B-5) when compared with other fish, as well as a significant decrease in SOD when compared with fish from the other two canals. The PCA of eight blood biochemistry parameters from carp captured at three locations produced three significant components. First two principal components (PCs), as shown in Figure 5a, explain 76.86 % of variation in our dataset. PC1 accounted for 43.48 % of variation and loaded mainly on stress variables (CHOL, TRIG, TP, ALB, GLU), PC2 accounted for 33.38 % of variation and loaded mainly on liver impairment parameters, such as ALP and ALT. Axes PC1 and PC3 (Figure 5b) explain 54.47 % of variation, where PC1 (42.26 % of variation) loaded mainly on stress variables, and PC3 which accounted for 12.21 % of variation, loaded mainly of renal impairment parameters, (CRE, URE, TP and ALT).

The mean haematocrit values were 33.60 ± 14.52, 42.00 ± 8.37, 47.14 ± 7.26 from unaffected, effluent-receiving and County canal, respectively. They all passed the Normality test, but showed no significant relationships between the groups (p>0.05).

Analyses of erythrocyte nuclear abnormalities revealed the highest frequency (8.96 ‰) in fish sampled from effluent-receiving canal (B-5), while the frequencies of erythrocyte nuclear anomalies in unaffected stream (A-1) and County canal downstream before the Drava river (C-9) were much lower (2.89 ‰ and 1.93 ‰, respectively). However, these differences were not significant (p<0.05). Micronucleated erythrocytes were recorded in four fish (1 from the site A-1, 1 from the site B-5 and 2 from the site C-9) with very low frequency (0.33 ‰ per fish) and not significant (p>0.05) differences.

3.5. MXR activity in earthworms

Measurement of accumulated R123 after 24h exposure of earthworms to the raw (2) and treated wastewater (4) and sludge (10) (as not diluted, diluted 100x, and diluted 10,000x) showed significantly higher accumulation of R123 in the whole earthworm tissue, relative to the control (Figure 6) indicating the presence of the MXR inhibitors in water samples. The greatest accumulation of R123 was observed with exposure to active sludge (10), to waters where the effluent-receiving canal enters the County canal (7), and where the sugar plant wastewaters inflow to the WWTP (3) (208 %, 166 % and 144 %, respectively; p<0.03). After 100x dilution of all the samples, the greatest accumulation of the fluorescent substrate was observed in animals exposed to the unaffected stream waters (1), County canal waters downstream (6), and sugar plant wastewaters inflowing to the WWTP (3) (270 %, 254 % and 224 %, respectively).

Exposure of earthworms to a higher dilution (10,000x) of WWTP active sludge (10), waters from the County canal (8) and from the canal entering the County canal (7), increased the accumulation of R123 287 %, 251 % and 226 %, respectively.

4. Discussion

This wide-scale survey comprised an array of studies on the general quality of the water, sludge and sediment associated with the WWTP, as well as their microbiological and pharmaceutical assessment, related to the impact on the organismic biosensors, particularly fish and earthworms, demonstrating specifically and non-specifically the effluent-induced changes in their biological systems. Such a combined approach was considered due to possible misinterpretations resulting from the isolated analyses of each aspect and therefore inherently implies a broader ecological and, ultimately, human health insight. Although most environmental studies take into account only a limited amount of biological responses, parameters which individually show no distinctive response to a stressor can, when used in conjunction with other parameters, be useful indicators of the extent of the environmental impact (Bernet et al., 2000). This impact can be demonstrated as either direct chemical or physical damage to cells or tissues, modification of physiological and biochemical reactions, increased infection pressure by facultative pathogens or reduced resistance to infection (Schmidt-Posthaus et al., 2001), thus their determination at the whole organism level can detect sublethal and chronic effects on organisms and present integrative responses to complex mixtures of pollutants. The results of this study corroborate the hypotheses, indicating that antibiotic effects, bacterial pathogens, and

the changes of biological responses related to the WWTP reach far downstream from the WWTP.

4.1. Water quality

The sugar plant wastewaters (3) mostly surpassed the raw municipal wastewaters inflowing the WWTP (2) in values of many parameters, such as suspended solids, COD, COD-Mn, BODn, nitrate, nitrite, whilst they matched in ammonium and total nitrogen concentrations. In order to avoid the release of contaminants from the sugar plant to the environment, zero effluent discharge, involving water reuse, recycling and regeneration, is this sector's greatest challenge. Its environmental impact can further be assessed by measuring the environmental performance index which measures the levels of COD above the regulated values, and the environmental performance index which calculates the specific increase of COD in liquid effluents (Ingaramo et al., 2009). In this work COD decreased below the regulated borderline values in the effluent (4) and downstream waters (5-9).

When comparing nitrite, total nitrogen and total phosphorus concentrations in the waters from the unaffected stream (1), inflow of raw municipal waters to the WWTP (2) and inflow of sugar plant wastewaters to the WWTP (3) with the work of El-Shafai et al. (2004), our values were significantly higher, mainly due to the very high total nitrogen in all sites. Increased nitrogen and phosphorus may result in overgrowth of algae, which can decrease the dissolved oxygen content of the water, thereby harming or killing fish and other aquatic species. Although presumably unaffected by industrial or agricultural activity, our reference stream (1), albeit not

connected to the effluent receiving system, must have been impacted by some sort of fertilizer use or animal wastes.

4.2. Microbiological counts, bacterial identification and discrimination; microbial resistance; health risks

Fish in the water bodies downstream from the WWTP effluent discharge are fished and used by the local population (personal communication). Public health risks are the main concerns in such practice, although using treated wastewater in fish farming is not uncommon (El-Shafai et al., 2004; Harnisz and Tucholski, 2010). The microbial quality of fish-bearing waters is reflected in the microbial flora of fish. If the total counts of heterotrophic aerobes exceed 5×10^4 CFU/mL of water, they pose a risk of human pathogens penetrating fish edible tissues (Mara and Cairncross, 1989). Total bacterial counts and intestinal enterococci measured in this work were significantly higher in water from the canal receiving the effluent (B-5), when compared to the two other fish sampling sites (A-1, C-9). The number of fecal coliform bacteria should not exceed 10^3 CFU/100 mL water in order to render fish safe for consumption (WHO, 2006). In none of the locations from which fish were retrieved fecal coliforms amounted to such numbers, although in the unaffected stream (1) they were the highest of the three locations (3.2×10^2 CFU/100 mL). Relatively low levels of fecal coliforms could nevertheless accumulate in the fish digestive system, posing a risk of transmission to consumers by cross-contamination of tissues, in case of inadequate hygiene standards in fish preparation (El-Shafai et al., 2004; Harnisz and Tucholski, 2010)

API 20E and MALDI-TOF MS identification results for isolates from fish captured at three locations and respective water largely corresponded, inasmuch that the majority of readings indicated to *Aeromonas* spp. It was previously found that environmental *Aeromonas* isolates have a wider phenotypic diversity than isolates obtained from clinical samples (Donohue et al., 2007). Although API 20E showed a limited sensitivity in distinguishing species, dividing all environmental aeromonads between *A. hydrophila* group 1 and group 2, and had drawbacks regarding slow phenotypic reactions, it was a valid first step in preliminary bacterial identification. MALDI-TOF MS spectra demonstrated many interspecies similarities, in which each strain of *Aeromonas* was defined by its unique mass distribution corresponding to a fingerprint spectrum characteristic or its *m/z* signature. MALDI-TOF MS proved to be a highly reproducible method for rapid discrimination of *Aeromonas* spp. and related species, as well as capable for differentiation of closely related aeromonads, where the spectra of different isolates of the same species clustered together. A cluster analysis of the *m/z* signatures (Figure 4) graphically depicts how the strains relate to each other, as the fingerprints with the highest degree of association are joined together first, followed by those with the next highest degree of association (Donohue et al., 2006; Eddabra et al., 2012). The groupings are unique to the MS and bacterial culture procedures.

Mesophilic aeromonads persist in most aquatic environments, including chlorinated drinking water, raw sewage, groundwater and both polluted and unpolluted streams and rivers (Huddleston et al., 2006). Bearing in mind that most of the retrieved bacteria from fish, water and sludge in this work were aeromonads, it is important to stress that aeromonads have been associated with bacterial zoonoses. Also, the retrieved *A. salmonicida* ssp. *salmonicida* is a fish pathogen (Austin and Austin, 1999). As contact-borne, *A. hydrophyila*, *A. sobria*, and *A. caviae*

may cause fish handler disease through skin wound infections and systemic infections, while as food-borne, *A. hydrophyila* may lead to gastroenteritis, diarrhoea and systemic infections (Austin and Austin, 1999; Goni-Urriza et al., 2000; Lehane and Rawlin, 2000; Huddleston et al., 2006; Cabello et al., 2013). With the popularity of recreational fishing in watersheds downstream from the investigated WWTP, medical personnel may expect more aeromonad-related infections, whereas their diagnosis and treatment may be difficult, particularly taking into consideration the emerging antibiotic resistance of fish pathogens. Environmental contamination with antimicrobials and other antibiotic substances is an important contributor to the survival and spread of antibiotic resistance genes (Huddleston et al., 2006), particularly through spread of resistance plasmids between unrelated bacteria (Kostich and Lazorchak, 2008).

Of all aeromonads isolated from fish tissues in this work, 50 % (18 isolates) were resistant to sulfamethoxazole, and all were resistant to amoxicillin. Amoxicillin is closely related to ampicillin, towards which aeromonads have a natural resistance (Harnisz and Tucholski, 2010), therefore this result was expected. One possible reason for such a high resistance to SMX is that it alone is not very active against *Aeromonas* spp., but in combination with trimethoprim it is generally efficient (Goni-Urriza et al., 2000). The incidence of resistance of environmental *Aeromonas* spp. samples to SMX in this work was significantly higher than in comparable studies (Imzlin et al., 1996; Kampfer et al., 1999; Huddleston et al., 2006), most likely because our isolates have previously been exposed to antibiotics and chemotherapeutics due to a hospital discharge waters going through the WWTP. Possibly for the same reason 11 % of water and sludge aeromonads (3 isolates) were resistant to oxytetracycline and erythromycin. Indeed, Harnisz et al. (2011) found that tetracycline-resistant bacteria are a reliable indicator of antimicrobial resistance and the microbial quality of surface waters polluted due to human

activity. Table 4. demonstrates the subdivision of antimicrobial susceptibility over the sampling sites for all isolates, but overall resistance of all bacterial isolates irrespective of the location was shown to be the highest towards amoxicillin, sulfamethoxazole, erythromycin, and oxytetracycline (87.53 %, 63.10 %, 30.61 %, 21.34 %, respectively). The presence of bacteria resistant to antimicrobials may be more substantial than detected in this and other studies, since most analyses represent the resistance of culturable bacteria, which constitute only a small proportion of the total bacteria present in the aquatic environment (Cabello et al., 2013). Also, antibiotic resistance is crucial in emerging infectious diseases, while surface water pathways may represent a key route of dissemination of antibiotic resistance genes (Pruden et al., 2012). We have thus confirmed the hypothesis that resistant bacteria and potential fish and human pathogens will be identified from water and sludge.

4.3.Pharmaceuticals in water and sediment - sources of exposure

As hypothesized, the inflow of high concentrations of SMX, also found in the sediment, must have contributed to significantly higher resistance of environmental aeromonads towards this drug. Its mean of 50.70 ng/L corresponds with the mean of 48.40 ng/L found in waters affected by urban and rural activities in China (Xu et al., 2014). The same authors report the SMX mean of 16.10 ng/g for corresponding sediment, while our sediment values (sites 4, 5, 9) decreased with the distance from the WWTP (19.60>5.07>0.99 ng/g). In an area polluted with antibiotics (Xue et al., 2013), SMX water levels were up to 191 ng/L, but not detected in the sediment. The same authors report the 43.5 ng/L erythromycin concentration in the water, which is twice of our inflow (2) values, and proved SMX and erythromycin to pose a relatively high ecological risk to

sensitive aquatic species. Frequently sulfonamides, especially SMX, have the highest detection frequency and concentration over other antimicrobials tested in wastewaters (Chen and Zhou, 2014) however sediments are more informative of long-term accumulation and storage of drugs, being also a source of exposure to benthic and other aquatic organisms. Gibs et al. (2013) describe a likely cause for the declining trend of erythromycin and other antibiotics occurrence from the point source as a dilution from groundwater discharge and sorption to bottom sediments. The observed drug concentrations can be attributed to sorption from the concentration in the water column and subsequent sediment transport (Gibs et al., 2013). In the sedimentary phase, the total concentration of antibiotics in this work decreased from 144.55 to 18.58 ng/g with the distance from the WWTP, which is comparable with the work of Chen and Zhou (2014) (101.0 to 12.4 ng/g). Although it is presumed that hospitals are the major contributors of pharmaceuticals to municipal sewage, the community use is 70 % in the UK and 75 % in the US, and almost 70 % of the total antibiotics used are excreted unchanged as active compounds (Kümmerer, 2009). Frequently their metabolites are more soluble in water than their lipophilic parent compounds due to their biotransformation. That might explain our higher water rates of sulfa drugs and SMX in the morning time-points when compared to the mid-day and evening time-points. Such substances, if not eliminated, reach the environment and adversely affect aquatic and terrestrial organisms, and consequently impact the human population.

4.4. Fish health status, structural and functional tissue alterations

Fish in this study had similar external gross signs and necropsy findings over sampling sites, however those from a remote location from the WWTP (C-9) suffered from a heavier parasitic

infestation and demonstrated a greater variety in bacterial load than fish captured in the effluent-receiving canal (B-5), which could be attributed to their migration over the water bodies in pursuit of the more favorable feeding locations, but also to the lower concentration of antibiotics and pharmaceuticals found in the sediment furthest from the WWTP.

Structural and functional alterations found in the carp gill tissues, particularly in the effluent-receiving canal (B-5), relate extensively to the secondary lamellae, indicating to environmental pollution, as presumed in the first hypothesis of this work. Compactness in the secondary lamellae and formation of inter-lamellar bridges reduced the total available respiration surface area of the gill, resulting in reduction of the diffusing capacity which in turn decreased the gas exchange over the respiratory area. It is thought to be an adaptive physiological response during the longer exposure period in order to prevent irritants from entering the bloodstream (Munshi and Singh, 1971), occurring under the influence of xenobiotics due to dissociation of epithelial cells followed by reassociation and reaggregation in the form of interlamellar bridges. Flores-Lopes and Thomaz (2011) also observed several types of gill tissue changes such as epithelial lifting, necrosis, fusion, hypertrophy, hyperplasia and branchial gland proliferation, induced by different levels of environmental degradation.

Carp in the present study, captured in the effluent-receiving canal (B-5), were exposed to high levels of chromium, nickel and zinc (Table 6). A reduction of the respiratory area in carp exposed to heavy metals was previously reported (Zikic et al., 1997; Schjolden et al., 2007; Srivastava and Punia, 2011; Mustafa et al., 2012). The histopathological changes observed in gill tissues could be related to xenobiotics in the effluent, microbial load on gills, impaired water quality and increased heavy metal concentrations (sections *4.1.-4.3.*), and may serve as an important end-point in the discrimination between different polluted sites (Omar et al., 2013).

4.5. Fish plasma metabolites and enzymes, oxidative stress, genotoxicity

The hypothesis that the WWTP effluent will induce biological effects on organismic biosensors is further discussed in this section and the following one. Although carp's high tolerance to heavy metals is explained by its ability to avoid becoming hypoxic and resistance to severe loss of plasma ions (Schjolden et al., 2007), the impact of heavy metals might have contributed not only to structural and functional alterations of the gill tissues, but also to the increase of some plasma metabolites. A significant increase in values of URE, TP, ALB and TRIG was noted in the fish from the effluent-receiving canal (B-5), as well as a significant decrease in SOD when compared with fish from the other two canals (A-1, C-9). SOD is one of the main antioxidative enzymes serving to detoxify reactive oxygen species. Decreased SOD activity modifies the cell redox status by the inhibition of the superoxide radical formation (Ural, 2013), and was related to the toxic impact of pollutants in the aquatic environment (Karadag et al., 2014), and particularly to the carp cadmium exposure (Zikic et al., 1997). Furthermore, the extremely low dissolved oxygen content (0.39 mg/L) in the effluent-receiving canal (4), contributed to stress-related biological responses in fish tissues.

The excess energy reserves (in form of plasma GLU, CHOL, TRIG) are necessary to mediate the effects of stress, (Topić Popović et al., 2012), which could explain the increase of TRIG in effluent fish, serving as an energy buffer. The increase of URE in effluent fish were likely a consequence of inhibition of branchial excretion of nitrogenous waste products, therefore leading to the accumulation of URE in blood (Bernet et al., 2000), and also to histopathological gill lesions. Although also possibly affected by pollution, elevated TP could be associated with

nutritional fish status, as the discharge of treated effluent may have elevated nutrients, increased the biomass of the invertebrate community (Bernet et al., 2000), and thus contributed to the increase of both TP and ALB. The PCA allowed discrimination of the most relevant plasma mediators determining physiological responses of fish under assay. In the both PC1 and PC2, PC1 and PC3 axes (Figure 5), the first component was biologically consistent with parameters related to stress, and a clear effect was found for ALB and TP as well as for CHOL and GLU from fish originating from the effluent-receiving canal (B-5), which tend to group together and appear to have similar characteristics. There was a clear separation between the effluent fish and the unaffected stream fish, where stress impacted the liver impairment. Similar findings relating to stress-based tissue impairment were documented by Li et al. (2011). Renal impairment affected by stress reached downstream from the effluent, in the County canal (C-9), as evident from the CRE and URE parameters. Hematological and biochemical parameters in fish can significantly change in response to a variety of stressors, and although the changes found in this study suggest water quality as one of the important factors affecting overall physiology of investigated fish, their alterations are nonspecific to a wide range of substances, and thus should always be evaluated in concordance with other indicators and biomarkers. To that effect, micronucleus and erythrocyte nuclear abnormalities assays are frequently used for evaluation of the water pollution (Klobučar et al., 2003; Talapatra and Banerjee, 2007; Pavlica et al., 2008; Omar et al., 2012). The formation of micronuclei is the consequence of chromosomal damage or dysfunction of mitotic apparatus and represents acentral chromosomal fragments or whole chromosomes that have been excluded from the main nucleus during cell division (Udroiu, 2006). Although the mechanism of the erythrocyte nuclear abnormalities formation has not been fully explained, they have been considered as genotoxic analogues of micronuclei. In the present

study, micronuclei were detected in a few fish with very low frequencies from at all three sampling sites. Levels of the erythrocyte nuclear abnormalities were higher (although not significant) in fish sampled from the effluent-receiving canal (B-5). Higher ENA frequencies corresponded to higher concentrations of heavy metals in canal receiving the effluent, especially after the biological treatment plant (reed beds) (6). Discrepancy between MN and ENA appearance was also described by other authors (Güner and Muranh, 2011; Monteiro et al., 2011) who showed significant induction of nuclear abnormalities, but not micronuclei in fish exposed to copper, cadmium and lead.

4.6. Earthworms as bioindicators, MXR inhibition

Earthworms *E. fetida* are efficient species for bio-monitoring programs targeted at prevention of degradation of ecosystem due to anthropogenic activities (Tondoh et al., 2007). They can survive in highly contaminated environments due to their cell efflux of xenobiotics mediated by transporter proteins and detoxification enzymes. Although used mainly as diagnostic indicators of wastewater-derived anthropogenic organic contaminants in terrestrial environments (Kinney et al., 2010), it was found that they accumulate toxicants to levels above those reported for wastewater (Markman et al., 2007). The MXR defense system and accumulation of model fluorescent substrates in the earthworm tissue can be decreased by specific model inhibitors (Bošnjak et al., 2014). The protective function of the MXR mechanism can be inhibited by many pollutants present in the environment (Kurelec, 1997; Markman et al., 2007). The environmental presence of chemosensitizers or inhibitors of the MXR defense system in different organisms (aquatic, terrestrial) could cause an increase in intracellular accumulation and toxic effects of

other xenobiotics normally effluxed by MXR transport proteins [P-glycoprotein (P-gps), MRPs]. For example, ecotoxicological significance of MXR inhibition was supported in *in vivo* studies that demonstrated an increase in the production of mutagenic metabolites by mussels and an increase in the number of sea urchin embryos with apoptotic cells after exposure to model MXR inhibitors. It has also been demonstrated that MXR inhibitors are present among both conventional and emerging man-made pollutants: some pesticides and synthetic musk fragrances show extremely high MXR inhibitory potential at environmentally relevant concentrations. (Smital et al., 2004). This bioassay revealed the presence of MXR inhibitors in all examined samples, particularly in the diluted samples. We have thus confirmed the concept that the measurement of the MXR inhibition can be utilized as a valuable specific biomarker of environmental contamination (Epel et al., 2008; Hackenberger et al., 2012), and suggest the use of *E. fetida* exposure as a tool for constant bio-monitoring.

5. Limitations

The study was conducted once in spring of 2014, with limited number of fish, sampling days, and samples per location. The relatively low fish sample size per sampling site is a consequence of reduced accessibility to the posts appropriate for fishing and fish avoidance of the bait. The lack of correction for multiple testing, due to the preliminary nature of the study, is another of its limitations. The tested hypotheses relate to the particular WWTP under assay.

6. Conclusions

Treated wastewater discharged from the WWTP modified the environmental parameters and xenobiotic concentrations of the receiving surface waters, and played an important role in the antibiotic resistance scheme. The investigated histological, hematological and plasma biochemical parameters of fish indicated to significant changes related to a complexity of environmental stressors, contributing to their overall physiological status assessment. We established that *E. fetida* can serve as a sentinel organism for environmental pollutants, as the sensitive earthworm cellular efflux mechanisms mediated by the ABC transporters can thus be utilized as a biomarker of environmental contamination, and we suggest its use in complex bio-monitoring studies. However, biomarkers of species vulnerability still need to be developed, as well as identifying the most vulnerable life stages and populations, to understand the interactions between global environmental changes, nutritional status, pathogens and toxic chemicals, and to develop integrated approaches to manage vulnerability of aquatic systems to toxicants (Couillard et al., 2008).

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Table 1. Number of samples per sampling location for a) Prussian carp (*Carassius gibelio*) for necropsy, microbiological, blood plasma, genotoxicity, histopathology assessment b) water for physico-chemical, heavy metal, and microbiological analyses, c) sludge for physico-chemical, heavy metal, and microbiological analyses, d) water for analytical chemistry, e) sediment for analytical chemistry. Samples of water d) were taken at three time-points (morning 8:30, mid-day 14:00, evening 20:30 hrs) as a one-grab sample, while all other samples were taken in the early morning as a one-grab sample.

Locations:	1/A-1 Reference stream	2 Inflow of raw municipal water to WWTP	3 Inflow of sugar plant waters to WWTP	4 Treated water leaving WWTP	5/B-5 Canal receiving the effluent	6 Canal after the biological treatment plant	7 Canal entering the County canal	8 County canal	9/C-9 County canal before the river	10 WWTP active sludge	11 Sludge from the depot
a) Prussian carp	8				8				8		
b) Water	1	1	1	1	1	1	1	1	1		
c) Sludge										1	1
d) Water		3		3							
e) Sediment				1	1				1		

Table 2. Total viable counts and concentration of bacteria across all sample collection sites. Sites 1-9 refer to water as in the Table 1, sampling sites 10 and 11 refer to WWTP active sludge, and sludge from the depot, respectively.

	1	2	3	4	5	6	7	8	9	10	11 (CFU/g)
Counts at 37 °C (CFU/mL)	9×10^2	5.9×10^5	3.1×10^6	3.7×10^3	1.6×10^4	4×10^4	2.9×10^2	4×10^2	1.5×10^3	6.9×10^5	5.6×10^8
Counts at 22 °C (CFU/mL)	2.1×10^3	1.6×10^6	1.4×10^7	9×10^3	1.1×10^5	3×10^5	3×10^3	3×10^3	3.3×10^3	1.2×10^6	6×10^8
Fecal coliforms (CFU/100 mL)	3.2×10^2	2×10^6	1.3×10^3	3×10^2	39	1×10^2	6	26	40	3×10^5	6.4×10^6
Intestinal enterococci (CFU/100 mL)	92	1.4×10^6	1.3×10^4	8×10^2	1.4×10^2	31	4×10^3	52	40	4.3×10^5	2×10^7
E. coli (presence/absence)	+	+	+	+	+	+	+	+	+	+	+
Salmonella spp. (presence/absence)	-	-	-	-	-	-	-	-	-	-	-
L. monocytogenes (presence/absence)	-	-	-	-	-	-	-	-	-	-	-
Ps. aeruginosa (CFU/100 mL)	0	2×10^3	0	0	0	0	0	0	0	0	0
Sulphite reducing clostridia (CFU/100 mL)	0	1×10^3	10	10	1×10^2	2×10^2	1	10	20	6×10^2	3×10^7
Yeasts (CFU/mL)	70	6.5×10^2	3×10^2	0	35	25	0	10	0	7.9×10^3	0
Moulds (CFU/mL)	30	1×10^2	1×10^3	80	3×10^2	4×10^2	1×10^2	70	1.4×10^2	4×10^3	8.7×10^5

Table 3. Percentage of API 20E and MALDI-TOF MS identification results for Gram-negative isolates from fish captured at three locations: A-1: unaffected stream, B-5: effluent-receiving canal, entering the County canal, C-9: County canal downstream before the Drava river (corresponding with water sampling sites 1, 5, 9, respectively). Bacteria were retrieved from gill, kidney, liver and spleen tissues. MALDI-TOF MS scores ranged from 1.434 to 2.279, where 42.8 % of scores corresponded to 2.000 to 2.299: *Secure genus identification with probable species identification**. API 20E results ranged from *Unacceptable* to *Excellent identification*, with only 8 % of profiles corresponding to *Excellent identification to the genus***.

Bacterial isolates	A-1	B-5	C-9
MALDI-TOF MS (%)			
A. bestiarum (8.68)	4.34	2.17	4.34
A. media (10.87)	2.17	-	8.70
A. salmonicida ssp. *salmonicida* (2.17)	-	2.17	-
A. popoffii (4.34)	-	-	4.34
A. encheleia (2.17)	-	-	2.17
A. sobria (17.39)	-	-	17.39
A. veronii (6.52)	-	-	6.52
Shewanella baltica (10.85)	2.17	4.34	4.34
Acinetobacter johnsonii (4.34)	2.17	-	2.17
Ac. tandoii (2.17)	-	-	2.17
Ac. pittii (4.34)	-	-	4.34
Ac. guillouiae (2.17)	-	-	2.17
Ac. gerneri (2.17)	-	-	2.17
Citrobacter gillenii (2.17)	-	-	2.17
Pseudomonas fragi (4.34)	4.34	-	-
Ps. brenneri (2.17)	-	2.17	-
Rheinheimera soli (10.87)	-	2.17	8.70
Janthinobacterium lividum (2.17)	-	-	2.17
API 20E (%)			
A. hydrophila group 1 (20.41)	4.08	2.04	14.29
A. hydrophila group 2 (51.02)	6.12	6.12	38.78
Ps. fluorescens/putida (4.08)	4.08	-	-
Ps. aeruginosa (2.04)	-	-	2.04

Ochrobactrum anthropi (16.33)	-	2.04	14.29
Citrobacter youngae (2.04)	-	-	2.04
Unacceptable profile (4.08)	-	2.04	2.04

* MALDI-TOF MS score: 2.300 to 3.000: Highly probable species identification; 2.000 to 2.299: Secure genus identification with probable species identification; 1.700 to 1.999: Probable identification to the genus level; <1.700: Not reliable identification.

**API 20E result: Excellent identification (% id ≥ 99.9 and T index ≥ 0.75); Very good identification (% id ≥ 99.0 and T index ≥ 0.50); Good identification (% id ≥ 99.9 and T index ≥ 0.25); Acceptable identification (% id ≥ 80.0 and T index ≥ 0). The identification is not reliable if the sum of the % id proposed is less than 80.0; The profile is Doubtful if a taxon having several tests against the identification is present among those proposed; The profile is Unacceptable if the number of choices proposed is 0, all the gross frequencies being less than the threshold value.

Table 4. Antimicrobial susceptibility of the isolated strains (retrieved from water, sludge, fish) from all locations is presented as percentages of susceptible (S), intermediate (I) or resistant (R) strains. Antimicrobials: oxytetracycline (OTC), sulfamethoxazole (SMX), amoxicillin (AMC), florfenicol (FFC), erythromycin (E), oxolinic acid (OA), norfloxacin (NOR), flumequine (UB).

	1/A-1	2	3	4	5/B-5	6	7	8	9/C-9	10	11
OTC											
S	0/28.6	25.0	0	75.0	50.0/60.0	100.0	75.0	50.0	25.0/57.1	0	25.0
I	0/71.4	50.0	75.0	25.0	50.0/40.0	0	25.0	25.0	75.0/19.0	0	75.0
R	100.0/0	25.0	25.0	0	0/0	0	0	25.0	0/23.8	100.0	0
SMX											
S	0/0	0	0	25.0	50.0/20.0	0	25.0	50.0	33.3/4.8	0	0
I	0/42.9	25.0	0	25.0	0/40.0	33.3	25.0	25.0	33.3/42.9	0	25.0
R	100.0/57.1	75.0	100.0	50.0	50.0/40.0	66.6	59.0	25.0	33.3/52.4	100.0	75.0
AMC											
S	0/14.3	0	0	0	0/40.00	0	0	0	33.3/9.5	0	0
I	0/14.3	0	0	0	0/0	0	25.0	0	0/23.8	0	0
R	100.0/71.4	100.0	100.0	100.0	100.0/60.0	100.0	75.0	100.0	66.6/66.7	100.0	100.0
FFC											
S	100.0/42.9	75.0	100.0	100.0	100.0/60.0	100.0	100.0	100.0	100.0/66.7	50.0	25.0
I	0/42.9	25.0	0	0	0/40.0	0	0	0	0/28.6	50.0	0
R	0/14.3	0	0	0	0/0	0	0	0	0/4.8	0	75.0
E											
S	0/14.3	25.0	0	25.0	0/60.0	33.3	50.0	25.0	66.6/33.3	0	0
I	0/71.4	75.0	0	75.0	100/40.0	66.6	25.0	75.0	33.3/52.4	0	25.0
R	100/14.3	0	100.0	0	0/0	0	25.0	0	0/14.3	100.0	75.0
OA											
S	50.0/28.6	50.0	25.0	100.0	100/60.0	100.0	75.0	75.0	100.0/66.7	50.0	25.0
I	50.0/71.4	0	75.0	0	0/40.0	0	0	25.0	0/33.3	25.0	75.0
R	0/0	50.0	0.0	0	0/0	0	25.0	0	0/0	25.0	0
NOR											

S	50.0/71.4	50.0	25.0	100.0	100.0/80.0	100.0	75.0	75.0	100.0/71.4	50.0	25.0
I	0/28.6	0	75.0	0	0/20.0	0	25.0	25.0	0/28.6	25.0	75.0
R	50.0/0	50.0	0	0	0/0	0	0	0	0/0	25.0	0
UB											
S	100.0/71.4	50.0	25.0	100.0	100.0/80.0	100.0	75.0	100.0	100.0/85.7	75.0	25.0
I	0/28.6	50.0	75.0	0	0/20.0	0	25.0	0	0/14.3	0	75.0
R	0/0	0	0	0	0/0	0	0	0	0/0	25.0	0

Table 5. Physico-chemical properties of water from water sampling sites: 1: unaffected stream, not related to any industrial nor agricultural waters; 2: inflow of raw municipal wastewaters to the WWTP; 3: inflow of sugar plant wastewaters to the WWTP; 4: treated wastewater leaving the WWTP; 5: canal receiving the effluent; 6: canal after the biological treatment plant (reed beds); 7: canal entering the County canal; 8: County canal; 9: County canal downstream before the Drava river

	1	2	3	4	5	6	7	8	9
Temperature (°C)	13.90	13.90	-	20.60	18.60	18.60	13.90	15.60	16.50
Dissolved oxygen (mg O_2/L)	9.66	5.93	-	0.39	2.26	2.72	4.86	1.91	9.29
Oxygen saturation (%)	94.90	58.40	-	4.40	24.6	29.60	44.60	19.50	95.9
pH	8.13	7.74	6.97	7.80	7.89	7.87	7.42	7.54	7.65
El. conductivity (µS/cm)	529.00	1532.00	4980.00	1369.00	1412.00	1295.00	699.00	879.00	810.00
Suspended solids (mg/L)	37.70	126.80	295.00	31.10	171.00	172.30	3.60	3.60	8.60
COD	43.16	369.91	7536.00	42.63	57.55	71.96	32.49	22.20	18.49

(mg O_2/L)									
COD-Mn (mg O_2/L)	15.63	69.66	1678.00	20.63	30.64	31.64	8.85	16.93	11.93
BODn (mg O_2/L)	19.85	310.00	6153.00	36.75	48.26	58.23	25.00	15.07	12.84
Ammonium (mg/L)	0.02	59.00	50.50	1.25	8.40	8.60	<0.010	2.40	0.05
Nitrite (mg/L)	0.011	0.075	1.95	0.16	0.40	0.35	0.006	0.053	0.036
Nitrate (mg/L)	0.30	0.00	4.50	5.40	1.30	1.10	<0.10	0.30	1.20
Total nitrogen (mg/L)	1.74	63.00	63.20	8.49	11.80	12.28	1.63	5.43	10.70
Phosphate (mg/L)	0.086	4.69	0.19	0.32	0.97	0.87	0.161	0.223	0.092
Total phosphorus (mg/L)	0.166	7.50	1.45	0.47	1.49	1.89	0.371	0.418	0.145

Table 6. Total concentrations of heavy metals from water and sludge. Sampling sites 1-9 refer to water as in the Table 1, sampling sites 10 and 11 refer to WWTP active sludge, and sludge from the depot, respectively.

Concentration in µg/L	1	2	3	4	5	6	7	8	9	10	11
Iron	8388.00	30.00	9197.00	50.00	703.00	419.00	207.00	138.00	128.00	810.00	-
Cadmium	0.063	0.10	0.241	0.056	0.80	1.618	0.034	0.034	0.05	1.39	0.046
Chromium	6.08	14.87	17.56	14.81	7.698	12.77	7.84	11.14	12.91	36.13	4.06
Mercury	<0.20	6.25	4.23	0.50	0.87	1.21	<0.20	0.83	0.45	2.42	0.25
Lead	1.86	4.27	3.17	1.41	5.58	5.83	2.98	1.95	2.14	15.93	4.00
Nickel	10.00	6.00	76.00	14.00	31.00	38.00	19.00	10.00	8.00	100.00	2.25
Zinc	23.00	128.00	46.00	44.00	90.00	101.00	37.00	29.00	27.00	466.00	32.25

Copper	56.00	90.00	43.00	46.00	270.00	274.00	80.00	81.00	61.00	336.00	18.22

Table 7. Concentration of pharmaceuticals in water (ng/L) and sediment (ng/g). Water was sampled at three time-points (morning 8:30, mid-day 14:00, evening 20:30 hrs) from two locations: at the inflow of raw municipal water to the WWTP (site 2) and at the outflow of treated water leaving the WWTP (site 4). Sediment was sampled at one time-point (morning) from three locations: treated water leaving WWTP (4), canal receiving the effluent (5), County canal before the river (9). In sediment, of all sulfa drugs, only sulfamethoxazole was detected, while erythromycin was not detected.

	Water					
	Inflow of raw waters to WWTP			Outflow of treated waters		
	Morning	Mid-day	Evening	Morning	Mid-day	Evening
Pharmaceuticals	112,333.45	107,688.88	118,557.90	21,085.91	35,383.55	27,830.73
Antibiotics	5,479.82	14,287.57	26,334.96	7,545.84	7,949.07	7651.23
Sulfa drugs	90.88	87.88	49.39	65.71	60.12	68.44
Sulfamethoxazole	57.74	67.81	26.55	51.68	51.02	53.14
Erythromycin	2.27	43.94	12.50	0	0	0

	Sediment		
	Treated water leaving WWTP	Canal receiving the effluent	County canal
Pharmaceuticals	158,789.49	33,446.11	28,210.90
Antibiotics	144.54	17.51	18.58
Sulfamethoxazole	19.60	5.07	0.99

Table 8. Descriptive statistics of metabolite concentrations and enzyme activities in plasma of Prussian carp (*Carassius gibelio*) captured at three locations: A: unaffected stream (A-1), B: effluent-receiving canal (B-5), C: County canal downstream (C-9).

Plasma parameters		Mean	Median	SD	SE	Rng. Diff.	25 %	75 %	Skewness
GLU (mmol/L)									
	A	27.93	24.90	19.92	11.50	39.50	13.50	43.13	0.67
	B	11.35	11.35	3.47	2.45	4.90	8.90	13.8	-
	C	11.49	9.20	8.34	2.23	30.10	6.10	18.40	0.84
URE (mmol/L)									
	A	0.87	0.70	0.47	0.27	0.90	0.55	1.23	1.39
	B	1.15	1.15	0.07	0.05	0.10	1.10	1.20	-
	C	0.64	0.50	0.33	0.09	1.10	0.40	0.80	1.74
CRE (μmol/L)									
	A	50.00	57.00	17.58	10.15	33.0	36.75	61.50	-1.51
	B	26.00	26.00	11.31	8.00	16.00	18.00	34.00	-
	C	31.00	30.00	8.49	2.27	34.00	27.00	32.00	1.36
TP (g/L)									
	A	31.67	31.00	9.02	5.21	18.00	25.00	38.50	0.33
	B	48.00	48.00	22.63	16.00	32.00	32.00	64.00	-
	C	40.21	40.50	11.78	3.15	40.00	29.00	47.00	0.89
ALB (g/L)									
	A	10.00	9.00	4.58	2.65	9.00	6.75	13.50	0.94
	B	15.50	15.50	9.19	6.50	32.00	32.00	64.00	-

	C	12.64	11.00	5.24	1.40	19.00	9.00	14.00	1.45
TRIG (mmol/L)									
	A	2.87	2.40	1.17	0.68	2.20	2.10	3.75	1.51
	B	4.80	4.80	0	0	0	4.80	4.80	-
	C	2.86	2.45	1.42	0.38	5.30	1.80	3.70	1.07
CHOL (mmol/L)									
	A	3.97	3.90	0.40	0.23	0.80	3.68	4.28	0.72
	B	5.50	5.50	1.70	1.20	2.40	4.30	6.70	-
	C	5.75	5.95	2.10	0.56	9.00	4.60	6.50	1.48
ALT (U/L)									
	A	69.33	32.00	78.01	45.04	142.00	20.75	127.25	1.66
	B	20.50	20.50	14.85	10.50	21.00	10.00	31.00	-
	C	23.21	8.50	34.45	9.21	115.00	7.00	16.00	2.22
SOD (U/L)									
	A	502.45	331.12	327.77	189.24	584.52	304.68	743.07	1.71
	B	392.47	392.47	77.29	54.65	109.30	337.82	447.12	-
	C	485.94	360.59	340.29	90.95	1096.88	206.78	723.54	1.00

Abbreviations: GLU glucose, URE urea, CRE creatinine, TP total proteins, ALB albumin, TRIG triglyceride, CHOL cholesterol, ALT alanine aminotransferase, SOD superoxide dismutase; SD standard deviation, SE standard error of the mean, Rng. Diff, range difference.

Figure 1. Sampling sites for raw water, treated water and sludge drawn in the ArcGIS 10.1 program (location: NE Croatia, city of Virovitica and surroundings) 1: unaffected stream, not related to any industrial nor agricultural waters, considered as a reference site; 2: inflow of raw municipal wastewaters to the WWTP; 3: inflow of sugar plant wastewaters to the WWTP; 4: treated wastewater leaving the WWTP; 5: canal receiving the effluent; 6: canal after the biological treatment plant (reed beds); 7: canal entering the County canal; 8: County canal; 9: County canal downstream before the Drava river; 10: WWTP active sludge; 11: sludge from the depot. Symbols: BU (Biological Unit), circle with a dot (smaller inhabited area).

Figure 2. Prussian carp gill tissue. (a): from the effluent-receiving canal, entering the County canal; lamellar fusion and bridging (double-arrow) of the inter-lamellar space, reduced interlamellar spaces; (b): from the County canal: dilation of blood sinuses and enlargement of marginal blood channels of secondary lamellae (arrow).

Figure 3. MALDI-TOF MS mass spectra of *Aeromonas* cells isolated from water and sludge, 18 isolates (a) and fish, 23 isolates (b). Main masses between 4,000 and 12,000 Da are indicated.

Figure 4. Dendrogram of MALDI-TOF MS patterns and relationships from identified *Aeromonas* strains retrieved from water and sludge (a) and fish (b). Branch lengths are proportional to the number of *m/z* differences between the strains. The principal components were determined based on clustering.

Figure 5. PCA correlation loading biplots for carp plasma parameters. Abbreviations: (A_metabolite: fish from the unaffected stream, B_metabolite: fish from the effluent-receiving canal, C_metabolite: fish from the County canal).

Figure 6. Accumulation of Rhodamine 123 dye in earthworms *E. fetida* after 24h exposure to three concentrations of water and sludge. Locations 1-10 as described in the Figure 1. Symbols: ' = 100x dilution of the sample, " = 10,000x dilution of the sample, C = control, PC = positive control 50 $\mu molL^{-1}$ MK571. Data are expressed as the mean of triplicate ± SD, *p<0.03, t-test, n=5.

Impact of treated wastewater on organismic biosensors at various levels of biological organization

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ABSTRACT

Relating the treated wastewater quality and its impact on organismic biosensors (Prussian carp, *Carassius gibelio* and earthworm, *Eisenia fetida*) was the main objective of the study. The impact on health status of fish living downstream, microbiological contamination and antimicrobial resistance, fish tissue structure, blood biochemistry, oxidative stress, genotoxic effects, as well as multixenobiotic resistance mechanism (MXR) was assessed. Treated wastewater discharged from the WWTP modified the environmental parameters and xenobiotic concentrations of the receiving surface waters. Potential bacterial pathogens from fish and respective waters were found in relatively low numbers, although they comprised aeromonads with a zoonotic potential. High resistance profiles were determined towards the tested antimicrobial compounds, mostly sulfamethoxazole and erytrhomycin. Histopathology primarily revealed gill lamellar fusion and reduction of interlamellar spaces of effluent fish. A significant increase in plasma values of urea, total proteins, albumins and triglycerides and a significant decrease in the activity of plasma superoxide dismutase were noted in carp from the effluent-receiving canal. Micronucleus test did not reveal significant differences between the examined groups, but a higher frequency of erythrocyte nuclear abnormalities was found in fish sampled from the effluent-receiving canal. Earthworms indicated to the presence of MXR inhibitors in water and sludge samples, thus proving as a sensitive sentinel organism for environmental pollutants. The integrative approach of this study could serve as a guiding principle in conducting evaluations of the aquatic habitat health in complex bio-monitoring studies.

Keywords: WWTP; Effluent; Bacteria; Fish; Genotoxicity; Earthworm; MXR

1. Introduction

Safe drinking water and proper sanitation are indispensable factors for sustaining life (Naidoo and Olaniran, 2014), while treated wastewater discharged to a body of water modifies its environmental parameters, both qualitative and quantitative. The discharge of effluent from domestic and industrial sources has detrimental effects on the aquatic ecosystem as this outfall can deposit large amount of organic matter, nutrients and pollutants leading to eutrophication, oxygen deficits and accumulation of pollutants into receiving waterways (Bhatia and Goyal, 2013). Urban wastewater treatment plants (WWTP) were originally designed to reduce the biological oxygen demand, total suspended solids and nitrogen and phosphorus pollution, while the removal of pathogenic microorganisms has received less attention (Lucas et al., 2014). Although the primary and secondary treatments are able to remove up to 99 % of fecal indicator bacteria (Servais et al., 2007, Lucas et al., 2014), the quality required to use treated wastewaters might be insufficient to achieve the level required for recreational activities in the receiving water bodies.

Environmental change can increase the vulnerability of aquatic species to toxic chemicals by challenging an organism's capacity to respond or to repair toxic injury or by modifying animal behavior like migration or predation (Couillard et al., 2008). Also, xenobiotics may affect the capacity of aquatic species to adapt to environmental challenges that come with stressors, such as pathogens. Fish are very susceptible to environmental variations, and their physiological status can serve as an early indicator of the specific ecosystem's health (Kaur and Dua, 2014).

A sampling strategy was developed to retrieve representative water, sediment, sludge, and fish samples related to a Croatian WWTP processing municipal, hospital and sugar plant wastewaters. It is a mechanical and chemical-biological facility with activated sludge, encompassing primary and secondary treatments of influents, treating mainly municipal wastewater deriving from a small city of 20,000 residents. Frequently, hospital wastewater is pretreated, but on this location it is connected directly to a municipal sewer and treated at the municipal WWTP. Treatment of such wastewater at the source has advantages of avoiding dilution due to mixing with the urban sewage and avoiding losses into the environment caused by sewer leakage and overflows. The sugar plant, operating at the time of this investigation, is a significant contributor to the wastewater to be treated at the WWTP. Sugarcane industry is among those industries with the largest water demands and, in addition, is an important source of non-toxic organic pollution (Ingaramo et al., 2009).

There is a gap in fundamental understanding of the specific contribution of the WWTP effluent in observed changes in organisms residing in/exposed to effluent- receiving waters and sludge. Also, the effectiveness of the WWTP in eliminating bacteria and pollutants which are not organic (particularly antibiotics), needs to be elucidated. Consequently, the objective of this wide-scale work was to test the hypotheses that 1) the WWTP effluent will induce biological effects on organismic biosensors; 2) resistant bacteria and potential fish and human pathogens will be identified from water and sludge, and antibiotics will be retrieved from sediment. To test our hypotheses, we conducted a series of tests to measure the impact on health status of fish living downstream, microbiological contamination, fish tissue structure, blood biochemistry, oxidative stress, genotoxic effects and multixenobiotic resistance mechanism (MXR): (i) general fish health examination, necropsy and histopathology were performed (hypothesis 1); (ii) rapid

phenotypic tests and matrix assisted laser induced desorption ionization connected to the time of flight mass spectrometry (MALDI-TOF MS) were conducted on multiple samples of water, fish, and sludge (hypotheses 1, 2); (iii) fish blood biochemistry parameters and oxidative stress parameters were determined (1); (iv) erythrocytic nuclear abnormalities and micronuclei were enumerated and assessed (1); (v) cellular efflux mechanism mediated by ATP binding cassette (ABC) transporters that bind and actively remove toxic substrates from cells was analyzed post exposure to raw and treated water and sludge (1); (vi) physical-chemical characteristics and heavy metal contents were determined in water and sludge samples (1); (vii) antibiotic concentrations were measured in water and sediment (2).

The impact of effluent on fish and earthworms as toxicity biosensors was specifically addressed, especially in the view of active influence of all contributors to the wastewater volume (municipal, sugar industrial, hospital) as it is frequently omitted from investigation of performance of wastewater treatment plants. Such an approach encompasses an integrated overview of the impact of treated wastewater on key environmental and organismal parameters.

2. Materials and methods

2.1. WWTP and description of the sampling sites

The study was carried out in spring 2014, and the samplings of fish, water, sludge and sediment were conducted during the April 23 and 24 (Table 1). The samplings were carried out during the treatment process of a municipal WWTP serving about 20,000 inhabitants, also receiving

hospital and sugar plant wastewaters. Treatment includes primary and secondary processes, including settling tanks, grit chambers, activated sludge biological process, aeration tanks, secondary tanks for removing the biomass and other suspended particles. The resultant final treated effluent is discharged into the water canal. This canal further downstream receives additional communal treated water from a biological treatment plant (reed beds) serving a small suburb, widens to enter a County canal (agricultural landuse), which eventually ends up in a Drava river. Therefore, sampling sites for water and sludge are defined as follows: 1: unaffected stream, not related to any industrial nor agricultural waters, considered as a reference site; 2: inflow of raw municipal wastewaters to the WWTP; 3: inflow of sugar plant wastewaters to the WWTP; 4: treated wastewater leaving the WWTP; 5: canal receiving the effluent; 6: canal after the biological treatment plant (reed beds); 7: canal entering the County canal; 8: County canal; 9: County canal downstream before the Drava river; 10: WWTP active sludge; 11: sludge from the depot (Figure 1). Water and sludge were collected in sterile glass and polypropylene flasks, refrigerated transported to the lab and immediately analyzed.

2.2. Physical and chemical properties and heavy metals analyses

Physico-chemical properties of water were analyzed according to the international standards as follows: determination of electrical conductivity, pH, suspended solids, dissolved oxygen, permanganate index (COD-Mn), chemical oxygen demand (COD), biochemical oxygen demand after n days (BODn) by dilution and seeding with allylthiourea, ammonium and phosphorus with spectrometric method, nitrite, total nitrogen by persulfate digestion method, total phosphorus by ascorbic acid method, nitrate by colorimetry, and cadmium reduction (ISO 7888:1985, ISO

10523:2008, ISO 872:2005, ISO 5813:1983, ISO 8467:1993, ISO 15705:2002, ISO 5815:1989, ISO 7150-1:1984, ISO 6878:2004, ISO 6777:1984, SM 4500-NO3-E, respectively).

Total concentrations of heavy metals (Cd, Cr, Pb, Hg Ni, Zn, Cu, Fe) were analyzed according to the international standards as follows: determination of total cadmium, chromium, and lead by atomic absorption spectrometry-graphite furnace technique (ISO 15586:2003, ISO 15586:2004, ISO 15586:2006); determination of mercury by atomic absorption spectrometry (ISO 12846:2012); determination of nickel, zinc, and copper by inductively coupled plasma optical emission spectrometry (ISO 11885:2007, ISO 11885:2011, ISO 11885:2012); determination of iron by spectrometric method using 1,10-phenanthroline (ISO 6332:1988).

2.3. Animals and tissue processing

This work has been carried out in accordance with the European Council Directive 86/609/European Economic Community for animal experiments, and fish were manipulated by the competent authorized persons (licensed veterinarians) in accordance with the provisions of national legislation. Fish were caught by nets and angling at three locations: A-1: unaffected stream, B-5: effluent-receiving canal, entering the County canal, C-9: County canal downstream before the Drava river (corresponding with water sampling sites 1, 5, 9, respectively) (Table 1). A total of 24 Prussian carp (*Carassius gibelio*) of both sexes were subjected to examination, 8 from each site: A-1 (mean weight 155.20 ± 16.99 g, mean length 77.00 ± 27.01 mm), B-5 (mean weight 144.00 ± 8.74 g, mean length 55.60 ± 6.43 mm), C-9 (mean weight 259.07 ± 49.13 g, mean length 398.00 ± 213.45 mm).

Specimens were transported live to the laboratory and within few hours sacrificed by overdose of tricaine methane-sulfonate (MS-222, Sigma, St. Louis, Missouri, USA). Blood withdrawal and necropsy were performed immediately and tissues (gills, spleen, liver) were fixed in 4 % neutral buffered formalin, and after graded ethanol-xylene dehydration were embedded in paraplast. Sagital and transverse sections of 3-5 μm thickness were stained with hematoxylin/eosin (H&E). Microphotographs were taken with a digital camera DP 70 Olympus® connected to an Olympus® BX51 binocular microscope, and transferred to Microsoft® AnalySIS Soft Imaging System for interpretation.

The *Eisenia fetida* earthworms (Oligochaeta, Lumbricidae) were obtained from an earthworm farm (Donja Bistra, Croatia). Before experiment, adult clitellate specimens (180-260 mg) were stored in Petri dishes on moist filter paper for 24h (in the dark at 20 ± 1 °C) to empty the gut contents.

2.4. Pharmaceuticals in water and sediment

Water was sampled at the inflow of raw wastewaters to the WWTP (site 1) and outlet of the treated wastewater leaving the WWTP (site 4), at three time-points (morning, mid-day, evening). Sediment was collected from three locations: treated water leaving the WWTP (4), canal receiving the effluent (5), County canal before the river (9) (Table 1).

2.4.1. Reagents and sample preparation

All chemicals were of high purity grade. Suprapur formic acid was from Merck (Darmstadt, Germany). Water, acetone and methanol (all from J.T.Baker, Deventer, Netherlands) were of LC

MS grade. Ammonium formate was from Fischer Scientific (New Jersey, USA). Analytical standards of sulfa drugs (sulfachloropyridazine, sulfadimethoxine, sulfamethazine, sulfamethizole and sulfametoxazole) were from Supelco (Sigma Aldrich , Steinheim, Germany). Ciprofloxacin, erythromycin and trimethoprim were purchased from Restek (Bellafonte, USA), and azithromycin was from Dr. Ehrenstorfer (Germany). Polycarbonate bottles containing 250 mL of water and sediments were stored for 24 h at 4°C until analysis. Prior analyses of water samples were filtrated on 0,2µ PTFE filter. Ultrapure laboratory water samples were always processed in parallel with the environmental water samples.

2.4.2. Methods and analytical procedure

The direct injection method was used for the determination of pharmaceuticals in water. Calibration curve was obtained by direct injecting, in triplicate standard solutions at 7 concentration levels. Correlation coefficients >0.999 were used as linearity acceptance criterion. By testing blank samples spiked at three concentration levels, accuracy and precision were calculated, and for each concentration were evaluated in quintuplicate. Acceptance criteria was for accuracy = recoveries (between 70 % and 110 %) and for repeatability precision lower than 20 %. The method reported by Darwano et al. (2014) was used for sediment samples. Pharmaceuticals were determined by ultra high performance liquid chromatography quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS).

Analyses were performed on 1290 UHPLC system, Agilent Technologies, USA (G4226A autosampler, G4220B binary pump and G1316C thermostated column). The separation of analytes was done using the RP column ACQUITY UPLC, HSS T3 (150 mm x 2.1 mm, 1.8

μm). The gradient was from 100 % water to 100 % organic in 20 min run. The injection volume was 100 µL. The temperature of the column chamber was set to 50°C. In positive ESI (ESI(+)), the mobile phases were composed of solvent A (5 mM ammonium formate/formic acid , and B (100 % MeOH). Gradient elution with a flow rate of 0.4 mL/min was used. The analytes were detected using a 6550 i-Funnel Q-TOF-LC/MS (Agilent Technologies, USA) in 4 GHz detector rate, with a 40,000 resolving power and <2 ppm accuracy. QTOF mass spectrometer (Agilent 6550) operated in positive (ESI+) ion mode. Ions were generated using a dual AJS ESI (Agilent Jet Stream) ion source. Operation conditions were as follows: sheath gas temperature 375 °C, gas temperature 125°C, heat gas 12 L N2/min, drying gas 15 L N2/min, capillary voltages 3500 V, fragmentor 400V, and nebulizer 35 psig. The resolution power for ESI+ was 52296 at 922.009798 m/z and 21801 at 118,086255 m/z. Correction during measuring for any possible drift in the mass axis was done automatically with lock 2 mass ion software. Analyses were performed using MS and MS/MS mode with fixed collision energy and in mass range of 50–1,000 m/z. Fata were further processed with Agilent MassHunter Workstation software (Quantitative Analysis version B.07.00/Build 7.0.457.0 for QTOF, Agilent Technologies, USA).

2.5.Microbiological analyses

Samples were streaked on media for fastidious organisms, general, selective isolation and standard plate count media (all Oxoid Ltd, England, UK). Total viable counts and methods for detection and enumeration of bacteria were as follows: Enumeration of culturable micro-organisms - Colony count by inoculation in a nutrient agar culture medium (ISO 6222:1999); Detection and enumeration of *Escherichia coli* and coliform bacteria – Part 1: Membrane

filtration method (ISO 9308-1:2000/Corr.1:2008); Detection and enumeration of intestinal enterococci – Part 2: Membrane filtration method (ISO 7899-2:2000); Detection and enumeration of *Pseudomonas aeruginosa* by membrane filtration (ISO 16266:2008); Microbiology of food and animal feeding stuffs – Horizontal method for detection of *Salmonella* spp. (ISO 6579:2002/Corr. 1:2004); Microbiology of food and animal feeding stuffs – Horizontal method for detection and enumeration of *Listeria monocytogenes* – Part 1: Detection method (ISO 11290-1:1996); Microbiology of food and animal feeding stuff – Horizontal method for detection and enumeration of *Listeria monocytogenes* – Part 2: Enumeration method (ISO 11290-1:1996/A1:2004; Detection and enumeration of the spores of sulfite-reducing anaerobes (clostridia) - Part 2: Method by membrane filtration (ISO 6461-2:1986); Microbiology - General guidance for enumeration of yeasts and moulds - Colony count technique at 25 degrees C (ISO 21527-1:2008, ISO 21527-2:2008).

Samples of fish gills, kidney and liver were inoculated onto Tryptone Soya Agar, MacConkey Agar (Oxoid) and Blood Agar (Certifikat doo, Croatia). The plates were incubated at 22°C for 48-72 h. Representative colonies were isolated and reincubated on fresh media. Pure colonies were Gram-stained and subjected to morphological, physiological and biochemical tests. The taxonomic position of the isolates was determined by API 20E panels (bioMerieux, Marcy l'Etoile, France) and Bruker Biotyper MALDI-TOF MS (Bruker Daltonics, Billerica, MA). The API 20E tests were performed according to the manufacturer's instructions with a few alterations in order to adapt the system to the bacteria of freshwater fish as described in Topić Popović et al. (2007).

Antimicrobial susceptibility of the isolated strains was determined with Kirby-Bauer disk diffusion method on Mueller Hinton agar (all Oxoid). The following antimicrobials with

respective concentrations (μg) were used in the test: oxytetracycline (OTC_{30}), amoxicillin (AMC_{30}), oxolinic acid (OA_2), erythromycin (E_{15}), sulfamethoxazole (SMX_{50}), florfenicol (FFC_{30}), norfloxacin (NOR_{10}), flumequine (UB_{30}). The inoculum was prepared in sterile 5 mL of 0.85 % Suspension medium (bioMerieux, Marcy l'Etoile, France) and turbidity was adjusted to 0.5 MacFarland's standard. The diameter of the zones of inhibition was read and expressed by referring to the manufacturer's standard table and reported as susceptible (S), intermediate (I) or resistant (R).

2.5.1. Bruker Biotyper MALDI-TOF

Bacterial isolates (one loopful of each bacterial culture) were smeared to a 24-spot steel plate (Bruker Daltonics) in the form of the direct colony technique in two replicates, and were allowed to visibly dry at 22°C. A saturated solution of α-cyano-4-hydroxycinnamic acid in 50 % acetonitrile and 2.5 % trifluoroacetic acid (MALDI matrix, 2 μL) was added to the bacterial colony and dried. The ions generated with a 337-nm nitrogen laser were captured in the positive linear mode between 2 to 20 kDa of mass range. The linear mode of accelerating voltage (20 kV) extracted the positive ions. Two hundred laser shots in five different regions of every well yielded a spectrum comprising the sum of the respective ions. The MALDI Biotyper automation control and Bruker Biotyper 2.0 software (Bruker Daltonics) were used to analyze the captured spectra. For each 24-spot plate, a standard (bacterial test standard; Bruker Daltonics) was included to calibrate the instrument and validate the run. The criteria for identification were as follows: a score of 2.300 to 3.000 indicated highly probable species level identification, a score of 2.000 to 2.299 indicated secure genus identification with probable species identification, a

score 1.700 to 1.999 indicated probable identification to the genus level, and a score of <1.700 was considered to be unreliable. The data obtained with the two replicates were added to the calculation in order to minimize any random effect. The fingerprints for every isolate were identified as presence or absence of peaks and matched with the reference strain in the database.

2.6. Blood biochemistry, oxidative stress, and genotoxic analyses

Blood was withdrawn from all sampled fish by caudal artery and vein puncture, immediately after capture. Tubes were coated with lithium heparin as anticoagulant and centrifuged at 12,000*g* for 90 seconds. Plasma was separated from the corpuscular fraction and frozen at -80ºC until analysis. Heparinized microhaematocrit capillaries were centrifuged at 12,000*g* for 120 seconds within 20 minutes from collection. Haematocrit (Hct) was determined as the volume percentage of red blood cells in blood (Wedemeyer and Yasutake, 1977).

In blood plasma, concentrations of glucose (GLU), urea (URE), creatinine (CRE), cholesterol (CHOL), triglyceride (TRIG), total proteins (TP), albumin (ALB) and activity of alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT) were determined by Beckman Coulter commercial kits (Olympus Life and Material Science Europe, Ireland) on the Olympus AU 640 biochemistry analyzer (Olympus, Japan). The activity of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were determined by Randox commercial kits on the Olympus AU 640 biochemistry analyzer. Paraoxonase activity (PON 1) in serum was assayed by modified method of hydrolysis of paraoxon described by Charlton-Menys et al. (2006) on the Olympus AU 640. Enzyme activity was presented in U/L (1 μmol p-nitrophenol formed/min/L).

For the genotoxicity assessment (micronucleus – MN and erythrocyte nuclear abnormalities – ENA assays), three blood smears per fish were allowed to air dry, then were fixed in 95 % methanol for 3 min and stained with May Grünwald/Giemsa stains. One thousand erythrocytes per slide were scored on randomized and coded slides at 1000x magnification. Nuclear anomalies such as nuclear buds, blebbed, lobed, notched, vacuolated nuclei and bi-nuclei were considered together, while micronuclei were recorded separately and represented as n/1000 (‰).

2.7. MXR activity analyses

2.7.1. Filter paper contact test

In order to determine the presence of inhibitors of the MXR activity in water samples, the filter paper contact toxicity method was applied (OECD, 1984) based on the accumulation of the model fluorescent substrate in the whole body of the earthworm (Bošnjak et al., 2014). *E. fetida* specimens were exposed to three concentrations of raw and treated wastewater and sludge as undiluted, diluted 100x and diluted 10,000x. Exposure was performed as follows: the total volume of 1.2 mL per tested concentration was added to filter paper cut-out discs (80 to 85 g/m^2, approximately 0.2 mm thick, medium grade), which were placed in Petri dishes, changed and moistened with sample to be tested every day during the experiment. Five earthworms per one Petri dish were used. Positive and negative controls were run in parallel with MK571 sodium salt and distilled water. After the pre-treatment (24h), 10 µM fluorescent substrate Rhodamine 123 (Sigma-Aldrich, CAS No. 62669-70-9; R123) was added on filter paper for additional 24 h.

After the end of the exposure, earthworms were weighed and rinsed with distilled water, and then used for measurement of efflux pump activity. All Petri dishes were kept in the dark at (20 ± 1 °C). Experiments were run 3 times in duplicate (total of 270 specimens) and behavioral and morphological changes were recorded during every stage of experiment.

2.7.2. MXR inhibitory potential: determination of inhibitors of MXR mechanism

Accumulation of R123 was measured in the whole earthworm body 24 hours after the exposure. Earthworms exposed were weighed and then homogenized (for 30 sec) with Ultra-Turrax T18 homogenizer (IKA, Germany) in 1 mL distilled water and centrifuged for 10 min at 9,000*g*. After centrifugation, the supernatant was transferred to black 96-well microplates (Nunc, Thermo Fisher Scientific, Denmark). The amount of R123 in the supernatant was determined in triplicate using Tecan Infinite M200 plate reader (Tecan Trading AG, Männedorf, Switzerland) excitation at 490 nm, emission at 544 nm.

2.8. Statistical analysis

Statistical analyses were performed by SigmaStat and SigmaPlot Statistical Software ver. 11.0 (Jandel Corp., San Rafael, California). Analyses and correlations between blood parameters were conducted by *t*-test, Mann-Whitney Rank Sum Test and Spearman correlation coefficients. The significant differences were regarded at $p<0.05$. Differences in MN and ENA frequencies between groups were assessed using Kruskal-Wallis ANOVA on Ranks since the data were not normally distributed. The amount of the accumulated R123 in the whole earthworm body was

calculated in relation to its mass and shown as fluorescent units per mg of earthworm tissue (FU mg^{-1}). To determine differences between treatment groups and controls we used the unpaired *t*-test. The probability level for significance was $p<0.05$. Data are presented as mean ± SD. Principal component analysis (PCA) was applied to describe the main sources of variation and relationship among the selected blood biochemistry parameters. The results are presented graphically by a biplot. The software used was SPSS 13.0 for Windows. The dendrograms were created by MALDI Biotyper 3.0 (Bruker Daltonics, Bremen, Germany) with following settings: distance measure was set at *correlation* and linkage at *complete*. Spearman's rank correlation as column distance measure was applied with pair-wise complete-linkage as the clustering method. Distance values were relative and normalized to a maximum value of 1000.

3. Results

3.1. Fish health and histopathology

The external gross signs and necropsy findings from fish captured in the three representative water bodies did not indicate to any manifest disease. However, fish from the unaffected stream (A-1) demonstrated a low parasitic infestation of gills with *Dactylogyrus* spp., while those from the County canal (C-9) carried a medium load, mainly *Dactylogyrus* spp. on gills and *Thelohanellus* spp. on opercula, skin and fins. Fish from the effluent-receiving canal (B-5) had no parasitic infestations. Histopathological findings of the Prussian carp gill tissues varied in fish sampled from the effluent-receiving canal (site 5), entering the County canal (site 7) and County

Canal (site 8) (Figure 2). Lamellar fusion was the most remarkable microscopic lesion (Fig. 2(a)), where the leading lateral edges of non-fused lamellae were turned towards the neighboring ones often possessing epithelial extensions, bridging the inter-lamellar space. Interlamellar spaces were reduced, with sparse mucus and undifferentiated cells. Epithelial hypertrophy and hyperplasia were also noted in several specimens, causing fusion of two or more lamellae. Fig. 2(b) demonstrates mild dilation of blood sinuses and enlargement of marginal blood channels of secondary lamellae.

3.2. Bacterial identification and antimicrobial susceptibility

In Table 2, total viable counts and concentration of bacteria across all sample collection sites are presented. Colony counts at both incubation temperatures retrieved the highest load from the active sludge (10) and sludge from the depot (11), as well as from the inflows of municipal (2) and sugar plant wastewaters to the WWTP (3). Interestingly, fecal coliforms and yeasts were even more abundant in the unaffected stream (1) than in the canals downstream from the effluent (5-9), while *E. coli* was retrievable from all the sampling sites. Gram-negative bacteria were recovered from all tissues under examination. The majority of isolates were retrieved from gills (61.22 %) and spleen (18.38 %), while less from liver (10.20 %) and kidney (10.20 %). Only 61.22 % of total isolates both identification systems (API 20E and MALDI-TOF MS) identified to the same genus, while most of the readings indicated to aeromonads (48.98 % MALDI-TOF MS; 71.43 % API 20E), particularly dominant in the canal C-9, as presented in Table 3. Of all aeromonads isolated from fish tissues, 50 % were resistant to sulfamethoxazole, which proved to

be the antimicrobial exerting the majority of resistance in all fish bacterial isolates, except amoxicillin, for which resistance was partly expected (Table 4).

From water and sludge, mainly Gram-negative bacteria were retrieved, whereas Gram-positives and Gram-variables (4.54 %) comprised *Bacillus simplex* and *Lysinibacillus fusiformis.* Overall, the most frequently identified genus from water and sludge, irrespective of the sampling site, was *Aeromonas* (38.64 % MALDI-TOF MS; 50.00 % API 20E). Of all the isolates, only 15.91 % matched completely in both API 20E and MALDI-TOF MS readings, while both panels identified 52.27 % of all isolates identically to the genus. For Gram-negative rods, the conventional method resulted in significantly fewer species identifications than with MALDI-TOF MS (11.36 % versus 81.82 %). With API 20E, the mean time to identification was 48 hours, whereas MALDI-TOF MS needed less than 10 minutes per bacterial isolate. MALDI-TOF MS spectra obtained from aeromonads isolated from water and fish from all locations are presented in Figure 3, showing characteristic fingerprints, with the highest intensity peaks ranging from 4,000 to 10,000 Da. Aeromonads retrieved from all samples were also separated by hierarchical clustering (Figure 4) which differentiated related *Aeromonas* species and clustered the closely related ones.

In Table 4, antimicrobial susceptibility of the isolated strains (fish, water, sludge) from all locations are presented as percentages of susceptible (S), intermediate (I) or resistant (R) strains. Except towards amoxicillin, most of the strains, even from the unaffected stream (1), showed resistance towards sulfamethoxazole and erythromycin. Resistance towards tested antimicrobials was most pronounced in the active sludge isolates (10, 11).

3.3. Physical and chemical properties of water; heavy metals; pharmaceuticals in water and sediment

Physico-chemical properties of water and total concentrations of heavy metals from water and sludge are presented in Tables 5 and 6, respectively. Of all pharmaceuticals entering the WWTP (2), antibiotics on average comprised 13.62 %, while at the outlet of treated wastewater (4) their portion increased to 27.45 %. The mean concentration of antibiotics at the inflow of raw wastewaters (2) was 15,367.45 ng/L, while at the outlet (4) it was 7,715.39 ng/L, of which sulfa drugs comprised 0.049 % (76.05 ng/L) and 0.839 % (64.76 ng/L), respectively (Table 7). The overall concentration of antibiotics entering the WWTP (2) increased five-fold from morning towards the evening time-points, but it remained constant over time at the outlet. By contrast, the concentration of sulfa drugs, sulfamethoxazole included, entering the WWTP decreased to half by the evening time-points, but also remained constant over time at the outlet (Table 7). The average SMX concentration in both locations was comparable, while erythromycin concentration (inflow at 19.57 ng/L) was not detectable at the outlet at any time. Erythromycin was also not detectable in any of the sediment tested, while the sediment SMX concentration decreased with the distance from the WWTP. Total pharmaceuticals in sediment also decreased, as well as total antibiotics, although not in the same proportion.

3.4. Fish plasma biochemistry, oxidative stress (SOD), MN, and ENA

A general comparison of the plasma values of GLU, URE, CRE, CHOL, TRIG, TP, ALB, ALT, ALP and SOD of Prussian carp are presented in Table 8. in the form of descriptive statistics. The values of ALP, GGT, GSH-Px and PON 1 were not measurable in most of the tested samples, probably due to haemolysis, and are therefore not presented. TRIG from fish captured in the effluent-receiving canal (B-5) failed the Normality test, as well as the URE, CRE, ALT and CHOL from fish captured in the County canal downstream. The Spearman Rank Order Correlation demonstrated that pairs of variables with *p* values below 0.050 and positive correlation coefficients (r_s), such as URE/ALT (0.5), URE/TRIG (1.0), URE/CHOL (0.5), CRE/SOD (1.0), TP/TRIG (0.56), TP/CHOL (0.74), ALB/TRIG (0.73), ALB/CHOL (0.68), ALT/TRIG (0.86), ALT/CHOL (0.67), ALT/SOD (0.68) ALP/TRIG (0.59), TRIG/CHOL (0.87), TRIG/SOD (0.56) from the fish captured in the County canal, C-9, tend to increase together. A significant increase in values of URE, TP, ALB and TRIG was noted in the fish from the effluent-receiving canal (B-5) when compared with other fish, as well as a significant decrease in SOD when compared with fish from the other two canals. The PCA of eight blood biochemistry parameters from carp captured at three locations produced three significant components. First two principal components (PCs), as shown in Figure 5a, explain 76.86 % of variation in our dataset. PC1 accounted for 43.48 % of variation and loaded mainly on stress variables (CHOL, TRIG, TP, ALB, GLU), PC2 accounted for 33.38 % of variation and loaded mainly on liver impairment parameters, such as ALP and ALT. Axes PC1 and PC3 (Figure 5b) explain 54.47 % of variation, where PC1 (42.26 % of variation) loaded mainly on stress variables, and PC3 which accounted for 12.21 % of variation, loaded mainly of renal impairment parameters, (CRE, URE, TP and ALT).

The mean haematocrit values were 33.60 ± 14.52, 42.00 ± 8.37, 47.14 ± 7.26 from unaffected, effluent-receiving and County canal, respectively. They all passed the Normality test, but showed no significant relationships between the groups (p>0.05).

Analyses of erythrocyte nuclear abnormalities revealed the highest frequency (8.96 ‰) in fish sampled from effluent-receiving canal (B-5), while the frequencies of erythrocyte nuclear anomalies in unaffected stream (A-1) and County canal downstream before the Drava river (C-9) were much lower (2.89 ‰ and 1.93 ‰, respectively). However, these differences were not significant (p<0.05). Micronucleated erythrocytes were recorded in four fish (1 from the site A-1, 1 from the site B-5 and 2 from the site C-9) with very low frequency (0.33 ‰ per fish) and not significant (p>0.05) differences.

3.5. MXR activity in earthworms

Measurement of accumulated R123 after 24h exposure of earthworms to the raw (2) and treated wastewater (4) and sludge (10) (as not diluted, diluted 100x, and diluted 10,000x) showed significantly higher accumulation of R123 in the whole earthworm tissue, relative to the control (Figure 6) indicating the presence of the MXR inhibitors in water samples. The greatest accumulation of R123 was observed with exposure to active sludge (10), to waters where the effluent-receiving canal enters the County canal (7), and where the sugar plant wastewaters inflow to the WWTP (3) (208 %, 166 % and 144 %, respectively; p<0.03). After 100x dilution of all the samples, the greatest accumulation of the fluorescent substrate was observed in animals exposed to the unaffected stream waters (1), County canal waters downstream (6), and sugar plant wastewaters inflowing to the WWTP (3) (270 %, 254 % and 224 %, respectively).

Exposure of earthworms to a higher dilution (10,000x) of WWTP active sludge (10), waters from the County canal (8) and from the canal entering the County canal (7), increased the accumulation of R123 287 %, 251 % and 226 %, respectively.

4. Discussion

This wide-scale survey comprised an array of studies on the general quality of the water, sludge and sediment associated with the WWTP, as well as their microbiological and pharmaceutical assessment, related to the impact on the organismic biosensors, particularly fish and earthworms, demonstrating specifically and non-specifically the effluent-induced changes in their biological systems. Such a combined approach was considered due to possible misinterpretations resulting from the isolated analyses of each aspect and therefore inherently implies a broader ecological and, ultimately, human health insight. Although most environmental studies take into account only a limited amount of biological responses, parameters which individually show no distinctive response to a stressor can, when used in conjunction with other parameters, be useful indicators of the extent of the environmental impact (Bernet et al., 2000). This impact can be demonstrated as either direct chemical or physical damage to cells or tissues, modification of physiological and biochemical reactions, increased infection pressure by facultative pathogens or reduced resistance to infection (Schmidt-Posthaus et al., 2001), thus their determination at the whole organism level can detect sublethal and chronic effects on organisms and present integrative responses to complex mixtures of pollutants. The results of this study corroborate the hypotheses, indicating that antibiotic effects, bacterial pathogens, and

the changes of biological responses related to the WWTP reach far downstream from the WWTP.

4.1. Water quality

The sugar plant wastewaters (3) mostly surpassed the raw municipal wastewaters inflowing the WWTP (2) in values of many parameters, such as suspended solids, COD, COD-Mn, BODn, nitrate, nitrite, whilst they matched in ammonium and total nitrogen concentrations. In order to avoid the release of contaminants from the sugar plant to the environment, zero effluent discharge, involving water reuse, recycling and regeneration, is this sector's greatest challenge. Its environmental impact can further be assessed by measuring the environmental performance index which measures the levels of COD above the regulated values, and the environmental performance index which calculates the specific increase of COD in liquid effluents (Ingaramo et al., 2009). In this work COD decreased below the regulated borderline values in the effluent (4) and downstream waters (5-9).

When comparing nitrite, total nitrogen and total phosphorus concentrations in the waters from the unaffected stream (1), inflow of raw municipal waters to the WWTP (2) and inflow of sugar plant wastewaters to the WWTP (3) with the work of El-Shafai et al. (2004), our values were significantly higher, mainly due to the very high total nitrogen in all sites. Increased nitrogen and phosphorus may result in overgrowth of algae, which can decrease the dissolved oxygen content of the water, thereby harming or killing fish and other aquatic species. Although presumably unaffected by industrial or agricultural activity, our reference stream (1), albeit not

connected to the effluent receiving system, must have been impacted by some sort of fertilizer use or animal wastes.

4.2. Microbiological counts, bacterial identification and discrimination; microbial resistance; health risks

Fish in the water bodies downstream from the WWTP effluent discharge are fished and used by the local population (personal communication). Public health risks are the main concerns in such practice, although using treated wastewater in fish farming is not uncommon (El-Shafai et al., 2004; Harnisz and Tucholski, 2010). The microbial quality of fish-bearing waters is reflected in the microbial flora of fish. If the total counts of heterotrophic aerobes exceed 5×10^4 CFU/mL of water, they pose a risk of human pathogens penetrating fish edible tissues (Mara and Cairncross, 1989). Total bacterial counts and intestinal enterococci measured in this work were significantly higher in water from the canal receiving the effluent (B-5), when compared to the two other fish sampling sites (A-1, C-9). The number of fecal coliform bacteria should not exceed 10^3 CFU/100 mL water in order to render fish safe for consumption (WHO, 2006). In none of the locations from which fish were retrieved fecal coliforms amounted to such numbers, although in the unaffected stream (1) they were the highest of the three locations (3.2×10^2 CFU/100 mL). Relatively low levels of fecal coliforms could nevertheless accumulate in the fish digestive system, posing a risk of transmission to consumers by cross-contamination of tissues, in case of inadequate hygiene standards in fish preparation (El-Shafai et al., 2004; Harnisz and Tucholski, 2010)

API 20E and MALDI-TOF MS identification results for isolates from fish captured at three locations and respective water largely corresponded, inasmuch that the majority of readings indicated to *Aeromonas* spp. It was previously found that environmental *Aeromonas* isolates have a wider phenotypic diversity than isolates obtained from clinical samples (Donohue et al., 2007). Although API 20E showed a limited sensitivity in distinguishing species, dividing all environmental aeromonads between *A. hydrophila* group 1 and group 2, and had drawbacks regarding slow phenotypic reactions, it was a valid first step in preliminary bacterial identification. MALDI-TOF MS spectra demonstrated many interspecies similarities, in which each strain of *Aeromonas* was defined by its unique mass distribution corresponding to a fingerprint spectrum characteristic or its *m/z* signature. MALDI-TOF MS proved to be a highly reproducible method for rapid discrimination of *Aeromonas* spp. and related species, as well as capable for differentiation of closely related aeromonads, where the spectra of different isolates of the same species clustered together. A cluster analysis of the *m/z* signatures (Figure 4) graphically depicts how the strains relate to each other, as the fingerprints with the highest degree of association are joined together first, followed by those with the next highest degree of association (Donohue et al., 2006; Eddabra et al., 2012). The groupings are unique to the MS and bacterial culture procedures.

Mesophilic aeromonads persist in most aquatic environments, including chlorinated drinking water, raw sewage, groundwater and both polluted and unpolluted streams and rivers (Huddleston et al., 2006). Bearing in mind that most of the retrieved bacteria from fish, water and sludge in this work were aeromonads, it is important to stress that aeromonads have been associated with bacterial zoonoses. Also, the retrieved *A. salmonicida* ssp. *salmonicida* is a fish pathogen (Austin and Austin, 1999). As contact-borne, *A. hydrophyila*, *A. sobria*, and *A. caviae*

may cause fish handler disease through skin wound infections and systemic infections, while as food-borne, *A. hydrophyila* may lead to gastroenteritis, diarrhoea and systemic infections (Austin and Austin, 1999; Goni-Urriza et al., 2000; Lehane and Rawlin, 2000; Huddleston et al., 2006; Cabello et al., 2013). With the popularity of recreational fishing in watersheds downstream from the investigated WWTP, medical personnel may expect more aeromonad-related infections, whereas their diagnosis and treatment may be difficult, particularly taking into consideration the emerging antibiotic resistance of fish pathogens. Environmental contamination with antimicrobials and other antibiotic substances is an important contributor to the survival and spread of antibiotic resistance genes (Huddleston et al., 2006), particularly through spread of resistance plasmids between unrelated bacteria (Kostich and Lazorchak, 2008).

Of all aeromonads isolated from fish tissues in this work, 50 % (18 isolates) were resistant to sulfamethoxazole, and all were resistant to amoxicillin. Amoxicillin is closely related to ampicillin, towards which aeromonads have a natural resistance (Harnisz and Tucholski, 2010), therefore this result was expected. One possible reason for such a high resistance to SMX is that it alone is not very active against *Aeromonas* spp., but in combination with trimethoprim it is generally efficient (Goni-Urriza et al., 2000). The incidence of resistance of environmental *Aeromonas* spp. samples to SMX in this work was significantly higher than in comparable studies (Imzlin et al., 1996; Kampfer et al., 1999; Huddleston et al., 2006), most likely because our isolates have previously been exposed to antibiotics and chemotherapeutics due to a hospital discharge waters going through the WWTP. Possibly for the same reason 11 % of water and sludge aeromonads (3 isolates) were resistant to oxytetracycline and erythromycin. Indeed, Harnisz et al. (2011) found that tetracycline-resistant bacteria are a reliable indicator of antimicrobial resistance and the microbial quality of surface waters polluted due to human

activity. Table 4. demonstrates the subdivision of antimicrobial susceptibility over the sampling sites for all isolates, but overall resistance of all bacterial isolates irrespective of the location was shown to be the highest towards amoxicillin, sulfamethoxazole, erythromycin, and oxytetracycline (87.53 %, 63.10 %, 30.61 %, 21.34 %, respectively). The presence of bacteria resistant to antimicrobials may be more substantial than detected in this and other studies, since most analyses represent the resistance of culturable bacteria, which constitute only a small proportion of the total bacteria present in the aquatic environment (Cabello et al., 2013). Also, antibiotic resistance is crucial in emerging infectious diseases, while surface water pathways may represent a key route of dissemination of antibiotic resistance genes (Pruden et al., 2012). We have thus confirmed the hypothesis that resistant bacteria and potential fish and human pathogens will be identified from water and sludge.

4.3.Pharmaceuticals in water and sediment - sources of exposure

As hypothesized, the inflow of high concentrations of SMX, also found in the sediment, must have contributed to significantly higher resistance of environmental aeromonads towards this drug. Its mean of 50.70 ng/L corresponds with the mean of 48.40 ng/L found in waters affected by urban and rural activities in China (Xu et al., 2014). The same authors report the SMX mean of 16.10 ng/g for corresponding sediment, while our sediment values (sites 4, 5, 9) decreased with the distance from the WWTP (19.60>5.07>0.99 ng/g). In an area polluted with antibiotics (Xue et al., 2013), SMX water levels were up to 191 ng/L, but not detected in the sediment. The same authors report the 43.5 ng/L erythromycin concentration in the water, which is twice of our inflow (2) values, and proved SMX and erythromycin to pose a relatively high ecological risk to

sensitive aquatic species. Frequently sulfonamides, especially SMX, have the highest detection frequency and concentration over other antimicrobials tested in wastewaters (Chen and Zhou, 2014) however sediments are more informative of long-term accumulation and storage of drugs, being also a source of exposure to benthic and other aquatic organisms. Gibs et al. (2013) describe a likely cause for the declining trend of erythromycin and other antibiotics occurrence from the point source as a dilution from groundwater discharge and sorption to bottom sediments. The observed drug concentrations can be attributed to sorption from the concentration in the water column and subsequent sediment transport (Gibs et al., 2013). In the sedimentary phase, the total concentration of antibiotics in this work decreased from 144.55 to 18.58 ng/g with the distance from the WWTP, which is comparable with the work of Chen and Zhou (2014) (101.0 to 12.4 ng/g). Although it is presumed that hospitals are the major contributors of pharmaceuticals to municipal sewage, the community use is 70 % in the UK and 75 % in the US, and almost 70 % of the total antibiotics used are excreted unchanged as active compounds (Kümmerer, 2009). Frequently their metabolites are more soluble in water than their lipophilic parent compounds due to their biotransformation. That might explain our higher water rates of sulfa drugs and SMX in the morning time-points when compared to the mid-day and evening time-points. Such substances, if not eliminated, reach the environment and adversely affect aquatic and terrestrial organisms, and consequently impact the human population.

4.4. Fish health status, structural and functional tissue alterations

Fish in this study had similar external gross signs and necropsy findings over sampling sites, however those from a remote location from the WWTP (C-9) suffered from a heavier parasitic

infestation and demonstrated a greater variety in bacterial load than fish captured in the effluent-receiving canal (B-5), which could be attributed to their migration over the water bodies in pursuit of the more favorable feeding locations, but also to the lower concentration of antibiotics and pharmaceuticals found in the sediment furthest from the WWTP.

Structural and functional alterations found in the carp gill tissues, particularly in the effluent-receiving canal (B-5), relate extensively to the secondary lamellae, indicating to environmental pollution, as presumed in the first hypothesis of this work. Compactness in the secondary lamellae and formation of inter-lamellar bridges reduced the total available respiration surface area of the gill, resulting in reduction of the diffusing capacity which in turn decreased the gas exchange over the respiratory area. It is thought to be an adaptive physiological response during the longer exposure period in order to prevent irritants from entering the bloodstream (Munshi and Singh, 1971), occurring under the influence of xenobiotics due to dissociation of epithelial cells followed by reassociation and reaggregation in the form of interlamellar bridges. Flores-Lopes and Thomaz (2011) also observed several types of gill tissue changes such as epithelial lifting, necrosis, fusion, hypertrophy, hyperplasia and branchial gland proliferation, induced by different levels of environmental degradation.

Carp in the present study, captured in the effluent-receiving canal (B-5), were exposed to high levels of chromium, nickel and zinc (Table 6). A reduction of the respiratory area in carp exposed to heavy metals was previously reported (Zikic et al., 1997; Schjolden et al., 2007; Srivastava and Punia, 2011; Mustafa et al., 2012). The histopathological changes observed in gill tissues could be related to xenobiotics in the effluent, microbial load on gills, impaired water quality and increased heavy metal concentrations (sections *4.1.-4.3.*), and may serve as an important end-point in the discrimination between different polluted sites (Omar et al., 2013).

4.5. Fish plasma metabolites and enzymes, oxidative stress, genotoxicity

The hypothesis that the WWTP effluent will induce biological effects on organismic biosensors is further discussed in this section and the following one. Although carp's high tolerance to heavy metals is explained by its ability to avoid becoming hypoxic and resistance to severe loss of plasma ions (Schjolden et al., 2007), the impact of heavy metals might have contributed not only to structural and functional alterations of the gill tissues, but also to the increase of some plasma metabolites. A significant increase in values of URE, TP, ALB and TRIG was noted in the fish from the effluent-receiving canal (B-5), as well as a significant decrease in SOD when compared with fish from the other two canals (A-1, C-9). SOD is one of the main antioxidative enzymes serving to detoxify reactive oxygen species. Decreased SOD activity modifies the cell redox status by the inhibition of the superoxide radical formation (Ural, 2013), and was related to the toxic impact of pollutants in the aquatic environment (Karadag et al., 2014), and particularly to the carp cadmium exposure (Zikic et al., 1997). Furthermore, the extremely low dissolved oxygen content (0.39 mg/L) in the effluent-receiving canal (4), contributed to stress-related biological responses in fish tissues.

The excess energy reserves (in form of plasma GLU, CHOL, TRIG) are necessary to mediate the effects of stress, (Topić Popović et al., 2012), which could explain the increase of TRIG in effluent fish, serving as an energy buffer. The increase of URE in effluent fish were likely a consequence of inhibition of branchial excretion of nitrogenous waste products, therefore leading to the accumulation of URE in blood (Bernet et al., 2000), and also to histopathological gill lesions. Although also possibly affected by pollution, elevated TP could be associated with

nutritional fish status, as the discharge of treated effluent may have elevated nutrients, increased the biomass of the invertebrate community (Bernet et al., 2000), and thus contributed to the increase of both TP and ALB. The PCA allowed discrimination of the most relevant plasma mediators determining physiological responses of fish under assay. In the both PC1 and PC2, PC1 and PC3 axes (Figure 5), the first component was biologically consistent with parameters related to stress, and a clear effect was found for ALB and TP as well as for CHOL and GLU from fish originating from the effluent-receiving canal (B-5), which tend to group together and appear to have similar characteristics. There was a clear separation between the effluent fish and the unaffected stream fish, where stress impacted the liver impairment. Similar findings relating to stress-based tissue impairment were documented by Li et al. (2011). Renal impairment affected by stress reached downstream from the effluent, in the County canal (C-9), as evident from the CRE and URE parameters. Hematological and biochemical parameters in fish can significantly change in response to a variety of stressors, and although the changes found in this study suggest water quality as one of the important factors affecting overall physiology of investigated fish, their alterations are nonspecific to a wide range of substances, and thus should always be evaluated in concordance with other indicators and biomarkers. To that effect, micronucleus and erythrocyte nuclear abnormalities assays are frequently used for evaluation of the water pollution (Klobučar et al., 2003; Talapatra and Banerjee, 2007; Pavlica et al., 2008; Omar et al., 2012). The formation of micronuclei is the consequence of chromosomal damage or dysfunction of mitotic apparatus and represents acentral chromosomal fragments or whole chromosomes that have been excluded from the main nucleus during cell division (Udroiu, 2006). Although the mechanism of the erythrocyte nuclear abnormalities formation has not been fully explained, they have been considered as genotoxic analogues of micronuclei. In the present

study, micronuclei were detected in a few fish with very low frequencies from at all three sampling sites. Levels of the erythrocyte nuclear abnormalities were higher (although not significant) in fish sampled from the effluent-receiving canal (B-5). Higher ENA frequencies corresponded to higher concentrations of heavy metals in canal receiving the effluent, especially after the biological treatment plant (reed beds) (6). Discrepancy between MN and ENA appearance was also described by other authors (Güner and Muranh, 2011; Monteiro et al., 2011) who showed significant induction of nuclear abnormalities, but not micronuclei in fish exposed to copper, cadmium and lead.

4.6. Earthworms as bioindicators, MXR inhibition

Earthworms *E. fetida* are efficient species for bio-monitoring programs targeted at prevention of degradation of ecosystem due to anthropogenic activities (Tondoh et al., 2007). They can survive in highly contaminated environments due to their cell efflux of xenobiotics mediated by transporter proteins and detoxification enzymes. Although used mainly as diagnostic indicators of wastewater-derived anthropogenic organic contaminants in terrestrial environments (Kinney et al., 2010), it was found that they accumulate toxicants to levels above those reported for wastewater (Markman et al., 2007). The MXR defense system and accumulation of model fluorescent substrates in the earthworm tissue can be decreased by specific model inhibitors (Bošnjak et al., 2014). The protective function of the MXR mechanism can be inhibited by many pollutants present in the environment (Kurelec, 1997; Markman et al., 2007). The environmental presence of chemosensitizers or inhibitors of the MXR defense system in different organisms (aquatic, terrestrial) could cause an increase in intracellular accumulation and toxic effects of

other xenobiotics normally effluxed by MXR transport proteins [P-glycoprotein (P-gps), MRPs]. For example, ecotoxicological significance of MXR inhibition was supported in *in vivo* studies that demonstrated an increase in the production of mutagenic metabolites by mussels and an increase in the number of sea urchin embryos with apoptotic cells after exposure to model MXR inhibitors. It has also been demonstrated that MXR inhibitors are present among both conventional and emerging man-made pollutants: some pesticides and synthetic musk fragrances show extremely high MXR inhibitory potential at environmentally relevant concentrations. (Smital et al., 2004). This bioassay revealed the presence of MXR inhibitors in all examined samples, particularly in the diluted samples. We have thus confirmed the concept that the measurement of the MXR inhibition can be utilized as a valuable specific biomarker of environmental contamination (Epel et al., 2008; Hackenberger et al., 2012), and suggest the use of *E. fetida* exposure as a tool for constant bio-monitoring.

5. Limitations

The study was conducted once in spring of 2014, with limited number of fish, sampling days, and samples per location. The relatively low fish sample size per sampling site is a consequence of reduced accessibility to the posts appropriate for fishing and fish avoidance of the bait. The lack of correction for multiple testing, due to the preliminary nature of the study, is another of its limitations. The tested hypotheses relate to the particular WWTP under assay.

6. Conclusions

Treated wastewater discharged from the WWTP modified the environmental parameters and xenobiotic concentrations of the receiving surface waters, and played an important role in the antibiotic resistance scheme. The investigated histological, hematological and plasma biochemical parameters of fish indicated to significant changes related to a complexity of environmental stressors, contributing to their overall physiological status assessment. We established that *E. fetida* can serve as a sentinel organism for environmental pollutants, as the sensitive earthworm cellular efflux mechanisms mediated by the ABC transporters can thus be utilized as a biomarker of environmental contamination, and we suggest its use in complex bio-monitoring studies. However, biomarkers of species vulnerability still need to be developed, as well as identifying the most vulnerable life stages and populations, to understand the interactions between global environmental changes, nutritional status, pathogens and toxic chemicals, and to develop integrated approaches to manage vulnerability of aquatic systems to toxicants (Couillard et al., 2008).

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Table 1. Number of samples per sampling location for a) Prussian carp (*Carassius gibelio*) for necropsy, microbiological, blood plasma, genotoxicity, histopathology assessment b) water for physico-chemical, heavy metal, and microbiological analyses, c) sludge for physico-chemical, heavy metal, and microbiological analyses, d) water for analytical chemistry, e) sediment for analytical chemistry. Samples of water d) were taken at three time-points (morning 8:30, mid-day 14:00, evening 20:30 hrs) as a one-grab sample, while all other samples were taken in the early morning as a one-grab sample.

Locations:	1/A-1 Reference stream	2 Inflow of raw municipal water to WWTP	3 Inflow of sugar plant waters to WWTP	4 Treated water leaving WWTP	5/B-5 Canal receiving the effluent	6 Canal after the biological treatment plant	7 Canal entering the County canal	8 County canal	9/C-9 County canal before the river	10 WWTP active sludge	11 Sludge from the depot
a) Prussian carp	8				8				8		
b) Water	1	1	1	1	1	1	1	1	1		
c) Sludge										1	1
d) Water		3		3							
e) Sediment				1	1				1		

Table 2. Total viable counts and concentration of bacteria across all sample collection sites. Sites 1-9 refer to water as in the Table 1, sampling sites 10 and 11 refer to WWTP active sludge, and sludge from the depot, respectively.

	1	2	3	4	5	6	7	8	9	10	11 (CFU/g)
Counts at 37 °C (CFU/mL)	9×10^2	5.9×10^5	3.1×10^6	3.7×10^3	1.6×10^4	4×10^4	2.9×10^2	4×10^2	1.5×10^3	6.9×10^5	5.6×10^8
Counts at 22 °C (CFU/mL)	2.1×10^3	1.6×10^6	1.4×10^7	9×10^3	1.1×10^5	3×10^5	3×10^3	3×10^3	3.3×10^3	1.2×10^6	6×10^8
Fecal coliforms (CFU/100 mL)	3.2×10^2	2×10^6	1.3×10^3	3×10^2	39	1×10^2	6	26	40	3×10^5	6.4×10^6
Intestinal enterococci (CFU/100 mL)	92	1.4×10^6	1.3×10^4	8×10^2	1.4×10^2	31	4×10^3	52	40	4.3×10^5	2×10^7
E. coli (presence/absence)	+	+	+	+	+	+	+	+	+	+	+
Salmonella spp. (presence/absence)	-	-	-	-	-	-	-	-	-	-	-
L. monocytogenes (presence/absence)	-	-	-	-	-	-	-	-	-	-	-
Ps. aeruginosa (CFU/100 mL)	0	2×10^3	0	0	0	0	0	0	0	0	0
Sulphite reducing clostridia (CFU/100 mL)	0	1×10^3	10	10	1×10^2	2×10^2	1	10	20	6×10^2	3×10^7
Yeasts (CFU/mL)	70	6.5×10^2	3×10^2	0	35	25	0	10	0	7.9×10^3	0
Moulds (CFU/mL)	30	1×10^2	1×10^3	80	3×10^2	4×10^2	1×10^2	70	1.4×10^2	4×10^3	8.7×10^5

Table 3. Percentage of API 20E and MALDI-TOF MS identification results for Gram-negative isolates from fish captured at three locations: A-1: unaffected stream, B-5: effluent-receiving canal, entering the County canal, C-9: County canal downstream before the Drava river (corresponding with water sampling sites 1, 5, 9, respectively). Bacteria were retrieved from gill, kidney, liver and spleen tissues. MALDI-TOF MS scores ranged from 1.434 to 2.279, where 42.8 % of scores corresponded to 2.000 to 2.299: *Secure genus identification with probable species identification**. API 20E results ranged from *Unacceptable* to *Excellent identification*, with only 8 % of profiles corresponding to *Excellent identification to the genus***.

Bacterial isolates	A-1	B-5	C-9
MALDI-TOF MS (%)			
A. bestiarum (8.68)	4.34	2.17	4.34
A. media (10.87)	2.17	-	8.70
A. salmonicida ssp. *salmonicida* (2.17)	-	2.17	-
A. popoffii (4.34)	-	-	4.34
A. encheleia (2.17)	-	-	2.17
A. sobria (17.39)	-	-	17.39
A. veronii (6.52)	-	-	6.52
Shewanella baltica (10.85)	2.17	4.34	4.34
Acinetobacter johnsonii (4.34)	2.17	-	2.17
Ac. tandoii (2.17)	-	-	2.17
Ac. pittii (4.34)	-	-	4.34
Ac. guillouiae (2.17)	-	-	2.17
Ac. gerneri (2.17)	-	-	2.17
Citrobacter gillenii (2.17)	-	-	2.17
Pseudomonas fragi (4.34)	4.34	-	-
Ps. brenneri (2.17)	-	2.17	-
Rheinheimera soli (10.87)	-	2.17	8.70
Janthinobacterium lividum (2.17)	-	-	2.17
API 20E (%)			
A. hydrophila group 1 (20.41)	4.08	2.04	14.29
A. hydrophila group 2 (51.02)	6.12	6.12	38.78
Ps. fluorescens/putida (4.08)	4.08	-	-
Ps. aeruginosa (2.04)	-	-	2.04

Ochrobactrum anthropi (16.33)	-	2.04	14.29
Citrobacter youngae (2.04)	-	-	2.04
Unacceptable profile (4.08)	-	2.04	2.04

* MALDI-TOF MS score: 2.300 to 3.000: Highly probable species identification; 2.000 to 2.299: Secure genus identification with probable species identification; 1.700 to 1.999: Probable identification to the genus level; <1.700: Not reliable identification.

**API 20E result: Excellent identification (% id ≥ 99.9 and T index ≥ 0.75); Very good identification (% id ≥ 99.0 and T index ≥ 0.50); Good identification (% id ≥ 99.9 and T index ≥ 0.25); Acceptable identification (% id ≥ 80.0 and T index ≥ 0). The identification is not reliable if the sum of the % id proposed is less than 80.0; The profile is Doubtful if a taxon having several tests against the identification is present among those proposed; The profile is Unacceptable if the number of choices proposed is 0, all the gross frequencies being less than the threshold value.

Table 4. Antimicrobial susceptibility of the isolated strains (retrieved from water, sludge, fish) from all locations is presented as percentages of susceptible (S), intermediate (I) or resistant (R) strains. Antimicrobials: oxytetracycline (OTC), sulfamethoxazole (SMX), amoxicillin (AMC), florfenicol (FFC), erythromycin (E), oxolinic acid (OA), norfloxacin (NOR), flumequine (UB).

	1/A-1	2	3	4	5/B-5	6	7	8	9/C-9	10	11
OTC											
S	0/28.6	25.0	0	75.0	50.0/60.0	100.0	75.0	50.0	25.0/57.1	0	25.0
I	0/71.4	50.0	75.0	25.0	50.0/40.0	0	25.0	25.0	75.0/19.0	0	75.0
R	100.0/0	25.0	25.0	0	0/0	0	0	25.0	0/23.8	100.0	0
SMX											
S	0/0	0	0	25.0	50.0/20.0	0	25.0	50.0	33.3/4.8	0	0
I	0/42.9	25.0	0	25.0	0/40.0	33.3	25.0	25.0	33.3/42.9	0	25.0
R	100.0/57.1	75.0	100.0	50.0	50.0/40.0	66.6	59.0	25.0	33.3/52.4	100.0	75.0
AMC											
S	0/14.3	0	0	0	0/40.00	0	0	0	33.3/9.5	0	0
I	0/14.3	0	0	0	0/0	0	25.0	0	0/23.8	0	0
R	100.0/71.4	100.0	100.0	100.0	100.0/60.0	100.0	75.0	100.0	66.6/66.7	100.0	100.0
FFC											
S	100.0/42.9	75.0	100.0	100.0	100.0/60.0	100.0	100.0	100.0	100.0/66.7	50.0	25.0
I	0/42.9	25.0	0	0	0/40.0	0	0	0	0/28.6	50.0	0
R	0/14.3	0	0	0	0/0	0	0	0	0/4.8	0	75.0
E											
S	0/14.3	25.0	0	25.0	0/60.0	33.3	50.0	25.0	66.6/33.3	0	0
I	0/71.4	75.0	0	75.0	100/40.0	66.6	25.0	75.0	33.3/52.4	0	25.0
R	100/14.3	0	100.0	0	0/0	0	25.0	0	0/14.3	100.0	75.0
OA											
S	50.0/28.6	50.0	25.0	100.0	100/60.0	100.0	75.0	75.0	100.0/66.7	50.0	25.0
I	50.0/71.4	0	75.0	0	0/40.0	0	0	25.0	0/33.3	25.0	75.0
R	0/0	50.0	0.0	0	0/0	0	25.0	0	0/0	25.0	0
NOR											

S	50.0/71.4	50.0	25.0	100.0	100.0/80.0	100.0	75.0	75.0	100.0/71.4	50.0	25.0
I	0/28.6	0	75.0	0	0/20.0	0	25.0	25.0	0/28.6	25.0	75.0
R	50.0/0	50.0	0	0	0/0	0	0	0	0/0	25.0	0
UB											
S	100.0/71.4	50.0	25.0	100.0	100.0/80.0	100.0	75.0	100.0	100.0/85.7	75.0	25.0
I	0/28.6	50.0	75.0	0	0/20.0	0	25.0	0	0/14.3	0	75.0
R	0/0	0	0	0	0/0	0	0	0	0/0	25.0	0

Table 5. Physico-chemical properties of water from water sampling sites: 1: unaffected stream, not related to any industrial nor agricultural waters; 2: inflow of raw municipal wastewaters to the WWTP; 3: inflow of sugar plant wastewaters to the WWTP; 4: treated wastewater leaving the WWTP; 5: canal receiving the effluent; 6: canal after the biological treatment plant (reed beds); 7: canal entering the County canal; 8: County canal; 9: County canal downstream before the Drava river

	1	2	3	4	5	6	7	8	9
Temperature (°C)	13.90	13.90	-	20.60	18.60	18.60	13.90	15.60	16.50
Dissolved oxygen (mg O_2/L)	9.66	5.93	-	0.39	2.26	2.72	4.86	1.91	9.29
Oxygen saturation (%)	94.90	58.40	-	4.40	24.6	29.60	44.60	19.50	95.9
pH	8.13	7.74	6.97	7.80	7.89	7.87	7.42	7.54	7.65
El. conductivity (µS/cm)	529.00	1532.00	4980.00	1369.00	1412.00	1295.00	699.00	879.00	810.00
Suspended solids (mg/L)	37.70	126.80	295.00	31.10	171.00	172.30	3.60	3.60	8.60
COD	43.16	369.91	7536.00	42.63	57.55	71.96	32.49	22.20	18.49

(mg O_2/L)									
COD-Mn (mg O_2/L)	15.63	69.66	1678.00	20.63	30.64	31.64	8.85	16.93	11.93
BODn (mg O_2/L)	19.85	310.00	6153.00	36.75	48.26	58.23	25.00	15.07	12.84
Ammonium (mg/L)	0.02	59.00	50.50	1.25	8.40	8.60	<0.010	2.40	0.05
Nitrite (mg/L)	0.011	0.075	1.95	0.16	0.40	0.35	0.006	0.053	0.036
Nitrate (mg/L)	0.30	0.00	4.50	5.40	1.30	1.10	<0.10	0.30	1.20
Total nitrogen (mg/L)	1.74	63.00	63.20	8.49	11.80	12.28	1.63	5.43	10.70
Phosphate (mg/L)	0.086	4.69	0.19	0.32	0.97	0.87	0.161	0.223	0.092
Total phosphorus (mg/L)	0.166	7.50	1.45	0.47	1.49	1.89	0.371	0.418	0.145

Table 6. Total concentrations of heavy metals from water and sludge. Sampling sites 1-9 refer to water as in the Table 1, sampling sites 10 and 11 refer to WWTP active sludge, and sludge from the depot, respectively.

Concentration in µg/L	1	2	3	4	5	6	7	8	9	10	11
Iron	8388.00	30.00	9197.00	50.00	703.00	419.00	207.00	138.00	128.00	810.00	-
Cadmium	0.063	0.10	0.241	0.056	0.80	1.618	0.034	0.034	0.05	1.39	0.046
Chromium	6.08	14.87	17.56	14.81	7.698	12.77	7.84	11.14	12.91	36.13	4.06
Mercury	<0.20	6.25	4.23	0.50	0.87	1.21	<0.20	0.83	0.45	2.42	0.25
Lead	1.86	4.27	3.17	1.41	5.58	5.83	2.98	1.95	2.14	15.93	4.00
Nickel	10.00	6.00	76.00	14.00	31.00	38.00	19.00	10.00	8.00	100.00	2.25
Zinc	23.00	128.00	46.00	44.00	90.00	101.00	37.00	29.00	27.00	466.00	32.25

Copper	56.00	90.00	43.00	46.00	270.00	274.00	80.00	81.00	61.00	336.00	18.22

Table 7. Concentration of pharmaceuticals in water (ng/L) and sediment (ng/g). Water was sampled at three time-points (morning 8:30, mid-day 14:00, evening 20:30 hrs) from two locations: at the inflow of raw municipal water to the WWTP (site 2) and at the outflow of treated water leaving the WWTP (site 4). Sediment was sampled at one time-point (morning) from three locations: treated water leaving WWTP (4), canal receiving the effluent (5), County canal before the river (9). In sediment, of all sulfa drugs, only sulfamethoxazole was detected, while erythromycin was not detected.

	Water					
	Inflow of raw waters to WWTP			Outflow of treated waters		
	Morning	Mid-day	Evening	Morning	Mid-day	Evening
Pharmaceuticals	112,333.45	107,688.88	118,557.90	21,085.91	35,383.55	27,830.73
Antibiotics	5,479.82	14,287.57	26,334.96	7,545.84	7,949.07	7651.23
Sulfa drugs	90.88	87.88	49.39	65.71	60.12	68.44
Sulfamethoxazole	57.74	67.81	26.55	51.68	51.02	53.14
Erythromycin	2.27	43.94	12.50	0	0	0

	Sediment		
	Treated water leaving WWTP	Canal receiving the effluent	County canal
Pharmaceuticals	158,789.49	33,446.11	28,210.90
Antibiotics	144.54	17.51	18.58
Sulfamethoxazole	19.60	5.07	0.99

Table 8. Descriptive statistics of metabolite concentrations and enzyme activities in plasma of Prussian carp (*Carassius gibelio*) captured at three locations: A: unaffected stream (A-1), B: effluent-receiving canal (B-5), C: County canal downstream (C-9).

Plasma parameters		Mean	Median	SD	SE	Rng. Diff.	25 %	75 %	Skewness
GLU (mmol/L)									
	A	27.93	24.90	19.92	11.50	39.50	13.50	43.13	0.67
	B	11.35	11.35	3.47	2.45	4.90	8.90	13.8	-
	C	11.49	9.20	8.34	2.23	30.10	6.10	18.40	0.84
URE (mmol/L)									
	A	0.87	0.70	0.47	0.27	0.90	0.55	1.23	1.39
	B	1.15	1.15	0.07	0.05	0.10	1.10	1.20	-
	C	0.64	0.50	0.33	0.09	1.10	0.40	0.80	1.74
CRE (μmol/L)									
	A	50.00	57.00	17.58	10.15	33.0	36.75	61.50	-1.51
	B	26.00	26.00	11.31	8.00	16.00	18.00	34.00	-
	C	31.00	30.00	8.49	2.27	34.00	27.00	32.00	1.36
TP (g/L)									
	A	31.67	31.00	9.02	5.21	18.00	25.00	38.50	0.33
	B	48.00	48.00	22.63	16.00	32.00	32.00	64.00	-
	C	40.21	40.50	11.78	3.15	40.00	29.00	47.00	0.89
ALB (g/L)									
	A	10.00	9.00	4.58	2.65	9.00	6.75	13.50	0.94
	B	15.50	15.50	9.19	6.50	32.00	32.00	64.00	-

	C	12.64	11.00	5.24	1.40	19.00	9.00	14.00	1.45
TRIG (mmol/L)									
	A	2.87	2.40	1.17	0.68	2.20	2.10	3.75	1.51
	B	4.80	4.80	0	0	0	4.80	4.80	-
	C	2.86	2.45	1.42	0.38	5.30	1.80	3.70	1.07
CHOL (mmol/L)									
	A	3.97	3.90	0.40	0.23	0.80	3.68	4.28	0.72
	B	5.50	5.50	1.70	1.20	2.40	4.30	6.70	-
	C	5.75	5.95	2.10	0.56	9.00	4.60	6.50	1.48
ALT (U/L)									
	A	69.33	32.00	78.01	45.04	142.00	20.75	127.25	1.66
	B	20.50	20.50	14.85	10.50	21.00	10.00	31.00	-
	C	23.21	8.50	34.45	9.21	115.00	7.00	16.00	2.22
SOD (U/L)									
	A	502.45	331.12	327.77	189.24	584.52	304.68	743.07	1.71
	B	392.47	392.47	77.29	54.65	109.30	337.82	447.12	-
	C	485.94	360.59	340.29	90.95	1096.88	206.78	723.54	1.00

Abbreviations: GLU glucose, URE urea, CRE creatinine, TP total proteins, ALB albumin, TRIG triglyceride, CHOL cholesterol, ALT alanine aminotransferase, SOD superoxide dismutase; SD standard deviation, SE standard error of the mean, Rng. Diff, range difference.

Figure 1. Sampling sites for raw water, treated water and sludge drawn in the ArcGIS 10.1 program (location: NE Croatia, city of Virovitica and surroundings) 1: unaffected stream, not related to any industrial nor agricultural waters, considered as a reference site; 2: inflow of raw municipal wastewaters to the WWTP; 3: inflow of sugar plant wastewaters to the WWTP; 4: treated wastewater leaving the WWTP; 5: canal receiving the effluent; 6: canal after the biological treatment plant (reed beds); 7: canal entering the County canal; 8: County canal; 9: County canal downstream before the Drava river; 10: WWTP active sludge; 11: sludge from the depot. Symbols: BU (Biological Unit), circle with a dot (smaller inhabited area).

Figure 2. Prussian carp gill tissue. (a): from the effluent-receiving canal, entering the County canal; lamellar fusion and bridging (double-arrow) of the inter-lamellar space, reduced interlamellar spaces; (b): from the County canal: dilation of blood sinuses and enlargement of marginal blood channels of secondary lamellae (arrow).

Figure 3. MALDI-TOF MS mass spectra of *Aeromonas* cells isolated from water and sludge, 18 isolates (a) and fish, 23 isolates (b). Main masses between 4,000 and 12,000 Da are indicated.

Figure 4. Dendrogram of MALDI-TOF MS patterns and relationships from identified *Aeromonas* strains retrieved from water and sludge (a) and fish (b). Branch lengths are proportional to the number of *m/z* differences between the strains. The principal components were determined based on clustering.

Figure 5. PCA correlation loading biplots for carp plasma parameters. Abbreviations: (A_metabolite: fish from the unaffected stream, B_metabolite: fish from the effluent-receiving canal, C_metabolite: fish from the County canal).

Figure 6. Accumulation of Rhodamine 123 dye in earthworms *E. fetida* after 24h exposure to three concentrations of water and sludge. Locations 1-10 as described in the Figure 1. Symbols: ' = 100x dilution of the sample, " = 10,000x dilution of the sample, C = control, PC = positive control 50 $\mu molL^{-1}$ MK571. Data are expressed as the mean of triplicate ± SD, *p<0.03, t-test, n=5.

Figure 1

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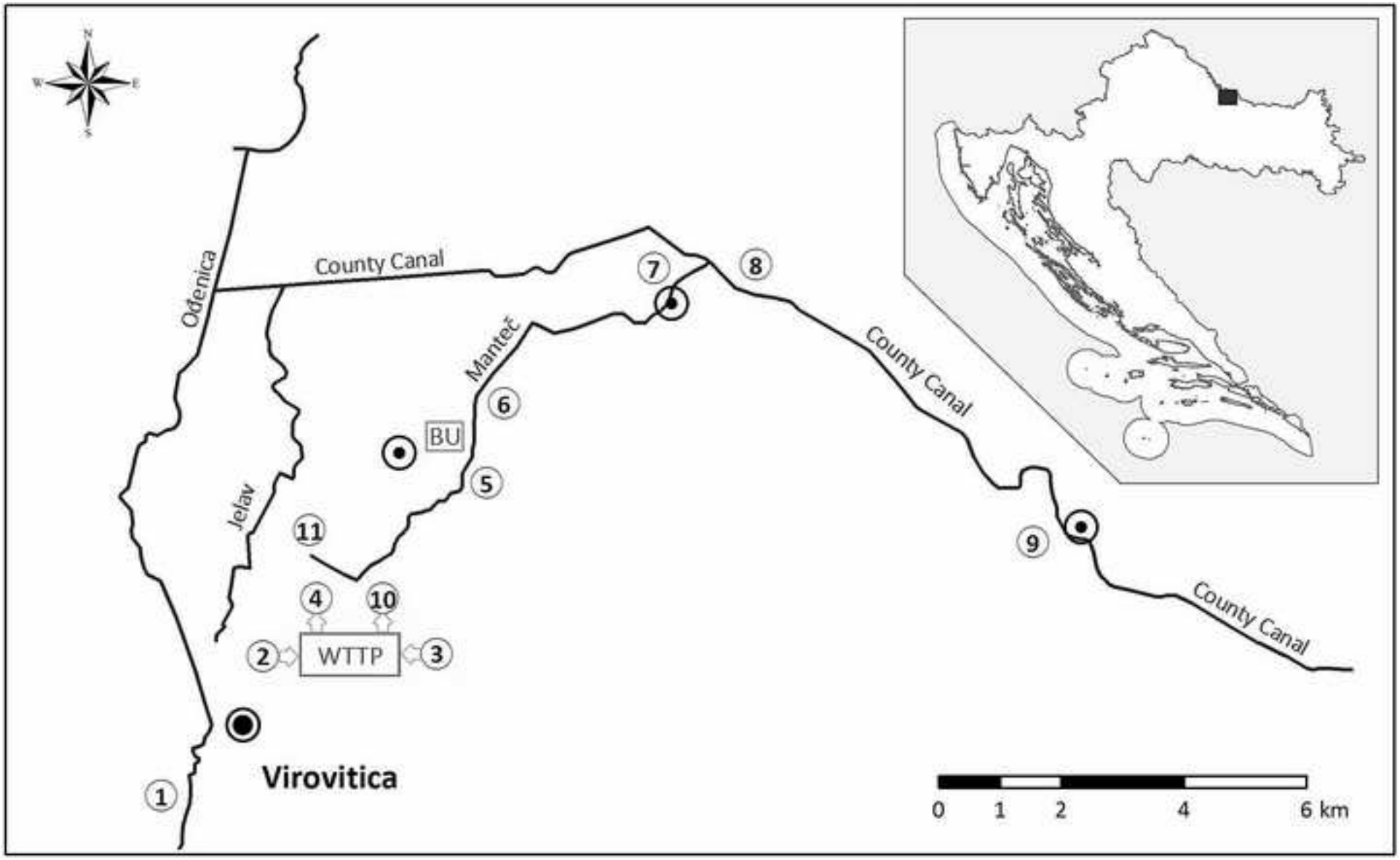

Figure 2

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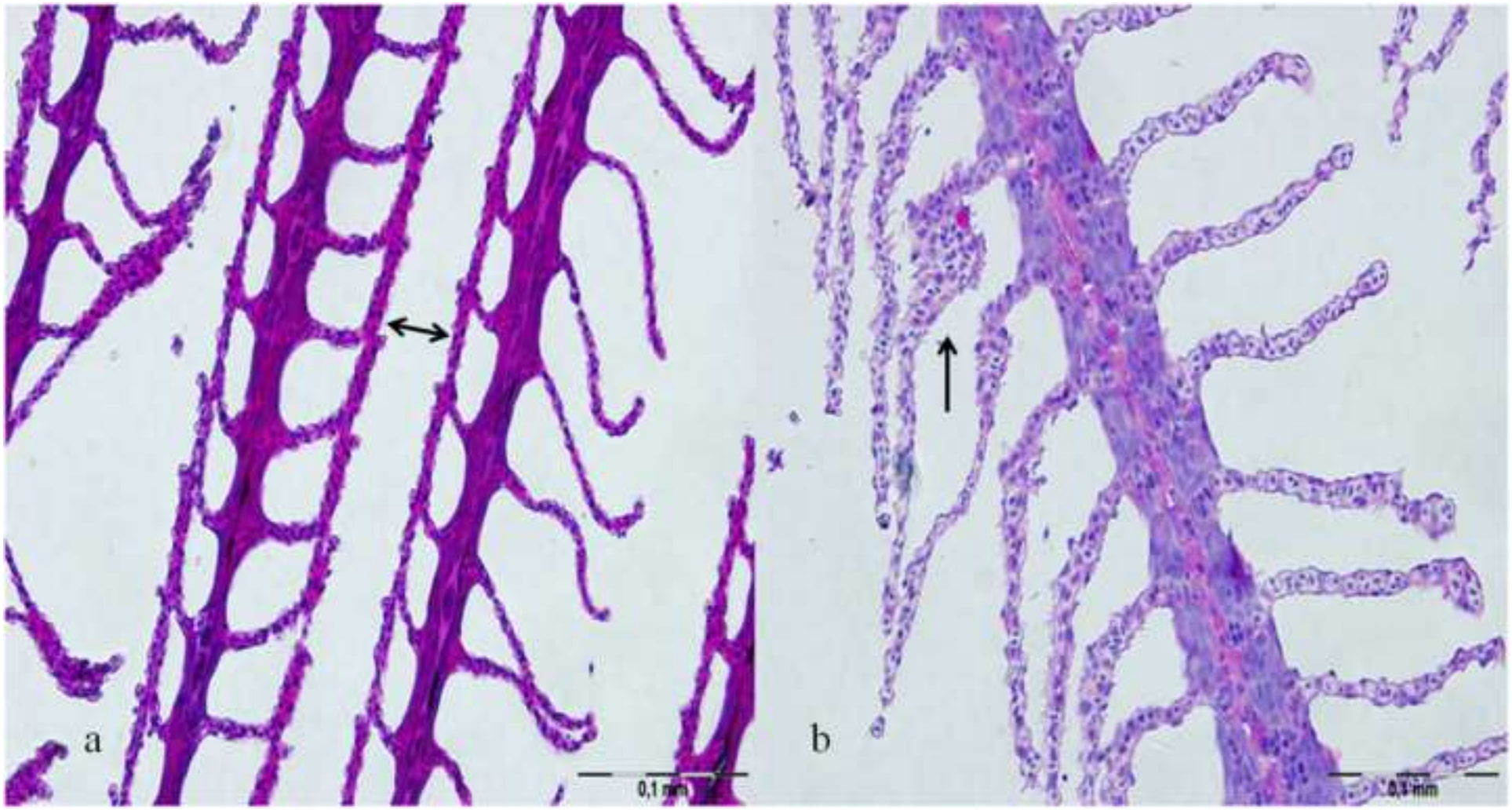

Figure 3

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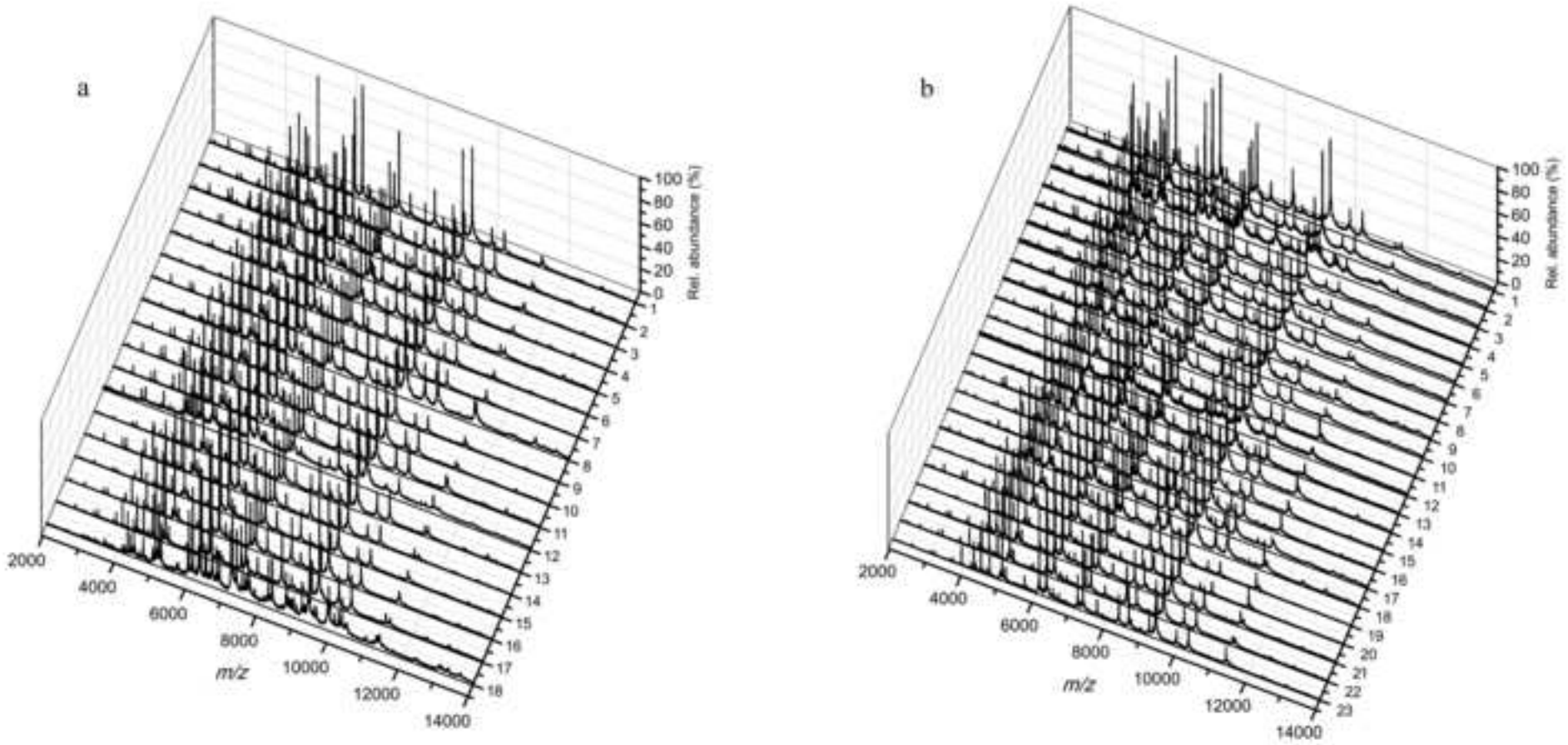

Figure 4

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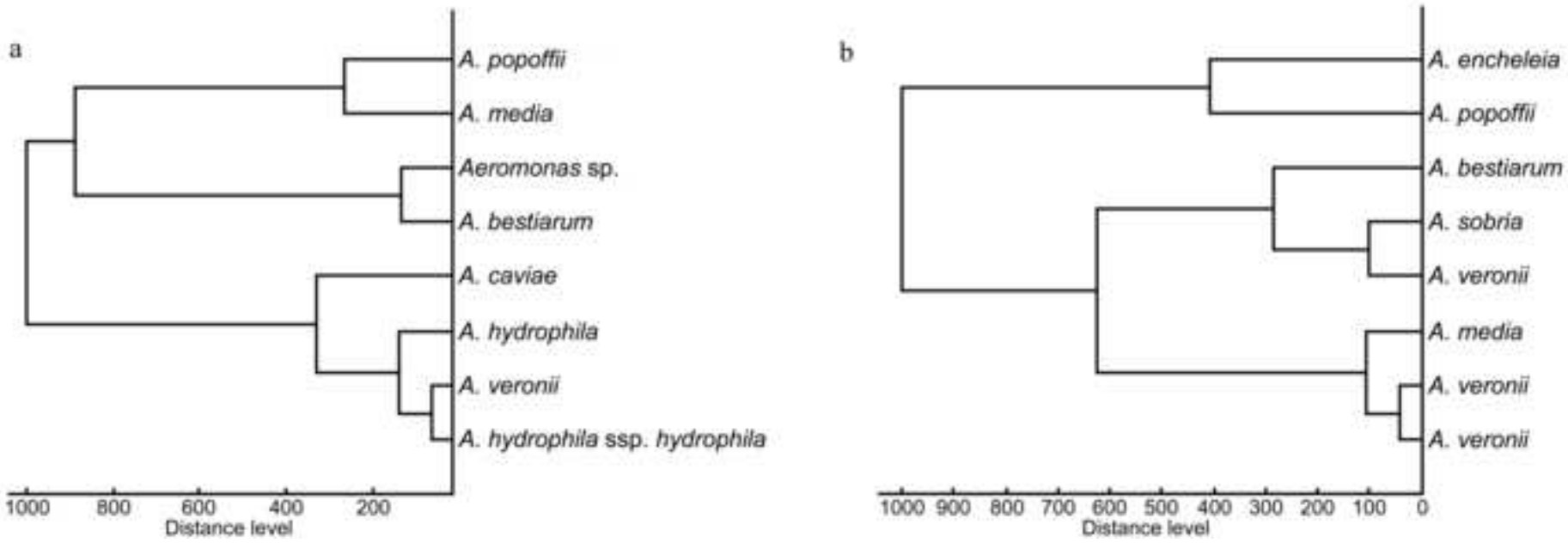

Figure 5

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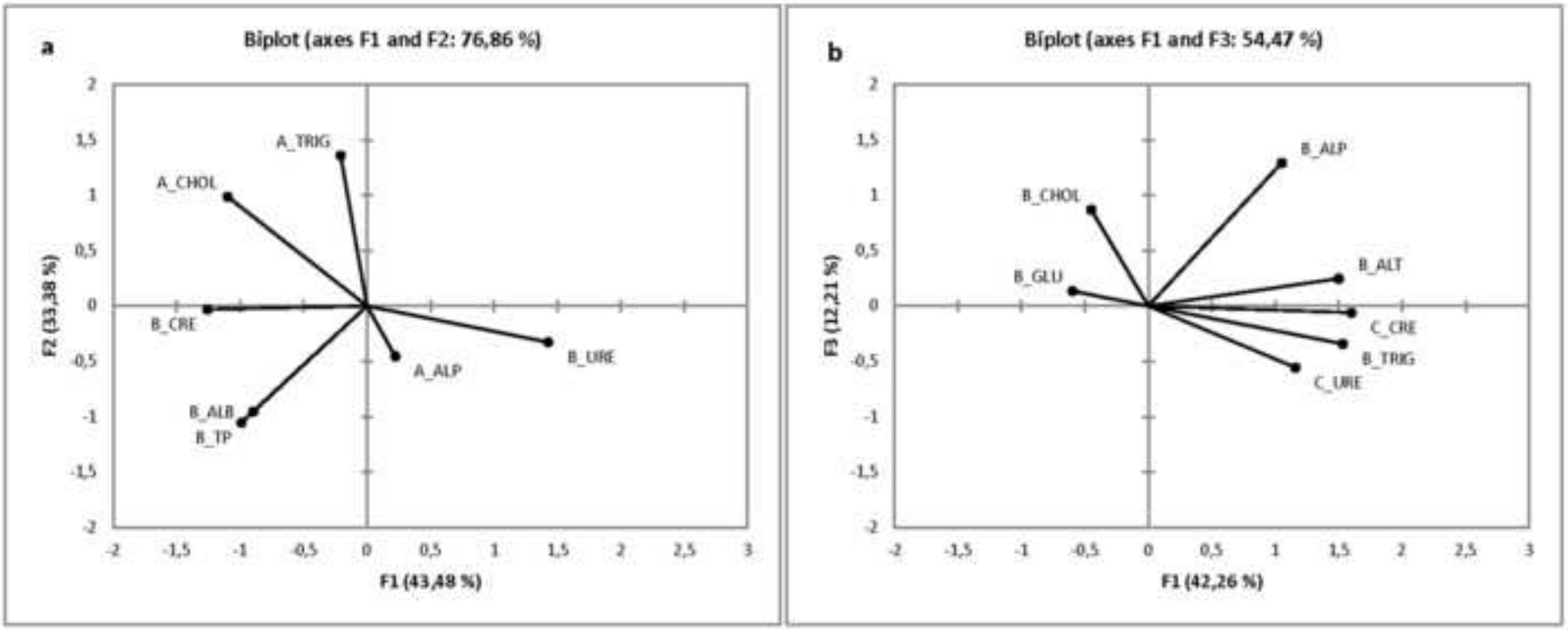

Figure 6

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