Review by Sophie Prud'homme, 09 May 2024 07:35

This article is remarkably well written and argued . The title is appropriate, and the abstract presents well the objectives and main finding of the article. The background and questions are clearly and synthetically presented, and the methods are precisely and clearly outlined. The exploration and interpretation of proteomic data and their relation to trace metal contamination data is thorough and well argued, making the approach convincing, which is not always the case in literature. It however lacks an information concerning the functional enrichment execution, that make for now impossible to judge about the relevance of the functional enrichment (see below, comment on L273).

The discussion of the data is enriched by a comparison with the available bibliography through an additional figure and table in supplementary materials, which provides transparency to the discussion.

I only have some recommendations and points of discussion on the manuscript :

> L114-115 : Summarizing liver function in terms of detoxification is a bit simplistic, and all liver functions should be listed.

We now provide a more comprehensive overview of liver functions :

L119-121 : "(ii) investigate the impact of trace element mixtures on the proteome of liver (the main organ implicated in xenobiotic metabolism as well as blood glucose regulation, protein synthesis, bile production, vitamin and mineral storage, steroid metabolism, and immune function)"

> L119 : Please define "LFQ"

Label-Free Quantification (LFQ) is a mass spectrometry technique used to determine the relative abundance of proteins in different samples without the need for labeling with isotopes or other tags. In LFQ, the quantification is based on the intensity of the detected peptide ions, which are used to infer the quantity of the corresponding proteins. We now include this definition in the manuscript for clarity : L200-201 "Protein quantification was performed using unique peptides only via the MaxLFQ option implemented in MaxQuant (Cox et al., 2014), which is a label-free quantification (LFQ) method that determines protein abundance based on the intensity of peptide signals."

> L273 – **"Functional enrichment analysis and pathway network" sectio**n: The background used to perform the functional enrichment on clusters have to be specified. Indeed, using a generic background (not organ specific) could lead to incorrect/biased pathway enrichment and lead to biased biological interpretation. (for example, discussed in Wijesooriya K, Jadaan SA, Perera KL, Kaur T, Ziemann M (2022) Urgent need for consistent standards in functional enrichment analysis. PLoS Comput Biol 18(3): e1009935. https://doi.org/10.1371/journal.pcbi.1009935)

According to the answer to this question, there may be some adjustments to perform to data analysis that may influence the content of the article.

We apologize for the lack of clarity in the original manuscript. We have addressed this potential bias by using the whole set of proteins identified in each tissue as the background for our functional enrichment analysis, thereby employing two distinct backgrounds for each tissue. We now specify the background used L284-287 : "The background used for each tissue consisted of the whole set of proteins identified in that specific tissue, ensuring two distinct backgrounds for the analysis. This

approach prevents biased enrichment from a generic background, ensuring accurate determination of the functional significance of the identified modules (Wijesooriya et al., 2022)."

> L280 : Can some arguments can be provided to justify the choice ton consider the top 10 enriched biological processes ?

The choice to consider the top 10 enriched biological processes relies on the use of the GOCircle function for visualization. It is optimized for displaying a smaller set of terms effectively. According to the documentation and guidelines for GOCircle, it is recommended to visualize around 10 to 12 terms to maintain clarity and readability of the plot (GOplot guidelines). Additionally, when numerous GO terms are significantly enriched, representing about 10 GO terms is a common practice in the literature (Banerjee et al., 2021; Ribeiro et al., 2019).

Banerjee, S. M., Stoll, J. A., Allen, C. D., Lynch, J. M., Harris, H. S., Kenyon, L., Connon, R. E., Sterling, E. J., Naro-Maciel, E., McFadden, K., Lamont, M. M., Benge, J., Fernandez, N. B., Seminoff, J. A., Benson, S. R., Lewison, R. L., Eguchi, T., Summers, T. M., Hapdei, J. R., … Komoroske, L. M. (2021). Species and population specific gene expression in blood transcriptomes of marine turtles. BMC Genomics, 22(1), 346. https://doi.org/10.1186/s12864-021-07656-5

Ribeiro, R. P., Ponz-Segrelles, G., Bleidorn, C., & Aguado, M. T. (2019). Comparative transcriptomics in Syllidae (Annelida) indicates that posterior regeneration and regular growth are comparable, while anterior regeneration is a distinct process. BMC Genomics, 20(1), 855. https://doi.org/10.1186/s12864-019-6223-y

> Results section "Physiological response to inorganic contamination": It would be important to provide, for each tissue, the total number of protein detected (not formally provided for muscle) and the proportion of this background included in significantly correlated modules.

We have now added the total number of proteins detected for each tissue and the proportion of these proteins included in significantly correlated modules:

- L363-366 : "Both mixtures 1 and 3 displayed significant positive correlations (P<0.05, encompassing approximately 31% of the detected proteins) with the same two co-expressed modules (turquoise and yellow, Figure 4), reflecting a general overexpression of proteins within these 2 modules when mixtures 1 and 3 increased in concentration"
- L394-395: "The WGCNA analysis of the red muscle proteome, containing 913 proteins, resulted in four modules namely blue (372 proteins), brown (119 proteins), turquoise (398 proteins), and grey (24 proteins)."
- L399-400 : " Only the blue module, which accounts for 41% of all detected proteins in the red muscle proteome, correlated (positively) with one contaminant mixture (i.e., mixture 1, Figure 6)."

> L489: I'm not convinced that the number of overexpressed pathways can be used to compare the intensity of organ response. It's more a reflection of the diversity or heterogeneity of the response. The absolute number of proteins included in clusters correlated with mixtures, or the part of detected proteins that are included in clusters correlated with mixtures in each tissue seems to be a better metric to compare the intensity of organ response – and should be considered by the authors.

We agree with the reviewer's comment and apologize if the message wasn't clear. We specify in the methods that we use that metric to compare the diversity of pathways involved and not this metric

as an absolute intensity in response. Indeed extrapolating an intensity of response would be over-interpreting our results and the limits of the methods used. We corrected in the referred lines the term intense by diverse L507.

>L513 - “Red muscle proteome response to mixture 1 was less marked than that of the liver proteome”: It is unclear on what criteria the categorization of “less marked” is based, and it should be specified.

We thank the reviewers for pointing out that out. As the previous comment made by reviewer, we modified in order to clarify that we consider the diversity of response in term of functional pathways.

>L548-549: Given that mTOR activity is dependent on several post-translational modifications, considering only its abundance as a biomarker of metals contamination may not fully respond the objectives - Authors should consider the characterization of mTOR post-translational modifications in addition to its abundance. (see for example Yin et al, Int. J. Mol. Sci. 2021, 22(4), 1784; https://doi.org/10.3390/ijms22041784)

We acknowledge that mTOR activity is indeed dependent on several post-translational modifications and that considering only its abundance as a biomarker may not fully address the objectives. In response to your suggestion, we have now included this information L565-568: “However, besides its abundance, post-translational modifications including phosphorylation, ubiquitination, acetylation, and glycosylation appears to be key regulators of mTOR signaling and should also be considered in further research (Yin et al., 2021).”

Review by Roberta Bettinetti, 03 Apr 2024 12:41

my comments

Title and abstract

Does the title clearly reflect the content of the article? Yes,
Does the abstract present the main findings of the study? Yes

Introduction

Are the research questions/hypotheses/predictions clearly presented? Yes,

Does the introduction build on relevant research in the field? Not at all. There are several points that you can explain better. Just in the last two years a new campaign on Mediterranean see has been condcted and more data are now available on metals and legacy pesticides. Pelase check and add also for comparisons.

Thank you for your valuable feedback. We are aware of the recent Mediterranean Sea campaign (SUCHIMED) and have now included a reference to this campaign in the introduction on line L103-106 : “However, little is known about a potential impact of contaminants on Mediterranean sardines, as few ecotoxicological studies have been performed so far on small pelagic fishes in the Gulf of Lions (but see COSTAS project; Tronczynski et al., 2013 and SUCHIMED campaign; Bouchoucha 2021).”. However, we cannot use these data for comparison as the contaminant levels were evaluated only

for anchovy (*Engraulis encrasicolus*) and sprat (*Sprattus sprattus*), not for sardine, which is the focus of our study.

You have also to consider the existence of legacy and emergent POP's and the cocktail is made also by them, not only by trace elements.

We agree with your observation regarding the importance of considering the existence of organic contaminants alongside trace elements. We have added the following information to the manuscript L492-499 : "Furthermore, it is important to consider the existence of organic and emerging pollutants (POPs, HAPs, PFOS, PFAS, etc.) that are becoming increasingly pervasive worldwide (Magulova & Priceputu, 2016). Given their persistence, long-range transportability, biomagnification in food chains, and bioaccumulation in humans and wildlife, their impact on individual health is becoming a growing concern (Magulova & Priceputu, 2016). The combined effects of trace elements and POPs create a complex chemical cocktail that can have synergistic or antagonistic impacts on marine life. Thus, there is a growing need for additional research to fully understand the scope of contamination, which requires considering both trace elements and POPs."

Please discuss in this section also the "problem" connnected with the realism of the use of biomarkers which is quite controversial.

We thank the reviewer for all the comments he/she made and her/his help to improve our manuscript. Unfortunately we are not sure to what “problem”, nor the section the reviewer refers to? Providing lines or specific section could help. Lines 72 to 92 we provided a whole section to discuss the power of considering multiple markers such as proteomic to integrate large scales response at the individual level compared to monitoring few biomarkers. We hope this sections addresses this remarks :

“ While experimental data under controlled conditions taught us a lot on the molecular and physiological costs of single contaminants (Isani et al., 2009; Souid et al., 2015; Wang et al., 2013), the challenge that ecologists now face is to understand the effects of trace element mixtures. Indeed, wildlife species are continuously and increasingly exposed to a large number of different chemicals at the same time (Heys et al., 2016). Therefore, the ecotoxicological impacts of trace element mixtures are relatively less understood. Even at low concentrations, trace elements mixed in the environment can have a combined toxicological effect (Heys et al., 2016) forming “reactive chemical cocktails” that explain the synergistic effects of the combination of distinct elements (Kaushal et al., 2018). Considering the effects of only one pollutant at a time can therefore lead to misinterpretation of biomarker data (Celander, 2011).

The emergence of omics tools has provided a potent means of analyzing complex and integrated responses to contaminants. These tools offer high sensitivity and excellent specificity in studying the molecular changes occurring in organisms (Denslow et al., 2005; Benninghoff 2007). Additionally, high-throughout shotgun proteomics (i.e. the direct and rapid analysis of the entire protein compartment within a complex mixture) provides a unique opportunity to comprehensively examine the expression of thousands of proteins in a specific tissue in a single experiment, using mass spectrometry and bioinformatics techniques instead of traditional biochemical methods (Sanchez et al., 2011; López-Pedrouso et al., 2020). This advance considerably improves our understanding at the protein level and further paves the way for identifying new biomarkers of exposure and effect, which can then be used to develop enhanced monitoring programs to better assess the impacts of pollutants on marine species (Apraiz et al., 2006; Benninghoff 2007).”

I would consider also the role of Temperature in time, during the last years as a probable factor causing the decreaase of number of fish (less or diverse preys as it is hppening in freshwater

environments). You should explain better this aspect, since of course (specify it better in the introduction) contamination of trace elements can't be the only cause (other fish? other equilibrium conditions?)

We acknowledge the role of temperature in affecting fish populations, particularly through its impact on prey availability. In the case of sardines in the Gulf of Lions, while the abundance of individuals has not significantly changed, there has been a drastic decrease of their size and body condition. We fully agree that increasing temperatures can play a key role as they lead to weaker planktonic blooms and favour smaller phytoplankton (Sommer & Lengfellner, 2008), which can impacts the quality and quantity of sardine preys. An extended work on the Mediterranean sardines as being one by Saraux et al., team over the last decade, ruling out temperature effect just by itself but highlighting that multifactorial environmental processes where involved in the decrease in size, body condition and a reduction of older individuals in the Gulf of Lion (Feuillolley et al., 2020, Queiros et al., 2019, Brosset et al., 2016, Queiros et al., 2024 for a review see Saraux et al., 2019).

We have now added these information this in the introduction by specifying the influence of mutlifactorial environmental changes' on L99-103: “The main hypothesis to explain the observed changes is a modification of the quality and/or quantity of sardine preys (such as copepods, Brosset et al., 2016) due to multifactorial environmental changes (SST, Upwelling, Stratification, Convection, WeMO, Chla concentration etc. Feuillolley et al., 2020), thus limiting fish energy resources (bottom-up control, Brosset et al., 2016; Saraux et al., 2019).”

Furthermore, we do not claim that the issues facing sardines are solely due to inorganic contamination. Instead, it appears to be a multifactorial problem on top of which inorganic contaminant may add up. Nonetheless, until now, the potential impacts of inorganic contamination had not been thoroughly explored and have therefore motivated this work. This is highlighted on L103-106 : 'However, little is known about a potential impact of contaminants on Mediterranean sardines, as few ecotoxicological studies have been performed so far on small pelagic fishes in the Gulf of Lions (but see COSTAS project; Tronczynski et al., 2013 and SUCHIMED campaign; Bouchoucha 2021).'

Additionally, on L613-620 : “The major driver proposed is the shift of sardine diet towards smaller planktonic prey observed in the Gulf of Lions due to multi-factorial environmental changes (Feuillolley et al., 2020, Brosset et al., 2016), leading to lower foraging efficiency and a reallocation trade-off toward reproduction instead of survival (Beauvieux et al., 2022, Queiros et al., 2019, 2024), […] Adding any additional energetic or immune burden, as those highlighted in response to contaminants in this study, might emphasize their reduced chance of survival (Queiros et al., 2021).”

Materials and methods

Are the methods and analyses sufficiently detailed to allow replication by other researchers? not at all: please explain how you measured wet weight of fish –

We measured the wet weight using a digital balance to ensure accuracy to the nearest 1g L134-135. We included this detail in the methodology section for clarity.

pleas esplain the method by Fold since it is quite old and not easy to find –

The methodology is old, but its use is still common. It is a basic chloroform-methanol extraction. We provided that information, and then refer to Sardenne et al., 2019 that investigated in detail this method.

explain why you did not take into account methyl mercury

As indicated in the Materials and Methods section, we measured total mercury in our samples. This encompasses all forms of mercury, including elemental mercury, inorganic mercury, and organic mercury compounds such as methylmercury. Total mercury analysis does not differentiate between these forms; instead, it provides a comprehensive measurement of the entire mercury content. Therefore, methylmercury, which is a significant and toxic form of mercury found in the environment, especially in aquatic systems, is included in this total measurement.

I'm not an expert of proteomi, so I've nothing to say about.

Are the methods and statistical analyses appropriate and well described? Yes

Results

In the case of negative results, is there a statistical power analysis (or an adequate Bayesian analysis or equivalence testing)? I don't know

Are the results described and interpreted correctly? Yes, - why did you look for the relationshiè between lenght and size and you corrected the calues? It is not clear to me

The relationship between weight and size was investigated to calculate the body condition of individuals. Body condition is largely considered in the literature as an indicator of the overall health and fitness of an organism, reflecting its energy reserves and nutritional status. It is typically assessed by comparing the observed weight of an individual to the expected weight for its length.

If you were referring to the relationship between the concentration of contaminants and size, it is important to consider this relationship as size may influence the uptake, distribution, and elimination of trace elements (Canli & Atli, 2003). This correction helps to normalize the data, ensuring that observed differences in contaminant concentrations are not merely due to variations in individual sizes but reflect genuine biological responses to contamination.

Canli, M., & Atli, G. (2003). The relationships between heavy metal (Cd, Cr, Cu, Fe, Pb, Zn) levels and the size of six Mediterranean fish species. Environmental Pollution, 121(1), 129–136. https://doi.org/10.1016/S0269-7491(02)00194-X

Discussion

Have the authors appropriately emphasized the strengths and limitations of their study/theory/methods/argument? Yes, even if you did not consider the importance of temperature variations. I wouldn't compare concentrations in seas which are so different and concentrations are to low and methods probably too different to be comparable.

We agree that temperature plays a significant role in the metabolism of xenobiotics. Temperature can influence the rate of enzymatic reactions involved in the detoxification processes, affecting how

organisms metabolize and eliminate contaminants. Therefore, higher temperatures can increase the metabolic rate, potentially leading to faster biotransformation of xenobiotics but also possibly increasing the formation of toxic metabolites (van der Oost et al., 2003; Noyes et al., 2009).

However, in this section of the discussion, we are not addressing the impact of contaminants on sardines from different regions worldwide. Instead, we are comparing their contaminant loads. This comparison is relevant regardless of temperature variations, as the contaminant load reflects the exposure level of the organisms.

please consider the size of the preys, thanks

The size of the preys is currently considered as the main driver of the Mediterranean sardines decline in size and body condition. We refer to that phenomenon several times through the manuscript referring to the work of Brosset et al., 2016, Queiros et al., 2019, 2024, Feuillolley et al., 2020, Beauvieux et al., 2022. See Saraux et al., 2019 for a review.

Review by anonymous reviewer 1, 15 Apr 2024 08:54

Beauvieux et al. provided data on the contamination of the European sardine and the relationship between the level of pollution with potential physiological responses in the Mediterranean sea. Considering that this basin has been longed reported as one of the most polluted sea at wordwide level, it is surely important to provides new updated data on marine ecotoxicology.

Despite its potential, this manuscript would surely benefict from an exhaustive bibliographic search as it is keepen very superficial throughtout its introduction and discussion as well. For instance:

We apologize if the bibliography appeared lacking. We have now added the references you pointed out to both the introduction and discussion sections to provide a more comprehensive context for our study.

line 44-45 need a reference;

Done

line 55-57: the authors mentioned multiple TE stating they can cause several adverse effects, however the cited work is only one and it is reffered to only copper. The authors need to cite proper references.

done

line 457-460: the levels of TE are potentially higher in the Med compared to other basins is likely linked to multiple sources of contamination, both natural and human's. It is much more complicated than just stating "higher levels because the Mediterranean throphic webs show enhanced abilities to better accumulate TE pollutants".

We agree that the higher levels of trace elements in the Mediterranean Sea are due to a complex interplay of multiple sources of contamination, both natural and anthropogenic. We have discussed these different sources in the introduction (L45-50) and will now incorporate this information into the discussion section L459-466 : “Trace metal concentrations in Mediterranean surface waters and top predators are generally higher than in the Atlantic Ocean (Boyle et al., 1985; Cossa and Coquery 2005). This phenomenon can be largely attributed to the Mediterranean's semi-enclosed geography,

which promotes pollutant accumulation. This accumulation is further exacerbated by significant atmospheric inputs, such as Saharan dust events, intense human activities (Bucchia et al., 2015; Pedrotti et al., 2016), and riverine discharges on continental shelves (Durrieu de Madron et al., 2011). Additionally, there is evidence suggesting that the Mediterranean pelagic food webs may have a heightened capacity to bioaccumulate certain trace elements, particularly Hg (Cossa and Coquery 2005; Harmelin-Vivien et al., 2009; Chouvelon et al., 2018).”

Other observations:

- authors need to be consistent throughout the manuscript. They need to use the acronym the first time the written about TE and then use either the acronym or the full name. However, in the discussion they still refer to Hg (mercury) etc. (Line 476).

We thank reviewer’s 3 for its helpful comments that we took in consideration to improve the manuscript.

- References need to be uniformed in style (e.g. something is "et al.," other time "et al.")

We homogenized accordingly throughout the manuscript.

Line 154: No need for "Trace-element", the authors could simply say "Trace element"

We modified accordingly throughout the manuscript.

Line 159 and 170 etc.: be careful in writing correctly (in style) the chemical formula as well as the unit

We modified accordingly throughout the manuscript.

Line 208: Latin name goes italics, moreover, if authors decide to use the common name or the Latin name is ok, but they need to be consistent.

We modified accordingly throughout the manuscript.