Oxidative stress ecology in brook trout (Salvelinus fontinalis) from a high-mountain lake (Cottian Alps)

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Title: Oxidative stress ecology in brook trout (Salvelinus fontinalis) from a high-mountain lake (Cottian Alps)

Article Type: Research Paper

Keywords: Alpine lakes, extreme ecosystems, oxidative stress biomarkers, trace elements

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Abstract: High-mountain lakes are pristine ecosystems characterized by extreme environmental conditions. The atmospheric transport of pollutants from lowlands may add further stress to organisms inhabiting these environments. We investigated the environmental stress pressure on brook trout (Salvelinus fontinalis) from a high-mountain lake in the Cottian Alps (Piedmont, northwest Italy). To do this, males and females of brook trout were sampled from Balma Lake in summer (August) and autumn (October) 2017 in order to assess the influence of trace elements accumulation and environmental parameters (physicochemical parameters and nutrient characteristics of water) on oxidative stress biomarkers. Bioaccumulation of Al, As, Cd, Cr, Cu, Fe, Hg, Pb, Ni, Se, and Zn and metallothionein levels were measured in muscle tissue of males and females. Liver, gills, kidney, and spleen tissue samples were analyzed for superoxide dismutase, catalase, total glutathione peroxidase, selenium-dependent glutathione peroxidase, glutathione reductase, and glutathione S-transferase activity. Analysis of environmental parameters showed changes in biomarker levels with seasonal variations. Water temperature was significantly higher in summer than autumn (Wilcoxon test; $p = 0.0078$), while pH was significantly higher in autumn than in summer (Wilcoxon test; $p = 0.0078$). Sex-related differences in oxidative stress biomarkers in tissues were unremarkable, whereas seasonal variability of oxidative stress biomarkers was observed, with major differences occurred for liver in summer and for gills, kidney, spleen and muscle in autumn. Positive correlations between environmental parameters and biomarkers were noted. Major fluctuations in water temperature, pH, Cu, Pb and Hg produced changes in biomarker levels; however, increased food intake during the ice-free season was probably the main factor that influenced changes in oxidative stress biomarker levels in brook trout in this extreme ecosystem.

Response to Reviewers: Response to Reviewer 5

Reviewer #5: In general, the authors improve the manuscript taking into account the comments/suggestions of the reviewers.
I recommend the publication of this study after some issues be addressed. The aim of the study is interesting and important/innovative data was obtained.
Authors' response: We are very pleased for your comments, thanks. We have studied your comments carefully and revised the manuscript accordingly. We hope our revision will meet with your approval.

Reviewer #5: In general, the English must be improved along the manuscript. Some parts of the manuscript are very confusing.
Authors' response: Thank you for your comment. The MS was revised by a professional English native speaker.

Reviewer #5: Specific comments: You refer along the manuscript that "…Liver, gills, kidney, spleen and muscle were analyzed for superoxide dismutase, catalase, glutathione peroxidases, glutathione reductase and S-transferase, and metallothionein for muscle..." You need to specify when you measure the levels and when you measure the activities.
Authors' response: Thank you for your comment. We measured the activity of SOD, CAT, GPx, Se-GPx, GR, GST and the levels of MTs. We pointed out it in the text.

Reviewer #5: An explanation about the evaluation of MT only in the muscle must also be included.
Authors' response: Thank you for your comment. In the present biomonitoring study, trace elements load and MTs were measured in muscle since the tissue represents the stable pool of trace elements for fish (Barwick and Maher, 2003). This last sentence was also added in the introduction.

Reviewer #5: Abstract. You need to be more specific and concise when you present your results.
Authors' response: Thank you for your comment. We revised the abstract accordingly.

Reviewer #5: Introduction. Improve the English: line 50-57. Improve the English along this section.
Authors' response: Thank you for your comment. Done.

Reviewer #5: Material and methods. In the Lines 175/176 substitute the complete names by the abbreviations: SOD, CAT…
Authors' response: Thank you for your comment. Done. Please, see lines 171-172.

Reviewer #5: Results. The presentation of the results is a little bit confusing. The authors must improve this section to be easier to understand the main results of the study.
Authors' response: Thank you for your comment. We revised the results section accordingly.

Reviewer #5: Line 247: Substitute "The mean concentration of trace elements in summer was…" by "The mean concentration of trace elements may be ranked as follow:.." The same for the Line 249.
Authors' response: Thank you for your comment. We rephrased the sentence.

Reviewer #5: The first time that you refer SeGPx is on the Results section. You need to refer this enzyme previously, at least on the Materials and Methods section.
Authors' response: Thank you for your comment. Done.

Reviewer #5: Sometimes along the Results section, it is difficult to understand if the results that you compare are significantly different or not. For example: Line: 260-262: "In spleen lower activity of SeGPx (70%) in females (Fig. 6), and GR and GST (up to 70%) in males (Fig. 7, 8) was measured in fish sampled 262 in autumn when compared to those of summer." It is significantly different? We need to see the figure to understand if it is or not. You need to clarify this along the text of the Results section when you compare the data.
Authors' response: Thank you for your comment. We revised this section accordingly.

Reviewer #5: I did not understand Line 290-291: "The separation between the organs from both seasons (summer on the right, 291 autumn on the left) suggested a difference in biomarkers concentration values." Can you clarify this?
Authors' response: Thank you for your comment. The biplot of loadings (variables) and score (observations) showed which organs (liver, gills, kidney, spleen and muscle) of brook trout is closest to them, and which variables (trace elements, biomarkers and physicochemical parameters) contributed to this grouping in the coordinate of Dim1 and Dim2. The separation between the organs from both seasons (summer on the right, autumn on the left) suggested a difference in biomarkers values.

Reviewer #5: Discussion. Improve the English along this section.
Authors' response: Thank you for your comment. Done.

Reviewer #5: Line 323: "The few studies on trace elements accumulation in biota from high-altitude lakes in general, and in Alpine lakes in particular, have mostly focused on fish." On Muscle of fish? Why you only measured on muscle?
Authors' response: Thank you for your comment. Yes, in fish muscle. We added this information in the text. We measured the trace element accumulation in muscle because it constitutes a stable pool of trace elements for fish (Barwick and Maher, 2003) (line 85).

Reviewer #5: Line 367/397: Did you measure the levels or the activity of CAT/SOD? I think that you measure activity but sometimes along the manuscript you refer levels.
Authors' response: Thank you for your comment. Yes, we measured the activity. We pointed out it throughout the text.

Reviewer #5: The Conclusion section does not show the main results of the study. The information of this section is too vague. You need to improve the section including the most relevant findings of the study, a take home message and future perspectives.
Authors' response: Thank you for your comment. Done. Please, see Conclusion section.

Reviewer #5: Figures. In Figure 3, is Superoxide dismutase concentration? It was activity, right? Put SOD on the caption of the figure to correspond to the complete name.
Authors' response: Thank you for your comment. Yes, activity. Done.

Reviewer #5: Figure 4: Put CAT on the caption of the figure to correspond to the complete name. The same for the figures 5-9.
Authors' response: Thank you for your comment. Done.

Response to Reviewer #6

Reviewer #6 1. Recommendation: major revision
2. Comments to Author:
Ms. Ref. No.: STOTEN-D-19-17682
Title: Oxidative stress ecology in brook trout (Salvelinus fontinalis) from a high-mountain lake (Cottian Alps). Overview and general recommendation:
Biomonitoring studies are constantly being developed field of research. More and more diagnostic tools are being proposed and monitoring of oxidative stress markers is part of this trend. The imbalance between production and elimination of Reactive Oxygen Species (ROS) leading to oxidative stress is valuable measure for organism metabolic and general health status. The methodology is accessible and well described, however, it has some limitations e.g. is species-specific; the results might be influenced by many factors. Including biochemical analysis into ecological research provides more comprehensive insight into the general problem aimed to be solved. The current study is quite well written. It describes several factors, which can affect the ecology of brook trout inhabiting extreme ecosystems, such as high-mountain lakes, and makes an attempt to verify this influence. In this case, the paper fits the journal scope. The authors put a big effort to carry out the studies in such remote site and conduct all the laboratory analysis, what should be highlighted and recognised. They distinguish between males and females, which is not common in oxidative stress research. The important support of their work is also quite detailed description of the study site as well as considering the diet composition. I find the research interesting and valuable, however, the authors did not avoid some major flaws. Although the introduction to the topic, study design and its performance generally meet the reviewer's expectations, the discussion of the obtained results is the weakest part of the manuscript. The authors indicate differences in measured parameters between tested tissues and sex, but they do not discuss it enough. There is almost no attempt to explain the cause of diverse results obtained for females compared to males, they are just mentioned. I am aware of the difficulty and effort this task requires; however, I think it would be more meaningful. Similar situation applies to the statement that food intake mainly affected the oxidative stress biomarkers. There is almost no discussion of the results as well as this issue and suddenly such conclusion appears. I encourage authors to rewrite the discussion part and consider the interpretation of obtained results. Therefore, I recommend the major revision of the paper.
Authors' response: We are very pleased for your comments, thanks. We have studied your comments carefully and revised the manuscript accordingly. Furthermore, the MS was revised by a professional English native speaker. We hope our revision will meet with your approval.

Reviewer #6: Detailed comments:
Title. As I mentioned in the general comments, if authors decide to regard oxidative stress ecology in their manuscript, the obtained results should be discussed in line with ecological explanation. If not, it would be better to change 'ecology' to 'parameters'.

Authors' response: Thank you for your comment. We decided to maintain the "ecology" in the tile, since we consider both biotic and abiotic parameters.

Reviewer #6: Keywords: brook trout and oxidative stress are words already mentioned in the title, thus I recommend exchanging them.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Highlights. Bullet points should capture the novel results of the research and/or new methods that were used. Now, the first three present what has been done in the study only (commonly used methods, nothing new or unique). The last one shows the results of the study, however, with the mistake (it should be food intake instead of feed intake).
Authors' response: Thank you for your comment. We revised the highlights following your suggestion.

Reviewer #6: Graphical abstract. Maybe it would be better to use another symbol for ice-free period than the leaf, especially when in the site description it is mentioned that the lake is located above the tree line. It is just a suggestion.
Authors' response: Thank you for your suggestion. We preferred to maintain the leaf since the emblem of the autumn.

Reviewer #6: Abstract. I recommend to change (finish after naming all the tested enzymes) the sentence starting in line 28 (Liver, gills, kidney, spleen and muscle were analyzed for superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and S-transferase.) and start the new sentence: Bioaccumulation of Al, As, Cd, Cr, Cu, Fe, Hg, Pb, Ni, Se and Zn and metallothionein level were investigated for both sexes in muscles. Without change, it can be confusing as oxidative stress parameters were measured in different tissues and metallothionein in muscle only. Combining measures of heavy metals and metallothionein together seems more appropriate as all were analyzed in muscle.
Authors' response: Thank you for your suggestion. Done.

Reviewer #6: Introduction.
Line 53: I suggest to change like to such as.
Line 62: I suggest to change like for e.g..
Line 66: Once in the organism,... Once what? Maybe Once introduced to the organism,...
Line 67: I suggest to change ensuing to leading.
Line 83: add activity after antioxidant enzymes.
Authors' response: Thank you for your suggestions. Done.

Reviewer #6: Line 86: what about other factors such as temperature?
Authors' response: Thank you for your comment. We added few sentences following your suggestion. Please, see line 96-98.

Reviewer #6: Materials and Methods.
Line 99: remove is.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Line 100: remove in which.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Line 112: captured instead of capture.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Line 117: The homogeneity of fish sizes are uncertain as the range for each term and sex is more than 10 cm (extremally it reaches 18 cm in autumn for females).
Authors' response: Thank you for your comment. We collected fish entangled by gillnets. Thus, it was not possible to select fish for this study.

Reviewer #6: Line 123: change at level order for at order level and remove a before closer.
Line 128: at instead of in 5 sites.
Line 129: remove in before other.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Line 151: It would be good to mention what tissues were analyzed.
Authors' response: Thank you for your comment. We analysed Liver, gills, kidney, spleen and muscle. Please, see line 175.

Reviewer #6: Line 153: what about temperature during the centrifuging homogenates?
Line 168: The assay was measured in ... buffer.
Line 174: Please add the time and temperature of centrifuging.
Authors' response: Thank you for your comment. Temperature of centrifugation was 4°C.
Homogenates were centrifuged at 50,000 x g for 30 minutes. It was already inserted in the original MS. The sentence was completed as follows: "Homogenates were centrifuged at 50,000 x g for 30 minutes at 4°C". For enzymatic analysis samples were homogenized with an UltraTurrax homogenizer in 100 mM potassium-phosphate buffer, pH 7.5, added with 2.5% sodium chloride (NaCl), 0.008 TIU ml-1 aprotinin and 0.1 mg ml-1 bacitracin. It was already inserted in the original MS.

Reviewer #6: Results. Line 207: Physico-chemical (with a dash to stay consequent).
Authors' response: Thank you for your comment. Done.

Reviewer #6: Line 209 and 228: p value should be consequently presented either $p > 0.05$ or with particular result.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Lines 212-216: This description is unnecessary as all these informations are already presented in the table and are not so relevant for the studies.
Authors' response: Thank you for your comment, but we think that the information are important for readers.

Reviewer #6: Lines 216-218: The diet analysis is poorly described considering the activities showed in the Materials and methods section. Especially, that the main conclusion is based on the food intake of the brook trout. This part of the results section need to be extended and presented with more details (e.g. table of food categories with, at least, frequency of occurence).
Authors' response: Thank you for your comment. Done. Please see section 3.2.

Reviewer #6: Line 226: Add '>' between Ni (0.06) and Hg (0.02).
Authors' response: Thank you for your comment. Done.

Reviewer #6: Lines 235-237: I suggest to rewrite the sentence: In spleen, lower activity of SeGPx (70%) in females (Fig. 6), and GR and GST (up to 70%) in males (Fig. 7, 8) was recorded in fish from autumn samples compared to summer one.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Line 238: add comma after In muscle and rewrite the beginning of the sentence: instead of a marked lowering was measured for SOD... I suggest to write In muscle, significant decrease in SOD activity (up to 50%) in autumn was measured for females.
Line 240: add comma after tissue.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Discussion.
Lines 290 and 295: repetition of studied the, this could be easily solved.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Line 297: exchange how to that.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Reviewer #6: Lines 297-300: I do not understand the purpose for presenting the results of this particular studies with details. Why this one only (authors mention other papers on trace elements accumulation in fish)?
Authors' response: Thank you for your comment. We presented the results of other studies following the suggestion of other reviewers.

Reviewer #6: Line 303: change these for the one presented by other ...
Authors' response: Thank you for your comment. Done.

Reviewer #6: Line 305: I do not understand why the authors mention pedogenic sources as a source of contamination, especially when the sentence in line 313 partially contradict this statement.
Authors' response: Thank you for your comment. Since our results are in line with other studies on trace elements accumulation in high-mountain lakes, and no studies have been performed previously in Balma Lake, we supposed that trace elements amount detected in brook trout from Balma Lake have an origin both from anthropogenic and pedogenic sources. This sentence was added following other reviewer's suggestions.

Reviewer #6: Lines 307-309: This statement better suits materials and method section.
Authors' response: Thank you for your comment, but we think that this statement is essential to introduce the discussion about metallothionines.

Reviewer #6: Line 321: put in accordance with other studies in commas.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Line 330: in fish from autumn samples or sampled in autumn.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Line 349: strenghten is not appropriate and should be changed for strongly. I also recommend to move strongly after how (PCA analysis showed how strong samples from liver in the summer were related to oxidative....).

Authors' response: Thank you for your comment. Done.

Reviewer #6: Line 356: remove for.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Lines 359-360: Mentioned species is just an example, I would not say it is well known for the broad audience. This sentence should be changed.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Line 375: remove a.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Lines 377-383: Why? What are the basis and reasons of such assumption? Authors present it as a main result of their studies without any analysis and discussion.
Authors' response: Thank you for your comment. The results and discussion about food intake were implemented.

Reviewer #6: Line 382: food instead of feed.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Conclusions.
Line 386: was instead of is.
Line 388: tissues instead of organs.
Line 392: food instead of feed.
Authors' response: Thank you for your comment. Done.

Reviewer #6: References. All the papers from the reference list were cited, however, lack of consequence in their format is striking. According to the journal requirements, the journal titles should be abbreviated with dots. In some references, this rule is obeyed, in most not (lines to be corrected: 404, 407, 409, 415,417, 423, 467, 474, 490, 497, 508, 511, 520, 531, 533, 537, 545, 548, 551, 553, 562, 564).
Authors' response: Thank you for your comment. Done.

Reviewer #6: Table 2. Add ±SD to Total Length - TL mean (cm).
Authors' response: Thank you for your comment. Done.

Reviewer #6: All the tables and figures were cited in the text.
Authors' response: Thanks.

Response to Reviewer #7

Reviewer #7: The manuscript titled "Oxidative stress ecology in brook trout (Salvelinus fontinalis) from a high-mountain lake (Cottian Alps)" (MS Number STOTEN-D-19-17682R1) by Pastorino P and colleagues investigate the environmental stressors pressure on brook trout (Salvelinus fontinalis) from Balma Lake, located in Piedmont Region (Cottian Alps, Northwest Italy). Main Physical-chemical parameters and nutrients were analyzed in water samples. Beside fish growth measurement, in fish muscle, liver, kidney, gills and spleen tissue, biomarkers of oxidative stress (superoxide dismutase, catalase, glutathione peroxidases, glutathione reductase and S-transferase, and metallothionein in fish muscle tissue) were analyzed. Furthermore, bioaccumulation level of trace elements (Al, As, Cd, Cr, Cu, Fe, Hg, Pb, Ni, Se and Zn) was analyzed in fish muscle tissue. Authors concluded that increased feed intake during the ice-free season mainly influenced the oxidative stress biomarkers.

In general, I recommend accepting the paper in STOTEN after minor corrections and additions.
Authors' response: We are very pleased for your comments, thanks. We have studied your comments carefully and revised the manuscript accordingly. We hope our revision will meet with your approval.

Reviewer #7: General suggestions:
1. Exchange the term "Water nutrients" with "nutrients". Authors measured nutrients in the water!
Authors' response: Thank you for your comment. Done.

Reviewer #7: 2. Instead of "Physical-chemical", I would suggest the use of "Physico-chemical"
Authors' response: Thank you for your comment. Done.

Reviewer #7: HIGHLIGHTS Replace the order of the second and third line.
Authors' response: Thank you for your comment. The highlights were rewritten following the other reviewers' suggestions.

Reviewer #7: ABSTRACT. Line 23: additional stress instead of "additive stress"
Authors' response: Thank you for your comment. Done.

Reviewer #7: INTRODUCTION. Appropriate and acceptable, only a few small corrections:
Line 101 -102: see general suggestions, especially the term "nutrients characteristics of water" should be corrected
Authors' response: Thank you for your comment. Done.

Reviewer #7: Lines 102 and 104: in both lines you have "c)". In line 104 should be "d)"
Authors' response: Thank you for your comment. Done.

Reviewer #7: MATERIALS AND METHODS. Appropriate and acceptable, only a few small corrections and one more explanation:
LINES 114-115. Correct the sentence (English is not correct; so the sentence is not clear enough)
Authors' response: Thank you for your comment. Done.

Reviewer #7: Line 126: "in 3 deep zone ones"??? What is this? Is it "at three depths"?
Authors' response: Thank you for your comment. We rephrased it.

Reviewer #7: Lines 134-139: three methods are cited, but measurement was made "by an adoption" of these three methods. If something is changed in methodology - it should be described. So, these adoptions of the methods should be explained! Otherwise, these methods could not be performed by others.
Authors' response: Thank you for your comment. We have taken this methodology from the instruction manual of spectrophotometer (Hanna Instrument). We didn't know other information.

Reviewer #7: Line 141: Delete "campaigns", "fish sampling" is just enough
Authors' response: Thank you for your comment. Done.

Reviewer #7: Lines 142-144: The sentence "The choice of the two sesons was prescribed by the necessity to reach on foot the site during the ice free period." (Write the sentence in better English!)
Authors' response: Thank you for your comment. Done.

Reviewer #7: Line 145: captured (add "d")
Authors' response: Thank you for your comment. Done.

Reviewer #7: Line 174: instead of "in single" use "individually"
Authors' response: Thank you for your comment. Done.

Reviewer #7: Line 177: use "enzymatic analysis" instead of "enzymes analysis"
Authors' response: Thank you for your comment. Done.

Reviewer #7: RESULTS. The results are presented in a clear manner. The figures used to show them are adequate. However, I would recommend:
Line 225: exchange "Physical-chemical and nutrients characteristics of water" with "Physico-chemical parameter and nutrient levels in water"
Authors' response: Thank you for your comment. Done.

Reviewer #7: Lines 259- 260: add "same" in front of seasons
Authors' response: Thank you for your comment. Done.

Reviewer #7: Line 270: delete water in front of nutrients
Authors' response: Thank you for your comment. Done.

Reviewer #7: DISCUSSION Line 329: better use trace elements instead of heavy metals, because Se is not Heavy metal (listed in line 331)
Authors' response: Thank you for your comment. Done.

Reviewer #7: Lines 359-361: Comment: Alkalinity is not same as pH, so this statement ("explanation of this outcome") is not proper!
Authors' response: Thank you for your comment. We corrected it.

Reviewer #7: 411: same comment as for the line 270
Authors' response: Thank you for your comment. Done.

Reviewer #7: CONCLUSIONS. Clear. Hope my comments will help authors to improve their manuscript!!!
Authors' response: Thank you for your suggestions.

Ref: STOTEN-D-19-17682R1

To the Editor-in-Chief

Damià Barceló

Science of the Total Environment

Dear Editor-in-Chief,

on behalf of my co-authors, I would like to thank the reviewers for the positive and constructive suggestions and for the opportunity to revise our manuscript entitled "Oxidative stress ecology in brook trout (*Salvelinus fontinalis*) from a high-mountain lake (Cottian Alps)" for publication in *Science of the Total Environment*. We have studied the comments carefully and revised the manuscript accordingly, which we hope will meet with your final approval. In the section "Responses to Reviewers Comments", we have provided detailed responses to the reviewers' comments and illustrated the corrections made in the paper. All new changes are underlined in red in the manuscript. Furthermore, the manuscript was carefully read and revised by an English native speaker.

We are confident that the manuscript in the present form is ready for publication.

We remain at complete disposal for any further information you might require.

Kind regards

Paolo Pastorino

Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta
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*Title Page

Oxidative stress ecology in brook trout (*Salvelinus fontinalis*) from a high-mountain lake (Cottian Alps)

Paolo Pastorino[1,2*], Antonia Concetta Elia[3**], Barbara Caldaroni[3], Vasco Menconi[2], Maria Cesarina Abete[2], Paola Brizio[2], Marco Bertoli[1], Annalisa Zaccaroni[4], Magara Gabriele[3], Ambrosius Josef Martin Dörr[3], Elisabetta Pizzul[1], Marino Prearo[1]

[1] Department of Life Sciences, University of Trieste, via Giorgieri 10, 34127 Trieste, Italy

[2] The Veterinary Medical Research Institute for Piemonte, Liguria and Valle d'Aosta, via Bologna 148, 10154 Torino, Italy

[3] Department of Chemistry Biology and Biotechnology, University of Perugia, via Elce di Sotto 8, 06123 Perugia, Italy

[4] Department of Veterinary Medical Science, University of Bologna, viale Vespucci 2, 47042 Cesenatico (FC), Italy

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** Corresponding author: Antonia Concetta Elia, e-mail address: antonia.elia@unipg.it (A.C. Elia)

Response to Reviewer 5

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I recommend the publication of this study after some issues be addressed. The aim of the study is interesting and important/innovative data was obtained.
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Authors' response: Thank you for your comment. We measured the activity of SOD, CAT, GPx, Se-GPx, GR, GST and the levels of MTs. We pointed out it in the text.

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Authors' response: Thank you for your comment. We revised the abstract accordingly.

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Authors' response: Thank you for your comment. Done.

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Authors' response: Thank you for your comment. We revised this section accordingly.

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Reviewer #5: The Conclusion section does not show the main results of the study. The information of this section is too vague. You need to improve the section including the most relevant findings of the study, a take home message and future perspectives.
Authors' response: Thank you for your comment. Done. Please, see Conclusion section.

Reviewer #5: Figures. In Figure 3, is Superoxide dismutase concentration? It was activity, right? Put SOD on the caption of the figure to correspond to the complete name.
Authors' response: Thank you for your comment. Yes, activity. Done.

Reviewer #5: Figure 4: Put CAT on the caption of the figure to correspond to the complete name. The same for the figures 5-9.
Authors' response: Thank you for your comment. Done.

Response to Reviewer #6

Reviewer #6 1. Recommendation: major revision

2. Comments to Author:
Ms. Ref. No.: STOTEN-D-19-17682
Title: Oxidative stress ecology in brook trout (Salvelinus fontinalis) from a high-mountain lake (Cottian Alps). Overview and general recommendation:
Biomonitoring studies are constantly being developed field of research. More and more diagnostic tools are being proposed and monitoring of oxidative stress markers is part of this trend. The imbalance between production and elimination of Reactive Oxygen Species (ROS) leading to oxidative stress is valuable measure for organism metabolic and general health status. The methodology is accessible and well described, however, it has some limitations e.g. is species-specific; the results might be influenced by many factors. Including biochemical analysis into ecological research provides more comprehensive insight into the general problem aimed to be solved. The current study is quite well written. It describes several factors, which can affect the ecology of brook trout inhabiting extreme ecosystems, such as high-mountain lakes, and makes an attempt to verify this influence. In this case, the paper fits the journal scope. The authors put a big effort to carry out the studies in such remote site and conduct all the laboratory analysis, what should be highlighted and recognised. They distinguish between males and females, which is not common in oxidative stress research. The important support of their work is also quite detailed description of the study site as well as considering the diet composition. I find the research interesting and valuable, however, the authors did not avoid some major flaws. Although the introduction to the topic, study design and its performance generally meet the reviewer's expectations, the discussion of the obtained results is the weakest part of the manuscript. The authors indicate differences in measured parameters between tested tissues and sex, but they do not discuss it enough. There is almost no attempt to explain the cause of diverse results obtained for females compared to males, they are just mentioned. I am aware of the difficulty and effort this task requires; however, I think it would be more meaningful. Similar situation applies to the statement that food intake mainly affected the oxidative stress biomarkers. There is almost no discussion of the results as well as this issue and suddenly such conclusion appears. I encourage authors to rewrite the discussion part and consider the interpretation of obtained results. Therefore, I recommend the major revision of the paper.
Authors' response: We are very pleased for your comments, thanks. We have studied your comments carefully and revised the manuscript accordingly. Furthermore, the MS was revised by a professional English native speaker. We hope our revision will meet with your approval.

Reviewer #6: Detailed comments:
Title. As I mentioned in the general comments, if authors decide to regard oxidative stress ecology in their manuscript, the obtained results should be discussed in line with ecological explanation. If not, it would be better to change 'ecology' to 'parameters'.
Authors' response: Thank you for your comment. We decided to maintain the "ecology" in the tile, since we consider both biotic and abiotic parameters.

Reviewer #6: Keywords: brook trout and oxidative stress are words already mentioned in the title, thus I recommend exchanging them.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Highlights. Bullet points should capture the novel results of the research and/or new methods that were used. Now, the first three present what has been done in the study only (commonly used methods, nothing new or unique). The last one shows the results of the study, however, with the mistake (it should be food intake instead of feed intake).
Authors' response: Thank you for your comment. We revised the highlights following your suggestion.

Reviewer #6: Graphical abstract. Maybe it would be better to use another symbol for ice-free period than the leaf, especially when in the site description it is mentioned that the lake is located above the tree line. It is just a suggestion.
Authors' response: Thank you for your suggestion. We preferred to maintain the leaf since the emblem of the autumn.

Reviewer #6: Abstract. I recommend to change (finish after naming all the tested enzymes) the sentence starting in line 28 (Liver, gills, kidney, spleen and muscle were analyzed for superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and S-transferase.) and start the new sentence: Bioaccumulation of Al, As, Cd, Cr, Cu, Fe, Hg, Pb, Ni, Se and Zn and metallothionein level were investigated for both sexes in muscles. Without change, it can be confusing as oxidative stress parameters were measured in different tissues and metallothionein in muscle only. Combining measures of heavy metals and metallothionein together seems more appropriate as all were analyzed in muscle.
Authors' response: Thank you for your suggestion. Done.

Reviewer #6: Introduction.
Line 53: I suggest to change like to such as.
Line 62: I suggest to change like for e.g..
Line 66: Once in the organism,... Once what? Maybe Once introduced to the organism,...
Line 67: I suggest to change ensuing to leading.
Line 83: add activity after antioxidant enzymes.
Authors' response: Thank you for your suggestions. Done.

Reviewer #6: Line 86: what about other factors such as temperature?
Authors' response: Thank you for your comment. We added few sentences following your suggestion. Please, see line 96-98.

Reviewer #6: Materials and Methods.
Line 99: remove is.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Line 100: remove in which.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Line 112: captured instead of capture.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Line 117: The homogeneity of fish sizes are uncertain as the range for each term and sex is more than 10 cm (extremally it reaches 18 cm in autumn for females).
Authors' response: Thank you for your comment. We collected fish entangled by gillnets. Thus, it was not possible to select fish for this study.

Reviewer #6: Line 123: change at level order for at order level and remove a before closer.
Line 128: at instead of in 5 sites.
Line 129: remove in before other.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Line 151: It would be good to mention what tissues were analyzed.
Authors' response: Thank you for your comment. We analysed Liver, gills, kidney, spleen and muscle. Please, see line 175.

Reviewer #6: Line 153: what about temperature during the centrifuging homogenates?
Line 168: The assay was measured in ... buffer.
Line 174: Please add the time and temperature of centrifuging.
Authors' response: Thank you for your comment. Temperature of centrifugation was 4°C. Homogenates were centrifuged at 50,000 x g for 30 minutes. It was already inserted in the original MS. The sentence was completed as follows: "Homogenates were centrifuged at 50,000 x g for 30 minutes at 4°C". For enzymatic analysis samples were homogenized with an UltraTurrax homogenizer in 100 mM potassium-phosphate buffer, pH 7.5, added with 2.5% sodium chloride (NaCl), 0.008 TIU ml^{-1} aprotinin and 0.1 mg ml^{-1} bacitracin. It was already inserted in the original MS.

Reviewer #6: Results. Line 207: Physico-chemical (with a dash to stay consequent).
Authors' response: Thank you for your comment. Done.

Reviewer #6: Line 209 and 228: p value should be consequently presented either $p > 0.05$ or with particular result.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Lines 212-216: This description is unnecessary as all these informations are already presented in the table and are not so relevant for the studies.
Authors' response: Thank you for your comment, but we think that the information are important for readers.

Reviewer #6: Lines 216-218: The diet analysis is poorly described considering the activities showed in the Materials and methods section. Especially, that the main conclusion is based on the food intake of the brook trout. This part of the results section need to be extended and presented with more details (e.g. table of food categories with, at least, frequency of occurence).
Authors' response: Thank you for your comment. Done. Please see section 3.2.

Reviewer #6: Line 226: Add '>' between Ni (0.06) and Hg (0.02).
Authors' response: Thank you for your comment. Done.

Reviewer #6: Lines 235-237: I suggest to rewrite the sentence: In spleen, lower activity of SeGPx (70%) in females (Fig. 6), and GR and GST (up to 70%) in males (Fig. 7, 8) was recorded in fish from autumn samples compared to summer one.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Line 238: add comma after In muscle and rewrite the beginning of the sentence: instead of a marked lowering was measured for SOD... I suggest to write In muscle, significant decrease in SOD activity (up to 50%) in autumn was measured for females.
Line 240: add comma after tissue.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Discussion.
Lines 290 and 295: repetition of studied the, this could be easily solved.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Line 297: exchange how to that.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Reviewer #6: Lines 297-300: I do not understand the purpose for presenting the results of this particular studies with details. Why this one only (authors mention other papers on trace elements accumulation in fish)?
Authors' response: Thank you for your comment. We presented the results of other studies following the suggestion of other reviewers.

Reviewer #6: Line 303: change these for the one presented by other ...
Authors' response: Thank you for your comment. Done.

Reviewer #6: Line 305: I do not understand why the authors mention pedogenic sources as a source of contamination, especially when the sentence in line 313 partially contradict this statement.
Authors' response: Thank you for your comment. Since our results are in line with other studies on trace elements accumulation in high-mountain lakes, and no studies have been performed previously in Balma Lake, we supposed that trace elements amount detected in brook trout from Balma Lake have an origin both from anthropogenic and pedogenic sources. This sentence was added following other reviewer's suggestions.

Reviewer #6: Lines 307-309: This statement better suits materials and method section.
Authors' response: Thank you for your comment, but we think that this statement is essential to introduce the discussion about metallothionines.

Reviewer #6: Line 321: put in accordance with other studies in commas.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Line 330: in fish from autumn samples or sampled in autumn.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Line 349: strenghten is not appropriate and should be changed for strongly. I also recommend to move strongly after how (PCA analysis showed how strong samples from liver in the summer were related to oxidative....).
Authors' response: Thank you for your comment. Done.

Reviewer #6: Line 356: remove for.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Lines 359-360: Mentioned species is just an example, I would not say it is well known for the broad audience. This sentence should be changed.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Line 375: remove a.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Lines 377-383: Why? What are the basis and reasons of such assumption? Authors present it as a main result of their studies without any analysis and discussion.
Authors' response: Thank you for your comment. The results and discussion about food intake were implemented.

Reviewer #6: Line 382: food instead of feed.
Authors' response: Thank you for your comment. Done.

Reviewer #6: Conclusions.

Line 386: was instead of is.
Line 388: tissues instead of organs.
Line 392: food instead of feed.
Authors' response: Thank you for your comment. Done.

Reviewer #6: References. All the papers from the reference list were cited, however, lack of consequence in their format is striking. According to the journal requirements, the journal titles should be abbreviated with dots. In some references, this rule is obeyed, in most not (lines to be corrected: 404, 407, 409, 415,417, 423, 467, 474, 490, 497, 508, 511, 520, 531, 533, 537, 545, 548, 551, 553, 562, 564).
Authors' response: Thank you for your comment. Done.

Reviewer #6: Table 2. Add ±SD to Total Length - TL mean (cm).
Authors' response: Thank you for your comment. Done.

Reviewer #6: All the tables and figures were cited in the text.
Authors' response: Thanks.

Response to Reviewer #7

Reviewer #7: The manuscript titled "Oxidative stress ecology in brook trout (Salvelinus fontinalis) from a high-mountain lake (Cottian Alps)" (MS Number STOTEN-D-19-17682R1) by Pastorino P and colleagues investigate the environmental stressors pressure on brook trout (Salvelinus fontinalis) from Balma Lake, located in Piedmont Region (Cottian Alps, Northwest Italy). Main Physical-chemical parameters and nutrients were analyzed in water samples. Beside fish growth measurement, in fish muscle, liver, kidney, gills and spleen tissue, biomarkers of oxidative stress (superoxide dismutase, catalase, glutathione peroxidases, glutathione reductase and S-transferase, and metallothionein in fish muscle tissue) were analyzed. Furthermore, bioaccumulation level of trace elements (Al, As, Cd, Cr, Cu, Fe, Hg, Pb, Ni, Se and Zn) was analyzed in fish muscle tissue. Authors concluded that increased feed intake during the ice-free season mainly influenced the oxidative stress biomarkers. In general, I recommend accepting the paper in STOTEN after minor corrections and additions.
Authors' response: We are very pleased for your comments, thanks. We have studied your comments carefully and revised the manuscript accordingly. We hope our revision will meet with your approval.

Reviewer #7: General suggestions:
1. Exchange the term "Water nutrients" with "nutrients". Authors measured nutrients in the water!
Authors' response: Thank you for your comment. Done.

Reviewer #7: 2. Instead of "Physical-chemical", I would suggest the use of "Physico-chemical"
Authors' response: Thank you for your comment. Done.

Reviewer #7: HIGHLIGHTS Replace the order of the second and third line.
Authors' response: Thank you for your comment. The highlights were rewritten following the other reviewers' suggestions.

Reviewer #7: ABSTRACT. Line 23: additional stress instead of "additive stress"
Authors' response: Thank you for your comment. Done.

Reviewer #7: INTRODUCTION. Appropriate and acceptable, only a few small corrections:
Line 101 -102: see general suggestions, especially the term "nutrients characteristics of water" should be corrected
Authors' response: Thank you for your comment. Done.

Reviewer #7: Lines 102 and 104: in both lines you have "c)". In line 104 should be "d)"
Authors' response: Thank you for your comment. Done.

Reviewer #7: MATERIALS AND METHODS. Appropriate and acceptable, only a few small corrections and one more explanation:
LINES 114-115. Correct the sentence (English is not correct; so the sentence is not clear enough)
Authors' response: Thank you for your comment. Done.

Reviewer #7: Line 126: "in 3 deep zone ones"??? What is this? Is it "at three depths"?
Authors' response: Thank you for your comment. We rephrased it.

Reviewer #7: Lines 134-139: three methods are cited, but measurement was made "by an adoption" of these three methods. If something is changed in methodology - it should be described. So, these adoptions of the methods should be explained! Otherwise, these methods could not be performed by others.
Authors' response: Thank you for your comment. We have taken this methodology from the instruction manual of spectrophotometer (Hanna Instrument). We didn't know other information.

Reviewer #7: Line 141: Delete "campaigns", "fish sampling" is just enough
Authors' response: Thank you for your comment. Done.

Reviewer #7: Lines 142-144: The sentence "The choice of the two sesons was prescribed by the necessity to reach on foot the site during the ice free period." (Write the sentence in better English!)
Authors' response: Thank you for your comment. Done.

Reviewer #7: Line 145: captured (add "d")
Authors' response: Thank you for your comment. Done.

Reviewer #7: Line 174: instead of "in single" use "individually"
Authors' response: Thank you for your comment. Done.

Reviewer #7: Line 177: use "enzymatic analysis" instead of "enzymes analysis"
Authors' response: Thank you for your comment. Done.

Reviewer #7: RESULTS. The results are presented in a clear manner. The figures used to show them are adequate. However, I would recommend:
Line 225: exchange "Physical-chemical and nutrients characteristics of water" with "Physico-chemical parameter and nutrient levels in water"
Authors' response: Thank you for your comment. Done.

Reviewer #7: Lines 259- 260: add "same" in front of seasons
Authors' response: Thank you for your comment. Done.

Reviewer #7: Line 270: delete water in front of nutrients
Authors' response: Thank you for your comment. Done.

Reviewer #7: DISCUSSION Line 329: better use trace elements instead of heavy metals, because Se is not Heavy metal (listed in line 331)
Authors' response: Thank you for your comment. Done.

Reviewer #7: Lines 359-361: Comment: Alkalinity is not same as pH, so this statement ("explanation of this outcome") is not proper!
Authors' response: Thank you for your comment. We corrected it.

Reviewer #7: 411: same comment as for the line 270
Authors' response: Thank you for your comment. Done.

Reviewer #7: CONCLUSIONS. Clear. Hope my comments will help authors to improve their manuscript!!!
Authors' response: Thank you for your suggestions.

Oxidative stress ecology in brook trout (*Salvelinus fontinalis*) from a high-mountain lake (Cottian Alps)

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Abstract

High-mountain lakes are pristine ecosystems characterized by extreme environmental conditions. The atmospheric transport of pollutants from lowlands may add further stress to organisms inhabiting these environments. We investigated the environmental stress pressure on brook trout (*Salvelinus fontinalis*) from a high-mountain lake in the Cottian Alps (Piedmont, northwest Italy). To do this, males and females of brook trout were sampled from Balma Lake in summer (August) and autumn (October) 2017 in order to assess the influence of trace elements accumulation and environmental parameters (physicochemical parameters and nutrient characteristics of water) on oxidative stress biomarkers. Bioaccumulation of Al, As, Cd, Cr, Cu, Fe, Hg, Pb, Ni, Se, and Zn and metallothionein levels were measured in muscle tissue of males and females. Liver, gills, kidney, and spleen tissue samples were analyzed for superoxide dismutase, catalase, total glutathione peroxidase, selenium-dependent glutathione peroxidase, glutathione reductase, and glutathione S-transferase activity. Analysis of environmental parameters showed changes in biomarker levels with seasonal variations. Water temperature was significantly higher in summer than autumn (Wilcoxon test; $p = 0.0078$), while pH was significantly higher in autumn than in summer (Wilcoxon test; $p =$

0.0078). Sex-related differences in oxidative stress biomarkers in tissues were unremarkable, whereas seasonal variability of oxidative stress biomarkers was observed, with major differences occurred for liver in summer and for gills, kidney, spleen and muscle in autumn. Positive correlations between environmental parameters and biomarkers were noted. Major fluctuations in water temperature, pH, Cu, Pb and Hg produced changes in biomarker levels; however, increased food intake during the ice-free season was probably the main factor that influenced changes in oxidative stress biomarker levels in brook trout in this extreme ecosystem.

Keywords: Alpine lakes; extreme ecosystems; oxidative stress biomarkers; trace elements

1. Introduction

Alpine lakes are remote, extreme ecosystems under harsh climatic conditions (Catalán et al., 2006). The ice-free season lasts for few months, generally from mid-June to late October. During this brief period of ideal conditions, some aquatic organisms can complete their life cycle before the snow covers the lakes again. Oligotrophic water conditions, UV radiation intensity, together with extreme temperatures allow for the development of a few dominant but well-adapted species (Sommaruga, 2001; Füreder et al., 2006; Pastorino et al., 2019a). These characteristics underlie the negative correlation between altitude and biodiversity (Rott, 1988; Starkweather, 1990). Due to their remote location, Alpine lakes are often considered pristine, unpolluted ecosystems (Pastorino et al., 2019b). Since the 1980s, however, they have been affected by the global anthropogenic impact of pollutants transported from lowland emission sources and the introduction of alien species (Tiberti et al., 2014; Pastorino et al., 2020). These ecosystems have low resilience to disturbances and can be particularly sensitive to the release of fish species for recreational angling, with important consequences along the entire trophic chain (Tiberti et al., 2014; Milardi et al., 2016; Perrine, 2017; Raposeiro et al., 2017). For example, the release of salmonids, especially brook trout (*Salvelinus fontinalis*), has had a dramatic outcome for zooplanktonic, macrobenthic, and amphibian communities in several Alpine lakes (Tiberti et al., 2014).

Alpine lakes are also a receptor for medium atmospheric transported (MRAT) contaminants (Ferrario et al., 2017), as observed in the Arctic (Hung et al., 2016), which is subject to the long-range transport potential (LRTP) of many chemicals. Altitudinal transport in the European Alps can occur over relatively short distances from sources of pollution in the industrialized areas of Germany, Switzerland, Austria, and northern Italy (Poma et al., 2017). The input of contaminant compounds into Alpine lakes is controlled by atmospheric deposition and condensation (Schmid et

al., 2007). The contaminants are bioaccumulated by the organisms inhabiting these ecosystems. Because fish occupy the uppermost trophic level, they provide an excellent bioindicator for the atmospheric medium-long range input of persistent organic compounds such as pesticides, brominated flame retardants (Schmid et al., 2007), and trace elements (Rognerud et al., 2002; Yang et al., 2007).

Moreover, fish are used as sentinel organisms to detect environmental contamination (Squadrone et al., 2013, 2014, 2016). They provide a useful model for assessing the impact of pollutants on biological functions such as detoxification (Elia et al., 2010). Assessment of contaminants in aquatic organisms can estimate and quantify the bioavailable fraction that might have the potential to induce an effect. However, because determination of body concentrations alone does not provide valuable data about the effects, quantification of some biological responses is necessary to evaluate the health state of contaminant-exposed organisms (Elia et al., 2010). Metals, for instance, are pro-oxidants that exert oxidative stress via reactive oxygen species (ROS) production and cause critical changes in cellular biotransformation/detoxification pathways (Lushchak, 2016). Biomarker levels can be also influenced by abiotic factors such as pH, dissolved oxygen content, and water temperature (Sroda and Cossu-Leguille, 2011). Water temperature is a major factor in physiological processes in fish and can induce the production of ROS (Lushchak, 2011).

Oxidative stress results from an imbalance between pro-oxidants such as ROS and the protective antioxidant system. Mechanisms involve the activity of numerous antioxidant enzymes, including superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), selenium-dependent glutathione peroxidase (Se-GPx, EC 1.11.1.9), glutathione reductase (GR, EC 1.6.4.2), glutathione S-transferases (GST, EC 2.5.1.18), as well as the rates of metal-trapping molecules such as reduced glutathione and metallothioneins (MTs). They are important protective metabolic pathways that are used as biomarkers of pollutant-induced oxidative stress. Selected oxidative stress biomarkers have proven useful to assess the impact of a range of metals in aquatic organisms (Al Kaddissi et al., 2014; Cozzari et al., 2015; Elia et al., 2006, 2007a, 2007b, 2010). Furthermore, contaminant levels and antioxidant enzyme activity in aquatic organisms may change with the season and in response to biological and environmental pressures (Monserrat et al., 2007). This poses a limitation on field studies, because biochemical response may be linked to either fish physiology or exposure to contaminants.

To our best knowledge, no studies are available about oxidative stress in fish from Balma Lake, an Alpine lake in Piedmont (Cottian Alps, northwest Italy). Originally, the lake was fishless and brook trout was released for recreational fishing. With the present study we investigated: a) the physicochemical parameters and nutrient characteristics of lake water; b) the biometric

characteristics and stomach contents of brook trout (*S. fontinalis*); c) the trace element accumulation in muscle because it constitutes a stable pool of trace elements for fish (Barwick and Maher, 2003); d) the biomarkers of oxidative stress in muscle, liver, kidney, gills, and spleen in male and female individuals captured in Balma Lake in summer and autumn 2017.

2. Materials and Methods

2.1 Study site

Balma Lake (45° 02' 14'' N; 07° 10' 52'' E) is located at 2100 m above sea level in the municipality of Coazze, a small town 40 kilometers from Turin (Piedmont, northwest Italy). It is a typical glacial-origin lake in the Cottian Alps, within the SCI/ZSC IT1110006 - Orsiera Rocciavrè. The lake is located above the tree line and is covered by ice from November to early June. Originally, the lake was fishless, and *S. fontinalis* was released for recreational fishing during the 1970s (Pastorino et al., 2020). The main substrate of the area is composed of ophiolite metamorphic bedrock. The main source of impact is the medium-long distance transport of pollutants from the plain, grazing activities, and angling during the ice-free period. No previous studies or data about the lake's hydrochemistry, taxa composition, and trace element accumulation are available. During summer 2017 morphometric and bathymetric survey of the lake was carried out by GeoStudio RC (Giaveno, Italy) using flying and floating drones. The lake measures 414 m in perimeter, 1.21 ha in surface area, and 6.42 m maximum depth in the central zone (Fig. 1).

2.2 Physicochemical parameters and nutrients of lake water

During both sampling periods the main physicochemical parameters were monitored at 5 sites in the littoral zone (in the upper centimeters of water) and 3 sites in the deep zone (in the water column) (Fig. 1). Water temperature (°C), dissolved oxygen (% saturation; mg L^{-1}), conductivity (µS cm^{-1}), and pH were recorded using field meters (HI 9033 conductivity meter, HI 9125 pH/ORP meter, HI 9147 oximeter, Hanna Instruments Inc. Woonsocket, RI, USA). Three replicates were carried out for each parameter. Water samples were collected in sterile containers (three 1-L bottles for each site), taking care not to include sediment particles, and then brought to the laboratory in a refrigerated container within a few hours. Concentrations of NH_4^+ (mg L^{-1}), NO_3^- (mg L^{-1}), and PO_4^{3-} (mg L^{-1}) were measured using a multi-parameter benchtop photometer (HI 83200-02, Hanna Instruments Inc.). NO_3^- (mg L^{-1}) concentration was obtained by measuring absorbance at 525 nm via an adaptation of the cadmium reduction method (APHA et al., 1998); NH_4^+ (mg L^{-1}) concentration was obtained by measuring the absorbance at 420 nm (ASTM, 2015) via adaptation

of the Nessler method; finally, PO_4^{3-} (mg L^{-1}) concentration was obtained by measuring absorbance at 610 nm via adaptation of the ascorbic acid method (APHA et al., 1998).

2.3 Fish sampling

Fish sampling campaigns were carried out during summer (August) and autumn (October) 2017. These months were chosen so that we could reach the sampling site on foot during the ice-free period. Permission for sampling was granted by the competent authority (Città Metropolitana di Torino; authorization n. 176-19040/2017). Fish were captured using 4 multimesh gillnets (36 x 1.8 m) divided into 6 panels of different mesh size (10 to 38 mm) to capture all size classes indiscriminately, except for offspring. The gillnets were randomly placed in the lake (Fig. 1) for 3 hours and then recovered. For each sampling period, 20 individuals (Table 2) were sacrificed after deep anesthesia with a lethal concentration (200 mg kg^{-1}) of tricaine methanesulfonate (MS-222) dissolved in water. The fish were necropsied, sexed, weighed, and measured for total length in the field. Immediately thereafter, samples of gill, liver, spleen, kidney, and muscle of each specimen were collected, packed in dry ice, and transported to the laboratory.

Stomach contents analysis was performed to obtain information about fish diet and to characterize seasonal variations. Stomachs were preserved in 70% alcohol, and the contents were inspected by stereomicroscopy (Zeiss Stemis V8, Jena, Germany). The ingested prey was identified to the order or family level, since closer identification was precluded by the digestion status of the organisms. To describe the diet, prey frequency of occurrence (Fi) (Tiberti et al., 2016) was calculated for both seasons.

2.4 Trace elements in fish muscle

Trace elements in fish muscle from males and females were analyzed by inductively coupled plasma-mass spectrometry (ICP-MS Xseries II, Thermo Scientific, Bremen, Germany). Determination of Al, As, Cd, Cr, Cu, Fe, Ni, Pb, Se, and Zn was performed following protocols reported by Squadrone et al. (2016). Hg concentration was determined on a direct mercury analyzer (DMA-80 Analyzer, Milestone, Shelton, CT, USA). Analytical performance was verified by processing certified reference materials (Oyster Tissue - SRM 1566b from the National Institute of Standard and Technology), along with blank reagents in each analytical session. Table S1 presents the reference material values and the percentages of recovery. The analytical method was validated according to ISO/IEC 17025 (general requirements for the competence of testing and calibration laboratories).

2.5 Biochemical analyses

A total of 40 specimens of *S. fontinalis*, 26 females (12 in August and 14 in October) and 14 males (8 in summer and 6 in autumn) were examined individually for oxidative stress biomarkers. Liver, gills, kidney, spleen, and muscle were analyzed for SOD, CAT, GPx, Se-GPx, GR, and GST activity, and MT levels only for muscle. For enzymatic analysis, the samples were homogenized with an UltraTurrax homogenizer in 100 mM potassium-phosphate buffer, pH 7.5, added with 2.5% sodium chloride (NaCl), 0.008 TIU ml^{-1} aprotinin and 0.1 mg ml^{-1} bacitracin. The homogenates were centrifuged at 50,000 x g for 30 minutes at 4°C. Cytosolic fractions were used to determine antioxidant biomarker activity. Biochemical analyzes were performed according to the methods reported in Elia et al. (2017). Briefly, SOD activity was assessed at 550 nm in 50 mM Na_2CO_3 buffer, pH 10, 0.1 mM EDTA, 500 mM cytochrome C and 1 mM hypoxanthine and xanthine oxidase. Cytochrome C reduction by the xanthine/hypoxanthine complex was evaluated by comparison with a standard SOD unit curve. CAT activity was measured at 240 nm after the decrease in absorbance following the consumption of H_2O_2. The assay was carried out in NaH_2PO_4 buffer + Na_2HPO_4 100 mM pH 7 and H_2O_2 24 mM. Total glutathione peroxidase (GPx) and selenium-dependent glutathione peroxidase (Se-GPx) activity was determined at 340 nm in NaH_2PO_4 + Na_2HPO_4 100 mM buffer, pH 7.5, 1 mM EDTA, 0.12 mM NADPH (b-nicotinamide adenine dinucleotide), 2 mM GSH, 1 U of GR (glutathione reductase), 1 mM NaN_3 and H_2O_2 0.6 mM for Se-GPx or 1 mM DTT and 0.8 mM cumene hydroperoxide for GPx. GR activity was measured at 340 nm in NaH_2PO_4 + Na_2HPO_4 100 mM buffer, pH 7, 1 mM GSSG (oxidized glutathione), and 0.06 mM NADPH. GST activity was measured at 340 nm using CDNB (1-chloro-2,4-dinitrobenzene) as substrate. The assay was carried out in 100 mM NaH_2PO_4 + Na_2HPO_4 100 mM buffer, pH 6.5, 2 mM GSH and 2 mM CDNB. Concentration of cytosolic proteins was determined according to the method of Lowry et al. (1951) and used to normalize biomarker activity.

Metallothionein (MT) levels were measured in the muscle tissue of individuals of both sexes. Samples were homogenized (1:4) in a buffer containing 0.02 M TRIS/HCl, 0.5 M sucrose, 0.1 mg ml^{-1} bacitracin, 0.008 tiu ml^{-1} aprotinin, 87 µg ml^{-1} phenylmethylsulfonyl fluoride (PMSF), and 0.1 µl ml^{-1} α-mercaptoethanol. The homogenates were centrifuged at 14,500 x g at 4°C to obtain the cytosolic fraction. The supernatants were purified using a chloroform/ethanol solution and then HCl/ethanol to obtain the partially purified MT fraction. The pellets were washed with ethanol/chloroform/TRIS/HCl (87/1/12) solution and suspended in 0.25 M NaCl. A destabilizing solution (HCl 1N + EDTA 4 mM) and Ellman's reagent (DTNB: 5,5 dithiobis-2-nitrobenzoic acid) were added to each sample. Sulphydril residue contents (-SH) were spectrophotometrically

quantified. Absorbance was measured at 412 nm and compared to that obtained from a standard curve with reduced glutathione (1 mg ml^{-1} GSH). All biochemical analyses were performed in triplicate for each sample on a Varian spectrophotometer (Cary 50 Thermostat Cell Holder) at a constant temperature of 25°C.

2.6 Statistical analyses

Spearman's rank correlation coefficient (ρS) was used to test for correlations between trace element concentration in fish muscle, water physicochemical parameters, nutrients, and oxidative stress biomarkers. Differences in the concentration of physicochemical parameters, nutrients, and trace elements between seasons were tested using the Wilcoxon test. Data from the biochemical analysis are reported as the mean and standard deviation (SD). Significant differences between sexes and months were tested using one-way ANOVA followed by Tukey's multiple comparison test. Homogeneity of variance was checked using Bartlett's test. The criterion for significance was set at $p < 0.05$. Principal component analysis (PCA) was performed to check for trends in trace elements, biomarkers, and physicochemical values between the sampling seasons (summer and autumn). Statistical analyses were performed using open source data analysis software RStudio® version 1.1.463 (RStudio, Inc.).

3. Results

3.1 Physicochemical and nutrient characteristics of lake water

Lake water temperature was significantly lower in autumn (range 7.20-8.80°C) than summer (range 14.60-16.10°C) (Wilcoxon test; $p = 0.0078$) in agreement with seasonal trends; no thermal stratification in the water column was observed, since the temperatures recorded at the deep sites were similar to those of the littoral sites in both seasons. pH values were significantly higher in autumn (range 7.53-7.90) than summer (range 6.52-7.31) (Wilcoxon test; $p = 0.0078$). No differences in oxygen saturation were recorded between summer (range 77-103%) and autumn (range 101-107%) (Wilcoxon test; $p > 0.05$). Water conductivity remained constant between summer and autumn (range 17-21 µS cm^{-1}) (Wilcoxon test; $p > 0.05$). PO_4^{3-} concentration was ≤ 0.02 mg L^{-1} in both seasons. NH_4^+ level was < 0.14 mg L^{-1} at all sites, except for station 4 (0.20 mg L^{-1}) in summer. NO_3^- level was < 9.20 mg L^{-1} at all sites, except for site 4 (12 mg L^{-1}) in summer. There were no significant differences in PO_4^{3-}, NH_4^+, and NO_3^- levels between seasons (Wilcoxon test; $p > 0.05$). Table 1 presents the changes in physicochemical and nutrient data (mean ± SD) .

3.2 Fish and stomach contents

Table 2 presents the average total length and weight of fish captured during summer and autumn 2017. Stomach contents analysis revealed the almost exclusive presence of terrestrial insect preys in both summer (94.19%) and autumn (81.04%). Preys belonged to the order Hymenoptera (77.74% and 66.39% in summer and autumn, respectively) and Coleoptera (16.45% and 14.65% in summer and autumn, respectively). Other taxa were present in extremely low proportions (Diptera Chironomidae larvae: 5.18% in summer and 2.79% in autumn).

3.3 Trace elements in fish muscle

Figure 2 presents the mean concentration and the relative standard deviation of each trace element detected in muscle tissue in summer and autumn. The mean concentration of trace elements was in the order: Zn (16.25) > Fe (8.78) > Al (1.49) > Se (0.67) > Cu (0.60) > Cr (0.14) > As (0.06) > Ni (0.06) > Pb (0.05) > Hg (0.01) mg Kg^{-1}. In autumn the mean concentration of trace elements was in the order: Zn (16.13) > Fe (10.63) > Al (2.03) > Se (0.49) > Cu (0.36) > Cr (0.16) > As (0.10) > Pb (0.10) > Ni (0.06) > Hg (0.02) mg Kg^{-1}. Cd was < LOQ (0.02 mg Kg^{-1}) in both seasons. There were no significant differences in trace element concentration between seasons (Wilcoxon test; $p > 0.05$ for all elements).

3.4 Biochemical analyses

In the liver, the activity of SOD, CAT, and GR was significantly lower in autumn (up to 70%) than summer in males and females (Tukey's test; $p < 0.05$) (Figs. 3-4, 7). In the gills, GPx activity was significantly higher in autumn (90%) mainly in the females (Tukey's test; $p < 0.05$); Se-GPx and GST activity was significantly higher (up to 2-fold) in males and females in autumn (Tukey's test; $p < 0.05$) (Figs. 5, 6, 8). In the kidney, only Se-GPx activity was significantly different between the seasons, being higher (up to 40%) in autumn (Tukey's test; $p < 0.05$) (Fig. 6). In the spleen, Se-GPx activity was significantly lower (70%) in the females (Tukey's test; $p < 0.05$) (Fig. 6). GR and GST activity was significantly higher (up to 70%) in males in autumn than in summer (Tukey's test; $p < 0.05$) (Fig. 7, 8). In muscle, SOD activity was significantly lower (up to 50%) in females in autumn (Tukey's test; $p < 0.05$), whereas CAT activity showed an opposite trend and was significantly higher in autumn (up to one-fold) (Tukey's test; $p < 0.05$) (Figs. 3-4). MT level was higher in autumn (up to one-fold) than in summer (Tukey's test; $p < 0.05$) (Fig. 9).

3.5 Spearman correlation matrix

Spearman correlation matrix revealed correlations between environmental parameters (trace elements, physicochemical parameters, nutrients) and oxidative stress biomarkers in muscle, gills,

liver, spleen, and kidney tissue for both seasons. Due to the multiplicity of positive correlations, Table S2 presents the correlation matrices (one for each organ). Only the most informative outcomes are presented and discussed for interpretation of biological response of *S. fontinalis* to environmental parameters. In detail, a significant positive correlation was found between MTs and Hg (ρS 0.787), MTs and Pb (ρS 0.787), MTs and Cu (ρS 0.683), MTs and pH (ρS 0.650), and CAT and pH (ρS 0.737) in muscle tissues of females captured in autumn; SOD and Cr (ρS 0.837) in the liver tissue of females captured in autumn; SOD and NO_3^- (ρS 0.750), Se-GPx and NO_3^- (ρS 0.750) in muscle and kidney tissue, respectively, of females captured in summer.

3.6 Principal Component Analysis (PCA)

The first two principal components (Dim1; Dim2) accounted for meaningful amounts of the total variance (58.2%), while the other components accounted for a relatively smaller fraction (Fig. 10). Dim1 accounted for 35.2% of the total variance and was positively correlated with the variables Ni, Se, temperature, oxygen, conductivity, and NO_3^- and negatively correlated with Al, Cr, Fe, As, Pb, Hg, and pH. Dim2 accounted for 23% of the total variance and was positively correlated with the variables GST, CAT, GPx, Se-GPx, SOD, and GR. The biplot of loadings (variables) and score (observations) shows which organs (liver, gills, kidney, spleen, and muscle) of brook trout are closest to them, and which variables (trace elements, biomarkers and physicochemical parameters) contributed to this grouping in the coordinate of Dim1 and Dim2. Separation of organs by season (summer on the right, autumn on the left) suggested a difference in biomarker values. In detail, the autumn samples of spleen, gill, muscle, kidney, and liver tissue are on the left in order of increasing value of Al, Cr, Fe, As, Pb, Hg, and pH. The summer samples of spleen, gill, muscle, kidney tissue are on the right in order of increasing value of NH_4^+, NO_3^-, conductivity, oxygen, temperature, Zn, Cu, Ni, and Se. Remarkably, summer samples of liver tissue are well separated from other organs, following the trend in biomarker values.

4. Discussion

The environmental parameters of water oxygenation, pH, conductivity, and temperature are in line with those reported by Tiberti et al. (2010) for 12 alpine lakes in the Gran Paradiso National Park (Western Alps, Italy). Temperatures were lower in autumn than in summer according to seasonality. The temperature data recorded at the deep sites revealed no vertical layering in the lake because the shallow maximum depth (6.42 m) does not allow for the formation of a thermocline layer. The pH values are related to rock composition. Since Lake Bama lies over a granite bedrock, its pH values are lower than lakes on limestone or sandstone bedrock (Camarero et al., 2009). The pH values

were in line with the literature reported for high-altitude environments (Boggero et al., 2006; Füreder et al., 2006; Fjellheim et al., 2009). As expected for mountain lakes, the oxygenation levels were high. The water conductivity values were in line with published literature, as conductivity of silty-like lakes tends to be < 50 µS cm^{-1} (Boggero et al., 2006; Füreder et al., 2006). No decrease in oxygen values correlated with depth was observed owing to the absence of temperature stratification. Nutrient levels (PO_4^{3-}, NO_3^- and NH_4^+) were in line with previous studies of Alpine lakes (Füreder et al., 2006; Camarero et al., 2009) and revealed an oligotrophic condition.

We observed a positive correlation between environmental parameters and biological response in the *S. fontinalis* from Balma Lake. The antioxidant response was tissue-specific; indeed, aerobic tissues such as the kidney, gills, spleen, and the liver in particular have a high potential for ROS production which is offset by protective mechanisms. Differently, muscle, which has a low content of mitochondria and a low-intensive oxidative metabolism, showed mild response to oxidative/reductive conditions. Moreover, the level of several biomarkers was related to the seasonal changes in the trace element concentration in some tissues.

The few studies on trace element accumulation in the biota from high-altitude lakes in general, and in Alpine lakes in particular, have focused largely on fish. Köck et al. (1996) studied the concentrations of Cd, Pb, Zn, and Cu in the muscle of arctic char (*Salvelinus alpinus*) from five oligotrophic Alpine lakes in northern Tyrol (Austria). Yang et al. (2007) studied the accumulation of trace elements in muscle of fish of the genus *Gymoncypris* (Cyprinidae) from high-mountain lakes in the Tibetan Plateau. Ni ranged from 0.094 to 0.12 mg kg^{-1}, Cu from 1.1 to 2.0 mg kg^{-1}, Zn from 4.4. to 6.9 mg kg^{-1}, As from 0.24 to 0.27 mg kg^{-1}, Se from 0.36 to 1.0 mg kg^{-1}, Cd from 0.024 to 0.025 mg kg^{-1}, and Pb from 0.047 to 0.079 mg kg^{-1}. Rognerud et al. (2002) found that Hg concentrations in fish muscle from high-mountain lakes in Europe ranged from 0.021 to 0.179 mg kg^{-1}. These data from previous studies demonstrate that high-mountain lakes function as a regional contaminant convergence zone for the medium and long-range atmospheric transport of contaminants. Since our results are in line with these findings, and because no previous studies have been performed to date, we assume that the trace element concentrations we detected in the brook trout from Balma Lake have both anthropogenic and pedogenic sources as their origin.

Metallothioneins have been widely considered as valuable biomarkers that reflect the level of trace elements in aquatic environments, where they act as metal trappers mainly of Cd, Hg, Pb, Cu, and Zn in fish (Bourdineaud et al., 2006; Morcillo et al., 2016). The higher levels of MTs we measured in autumn were related to the increased trace element concentration in this tissue, and we noted a strong correlation with Cu, Hg, and Pb levels in the females. This may suggest an alarming scenario, as it would signal an increased concentration of these elements in the environment.

340 Although no chemical analyses of the lake water were carried out, because of the peculiar
341 geomorphological characteristics of Balma Lake we can exclude an increase in such contaminants
342 during autumn.
343 Seasonal variation in metal concentrations in fish can be related to environmental factors such as
344 food availability, temperature, and biological cycle (Hermesz et al., 2001; Amiard et al., 2006;
345 Dragun et al., 2009). Furthermore, fish size may also play a key role in metal uptake (Wright and
346 Mason, 1999). In the present study, an increase in weight and length was measured in the fish from
347 both sampling seasons, and the frequency of occurrence of terrestrial invertebrates in the diet during
348 the ice-free season was in accordance with other studies performed on brook trout in other Alpine
349 lakes (Sotiropoulos et al., 2006; Tiberti et al., 2016). It is possible that fish growth due to a higher
350 intake of food in summer could have favored the uptake of heavy metals such as Pb and Hg.
351 This hypothesis is corroborated by a previous study on black scabbardfish *Aphanopus carbo*
352 (Trichiuridae), in which an exponential increase in total Hg load was found in all fish tissues in a
353 length-dependent manner (Bebianno et al., 2007). Furthermore, previous studies showed that
354 fluctuations toward high pH values can also play a key role in modulating metals uptake, affecting
355 their speciation and bioavailability (Playle, 1998). This outcome may explain the strong correlation
356 between pH and the two hydroxyl scavengers MTs and CAT in the female muscle tissue in autumn.
357 Trace elements also affected the activity of several enzymes in different tissues in the autumn fish
358 samples. We noted a strong correlation between Cr and SOD activity in the liver, as reported in
359 previous study in rock fish *Sebastes schlegelii* (Kim and Kang, 2016). Furthermore, a recent study
360 showed that Cr can induce conformational changes of CAT enzyme and reduce its activity
361 depending on its valence states and concentration (Chen et al., 2018). These findings may explain
362 the seasonal difference in CAT activity in the female muscle tissue.
363 In general, numerous elements can influence the activity of this biomarker, and As and Fe were the
364 elements most involved in modulating CAT enzyme activity in the tissues. Arsenic is a global
365 contaminant derived from natural or anthropogenic sources and a cause of great concern for
366 terrestrial and aquatic ecosystems (Elia et al., 2018). At high concentrations, arsenic may induce
367 oxidative stress by interacting with antioxidants and result in the accumulation of free radicals in
368 cells. Arsenite species can interact with sulfhydryl groups of biomolecules such as enzymes or
369 reduced glutathione (Elia et al., 2018). Furthermore, redox active metals such as iron generate ROS
370 or are involved in the Fenton route, leading to lipid peroxidation (Mahboob, 2013). Despite the
371 increase in trace element amount in autumn, the concentration of Pb, Hg, and Cd in fish muscle
372 mentioned in Regulation 1881/2006 (European Commission, 2006) was far below the established
373 threshold limit. This fact should be taken into account and may suggest the adaption of *S. fontinalis*

to seasonal changes rather than to oxidative stress. PCA analysis showed that summer samples of liver tissue were strongly related to oxidative biomarker level, since liver tissue is the site of multiple oxidative reactions and maximal free radical generation (Gul et al., 2004; Avci et al., 2005).

For ectothermic organisms, temperature is a crucial abiotic factor (Hassan et al., 2017). Daily temperature fluctuations and seasonal variations differ in their influence on fish (Buckley et al., 2006; Place and Hofmann, 2004; Podrabsky and Somero, 2004). Wellness and growth are optimal within a well-defined temperature range depending on the species (Godowsky and Caddell, 1991). Temperatures deviating excessively from the optimum can exert harmful effects and induce mortality (Rijnsdorp et al., 2009). In the present study, the marked drop in water temperature in autumn was related to changes mainly in GPx activity in the male kidney and in the female spleen. Moreover, higher temperature results in increased GPx activity in other fish such as the Antarctic *Pagothenia borchgrevinki* (Almroth et al., 2015). Thermal changes have also been associated with the up regulation of the NRF2 transcription factor, which is involved in the expression of antioxidants via binding to the antioxidant responsive element (ARE) (Almroth et al., 2015). Elevated GPx activity during summer may indicate strengthening of this fundamental defense line against ROS. However, the lack of change in SOD and CAT activity, as well as the lower concentration of trace elements in tissue, and the constant levels of the main physicochemical parameters of water, except for pH and temperature, preclude an oxidative pressure scenario and suggest an adaptive ability of *S. fontinalis* to higher temperature instead. On the other hand, increased SOD activity in the muscle tissue of males and females is linked to an abiotic factor, such as NO_3^-, in summer. Furthermore, nutrients also influenced SOD concentration in female muscle and Se-GPx activity in female kidney in summer. In the aquatic environment, it is not unusual that fish are simultaneously challenged by different abiotic factors. Conversely, at very high concentrations, ammonia can induce a range of toxicological effects in fish, such as altered metabolism, lack of growth, and mortality (Dosdat et al., 2003; Sinha et al., 2012, 2015). Ammonia exposure can also lead to oxidative stress in fish (Sun et al., 2012; Sinha et al., 2014). A previous study showed that conductivity plays a crucial role in maintaining the ammonia ionization equilibrium (NH_3 and the non-toxic form NH_4^+) in aquatic environments (Sinha et al., 2015). In the present study, the lack of change in conductivity and nutrients (e.g., phosphorus) between the seasons suggests that the changes in antioxidant parameters may be related to food intake (which indirectly promotes metals uptake) rather than to nutrient concentration. In their habitats, high-mountain lakes included, fish are often exposed to periods of food insufficiency in response to factors such as temperature, conductivity, and biological cycle (Pérez-Jiménez et al., 2007; Furné et

al., 2009). The increase in food intake during summer may also explain the fluctuation in biomarkers of oxidative stress.

5. Conclusion

With this study we investigated the influence of trace element accumulation and environmental parameters on oxidative stress biomarkers in male and female individuals of *S. fontinalis* from a high-mountain lake during the ice-free period. Generally, positive correlations were found between several environmental parameters and biomarkers. While oxidative stress biomarker levels were similar for males and females, significant fluctuation between seasons due to biological and environmental pressures was noted for several biomarkers. Although there was greater fluctuation in temperature, pH, and trace elements (e.g., Cu, Pb and Hg) between seasons, which certainly contributed to changes in biomarker levels, our findings indicate that increased food intake during the ice-free season was probably the main factor that affected the oxidative stress response. Future studies are needed to investigate other factors responsible for the changes in oxidative stress biomarkers.

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Table 1. Mean and standard deviation (three replicates) of physicochemical parameters and nutrients measured in Balma Lake in summer (August) and autumn (Autumn) 2017.

	Summer (August)	Autumn (October)
Temperature (°C)	15.62 ± 0.77	8.24 ± 0.53
Dissolved oxygen concentration (mg L^{-1})	7.40 ± 0.89	7.29 ± 1.92
Oxygen saturation (%)	92.88 ± 8.52	104.75 ± 2.43
pH	6.64 ± 0.28	7.69 ± 0.12
Conductivity (µS cm^{-1})	18.29 ± 1.12	18.56 ± 1.05
NH_4^+ (mg L^{-1})	0.09 ± 0.07	0.09 ± 0.05
NO_3^- (mg L^{-1})	8.55 ± 2.73	6.82 ± 2.14
PO_4^{3-} (mg L^{-1})	0.01 ± 0.01	0.02 ± 0.01

Table 2. Biometric values of females and males of brook trout (*Salvelinus fontinalis*) from Balma Lake in summer and autumn 2017.

	Summer (August)		Autumn (October)	
Sex	Males	Females	Males	Females
Number of individuals	8	12	6	14
Weight - W mean ± SD (g)	69.9 ± 30.34	71.20 ± 28.34	88.47 ± 56.23	90.05 ± 60.03
W min. (g)	15.00	15.36	10.23	11.00
W max. (g)	146.00	150.00	187.00	193.00
Total Length - TL mean ± SD (cm)	17.77 ± 3.90	18.56 ± 4.20	19.23 ± 4.66	19.35 ± 5.72
TL min. (cm)	11.50	11.00	7.50	8.00
TL max. (cm)	23.00	24.00	25.50	26.00

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707 **Figure 1.** Balma Lake: bathymetry and sampling sites for physicochemical parameters (L = littoral
708 sites; B = deep sites). Red rectangles indicate the placement of gillnets to sample fish in summer
709 (August) and autumn (October) 2017.

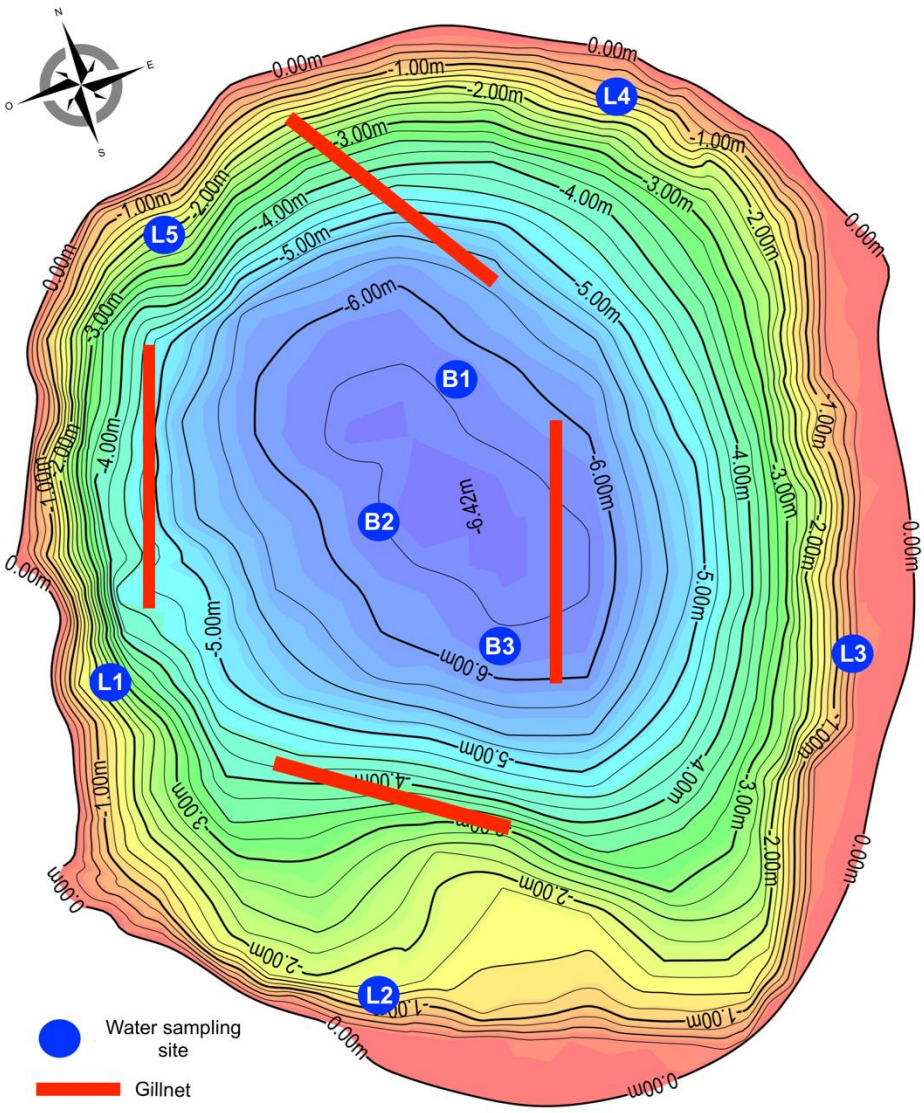

Figure 2. Trace elements (Log_{10} concentration; mg kg^{-1}) detected in the muscle tissue of brook trout in summer (August) and autumn (October) 2017.

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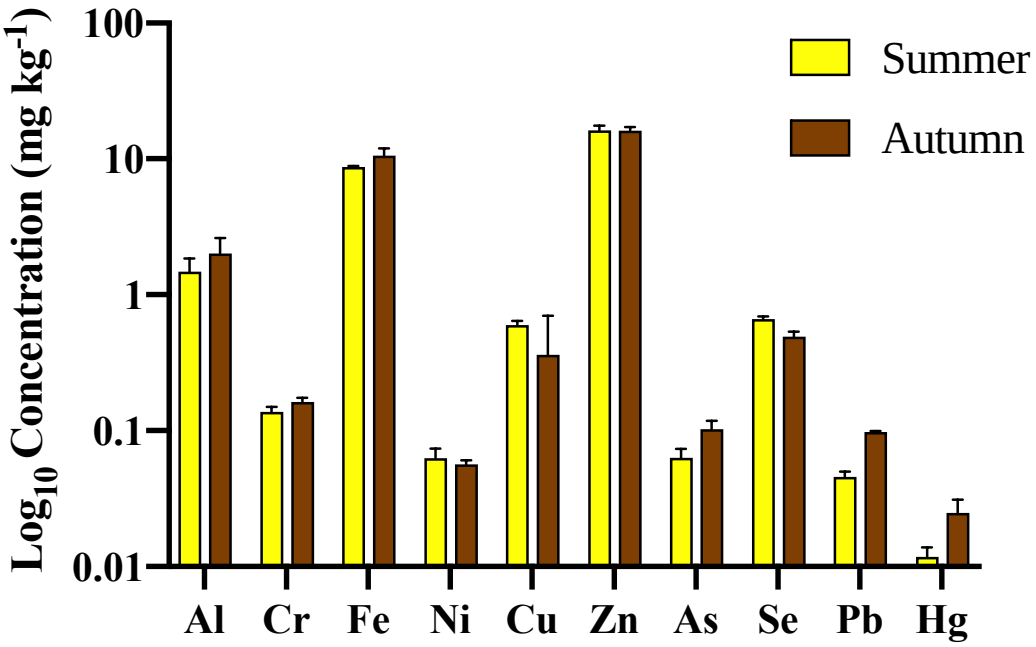

Log10 Concentration (mg kg-1)
100
10
1
0.1
0.01
Summer
Autumn
Al
Cr
Fe
Ni
Cu
Zn
As
Se
Pb
Hg

Figure 3. Superoxide dismutase (SOD) activity in the liver, gills, kidney, spleen, and muscle tissue of female and male brook trout. Data are reported as mean ± standard deviation. Summer (S); Autumn (A); females (F); males (M). Lower case letters indicate statistically significant differences (Tukey test, $p < 0.05$) between the sexes and the sampling months for each tissue type.

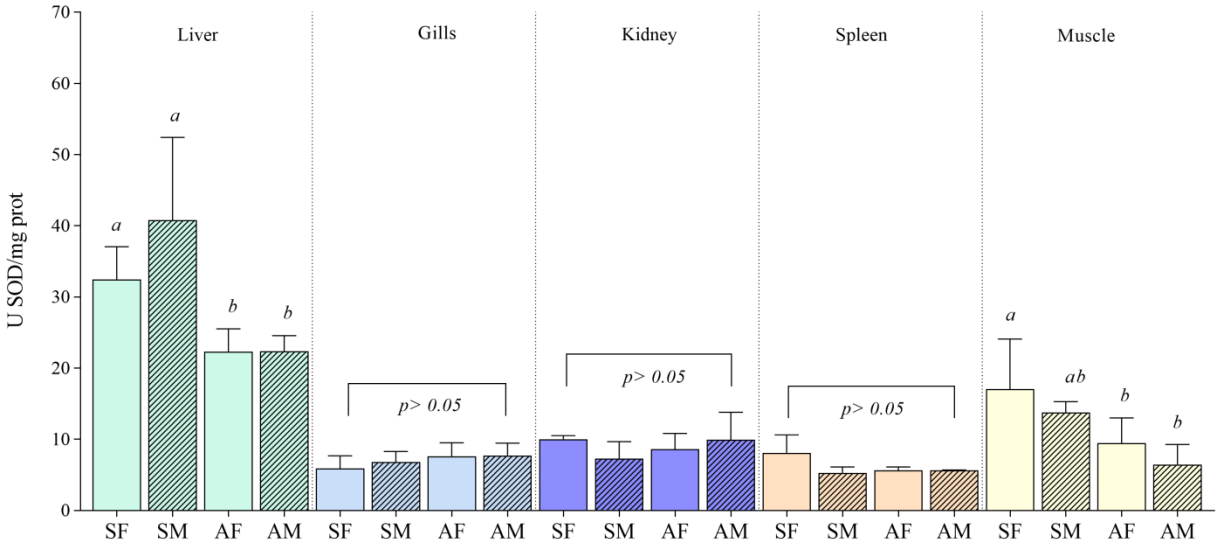


Figure 4. Catalase (CAT) activity in the liver, gills, kidney, spleen, and muscle tissue of female and male brook trout. Data are reported as mean ± standard deviation. Summer (S); Autumn (A); females (F); males (M). Lower case letters indicate statistically significant differences (Tukey test, $p < 0.05$) between the sexes and the sampling months for each tissue type.

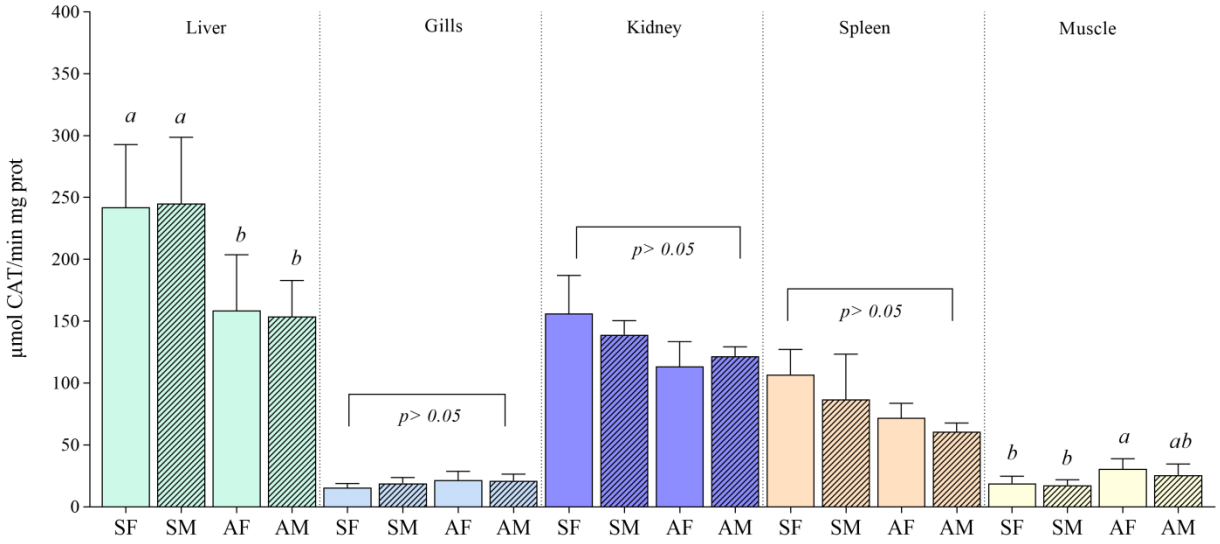


Figure 5. Total glutathione peroxidase (GPx) activity in the liver, gills, kidney, spleen, and muscle tissue of female and male brook trout. Data are reported as mean ± standard deviation. Summer (S); Autumn (A); females (F); males (M). Lower case letters indicate statistically significant differences (Tukey test, $p < 0.05$) between the sexes and the sampling months for each tissue type.

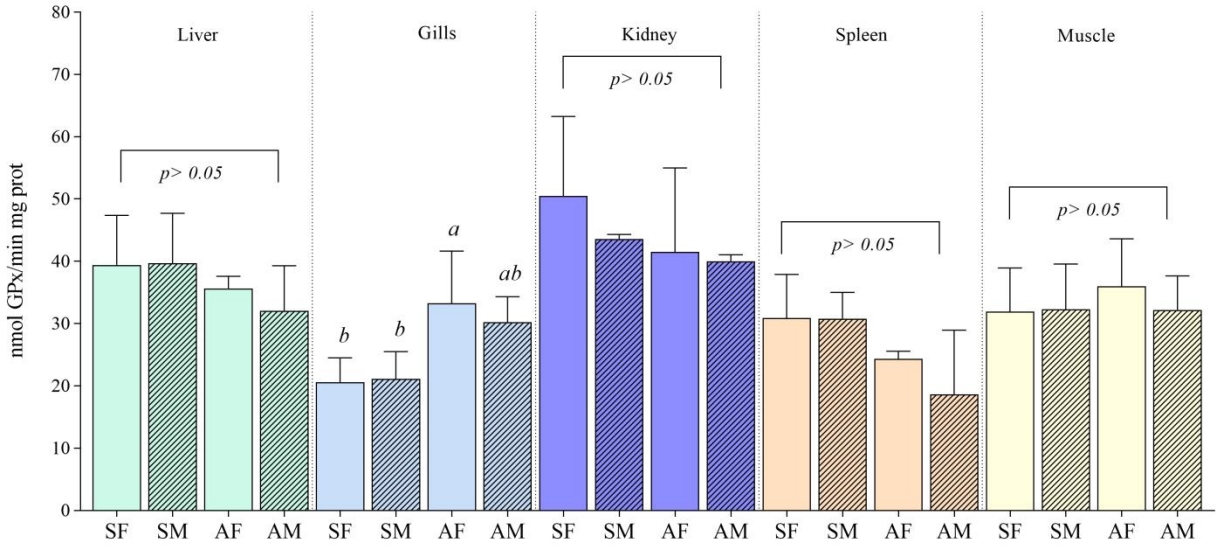


Figure 6. Selenium-dependent glutathione peroxidase (Se-GPx) activity in the liver, gills, kidney, spleen, and muscle tissue of female and male brook trout. Data are reported as mean ± standard deviation. Summer (S); Autumn (A); females (F); males (M). Lower case letters indicate statistically significant differences (Tukey test, $p < 0.05$) between the sexes and the sampling months for each tissue type.

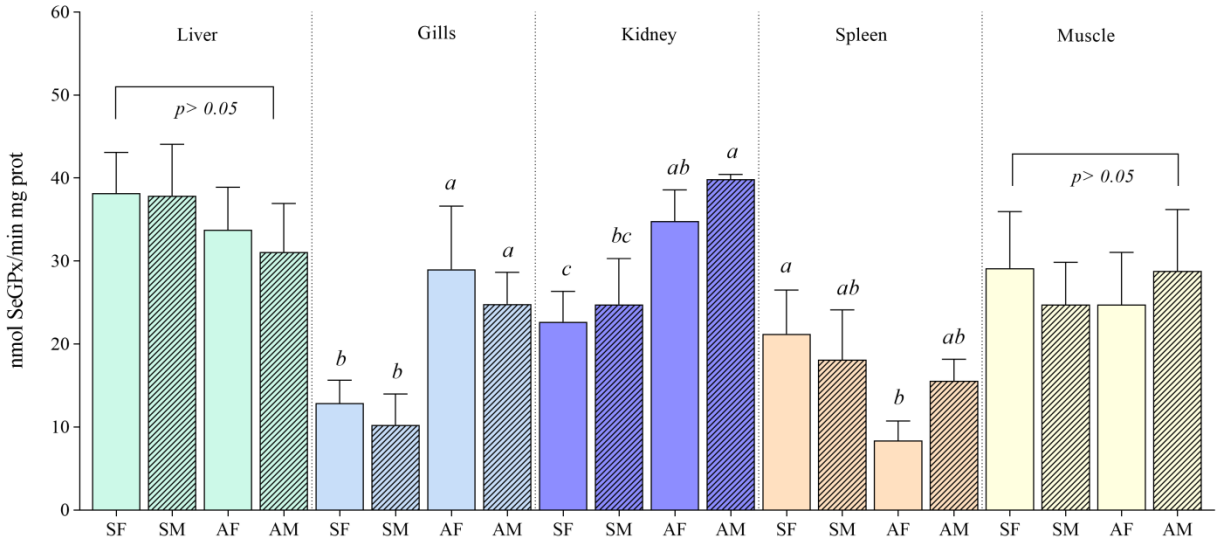


Figure 7. Glutathione reductase (GR) activity in the liver, gills, kidney, spleen, and muscle tissue of female and male brook trout. Data are reported as mean ± standard deviation. Summer (S); Autumn (A); females (F); males (M). Lower case letters indicate statistically significant differences (Tukey test, $p < 0.05$) between the sexes and the sampling months for each tissue type.

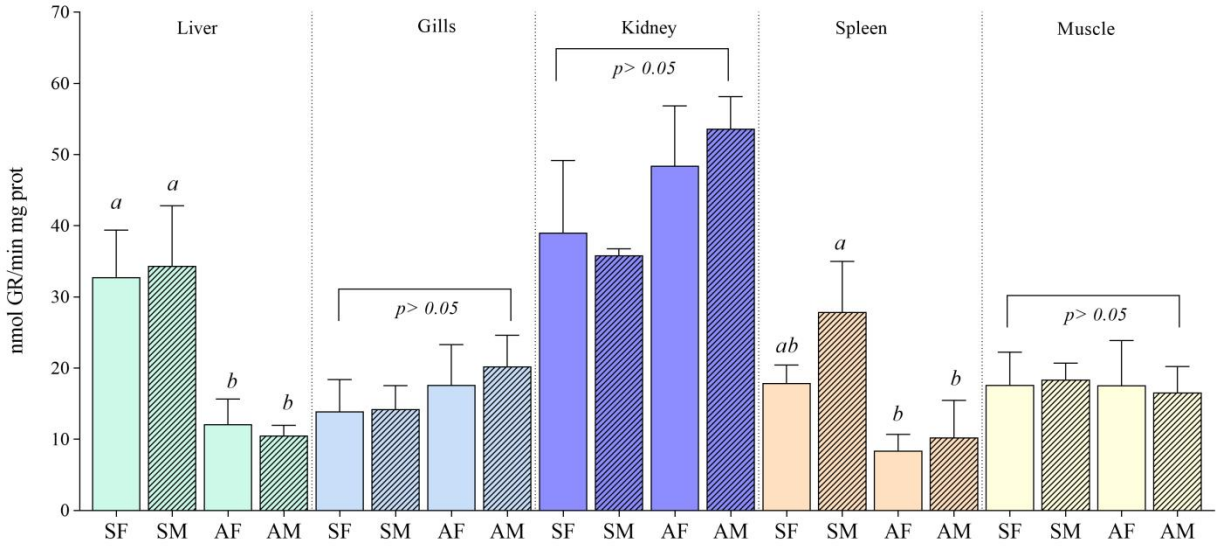


Figure 8. Glutathione S-transferase (GST) activity in the liver, gills, kidney, spleen, and muscle tissue of female and male brook trout. Data are reported as mean ± standard deviation. Summer (S); Autumn (A); females (F); males (M). Lower case letters indicate statistically significant differences (Tukey test, $p < 0.05$) between the sexes and the sampling months for each tissue type.

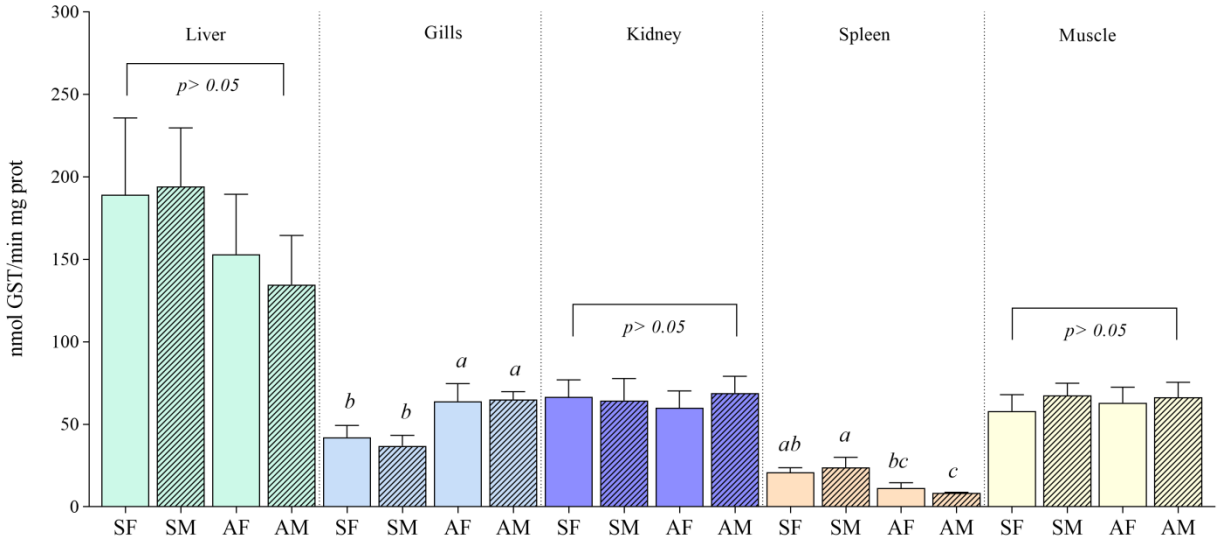
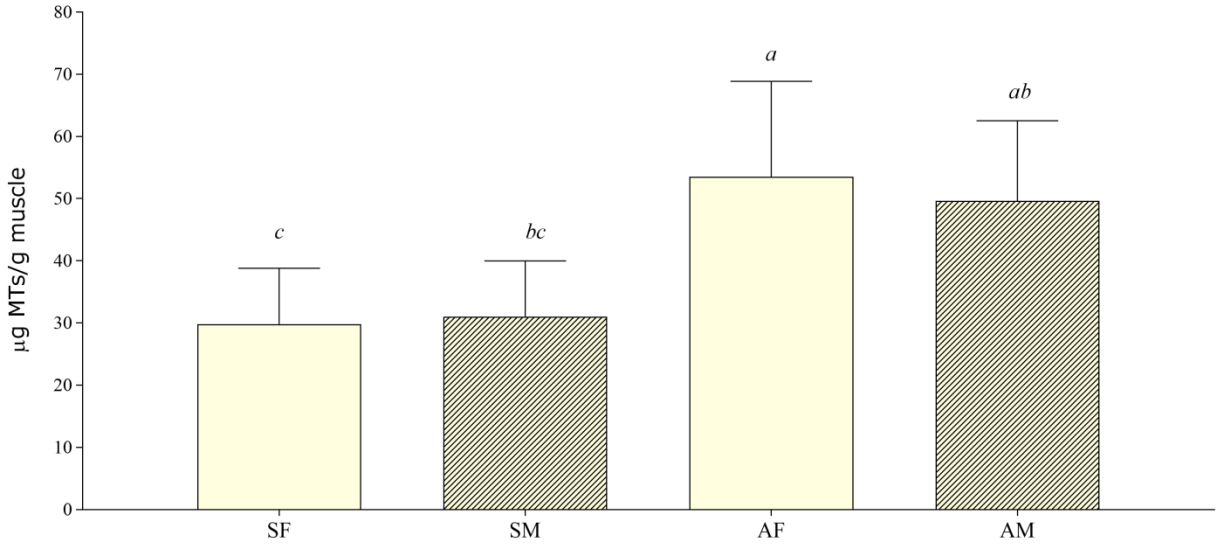
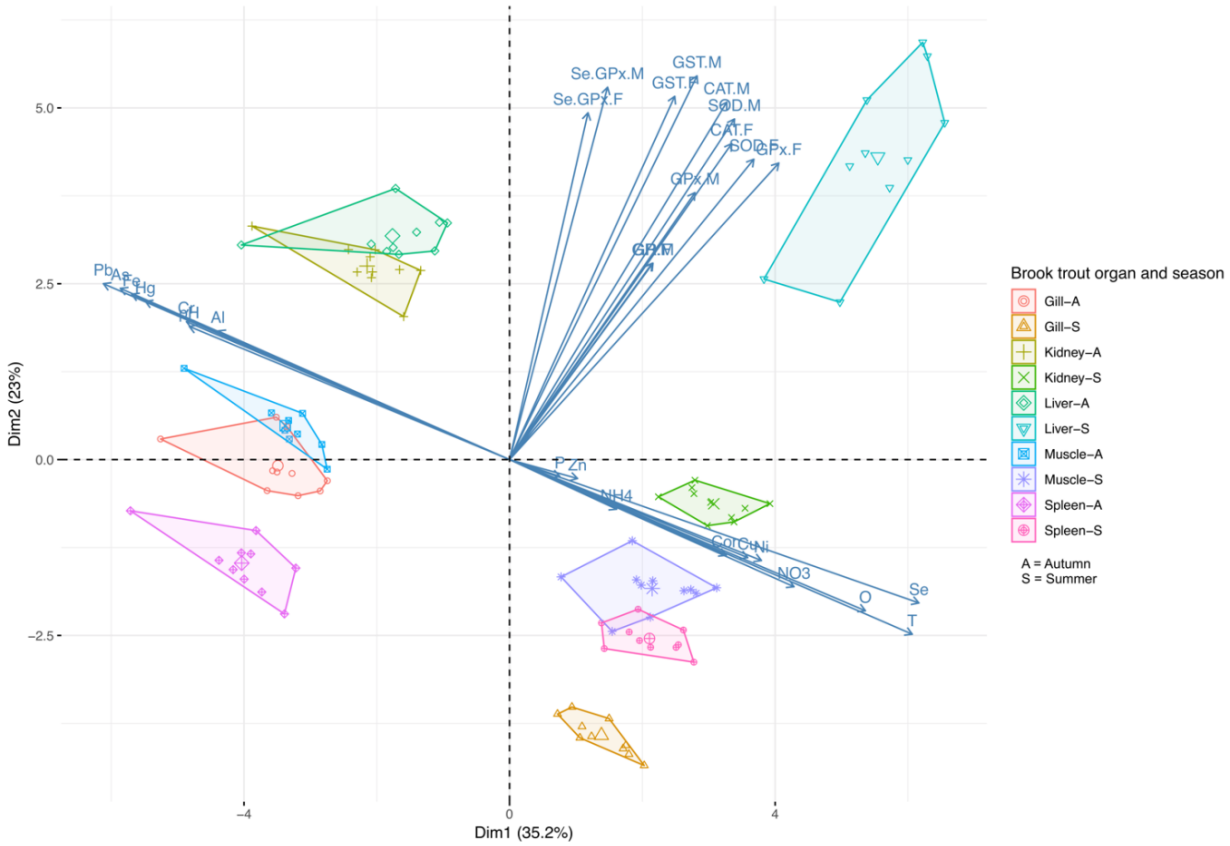


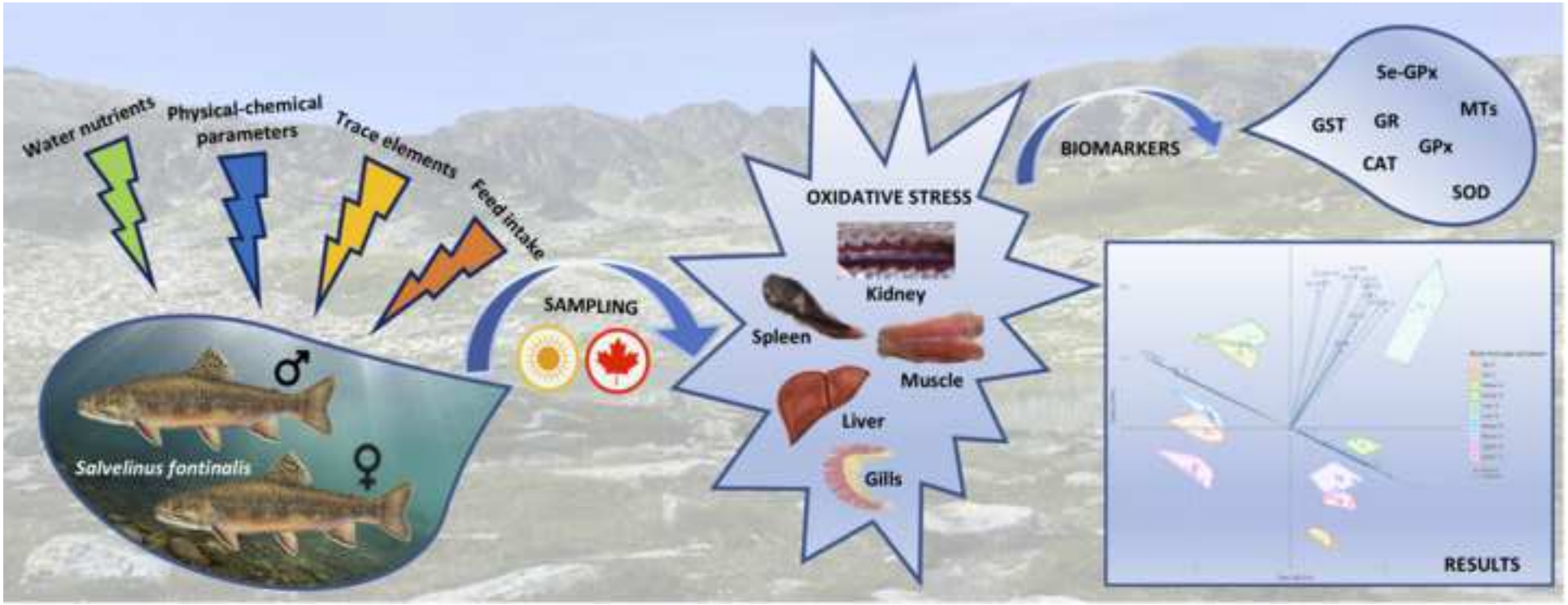
Figure 9. Metallothionein (MTs) concentration in the muscle tissue of female and male brook trout. Data are reported as mean ± standard deviation. Summer (S); Autumn (A); females (F); males (M). Lower case letters indicate statistically significant differences (Tukey test, $p < 0.05$) between the sexes and the sampling months for each tissue type.



844 **Figure 10.** Biplot of score and loadings from principal component analysis. The scores of each
845 organ (gill, kidney, liver, muscle, and spleen) are denoted by a color and a symbol (largest symbol =
846 average value). Confidence ellipses plot convex hull values of each organ.



*Graphical Abstract



*Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)

Highlights

- The influence of environmental factors on oxidative stress biomarkers was assessed
- Temperature, pH, Cu, Pb and Hg showed the major fluctuation during seasons
- A seasonal-linked variability of oxidative stress biomarkers level was recorded
- Both sexes showed similar level of oxidative stress biomarkers
- Food intake in summer mainly affected oxidative stress biomarkers

Oxidative stress ecology in brook trout (*Salvelinus fontinalis*) from a high-mountain lake (Cottian Alps)

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Abstract

High-mountain lakes are pristine ecosystems characterized by extreme environmental conditions. The atmospheric transport of pollutants from lowlands may add further stress to organisms inhabiting these environments. We investigated the environmental stress pressure on brook trout (*Salvelinus fontinalis*) from a high-mountain lake in the Cottian Alps (Piedmont, northwest Italy). To do this, males and females of brook trout were sampled from Balma Lake in summer (August) and autumn (October) 2017 in order to assess the influence of trace elements accumulation and

environmental parameters (physicochemical parameters and nutrient characteristics of water) on oxidative stress biomarkers. Bioaccumulation of Al, As, Cd, Cr, Cu, Fe, Hg, Pb, Ni, Se, and Zn and metallothionein levels were measured in muscle tissue of males and females. Liver, gills, kidney, and spleen tissue samples were analyzed for superoxide dismutase, catalase, total glutathione peroxidase, selenium-dependent glutathione peroxidase, glutathione reductase, and glutathione S-transferase activity. Analysis of environmental parameters showed changes in biomarker levels with seasonal variations. Water temperature was significantly higher in summer than autumn (Wilcoxon test; $p = 0.0078$), while pH was significantly higher in autumn than in summer (Wilcoxon test; $p = 0.0078$). Sex-related differences in oxidative stress biomarkers in tissues were unremarkable, whereas seasonal variability of oxidative stress biomarkers was observed, with major differences occurred for liver in summer and for gills, kidney, spleen and muscle in autumn. Positive correlations between environmental parameters and biomarkers were noted. Major fluctuations in water temperature, pH, Cu, Pb and Hg produced changes in biomarker levels; however, increased food intake during the ice-free season was probably the main factor that influenced changes in oxidative stress biomarker levels in brook trout in this extreme ecosystem.

Keywords: Alpine lakes; extreme ecosystems; oxidative stress biomarkers; trace elements

1. Introduction

Alpine lakes are remote, extreme ecosystems under harsh climatic conditions (Catalán et al., 2006). The ice-free season lasts for few months, generally from mid-June to late October. During this brief period of ideal conditions, some aquatic organisms can complete their life cycle before the snow covers the lakes again. Oligotrophic water conditions, UV radiation intensity, together with extreme temperatures allow for the development of a few dominant but well-adapted species (Sommaruga, 2001; Füreder et al., 2006; Pastorino et al., 2019a). These characteristics underlie the negative

correlation between altitude and biodiversity (Rott, 1988; Starkweather, 1990). Due to their remote location, Alpine lakes are often considered pristine, unpolluted ecosystems (Pastorino et al., 2019b). Since the 1980s, however, they have been affected by the global anthropogenic impact of pollutants transported from lowland emission sources and the introduction of alien species (Tiberti et al., 2014; Pastorino et al., 2020). These ecosystems have low resilience to disturbances and can be particularly sensitive to the release of fish species for recreational angling, with important consequences along the entire trophic chain (Tiberti et al., 2014; Milardi et al., 2016; Perrine, 2017; Raposeiro et al., 2017). For example, the release of salmonids, especially brook trout (*Salvelinus fontinalis*), has had a dramatic outcome for zooplanktonic, macrobenthic, and amphibian communities in several Alpine lakes (Tiberti et al., 2014).

Alpine lakes are also a receptor for medium atmospheric transported (MRAT) contaminants (Ferrario et al., 2017), as observed in the Arctic (Hung et al., 2016), which is subject to the long-range transport potential (LRTP) of many chemicals. Altitudinal transport in the European Alps can occur over relatively short distances from sources of pollution in the industrialized areas of Germany, Switzerland, Austria, and northern Italy (Poma et al., 2017). The input of contaminant compounds into Alpine lakes is controlled by atmospheric deposition and condensation (Schmid et al., 2007). The contaminants are bioaccumulated by the organisms inhabiting these ecosystems. Because fish occupy the uppermost trophic level, they provide an excellent bioindicator for the atmospheric medium-long range input of persistent organic compounds such as pesticides, brominated flame retardants (Schmid et al., 2007), and trace elements (Rognerud et al., 2002; Yang et al., 2007).

Moreover, fish are used as sentinel organisms to detect environmental contamination (Squadrone et al., 2013, 2014, 2016). They provide a useful model for assessing the impact of pollutants on biological functions such as detoxification (Elia et al., 2010). Assessment of contaminants in aquatic organisms can estimate and quantify the bioavailable fraction that might have the potential to induce an effect. However, because determination of body concentrations alone does not provide

valuable data about the effects, quantification of some biological responses is necessary to evaluate the health state of contaminant-exposed organisms (Elia et al., 2010). Metals, for instance, are pro-oxidants that exert oxidative stress via reactive oxygen species (ROS) production and cause critical changes in cellular biotransformation/detoxification pathways (Lushchak, 2016). Biomarker levels can be also influenced by abiotic factors such as pH, dissolved oxygen content, and water temperature (Sroda and Cossu-Leguille, 2011). Water temperature is a major factor in physiological processes in fish and can induce the production of ROS (Lushchak, 2011).

Oxidative stress results from an imbalance between pro-oxidants such as ROS and the protective antioxidant system. Mechanisms involve the activity of numerous antioxidant enzymes, including superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), selenium-dependent glutathione peroxidase (Se-GPx, EC 1.11.1.9), glutathione reductase (GR, EC 1.6.4.2), glutathione S-transferases (GST, EC 2.5.1.18), as well as the rates of metal-trapping molecules such as reduced glutathione and metallothioneins (MTs). They are important protective metabolic pathways that are used as biomarkers of pollutant-induced oxidative stress. Selected oxidative stress biomarkers have proven useful to assess the impact of a range of metals in aquatic organisms (Al Kaddissi et al., 2014; Cozzari et al., 2015; Elia et al., 2006, 2007a, 2007b, 2010). Furthermore, contaminant levels and antioxidant enzyme activity in aquatic organisms may change with the season and in response to biological and environmental pressures (Monserrat et al., 2007). This poses a limitation on field studies, because biochemical response may be linked to either fish physiology or exposure to contaminants.

To our best knowledge, no studies are available about oxidative stress in fish from Balma Lake, an Alpine lake in Piedmont (Cottian Alps, northwest Italy). Originally, the lake was fishless and brook trout was released for recreational fishing. With the present study we investigated: a) the physicochemical parameters and nutrient characteristics of lake water; b) the biometric characteristics and stomach contents of brook trout (*S. fontinalis*); c) the trace element accumulation in muscle because it constitutes a stable pool of trace elements for fish (Barwick and Maher, 2003);

d) the biomarkers of oxidative stress in muscle, liver, kidney, gills, and spleen in male and female individuals captured in Balma Lake in summer and autumn 2017.

2. Materials and Methods

2.1 Study site

Balma Lake (45° 02’ 14’’ N; 07° 10’ 52’’ E) is located at 2100 m above sea level in the municipality of Coazze, a small town 40 kilometers from Turin (Piedmont, northwest Italy). It is a typical glacial-origin lake in the Cottian Alps, within the SCI/ZSC IT1110006 - Orsiera Rocciavrè. The lake is located above the tree line and is covered by ice from November to early June. Originally, the lake was fishless, and *S. fontinalis* was released for recreational fishing during the 1970s (Pastorino et al., 2020). The main substrate of the area is composed of ophiolite metamorphic bedrock. The main source of impact is the medium-long distance transport of pollutants from the plain, grazing activities, and angling during the ice-free period. No previous studies or data about the lake’s hydrochemistry, taxa composition, and trace element accumulation are available. During summer 2017 morphometric and bathymetric survey of the lake was carried out by GeoStudio RC (Giaveno, Italy) using flying and floating drones. The lake measures 414 m in perimeter, 1.21 ha in surface area, and 6.42 m maximum depth in the central zone (Fig. 1).

2.2 Physicochemical parameters and nutrients of lake water

During both sampling periods the main physicochemical parameters were monitored at 5 sites in the littoral zone (in the upper centimeters of water) and 3 sites in the deep zone (in the water column) (Fig. 1). Water temperature (°C), dissolved oxygen (% saturation; mg L^{-1}), conductivity (µS cm^{-1}), and pH were recorded using field meters (HI 9033 conductivity meter, HI 9125 pH/ORP meter, HI 9147 oximeter, Hanna Instruments Inc. Woonsocket, RI, USA). Three replicates were carried out for each parameter. Water samples were collected in sterile containers (three 1-L bottles for each site), taking care not to include sediment particles, and then brought to the laboratory in a

refrigerated container within a few hours. Concentrations of NH_4^+ (mg L^{-1}), NO_3^- (mg L^{-1}), and PO_4^{3-} (mg L^{-1}) were measured using a multi-parameter benchtop photometer (HI 83200-02, Hanna Instruments Inc.). NO_3^- (mg L^{-1}) concentration was obtained by measuring absorbance at 525 nm via an adaptation of the cadmium reduction method (APHA et al., 1998); NH_4^+ (mg L^{-1}) concentration was obtained by measuring the absorbance at 420 nm (ASTM, 2015) via adaptation of the Nessler method; finally, PO_4^{3-} (mg L^{-1}) concentration was obtained by measuring absorbance at 610 nm via adaptation of the ascorbic acid method (APHA et al., 1998).

2.3 Fish sampling

Fish sampling campaigns were carried out during summer (August) and autumn (October) 2017. These months were chosen so that we could reach the sampling site on foot during the ice-free period. Permission for sampling was granted by the competent authority (Città Metropolitana di Torino; authorization n. 176-19040/2017). Fish were captured using 4 multimesh gillnets (36 x 1.8 m) divided into 6 panels of different mesh size (10 to 38 mm) to capture all size classes indiscriminately, except for offspring. The gillnets were randomly placed in the lake (Fig. 1) for 3 hours and then recovered. For each sampling period, 20 individuals (Table 2) were sacrificed after deep anesthesia with a lethal concentration (200 mg kg^{-1}) of tricaine methanesulfonate (MS-222) dissolved in water. The fish were necropsied, sexed, weighed, and measured for total length in the field. Immediately thereafter, samples of gill, liver, spleen, kidney, and muscle of each specimen were collected, packed in dry ice, and transported to the laboratory.

Stomach contents analysis was performed to obtain information about fish diet and to characterize seasonal variations. Stomachs were preserved in 70% alcohol, and the contents were inspected by stereomicroscopy (Zeiss Stemis V8, Jena, Germany). The ingested prey was identified to the order or family level, since closer identification was precluded by the digestion status of the organisms. To describe the diet, prey frequency of occurrence (Fi) (Tiberti et al., 2016) was calculated for both seasons.

157

158 *2.4 Trace elements in fish muscle*

159 Trace elements in fish muscle from males and females were analyzed by inductively coupled
160 plasma-mass spectrometry (ICP-MS Xseries II, Thermo Scientific, Bremen, Germany).
161 Determination of Al, As, Cd, Cr, Cu, Fe, Ni, Pb, Se, and Zn was performed following protocols
162 reported by Squadrone et al. (2016). Hg concentration was determined on a direct mercury analyzer
163 (DMA-80 Analyzer, Milestone, Shelton, CT, USA). Analytical performance was verified by
164 processing certified reference materials (Oyster Tissue - SRM 1566b from the National Institute of
165 Standard and Technology), along with blank reagents in each analytical session. Table S1 presents
166 the reference material values and the percentages of recovery. The analytical method was validated
167 according to ISO/IEC 17025 (general requirements for the competence of testing and calibration
168 laboratories).

169

170 *2.5 Biochemical analyses*

171 A total of 40 specimens of *S. fontinalis*, 26 females (12 in August and 14 in October) and 14 males
172 (8 in summer and 6 in autumn) were examined individually for oxidative stress biomarkers. Liver,
173 gills, kidney, spleen, and muscle were analyzed for SOD, CAT, GPx, Se-GPx, GR, and GST
174 activity, and MT levels only for muscle. For enzymatic analysis, the samples were homogenized
175 with an UltraTurrax homogenizer in 100 mM potassium-phosphate buffer, pH 7.5, added with 2.5%
176 sodium chloride (NaCl), 0.008 TIU ml^{-1} aprotinin and 0.1 mg ml^{-1} bacitracin. The homogenates
177 were centrifuged at 50,000 x g for 30 minutes at 4°C. Cytosolic fractions were used to determine
178 antioxidant biomarker activity. Biochemical analyzes were performed according to the methods
179 reported in Elia et al. (2017). Briefly, SOD activity was assessed at 550 nm in 50 mM Na_2CO_3
180 buffer, pH 10, 0.1 mM EDTA, 500 mM cytochrome C and 1 mM hypoxanthine and xanthine
181 oxidase. Cytochrome C reduction by the xanthine/hypoxanthine complex was evaluated by
182 comparison with a standard SOD unit curve. CAT activity was measured at 240 nm after the

183 decrease in absorbance following the consumption of H_2O_2. The assay was carried out in NaH_2PO_4
184 buffer + Na_2HPO_4 100 mM pH 7 and H_2O_2 24 mM. Total glutathione peroxidase (GPx) and
185 selenium-dependent glutathione peroxidase (Se-GPx) activity was determined at 340 nm in
186 NaH_2PO_4 + Na_2HPO_4 100 mM buffer, pH 7.5, 1 mM EDTA, 0.12 mM NADPH (b-nicotinamide
187 adenine dinucleotide), 2 mM GSH, 1 U of GR (glutathione reductase), 1 mM NaN_3 and H_2O_2 0.6
188 mM for Se-GPx or 1 mM DTT and 0.8 mM cumene hydroperoxide for GPx. GR activity was
189 measured at 340 nm in NaH_2PO_4 + Na_2HPO_4 100 mM buffer, pH 7, 1 mM GSSG (oxidized
190 glutathione), and 0.06 mM NADPH. GST activity was measured at 340 nm using CDNB (1-chloro-
191 2,4-dinitrobenzene) as substrate. The assay was carried out in 100 mM NaH_2PO_4 + Na_2HPO_4 100
192 mM buffer, pH 6.5, 2 mM GSH and 2 mM CDNB. Concentration of cytosolic proteins was
193 determined according to the method of Lowry et al. (1951) and used to normalize biomarker
194 activity.
195 Metallothionein (MT) levels were measured in the muscle tissue of individuals of both sexes.
196 Samples were homogenized (1:4) in a buffer containing 0.02 M TRIS/HCl, 0.5 M sucrose, 0.1 mg
197 ml^{-1} bacitracin, 0.008 tiu ml^{-1} aprotinin, 87 µg ml^{-1} phenylmethylsulfonyl fluoride (PMSF), and 0.1
198 µl ml^{-1} α-mercaptoethanol. The homogenates were centrifuged at 14,500 x g at 4°C to obtain the
199 cytosolic fraction. The supernatants were purified using a chloroform/ethanol solution and then
200 HCl/ethanol to obtain the partially purified MT fraction. The pellets were washed with
201 ethanol/chloroform/TRIS/HCl (87/1/12) solution and suspended in 0.25 M NaCl. A destabilizing
202 solution (HCl 1N + EDTA 4 mM) and Ellman's reagent (DTNB: 5,5 dithiobis-2-nitrobenzoic acid)
203 were added to each sample. Sulphydril residue contents (-SH) were spectrophotometrically
204 quantified. Absorbance was measured at 412 nm and compared to that obtained from a standard
205 curve with reduced glutathione (1 mg ml^{-1} GSH). All biochemical analyses were performed in
206 triplicate for each sample on a Varian spectrophotometer (Cary 50 Thermostat Cell Holder) at a
207 constant temperature of 25°C.
208

209 *2.6 Statistical analyses*

210 Spearman's rank correlation coefficient (ρS) was used to test for correlations between trace element
211 concentration in fish muscle, water physicochemical parameters, nutrients, and oxidative stress
212 biomarkers. Differences in the concentration of physicochemical parameters, nutrients, and trace
213 elements between seasons were tested using the Wilcoxon test. Data from the biochemical analysis
214 are reported as the mean and standard deviation (SD). Significant differences between sexes and
215 months were tested using one-way ANOVA followed by Tukey's multiple comparison test.
216 Homogeneity of variance was checked using Bartlett's test. The criterion for significance was set at
217 $p < 0.05$. Principal component analysis (PCA) was performed to check for trends in trace elements,
218 biomarkers, and physicochemical values between the sampling seasons (summer and autumn).
219 Statistical analyses were performed using open source data analysis software RStudio® version
220 1.1.463 (RStudio, Inc.).

221

222 **3. Results**

223 *3.1 Physicochemical and nutrient characteristics of lake water*

224 Lake water temperature was significantly lower in autumn (range 7.20-8.80°C) than summer (range
225 14.60-16.10°C) (Wilcoxon test; $p = 0.0078$) in agreement with seasonal trends; no thermal
226 stratification in the water column was observed, since the temperatures recorded at the deep sites
227 were similar to those of the littoral sites in both seasons. pH values were significantly higher in
228 autumn (range 7.53-7.90) than summer (range 6.52-7.31) (Wilcoxon test; $p = 0.0078$). No
229 differences in oxygen saturation were recorded between summer (range 77-103%) and autumn
230 (range 101-107%) (Wilcoxon test; $p > 0.05$). Water conductivity remained constant between
231 summer and autumn (range 17-21 µS cm^{-1}) (Wilcoxon test; $p > 0.05$). PO_4^{3-} concentration was ≤
232 0.02 mg L^{-1} in both seasons. NH_4^+ level was < 0.14 mg L^{-1} at all sites, except for station 4 (0.20 mg
233 L^{-1}) in summer. NO_3^- level was < 9.20 mg L^{-1} at all sites, except for site 4 (12 mg L^{-1}) in summer.

There were no significant differences in PO_4^{3-}, NH_4^+, and NO_3^- levels between seasons (Wilcoxon test; $p > 0.05$). Table 1 presents the changes in physicochemical and nutrient data (mean ± SD) .

3.2 Fish and stomach contents

Table 2 presents the average total length and weight of fish captured during summer and autumn 2017. Stomach contents analysis revealed the almost exclusive presence of terrestrial insect preys in both summer (94.19%) and autumn (81.04%). Preys belonged to the order Hymenoptera (77.74% and 66.39% in summer and autumn, respectively) and Coleoptera (16.45% and 14.65% in summer and autumn, respectively). Other taxa were present in extremely low proportions (Diptera Chironomidae larvae: 5.18% in summer and 2.79% in autumn).

3.3 Trace elements in fish muscle

Figure 2 presents the mean concentration and the relative standard deviation of each trace element detected in muscle tissue in summer and autumn. The mean concentration of trace elements was in the order: Zn (16.25) > Fe (8.78) > Al (1.49) > Se (0.67) > Cu (0.60) > Cr (0.14) > As (0.06) > Ni (0.06) > Pb (0.05) > Hg (0.01) mg Kg^{-1}. In autumn the mean concentration of trace elements was in the order: Zn (16.13) > Fe (10.63) > Al (2.03) > Se (0.49) > Cu (0.36) > Cr (0.16) > As (0.10) > Pb (0.10) > Ni (0.06) > Hg (0.02) mg Kg^{-1}. Cd was < LOQ (0.02 mg Kg^{-1}) in both seasons. There were no significant differences in trace element concentration between seasons (Wilcoxon test; $p > 0.05$ for all elements).

3.4 Biochemical analyses

In the liver, the activity of SOD, CAT, and GR was significantly lower in autumn (up to 70%) than summer in males and females (Tukey's test; $p < 0.05$) (Figs. 3-4, 7). In the gills, GPx activity was significantly higher in autumn (90%) mainly in the females (Tukey's test; $p < 0.05$); Se-GPx and GST activity was significantly higher (up to 2-fold) in males and females in autumn (Tukey's test; *p*

260 < 0.05) (Figs. 5, 6, 8). In the kidney, only Se-GPx activity was significantly different between the
261 seasons, being higher (up to 40%) in autumn (Tukey's test; $p < 0.05$) (Fig. 6). In the spleen, Se-GPx
262 activity was significantly lower (70%) in the females (Tukey's test; $p < 0.05$) (Fig. 6). GR and GST
263 activity was significantly higher (up to 70%) in males in autumn than in summer (Tukey's test; $p <$
264 0.05) (Fig. 7, 8). In muscle, SOD activity was significantly lower (up to 50%) in females in autumn
265 (Tukey's test; $p < 0.05$), whereas CAT activity showed an opposite trend and was significantly
266 higher in autumn (up to one-fold) (Tukey's test; $p < 0.05$) (Figs. 3-4). MT level was higher in
267 autumn (up to one-fold) than in summer (Tukey's test; $p < 0.05$) (Fig. 9).
268
269 *3.5 Spearman correlation matrix*
270 Spearman correlation matrix revealed correlations between environmental parameters (trace
271 elements, physicochemical parameters, nutrients) and oxidative stress biomarkers in muscle, gills,
272 liver, spleen, and kidney tissue for both seasons. Due to the multiplicity of positive correlations,
273 Table S2 presents the correlation matrices (one for each organ). Only the most informative
274 outcomes are presented and discussed for interpretation of biological response of *S. fontinalis* to
275 environmental parameters. In detail, a significant positive correlation was found between MTs and
276 Hg (ρS 0.787), MTs and Pb (ρS 0.787), MTs and Cu (ρS 0.683), MTs and pH (ρS 0.650), and CAT
277 and pH (ρS 0.737) in muscle tissues of females captured in autumn; SOD and Cr (ρS 0.837) in the
278 liver tissue of females captured in autumn; SOD and NO_3^- (ρS 0.750), Se-GPx and NO_3^- (ρS 0.750)
279 in muscle and kidney tissue, respectively, of females captured in summer.
280
281 *3.6 Principal Component Analysis (PCA)*
282 The first two principal components (Dim1; Dim2) accounted for meaningful amounts of the total
283 variance (58.2%), while the other components accounted for a relatively smaller fraction (Fig. 10).
284 Dim1 accounted for 35.2% of the total variance and was positively correlated with the variables Ni,
285 Se, temperature, oxygen, conductivity, and NO_3^- and negatively correlated with Al, Cr, Fe, As, Pb,

Hg, and pH. Dim2 accounted for 23% of the total variance and was positively correlated with the variables GST, CAT, GPx, Se-GPx, SOD, and GR. The biplot of loadings (variables) and score (observations) shows which organs (liver, gills, kidney, spleen, and muscle) of brook trout are closest to them, and which variables (trace elements, biomarkers and physicochemical parameters) contributed to this grouping in the coordinate of Dim1 and Dim2. Separation of organs by season (summer on the right, autumn on the left) suggested a difference in biomarker values. In detail, the autumn samples of spleen, gill, muscle, kidney, and liver tissue are on the left in order of increasing value of Al, Cr, Fe, As, Pb, Hg, and pH. The summer samples of spleen, gill, muscle, kidney tissue are on the right in order of increasing value of NH_4^+, NO_3^-, conductivity, oxygen, temperature, Zn, Cu, Ni, and Se. Remarkably, summer samples of liver tissue are well separated from other organs, following the trend in biomarker values.

4. Discussion

The environmental parameters of water oxygenation, pH, conductivity, and temperature are in line with those reported by Tiberti et al. (2010) for 12 alpine lakes in the Gran Paradiso National Park (Western Alps, Italy). Temperatures were lower in autumn than in summer according to seasonality. The temperature data recorded at the deep sites revealed no vertical layering in the lake because the shallow maximum depth (6.42 m) does not allow for the formation of a thermocline layer. The pH values are related to rock composition. Since Lake Bama lies over a granite bedrock, its pH values are lower than lakes on limestone or sandstone bedrock (Camarero et al., 2009). The pH values were in line with the literature reported for high-altitude environments (Boggero et al., 2006; Füreder et al., 2006; Fjellheim et al., 2009). As expected for mountain lakes, the oxygenation levels were high. The water conductivity values were in line with published literature, as conductivity of silty-like lakes tends to be < 50 $\mu S\ cm^{-1}$ (Boggero et al., 2006; Füreder et al., 2006). No decrease in oxygen values correlated with depth was observed owing to the absence of temperature

311 stratification. Nutrient levels (PO_4^{3-}, NO_3^- and NH_4^+) were in line with previous studies of Alpine
312 lakes (Füreder et al., 2006; Camarero et al., 2009) and revealed an oligotrophic condition.
313 We observed a positive correlation between environmental parameters and biological response in
314 the *S. fontinalis* from Balma Lake. The antioxidant response was tissue-specific; indeed, aerobic
315 tissues such as the kidney, gills, spleen, and the liver in particular have a high potential for ROS
316 production which is offset by protective mechanisms. Differently, muscle, which has a low content
317 of mitochondria and a low-intensive oxidative metabolism, showed mild response to
318 oxidative/reductive conditions. Moreover, the level of several biomarkers was related to the
319 seasonal changes in the trace element concentration in some tissues.
320 The few studies on trace element accumulation in the biota from high-altitude lakes in general, and
321 in Alpine lakes in particular, have focused largely on fish. Köck et al. (1996) studied the
322 concentrations of Cd, Pb, Zn, and Cu in the muscle of arctic char (*Salvelinus alpinus*) from five
323 oligotrophic Alpine lakes in northern Tyrol (Austria). Yang et al. (2007) studied the accumulation
324 of trace elements in muscle of fish of the genus *Gymoncypris* (Cyprinidae) from high-mountain
325 lakes in the Tibetan Plateau. Ni ranged from 0.094 to 0.12 mg kg^{-1}, Cu from 1.1 to 2.0 mg kg^{-1}, Zn
326 from 4.4. to 6.9 mg kg^{-1}, As from 0.24 to 0.27 mg kg^{-1}, Se from 0.36 to 1.0 mg kg^{-1}, Cd from 0.024
327 to 0.025 mg kg^{-1}, and Pb from 0.047 to 0.079 mg kg^{-1}. Rognerud et al. (2002) found that Hg
328 concentrations in fish muscle from high-mountain lakes in Europe ranged from 0.021 to 0.179 mg
329 kg^{-1}. These data from previous studies demonstrate that high-mountain lakes function as a regional
330 contaminant convergence zone for the medium and long-range atmospheric transport of
331 contaminants. Since our results are in line with these findings, and because no previous studies have
332 been performed to date, we assume that the trace element concentrations we detected in the brook
333 trout from Balma Lake have both anthropogenic and pedogenic sources as their origin.
334 Metallothioneins have been widely considered as valuable biomarkers that reflect the level of trace
335 elements in aquatic environments, where they act as metal trappers mainly of Cd, Hg, Pb, Cu, and
336 Zn in fish (Bourdineaud et al., 2006; Morcillo et al., 2016). The higher levels of MTs we measured

in autumn were related to the increased trace element concentration in this tissue, and we noted a strong correlation with Cu, Hg, and Pb levels in the females. This may suggest an alarming scenario, as it would signal an increased concentration of these elements in the environment. Although no chemical analyses of the lake water were carried out, because of the peculiar geomorphological characteristics of Balma Lake we can exclude an increase in such contaminants during autumn.

Seasonal variation in metal concentrations in fish can be related to environmental factors such as food availability, temperature, and biological cycle (Hermesz et al., 2001; Amiard et al., 2006; Dragun et al., 2009). Furthermore, fish size may also play a key role in metal uptake (Wright and Mason, 1999). In the present study, an increase in weight and length was measured in the fish from both sampling seasons, and the frequency of occurrence of terrestrial invertebrates in the diet during the ice-free season was in accordance with other studies performed on brook trout in other Alpine lakes (Sotiropoulos et al., 2006; Tiberti et al., 2016). It is possible that fish growth due to a higher intake of food in summer could have favored the uptake of heavy metals such as Pb and Hg.

This hypothesis is corroborated by a previous study on black scabbardfish *Aphanopus carbo* (Trichiuridae), in which an exponential increase in total Hg load was found in all fish tissues in a length-dependent manner (Bebianno et al., 2007). Furthermore, previous studies showed that fluctuations toward high pH values can also play a key role in modulating metals uptake, affecting their speciation and bioavailability (Playle, 1998). This outcome may explain the strong correlation between pH and the two hydroxyl scavengers MTs and CAT in the female muscle tissue in autumn.

Trace elements also affected the activity of several enzymes in different tissues in the autumn fish samples. We noted a strong correlation between Cr and SOD activity in the liver, as reported in previous study in rock fish *Sebastes schlegelii* (Kim and Kang, 2016). Furthermore, a recent study showed that Cr can induce conformational changes of CAT enzyme and reduce its activity depending on its valence states and concentration (Chen et al., 2018). These findings may explain the seasonal difference in CAT activity in the female muscle tissue.

In general, numerous elements can influence the activity of this biomarker, and As and Fe were the elements most involved in modulating CAT enzyme activity in the tissues. Arsenic is a global contaminant derived from natural or anthropogenic sources and a cause of great concern for terrestrial and aquatic ecosystems (Elia et al., 2018). At high concentrations, arsenic may induce oxidative stress by interacting with antioxidants and result in the accumulation of free radicals in cells. Arsenite species can interact with sulfhydryl groups of biomolecules such as enzymes or reduced glutathione (Elia et al., 2018). Furthermore, redox active metals such as iron generate ROS or are involved in the Fenton route, leading to lipid peroxidation (Mahboob, 2013). Despite the increase in trace element amount in autumn, the concentration of Pb, Hg, and Cd in fish muscle mentioned in Regulation 1881/2006 (European Commission, 2006) was far below the established threshold limit. This fact should be taken into account and may suggest the adaption of *S. fontinalis* to seasonal changes rather than to oxidative stress. PCA analysis showed that summer samples of liver tissue were strongly related to oxidative biomarker level, since liver tissue is the site of multiple oxidative reactions and maximal free radical generation (Gul et al., 2004; Avci et al., 2005).

For ectothermic organisms, temperature is a crucial abiotic factor (Hassan et al., 2017). Daily temperature fluctuations and seasonal variations differ in their influence on fish (Buckley et al., 2006; Place and Hofmann, 2004; Podrabsky and Somero, 2004). Wellness and growth are optimal within a well-defined temperature range depending on the species (Godowsky and Caddell, 1991). Temperatures deviating excessively from the optimum can exert harmful effects and induce mortality (Rijnsdorp et al., 2009). In the present study, the marked drop in water temperature in autumn was related to changes mainly in GPx activity in the male kidney and in the female spleen. Moreover, higher temperature results in increased GPx activity in other fish such as the Antarctic *Pagothenia borchgrevinki* (Almroth et al., 2015). Thermal changes have also been associated with the up regulation of the NRF2 transcription factor, which is involved in the expression of antioxidants via binding to the antioxidant responsive element (ARE) (Almroth et al., 2015).

389 Elevated GPx activity during summer may indicate strengthening of this fundamental defense line
390 against ROS. However, the lack of change in SOD and CAT activity, as well as the lower
391 concentration of trace elements in tissue, and the constant levels of the main physicochemical
392 parameters of water, except for pH and temperature, preclude an oxidative pressure scenario and
393 suggest an adaptive ability of *S. fontinalis* to higher temperature instead. On the other hand,
394 increased SOD activity in the muscle tissue of males and females is linked to an abiotic factor, such
395 as NO_3^-, in summer. Furthermore, nutrients also influenced SOD concentration in female muscle
396 and Se-GPx activity in female kidney in summer. In the aquatic environment, it is not unusual that
397 fish are simultaneously challenged by different abiotic factors. Conversely, at very high
398 concentrations, ammonia can induce a range of toxicological effects in fish, such as altered
399 metabolism, lack of growth, and mortality (Dosdat et al., 2003; Sinha et al., 2012, 2015). Ammonia
400 exposure can also lead to oxidative stress in fish (Sun et al., 2012; Sinha et al., 2014). A previous
401 study showed that conductivity plays a crucial role in maintaining the ammonia ionization
402 equilibrium (NH_3 and the non-toxic form NH_4^+) in aquatic environments (Sinha et al., 2015). In the
403 present study, the lack of change in conductivity and nutrients (e.g., phosphorus) between the
404 seasons suggests that the changes in antioxidant parameters may be related to food intake (which
405 indirectly promotes metals uptake) rather than to nutrient concentration. In their habitats, high-
406 mountain lakes included, fish are often exposed to periods of food insufficiency in response to
407 factors such as temperature, conductivity, and biological cycle (Pérez-Jiménez et al., 2007; Furné et
408 al., 2009). The increase in food intake during summer may also explain the fluctuation in
409 biomarkers of oxidative stress.
410
411 **5. Conclusion**
412 With this study we investigated the influence of trace element accumulation and environmental
413 parameters on oxidative stress biomarkers in male and female individuals of *S. fontinalis* from a
414 high-mountain lake during the ice-free period. Generally, positive correlations were found between

several environmental parameters and biomarkers. While oxidative stress biomarker levels were similar for males and females, significant fluctuation between seasons due to biological and environmental pressures was noted for several biomarkers. Although there was greater fluctuation in temperature, pH, and trace elements (e.g., Cu, Pb and Hg) between seasons, which certainly contributed to changes in biomarker levels, our findings indicate that increased food intake during the ice-free season was probably the main factor that affected the oxidative stress response. Future studies are needed to investigate other factors responsible for the changes in oxidative stress biomarkers.

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Table 1. Mean and standard deviation (three replicates) of physicochemical parameters and nutrients measured in Balma Lake in summer (August) and autumn (Autumn) 2017.

	Summer (August)	Autumn (October)
Temperature (°C)	15.62 ± 0.77	8.24 ± 0.53
Dissolved oxygen concentration (mg L^{-1})	7.40 ± 0.89	7.29 ± 1.92
Oxygen saturation (%)	92.88 ± 8.52	104.75 ± 2.43
pH	6.64 ± 0.28	7.69 ± 0.12
Conductivity (µS cm^{-1})	18.29 ± 1.12	18.56 ± 1.67
NH_4^+ (mg L^{-1})	0.09 ± 0.07	0.09 ± 0.05
NO_3^- (mg L^{-1})	8.55 ± 2.73	6.82 ± 2.14
PO_4^{3-} (mg L^{-1})	0.01 ± 0.01	0.02 ± 0.01

Table 2. Biometric values of females and males of brook trout (*Salvelinus fontinalis*) from Balma Lake in summer and autumn 2017.

	Summer (August)		Autumn (October)	
Sex	Males	Females	Males	Females
Number of individuals	8	12	6	14
Weight - W mean ± SD (g)	69.9 ± 30.34	71.20 ± 28.34	88.47 ± 56.23	90.05 ± 60.03
W min. (g)	15.00	15.36	10.23	11.00
W max. (g)	146.00	150.00	187.00	193.00
Total Length - TL mean ± SD (cm)	17.77 ± 3.90	18.56 ± 4.20	19.23 ± 4.66	19.35 ± 3.72
TL min. (cm)	11.50	11.00	7.50	8.00
TL max. (cm)	23.00	24.00	25.50	26.00

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711 **Figure 1.** Balma Lake: bathymetry and sampling sites for physicochemical parameters (L = littoral
712 sites; B = deep sites). Red rectangles indicate the placement of gillnets to sample fish in summer
713 (August) and autumn (October) 2017.

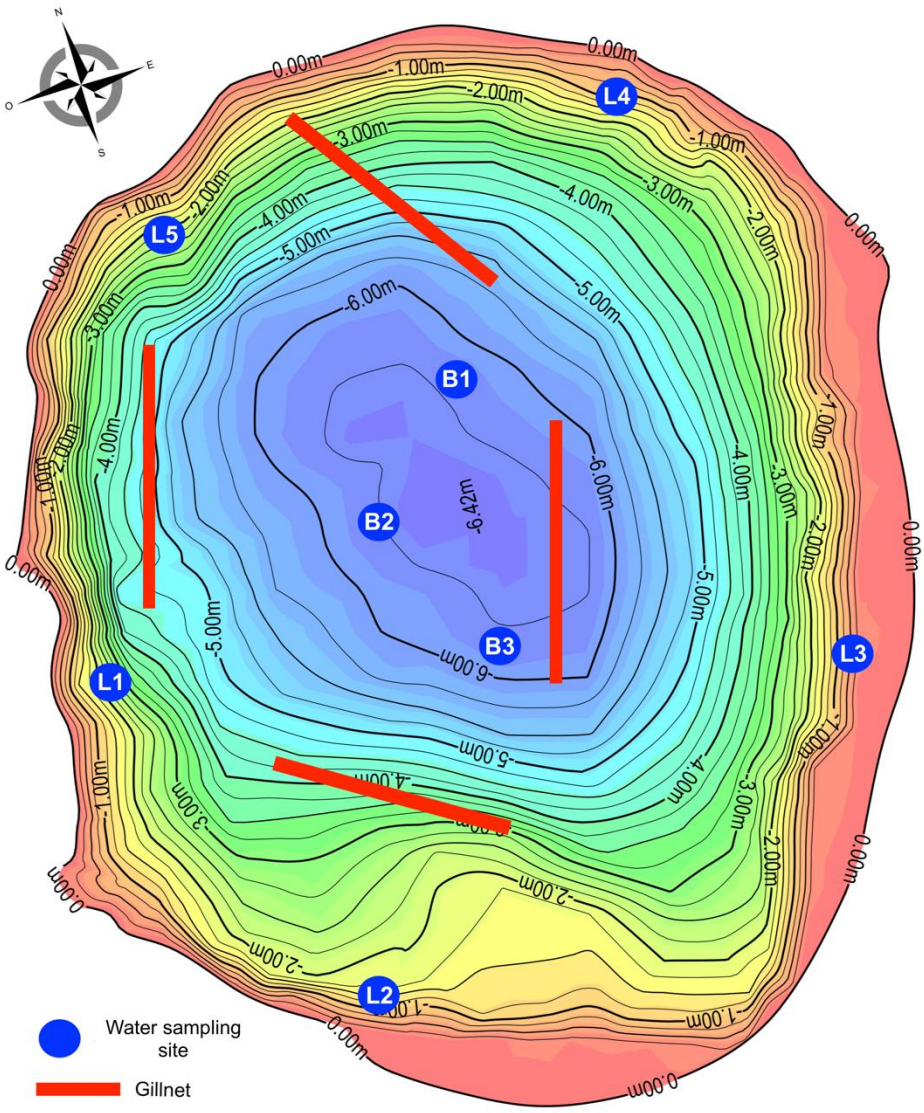

Figure 2. Trace elements (Log_{10} concentration; mg kg^{-1}) detected in the muscle tissue of brook trout in summer (August) and autumn (October) 2017.

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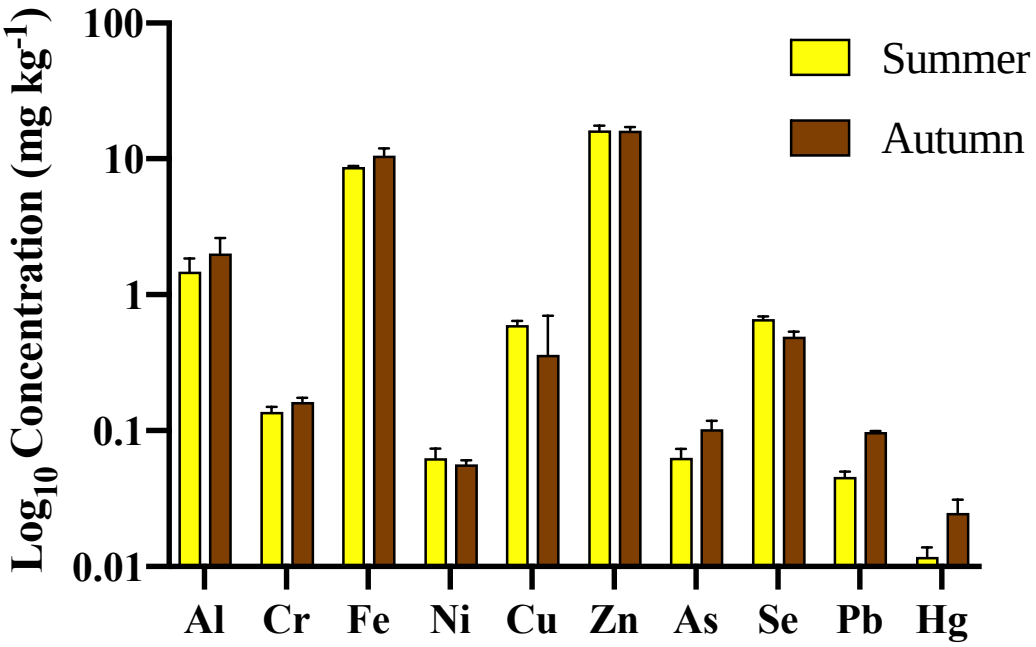
Log10 Concentration (mg kg-1)
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0.1
0.01
Summer
Autumn
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Cr
Fe
Ni
Cu
Zn
As
Se
Pb
Hg

Figure 3. Superoxide dismutase (SOD) activity in the liver, gills, kidney, spleen, and muscle tissue of female and male brook trout. Data are reported as mean ± standard deviation. Summer (S); Autumn (A); females (F); males (M). Lower case letters indicate statistically significant differences (Tukey test, $p < 0.05$) between the sexes and the sampling months for each tissue type.

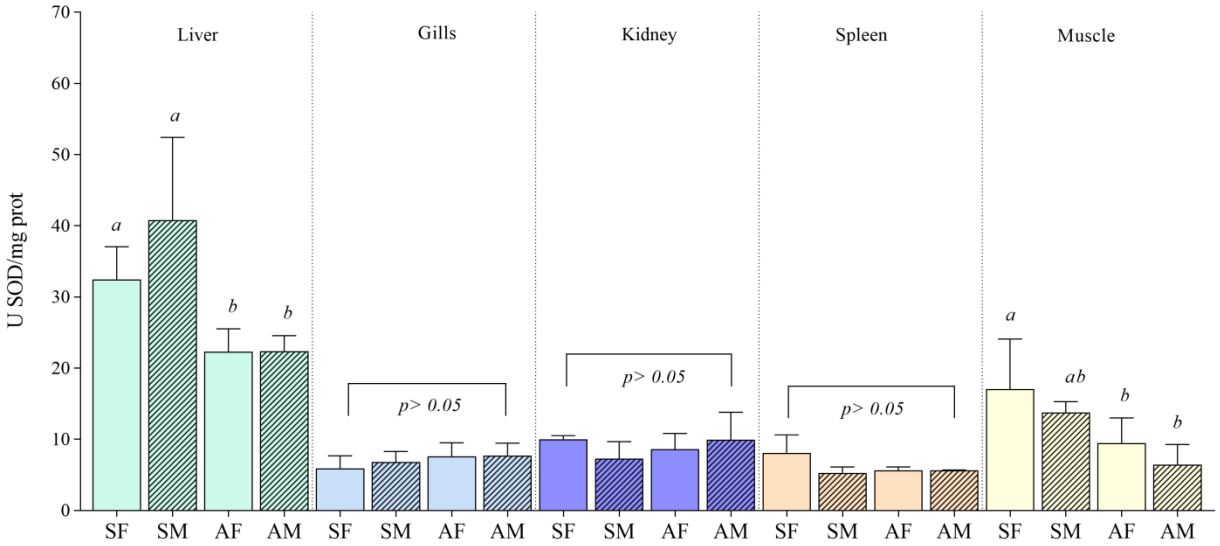
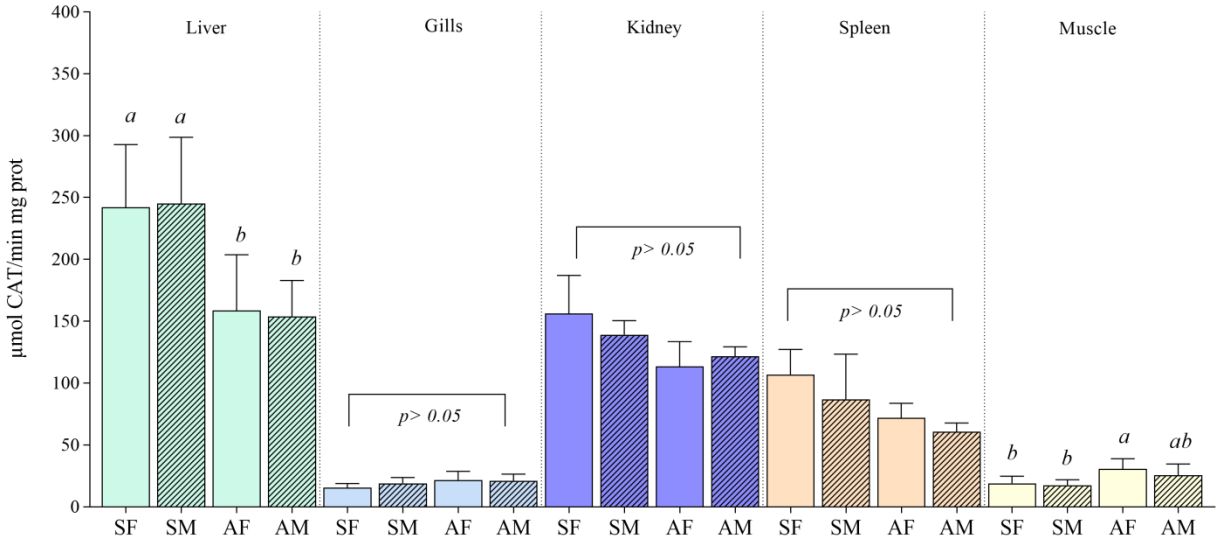


Figure 4. Catalase (CAT) activity in the liver, gills, kidney, spleen, and muscle tissue of female and male brook trout. Data are reported as mean ± standard deviation. Summer (S); Autumn (A); females (F); males (M). Lower case letters indicate statistically significant differences (Tukey test, $p < 0.05$) between the sexes and the sampling months for each tissue type.



764 **Figure 5.** Total glutathione peroxidase (GPx) activity in the liver, gills, kidney, spleen, and muscle
765 tissue of female and male brook trout. Data are reported as mean ± standard deviation. Summer (S);
766 Autumn (A); females (F); males (M). Lower case letters indicate statistically significant differences
767 (Tukey test, $p < 0.05$) between the sexes and the sampling months for each tissue type.

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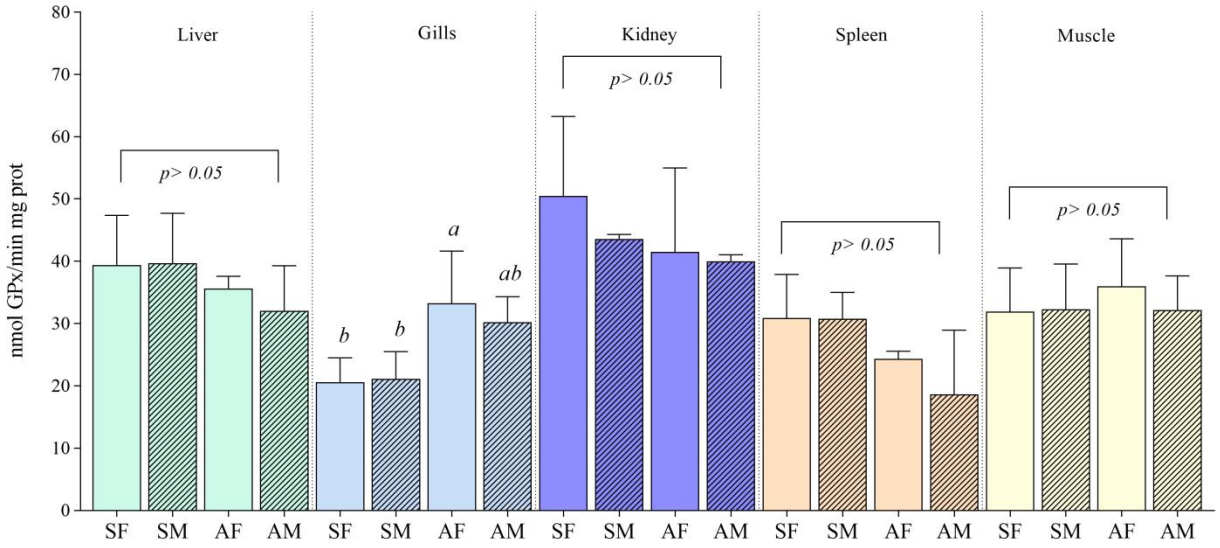

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Figure 6. Selenium-dependent glutathione peroxidase (Se-GPx) activity in the liver, gills, kidney, spleen, and muscle tissue of female and male brook trout. Data are reported as mean ± standard deviation. Summer (S); Autumn (A); females (F); males (M). Lower case letters indicate statistically significant differences (Tukey test, $p < 0.05$) between the sexes and the sampling months for each tissue type.

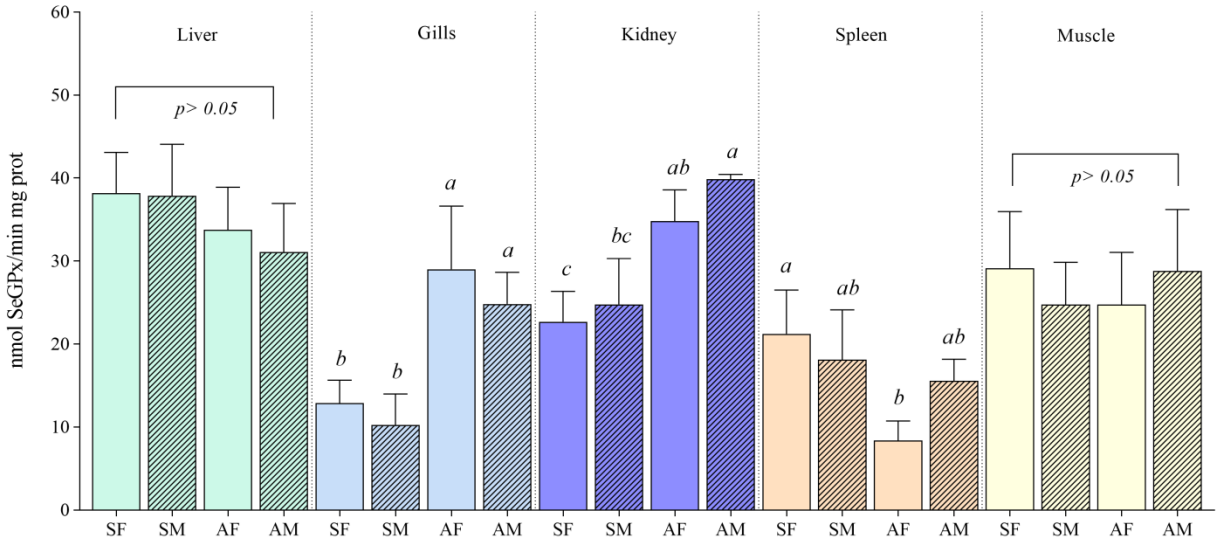

Figure 7. Glutathione reductase (GR) activity in the liver, gills, kidney, spleen, and muscle tissue of female and male brook trout. Data are reported as mean ± standard deviation. Summer (S); Autumn (A); females (F); males (M). Lower case letters indicate statistically significant differences (Tukey test, $p < 0.05$) between the sexes and the sampling months for each tissue type.

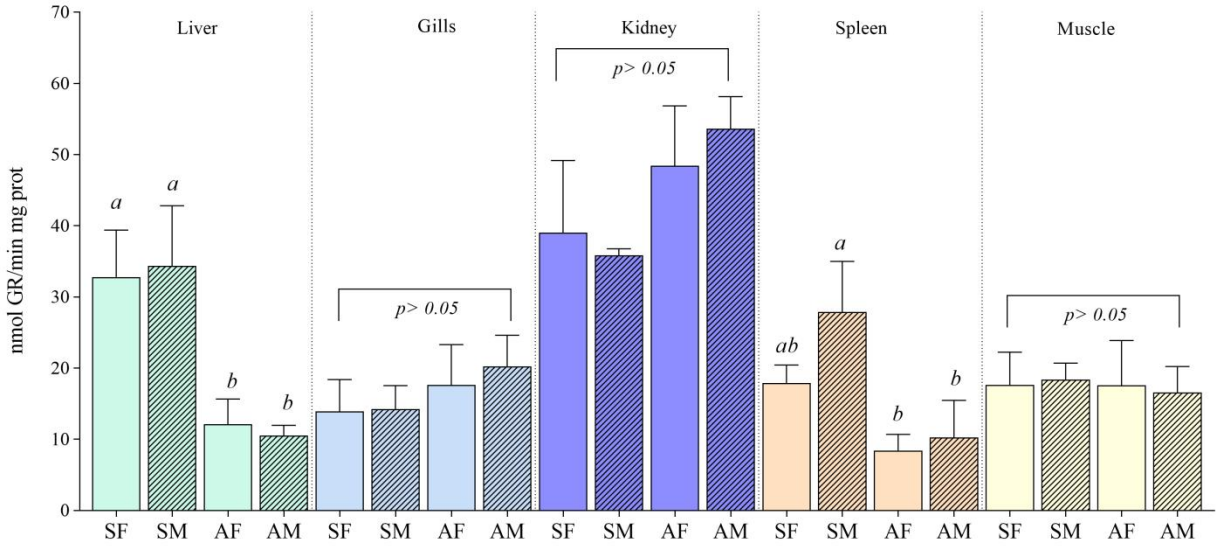

Figure 8. Glutathione S-transferase (GST) activity in the liver, gills, kidney, spleen, and muscle tissue of female and male brook trout. Data are reported as mean ± standard deviation. Summer (S); Autumn (A); females (F); males (M). Lower case letters indicate statistically significant differences (Tukey test, $p < 0.05$) between the sexes and the sampling months for each tissue type.

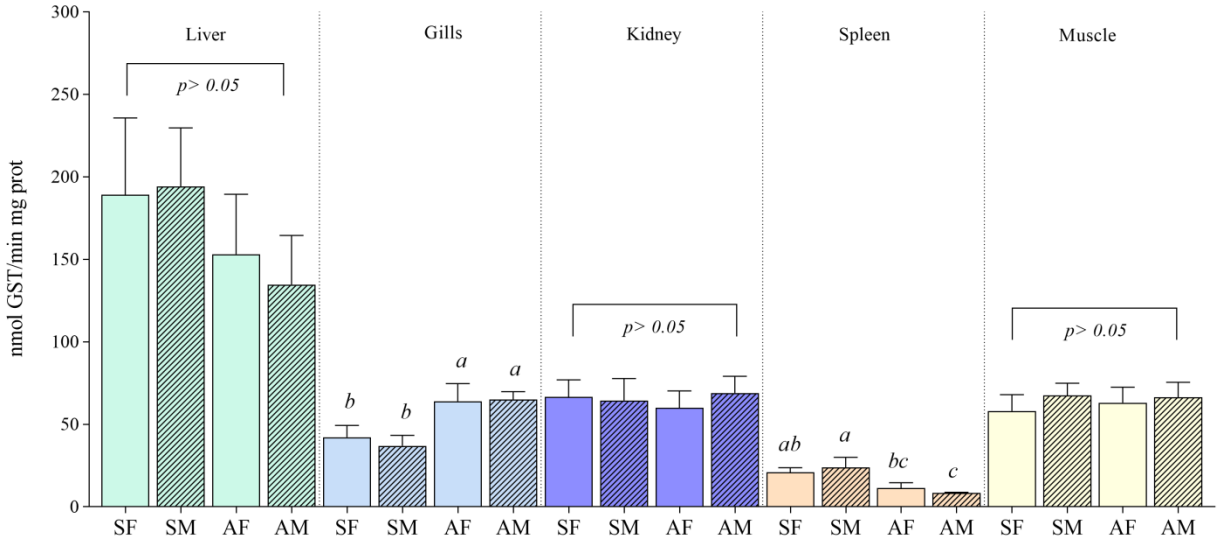

Figure 9. Metallothionein (MTs) concentration in the muscle tissue of female and male brook trout. Data are reported as mean ± standard deviation. Summer (S); Autumn (A); females (F); males (M). Lower case letters indicate statistically significant differences (Tukey test, $p < 0.05$) between the sexes and the sampling months for each tissue type.

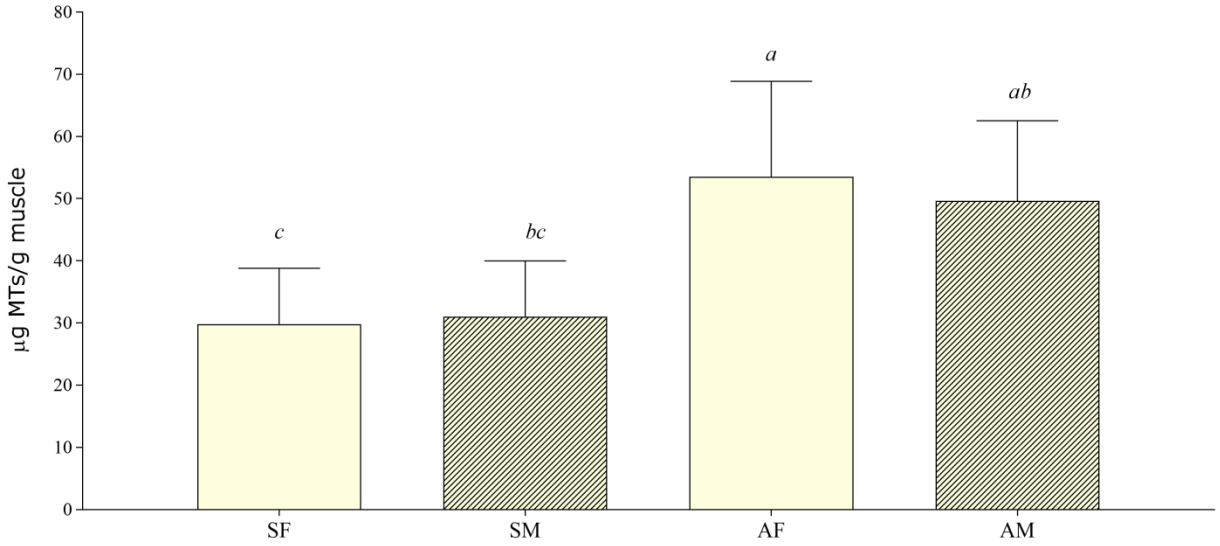

Figure 10. Biplot of score and loadings from principal component analysis. The scores of each organ (gill, kidney, liver, muscle, and spleen) are denoted by a color and a symbol (largest symbol = average value). Confidence ellipses plot convex hull values of each organ.

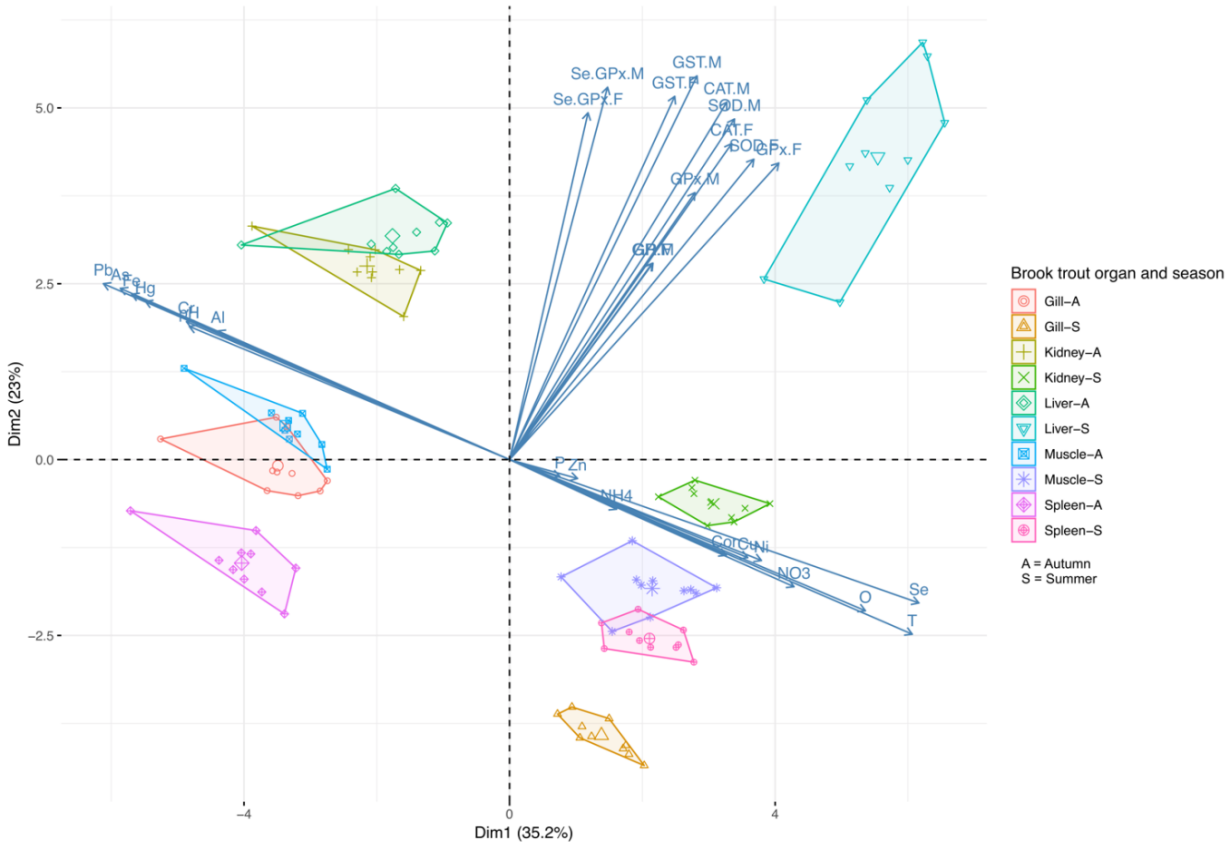

Figure 1

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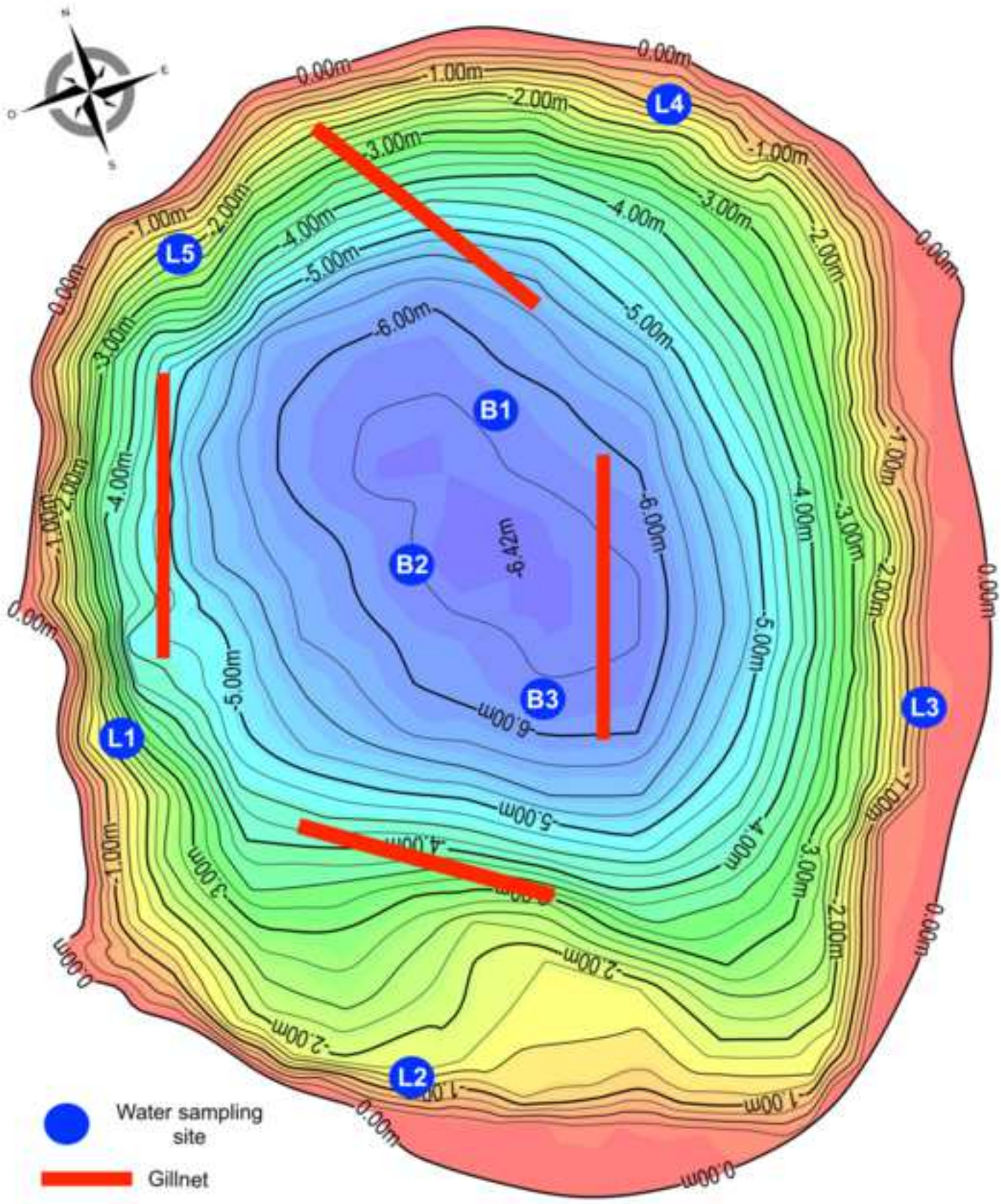

Figure 2

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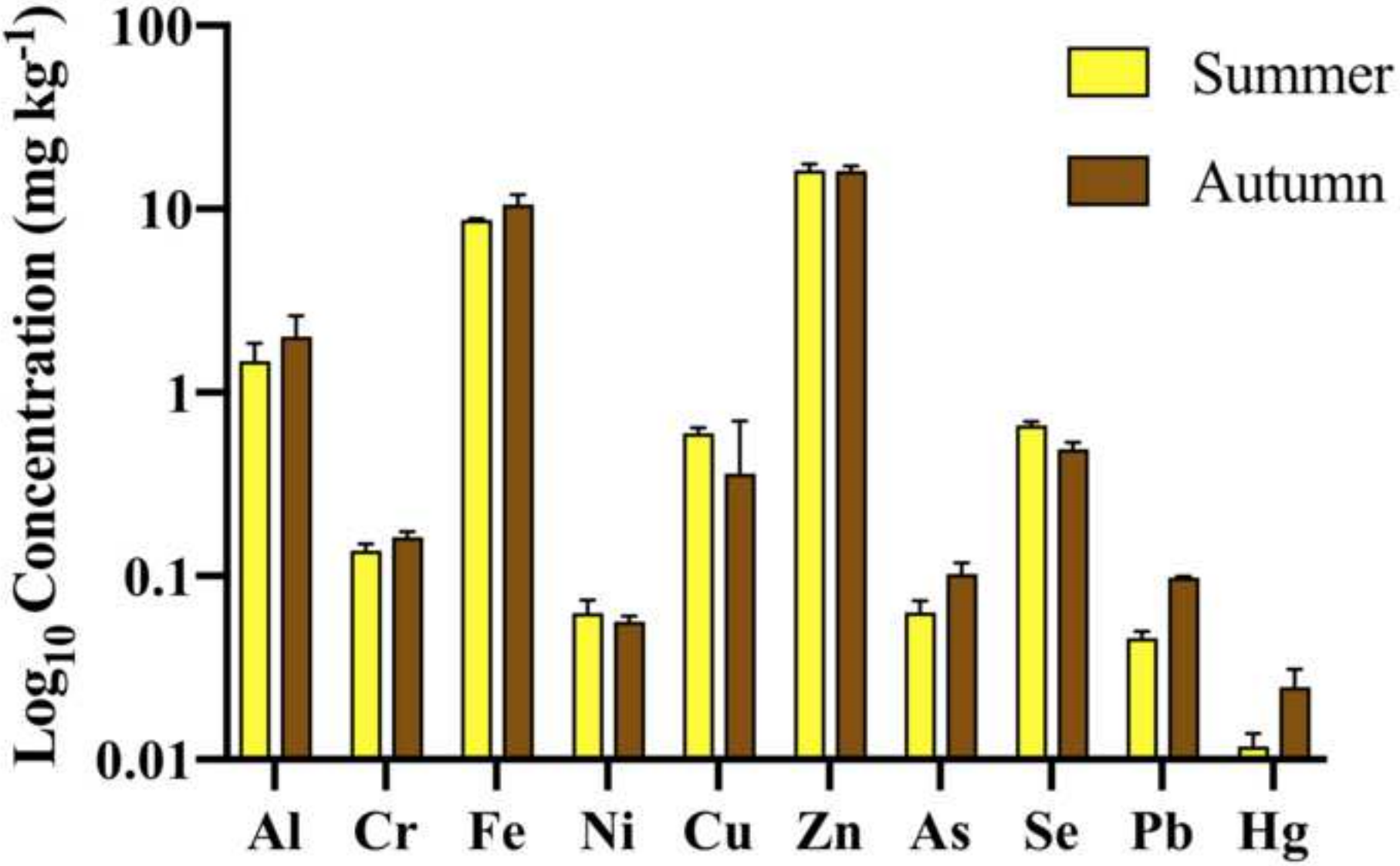

Figure 3

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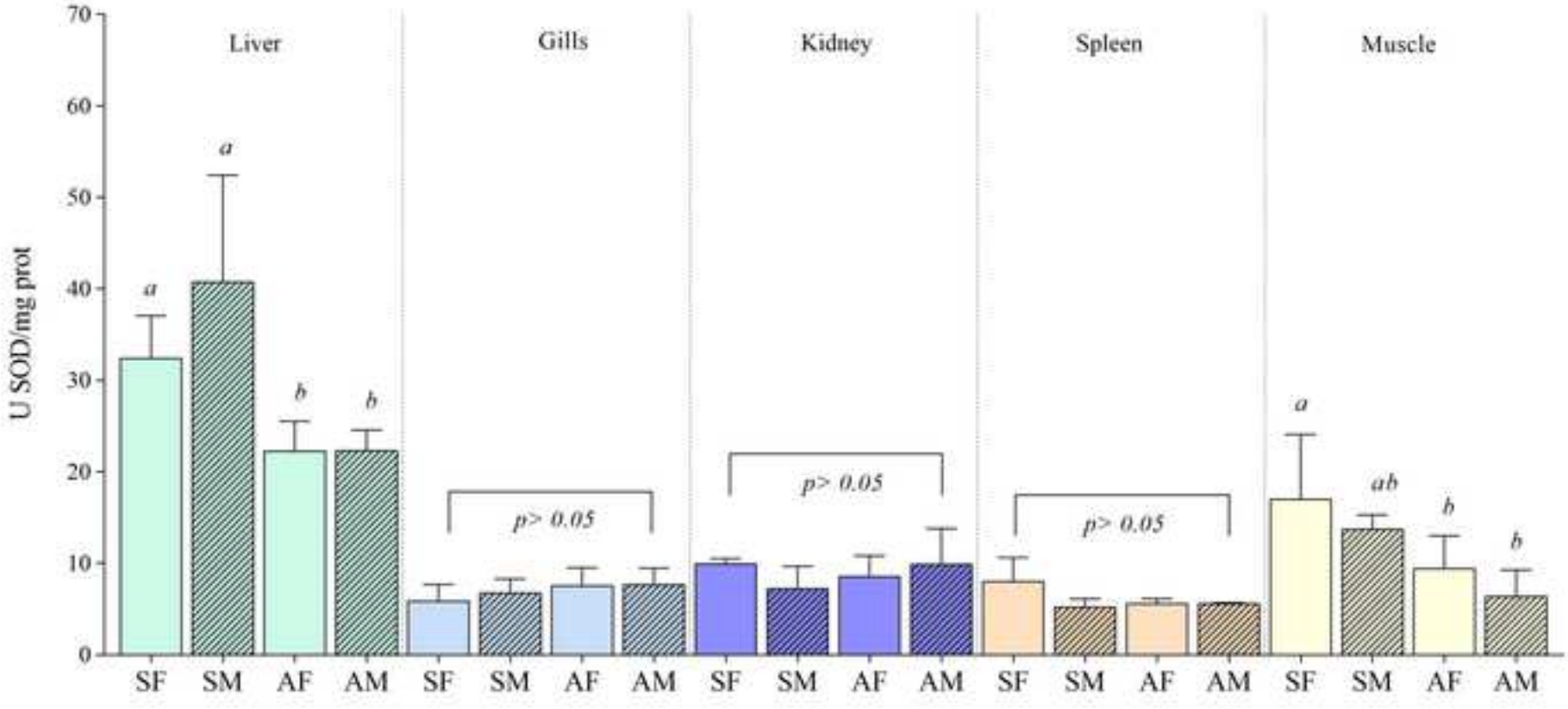

Figure 4

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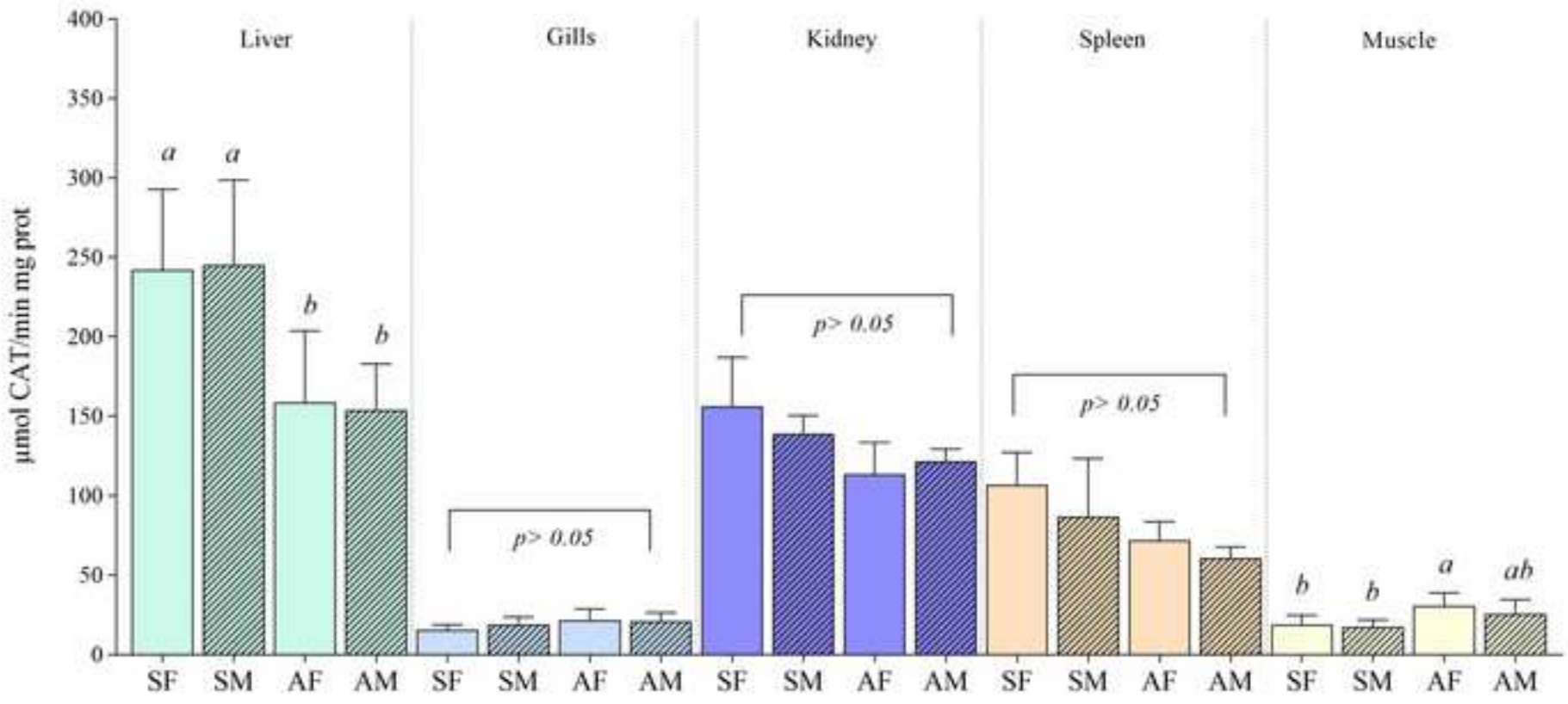

Figure 5

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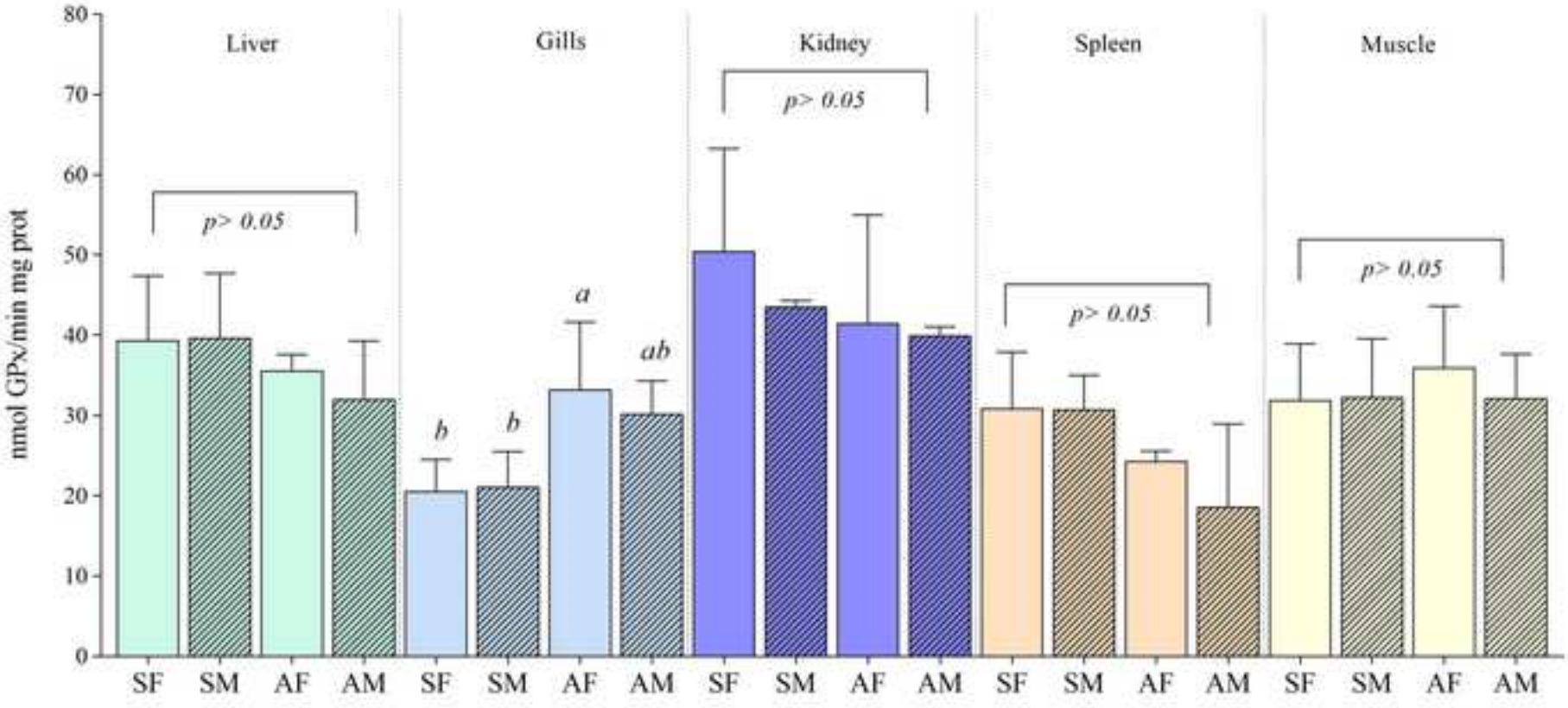

Figure 6

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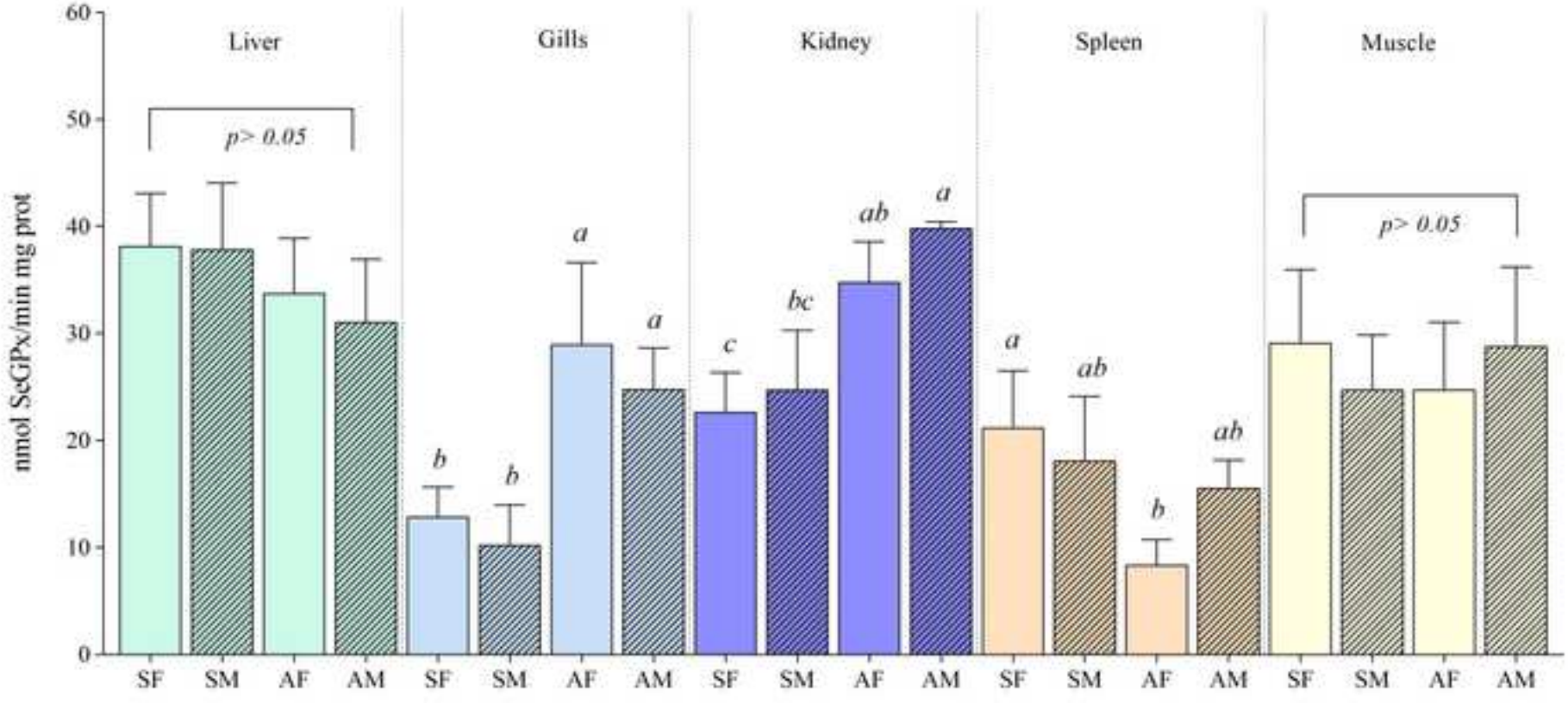

Figure 7

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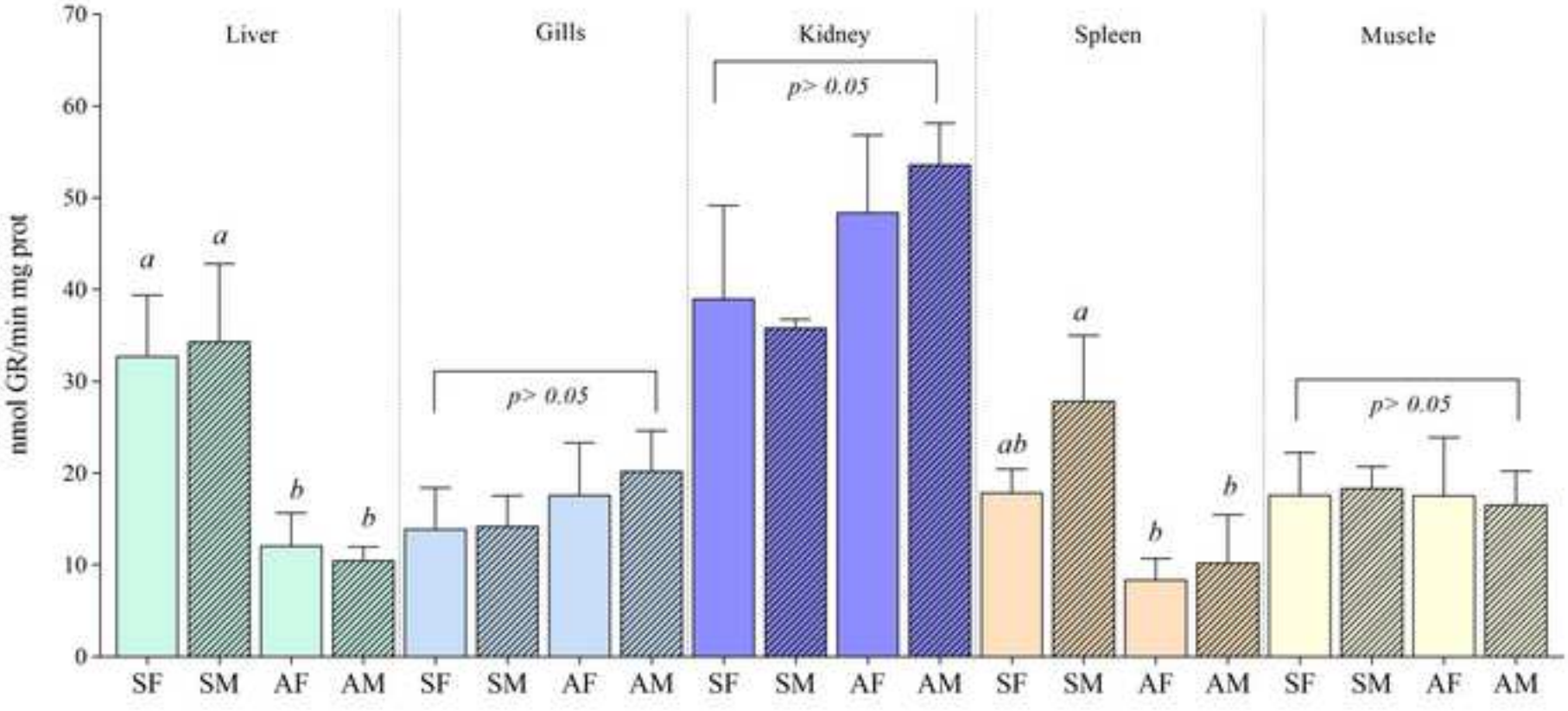

Figure 8

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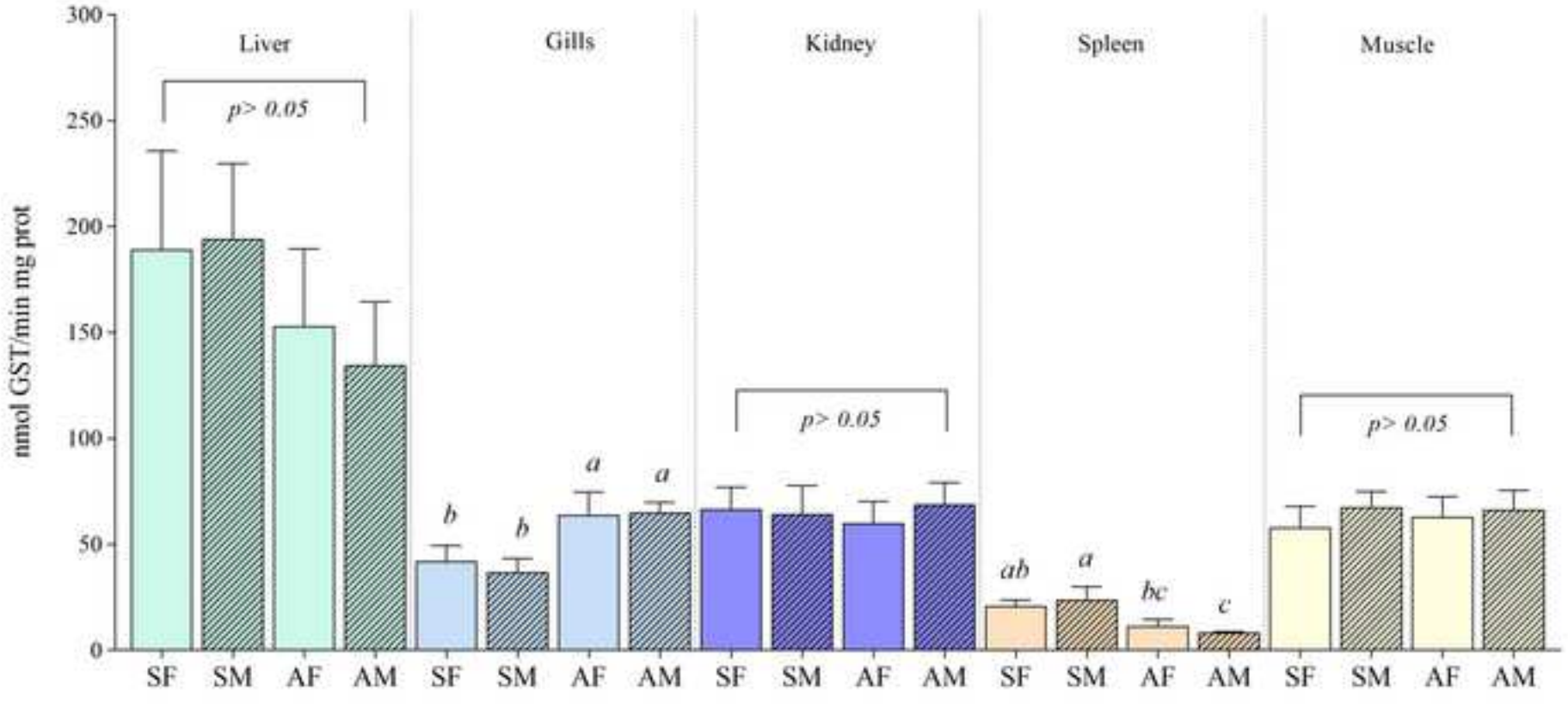

Figure 9

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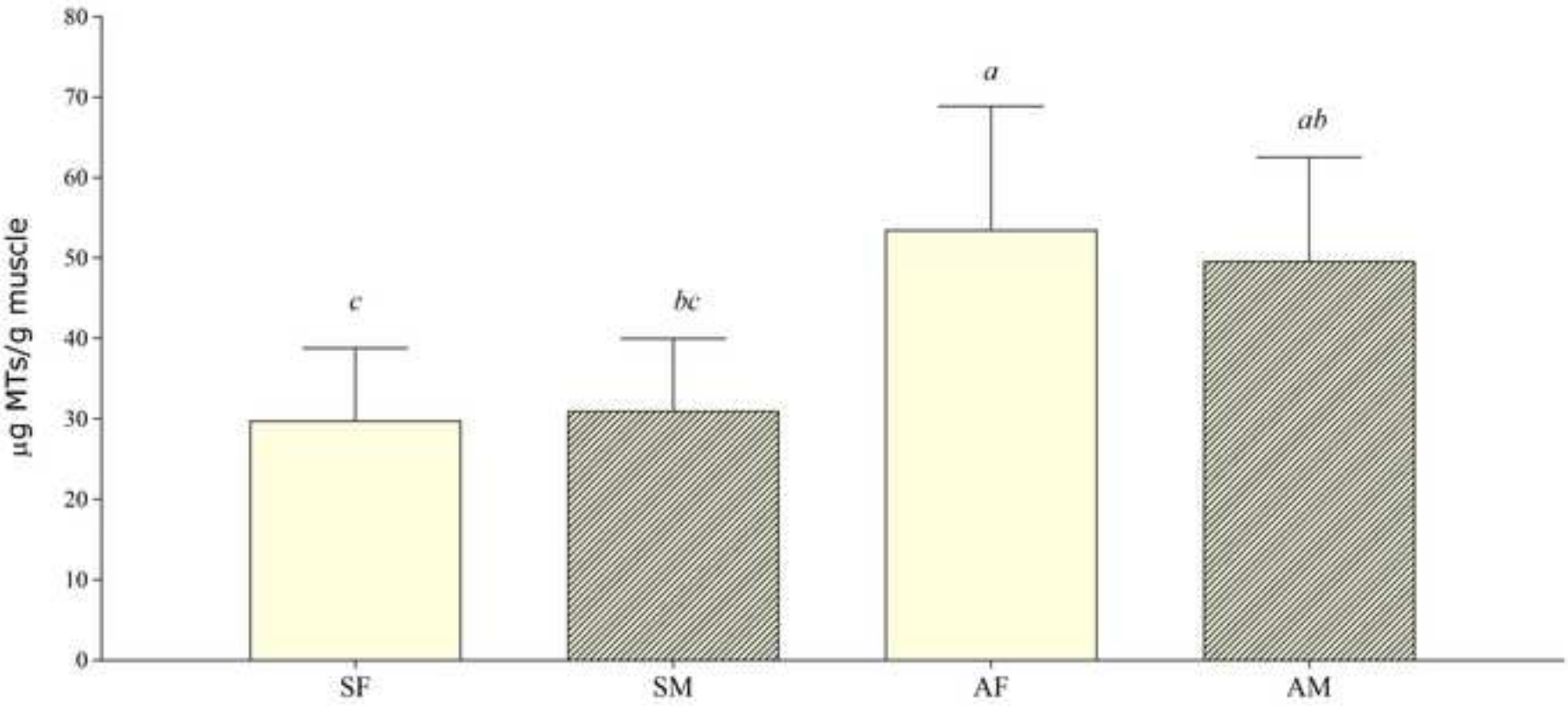

Figure 10

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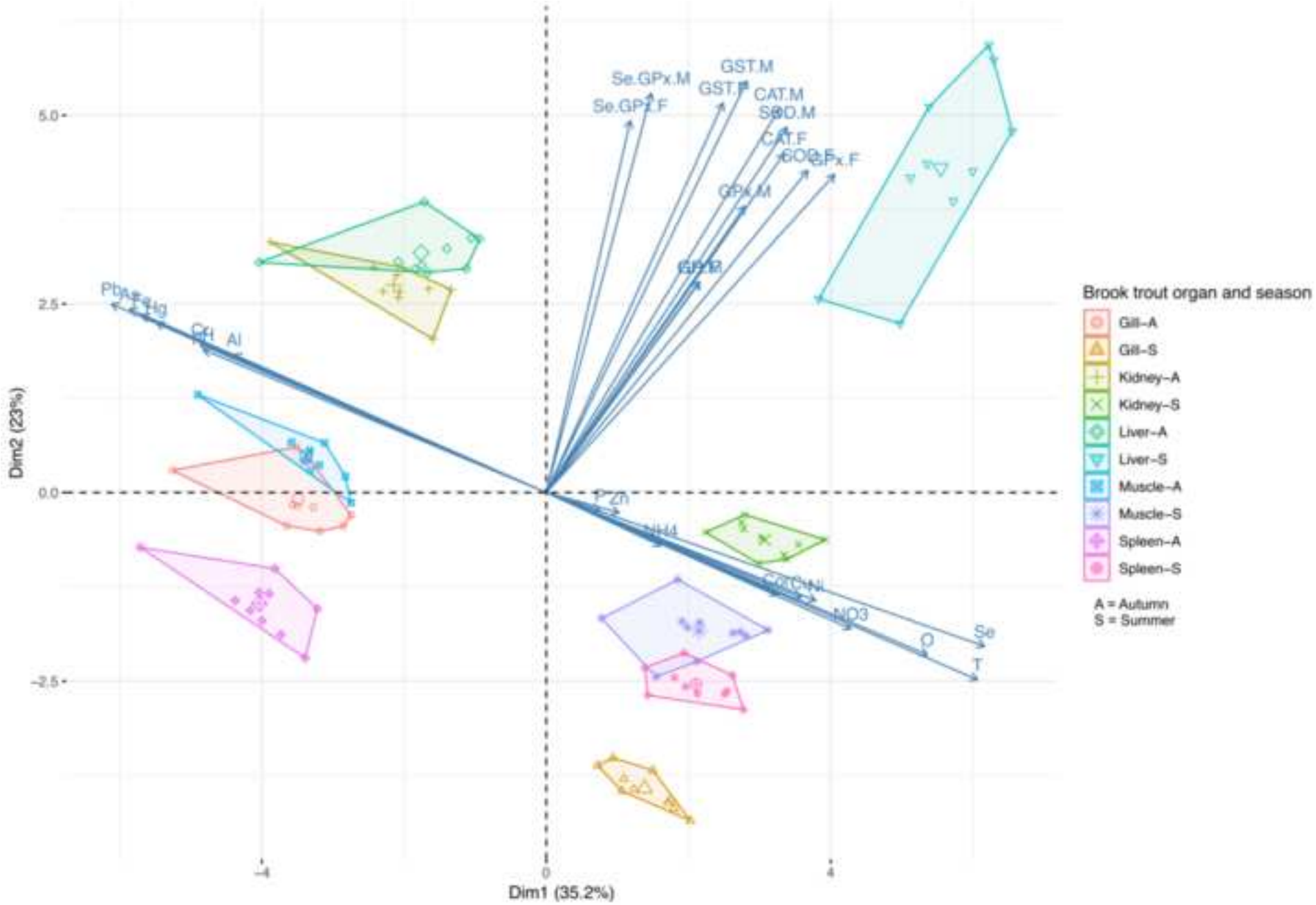

Table S1

Click here to download Supplementary material for on-line publication only: TableS1_recovery.doc

Table S2

Click here to download Supplementary material for on-line publication only: correlation matrices.xlsx

***conflict of Interest Statement**

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: