Answers to reviewers' comments

Dear Recommender,

We warmly thank you and the editorial board of PCI Ecotox Env Chem for providing us an opportunity to improve and re-submit our manuscript.

We also thank the reviewers for their fruitful remarks and advices.

We have carefully reworked the manuscript, following the comments of the reviewers.

Hereafter we answer in details to comments. In the revised version of the manuscript, changes are highlighted using the "track changes" option. Please note that the numbers of lines cited hereafter refer to the version of the manuscript with track changes.

Another version, the final "clean" one is also provided.

We thank you in advance for considering our new version.

Best,

Clémentine Fritsch

Reviewer's comments and responses

Reviewer 1, 29 Apr 2024 18:06

The authors have presented a nice study which measures the concentration of glyphosate, AMPA, and glufosinate in small mammals. Overall the article was a pleasure to read and the authors did a lot of work. It might be better to split this into two manuscripts, 1 focused on measuring the herbicides and metabolite and the second focused on dose reconstruction to assess risk.

- We warmly thank the reviewer for positive comments and suggestions.

The opportunity of splitting into 2 manuscripts have been discussed several times between the co-authors. We finally made the choice of presenting all the results together since the exploration of toxicological meaning constitutes a substantial added value for the readers and discussing risk analyses without detailed information about exposure makes less sense. With regards towards ethics and strategy in publishing practices in light of San Francisco Declaration on Research Assessment, we prefer to give priority to only one comprehensive manuscript rather than two separated publications.

We understand and agree that the manuscript can be considered as long and dense. We have made efforts to simplify and clarify the main text. Especially, the end of the Introduction section has been re-written to clarify the objectives. Since the objective of the study was not focused on the development and validation of the analytics of GLY, AMPA and GLUF in hair, detailed information about the methods have been moved as Supplementary Information (note that we have added the analytical and methodological details asked by the reviewers). We also have reworded and re-organized the

description of the statistics. The section Results & Discussion have been split into more subsections to enhance reading fluency and some sentences in the discussion have been deleted.

As you may note in my review below, I began to provide some very specific grammar edits, however there are numerous gramatical issues throughout the manuscript, and it should be throughly edited before submission. My review below does not point out all the gramatical issues.

- We thank the reviewer's for helping in improving grammar and typos. As suggested the MS has been submitted to English editing before re-submission (Services of American Journal Experts).

The overall flow of the manuscript was also a little challenging to read. There are a number of sections where information would be better in a different location. I.e. some of the information in the introduction should be moved to results/discussion and some of the methods should be moved to results/discussion, etc. I have pointed out some of them below, but have not given all specific line numbers for each section that the authors may consider moving.

- Thanks again for help in improving the text. We have addressed the recommendations in the revised version.

I also recommend that this manuscript is reviewed by an expert who can better review and provide feedback on the dose reconstruction which was described in sections 2.3 and 3.4.

- We are not sure to understand well the request of the reviewer and how to handle it. We acknowledge that reverse dosimetry, toxicokinetic/toxicodynamic modelling and risk assessment are not the core expertise of the authors. However, the authors have some competence and background about these issues. See for instance:

Coeurdassier M, Poirson C, Paul JP, Rieffel D, Michelat D, Reymond D, et al. 2012. The diet of migrant Red Kites *Milvus milvus* during a Water Vole *Arvicola terrestris* outbreak in eastern France and the associated risk of secondary poisoning by the rodenticide bromadiolone. Ibis 154:136–146; doi:10.1111/j.1474-919X.2011.01193.x.

Faÿs F, Palazzi P, Zeman F, Hardy EM, Schaeffer C, Rousselle C, et al. 2023. Incorporation of Fast-Elimination Chemicals in Hair Is Governed by Pharmacokinetics-Implications for Exposure Assessment. Environ Sci Technol 57:7336–7345; doi:10.1021/acs.est.2c06777.

Morrissey C, Fritsch C, Fremlin K, Adams W, Borgå K, Brinkmann M, et al. 2023. Advancing exposure assessment approaches to improve wildlife risk assessment. Integr Envir Assess & Manag 1–25; doi:10.1002/ieam.4743.

- Moreover, the authors of the manuscript, who gather skills in various fields of research including chemistry, ecophysiology, ecotoxicology, biomonitoring, pharmacology, and ecology have reviewed the approaches and the calculations. Asking an outside expert to review our work at this step of the process, and maybe add him or her as a co-author looks like unusual. We therefore considered that the peer-review process, handled by the recommender and several external reviewers, would tackle this issue. As a consequence, we did not take any further action about this recommendation.

Introduction.

Line 59-60, since multiple glyphosphate metabolites exist, it would help the reader to understand this is the main degredation product of GLY.

- Done (Lines 64)

Can you please clarify the statements in line 66 and 67, absence of shikimate pathway, that is GLY target, in animals and hydrophilic, low Kow, poorly metabolised and rapidly eliminated in mammals. For example, it would be helpful to clarify that the pathway is the target pathway in plants and that it doesn't exhist in animals and provide a reference for this statement. In line 67 Kow is used but should be explained for readers who aren't familiar with this.

- Done: lines 72-73 for the *shikimate pathway* and lines 74-77 for Kow

Line 75, They are associated to risks for chronic low-dose exposure in animals and human,.... Please revise as I think you mean there are risks related to chronic low-dose exposure. An example of a risk would be helpful to the reader.

- The meaning is that there is a risk of chronic exposure to low-dose, as justified from the statements in the previous sentences and in the references cited. We have re-phrased (lines 86-87).

Line 77-78, "Furthermore, GLY and 77 AMPA may be more bioaccumulative than predicted from their physico-chemical properties". Please try to rephrase "more bioaccumulative" as the sentence does not read smoothly.

- We have tried to change to make the sentence more readable (Line 89).

Lines 91-93, For those not in the EU, it is unclear what a regulatory exclusion is.

- For more clarity, ”regulatory exclusion” has been replaced by “withdrawal” (Line 105).

Line 107, seems like (rodents and shrews) should be after mammal species.

- We thought the two different taxonomic groups should refer to the different ecological traits, but we have moved in the sentence according to the reviewer’s suggestion (Line 145).

Line 116-117 If AMPA is in 93% of cropland soils vs 30% for GLY, how are small mammals exposed to GLY through more pathways than AMPA?

- We have added details at the end of the sentence to clarify that AMPA is not an active substance of plant protection products but a metabolite of GLY, it is not used as an herbicide and therefore the pathway of overspray is likely contrarily to GLY (Line 134).

Line 120, mobiles is the wrong tense.

- Corrected.

Line 122, the /// doesn't make sense.

- Replaced by “We expect species feeding on animal matter (i.e. omnivores, and carnivores, /insectivores or/ vermivores)” (Line 150).

Methods

Some of the methods belong in the results section (i.e. outcome of trapping efforts).

- We have modified according to the reviewer’s suggestion.

It may make more sense to place at the beginning how the farmland was classified before beginning the description of the trapping process.

- According to this comment and for more clarity, the first paragraph of the section "Material and Methods" describe the study design and the second one details the sampling process.

Please also for each acronym (i.e. NOEL) please make sure to spell it out the first time it is used so the reader can follow.

- We have checked, and as far as we did not miss a mistake, the full name is provided before the abbreviation (i.e "short-term dietary no observed effect level (short-term dietary NOEL)", "the chronic 21 days no observed adverse effect level (chronic NOAEL […]").

Line 146, the word alive is not needed.

- Deleted.

How were the organic fields confirmed not to be contaminated by run-off or draft as is pointed out in lines 176-177? While these lines refers to hedgerows, etc it still brings up the issue mentioned above.

- This cannot be confirmed here. We agree that such issues are indeed raised and this is discussed later in the manuscript. However, in this section the matter is to define how to classify the plots where the sampling was realized with regards towards agricultural practices. The classification as "managed under organic farming" or "managed under conventional farming" is obvious and informed for cultivated plots but we needed to adapt the classification for non-cultivated plots such as hedgerows. Please note that to limit interpretation bias we also conduct another classification as "not intentionally targeted by treatments (NT)" or "possibly targeted (T)" where hedgerows and plots cultivated under OF are classified the same as NT. Both classifications (OF *vs* CF and NT *vs* T) were investigated and the results are provided.
- For more clarity, we have reworded this part in order to limit any confusion (lines 216-218), and we have introduced the issue of unintentional contamination in the discussion (Lines 747-750).

Would it be better to follow these methods vs setting samples <LOD equal to 0? Critical Review Toward Improved Analysis of Concentration Data: Embracing Nondetects, https://setac.onlinelibrary.wiley.com/doi/full/10.1002/etc.4046?

- Overall our approach followed the recommendation provided in the reference cited by the reviewer, although some imputation approaches and statistics for censored data could not be used due to high frequency of non-detects and low sample size. Clarifications have been added in the M&M (lines 348-368), and the reference proposed by the reviewer has been cited and added to the bibliographic list.

Lines 219-236, are the methods provided elsewhere for chromotography analysis? Some important details such as the solvents used, flow rates, etc are not throughly described to allow replication. What grade were your reagents as only Methanol and water were provided with this level of detail. Also recommend you cite or provide the formulas for calculating your LOQ, etc.

- The requested information was added to the revised manuscript, in the main text (Lines 257-299) and in Sup Info Annex 1. Text A2.

Results.

This is where you should report the species you captured, numbers, etc.

- As requested we have moved information about capture success and sample size as a first section in the Results (Lines 473-483). We have modified Table 1 to provide the sample size (page 24).

Lines 330-331, clairfy that you mean all species and not the individual species.

- Done, see line 486.

Lines 331-332, quantities, was this grams, kgs, etc?

- These are ratios, so unitless. For more clarity we have added the units of original data in M&M line 240.

Lines 367-371, I recommend reviewing the article at the following https://www.intechopen.com/chapters/79317 as it describes the increased risks of pesticide exposure in bats. I also recommend you review the paper you cited about bats, as the authors did use internal standards (imicloprid-d4 and dicamba-d3) and calculated the recovery rates and matrix effects. A better hypothesis is that their washing method for the hair was inadequate vs what you suggest.

- Thanks for the recommendation. To follow the suggestion of the reviewer, we have added a reference dealing with ecotoxicology of PPP in wildlife in which the issues related to the vulnerability of bats are reviewed (Line 544).
- Several important reasons beyond analytical issues could explain the higher levels measured in bats and we lack evidence to prefer one hypothesis among others. The reviewer also asked to limit the length of the text. Therefore, we preferred to cite the non-exclusive hypothesis without further details.

Some of the figures are unable to be read due to low resolution. It is unclear if this is due to an artifact of the submission process or an actual problem with the resolution of the figures.

- Sorry about this. I guess that the remark concerns especially the former Figure 1. We made the most to get a better resolution of the picture to get a better figure (page 27) in the .pdf version, and performed the same for other figures in order to improve their resolution.

I would recommend considering which figures are in the manuscript vs which are in the appendix. Should table A.1 be in results as this is a really nice overview. Table A.2 I would also seriously consider putting into the manuscript as it shows the odds ratios which are discussed quite a bit in the manuscript. I bring this up as the majority of your results presented in the results/discussion reference the appendix and not the figures in the manuscript. It is cumbersome to keep going to the appendix and reviewing the results before continuing reading the manuscript.

- Table A1 essentially provide further details about sample size additionally to the text concerning study design, the captures of small mammals and animals included in the study. It is quite heavy. We prefer to keep Table A1 with full details as Supplementary Material but Table 1 (page 24) has been modified to provide information about sample size in the main text.
- Table A2 provide detailed statistical outputs that are described in the text and shown in Figure 2. In our first draft of the MS, Table A2 was included in main text. But as highlighted by the reviewers, our manuscript is quite long and dense, and we therefore preferred to use a figure to display statistically significant results rather than an extensive table (one full page A4).

Detailed results are therefore provided as supplementary information for the readers specifically interested in statistical outcomes.
Because all the reviewers mentioned that the main text must be simplified and only one of the reviewers asked to include the heavy tables in the manuscript we propose to maintain them in Sup Mat.

Could you please clarify if you are viewing a single species as omnivorous/granivorous, etc. It is difficult to tell the ecological traits and what species you are describing throughout the text and should be clarified.

- We have modified Table 1 to provide diet preference and home-range size for each species (page 24). The bank vole is often considered granivore, but the most recent literature showed omnivory in this species in agricultural areas. The wood mouse was also considered mostly granivore, but for a long time has been shown opportunistic and omnivore. To clarify we therefore considered the bank vole and the wood mouse as omnivorous species and updated accordingly throughout the manuscript.

Line 549, However, such an effect of a.s. sales on GLUF detection probabilities, what is a.s.?

- The abbreviation "a.s." means active substance, as described in M&M (first time mentioned line 240) and used earlier in Results.

Conclusion,

I recommended keeping paragraph 1 and 3, paragraph 2 is out of place here.

- We strongly believe that this is an important outcome of our study and that such paragraph is relevant in the conclusion. We have added a few words to highlight the limits of the approach and a scientific reference to justify its relevance. Please note that we have been contacted by the French national "phytopharmacovigilance" service who manages post-registration monitoring following the publication of a previous articles dealing with PPP multi-residue analyses in hair of small mammals. The scientific committee used our data in their phytopharmacovigilance works and asked for scientific support about the implementation of post-registration surveys based on measurements of PPP residues in wildlife hair. Based on these facts, we also added a few words to justify the relevance for post-registration regulation.

Review by anonymous reviewer 2, 08 Apr 2024 11:05

General comments

The authors studied the exposure of small mammals to three substances, the currently used herbicide glyphosate, its primary metabolite AMPA, and glufosinate an herbicide banned for three years when the fieldwork was conducted.

They show overall higher concentrations of GLY in comparison to AMPA and GLUF. With some concentrations exceeding ecotoxicological thresholds leading to health risk. Importantly they show that small mammals are exposed to these compounds despite the environmental measures used to protect the environment and biodiversity (hedgerows and organic farming).

These findings are alarming as they reveal that current surfaces of non-treated habitats within the agricultural landscapes are insufficient to mitigate the exposure of wildlife to GLY, AMPA and GLUF.

The manuscript is of great interest and well-written, the statistical analyses are robustly designed. Yet, I have a few suggestions which I believe could ease the reader's understanding and in some parts I would need additional clarifications on the data provided and its interpretation. For instance, the authors write that glyphosate concentrations are higher than AMPA or glufosinate concentrations, yet no statistical analyses were conducted among species to specifically conclude on this trend. The authors should remain cautious on this point.

At this stage I recommend minor revisions.

- We sincerely thank the reviewer for laudatory comments and for help in improving our manuscript. We have revised/added information as recommended.

Specific comments

l.101: please correct typo for "impediment"

- Sorry for the mistake. Thanks. We have corrected.

l.151: Please provide the complete name Microtus arvalis and Microtus agrestis in stead of M. arvalis and M. agrestis.

- Done

l. 164: It would be very helpful to provide a map showing the localisation of CF, OF and OF/CF, in addition to trapline locations and habitat type (cropland, hedgerows and woodlot). The readers could appreciate the proximity between each type of crop or habitat.

- As requested we have added a map as a new Figure 1 (page 20) in order to show the study design and the location of the samples over the area. We also understood that the sampling design description was not clear enough and updated M&M as necessary.

Can you please provide the home range of each species in supporting info, this would help understanding the exposure range of the targeted herbicides.

- We have modified Table 1 (page 24) to provide diet preference and home-range size for each species.

l.317-318: "The influence of species, habitat, farming practices and proxies for treatment intensity on the detection frequency of GLY, AMPA or GLUF".

This sentence is a bit difficult to understand as is. Maybe you could turn it other ways, for example: we tested whether the detection of GLY, AMPA or GLUF in small mammal's hair was dependent on species, habitat, farming practices and proxies for treatment intensity.

- Done, in the sentence for detection and also in the one for concentrations

l.350: "gastric content" correct typo

- Done.

Table 1: Be careful, there has been a shift in your column names for glufosinate. "All species" appears twice and the other species have shifted from one column.

- Thanks. We have corrected. Table 1 has been rebuilt to answer Reviewer#1.

l.361-363: According to the data provided in Table 1 only the common vole shows significantly higher concentrations of Gly than AMPA (probably resulting from the outlier), for the house mouse the differences are small, for the shrew non-significant and for the bank vole AMPA even seems higher. Without comparative statistics between GLU and AMPA concentrations for each species and for common voles with and without the outlier, I would remain cautious writing that GLY concentrations are higher than that of AMPA in hair.

- The reviewer is totally right. We have checked statistically whether concentrations of the three compounds significantly differed or not. We have added the test in M&M (Lines 369-372) and the outputs in the results (Lines 535-5379) and changed the text in the MS and the abstract accordingly. The raw detailed outputs of the tests have been added as Supplementary information (Text A4: Statistical outputs of the Tarone-Ware tests).

Table A2: correct typo in "the binomial" – In shrews and house mouse, Glufosinate was only detected in one individual, how did you compute confidence intervals for these two species?

- The probabilities of detection and CI were provided through the statistical tests, retrieved using the functions "tbl_regression" and "forest_model" applied on binomial GLMs. The packages used in statistics are listed lines 343-345.

l.409-411: How did you calculate slopes when for two species glufosinate was only detected in one individual?

- The outputs were obtained from the GLMs, since the species with only one detection did not differ statistically than some other species, the coefficients were computed from the several species belonging to the same statistical group. The outputs are provided in Tables A2 and A3.

l.428: correct typo "authorized"

- Done

l.444: correct typo "Statistical"

- Done

l.456: you can run the analyses with a random value between LOQ and LOD instead of setting LOD as 0

- We used all values between the LOQ and the lowest detected value. Nondetects (i.e., <LOD) were set to 0 for statistical analyses but LOD were not set at 0. We have made our best, in several relevant parts of the manuscript, to clarify our approach towards the limits of detection (see lines 287-299 and Appendix Text A2) and issues related to handling nondetects (lines 348-372).

- According to the remarks of the reviewers, we have modified the section about statistics in the M&M section in order to clarify the approach, justify why we could not apply imputation methods, and justify the reliability of the approach applied on our dataset (lines 348-415).

l.468: correct typo "comparison"

- Done

l.560-561: Did you consider the number of years since the crop was set as organic? The transition period for a field to become organic is three years, but some fields could be organic for a much longer period. It could be interesting to test if the duration in organic farming influences exposure, maybe the persistence of these compounds is much higher than previously thought.

- We could not test the effect of duration partly due to lack of data accurate enough, and because of low sample size.

- We considered as "OF" the plots where organic farming was applied since more than 3 years, and in the category OF/CF the ones that were under transition. This is specified lines 163-164. We did not detect any differences between these two categories. Glyphosate is supposed not persistent, and even if the half-life of AMPA seems longer than the one of GLY and according to the longest values provided in the literature, they would not persist longer than a few months. The main hypothesis in our study about the pervasive contamination by GLY and AMPA is related to the massive use and recurrent use over large surfaces (see line 1047 for instance). The case is different for GLUF which may be more persistent.

Could you also provide distances between OF and CF crops to have an idea of how close they are?

- We have included a map as requested by the reviewer (new Figure 1 page 20). Therefore, the distances between OF and CF crops are now visible over the whole area and in places where small mammals have been captured.

l.615: correct typo "agroecological"

- Done

l.635: correct typo "toxicological impairment"

- Done

l.693-694: Check also the studies led by F. Brischoux on the effects of AMPA exposure on spined toad tadpoles mortality and deformity

- Thanks. We have added the 2 references and associated remarks in the discussion (Lines 1019-1021).

l. 703: correct typo "soricidae"

- Done

Review by anonymous reviewer 3, 17 Apr 2024 08:28

GENERAL 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? No, please, see comments to author

- We have paid attention to clarify the text and simplify the reading of the MS.

Does the introduction build on relevant research in the field? Yes,

Materials and methods

Are the methods and analyses sufficiently detailed to allow replication by other researchers? No, please, see comments to author

- We have added details as requested by the reviewers (Lines 257-299 and Supporting Information: Appendix Text A2).

Are the methods and statistical analyses appropriate and well described? thease are confuse, please, see comments to author

- We have rewritten the sections.

Results

In the case of negative results, is there a statistical power analysis (or an adequate Bayesian analysis or equivalence testing)? please, see comments to author

- We have rewritten the sections to provide further details about the statistical analyses (Lines 345-415).

Are the results described and interpreted correctly? please, see comments to author

- We have reworked the M&M, the results and the discussion to answer the reviewer's remarks. Please see below as well as in the reply to the other reviewers.

Discussion

Have the authors appropriately emphasized the strengths and limitations of their study/theory/methods/argument? please, see comments to author

- We have reworked the M&M, the results and the discussion to answer the reviewer's remarks. Please see below as well as in the reply to the other reviewers.

Are the conclusions adequately supported by the results (without overstating the implications of the findings)? please, see comments to author

- We have reworked the M&M, the results and the discussion to answer the reviewer's remarks. Please see below as well as in the reply to the other reviewers.

Reviewer Blind Comments to Author

This manuscript addresses de exposure of glyphosate its first metabolite and glufosinate, herbicides widely used, in hair samples of small mammals, namely free-ranging rodents and shrews from treated and nontreated agricultural habitats. In addition, the study investigates the patterns of accumulation according to species, habitats, and treatment intensity at plot, landscape, or township scale. The study detects a generalized exposure of the tree compounds analysed. So far, few studies have addressed the study of these compounds in wildlife, thus, the study may therefore be of particular for researchers, environmental managers and conservationist. Despite this the manuscript is, in my opinion, too long and dense, which makes it difficult to follow and understand the work carried out properly, and thus the results that emerge from it. In this sense, I have described some crucial aspects that, in my opinion, should be clarified to improve the manuscript before publication.

- We warmly thank the reviewer for rave comments and for recommendations about our manuscript to improve the text and the description of our study.
- As emphasized earlier in responses to reviewers, we have made efforts to simplify and clarify the manuscript and to limit its length.

1. Introduction:

An example of the problem of density and lack of clarity can be found in the last paragraph related to the aim of the study. It is a one-page paragraph, in which the aim covers the first sentence: "The aim of this study was to investigate wildlife exposure in an arable landscape to GLY, AMPA and GLUF using a lowly invasive sampling method based on residue analyses in hair, and focusing on small mammal species having various ecological traits (rodents and shrews)." The main aim/objective of the study seems clear, but later the work appears to address more than that, e.g. Dose reconstruction to assess risk for deleterious effects in small mammals, which it difficult to extract from the indicated paragraph. I believe that more concrete objectives and/or sub-objectives would be necessary to follow and understand correctly all the work carried out under this manuscript.

- The end of the Introduction has been re-written (Lines 120-154).

2. Material and methods:

a. Sampling was carried out between may and june, were there any recaptures?

- we have added details in both the M&M (Lines 187-188) and in Results to address issues related to recaptures. The number of recaptures is now provided in the first section of the Results (see lines 476-478).

b. Was the food analysed to confirm the absence of transfer of any pesticides through this route?

- In case the reviewer asks about the food used to fill the traps, no we did not screen pesticides in the food and hay. Importantly, hay, vegetables, fruits and seeds originated from organic agriculture. We have added this information in M&M (line 179-180). Bias related to exposure to pesticides via the food provided in the traps for further analyses and interpretation about concentrations found in hair looked unlikely according to the toxicokinetic of the compounds, the fact that we used hair instead of

matrices such as gastric content or plasma and the duration of the stay in the traps that is less than 24h in comparison to the amount of time (weeks to months) during which the compound have been accumulated in hair when animals were free prior to the capture. Moreover, the fact that we found about 50% nondetects does not provide clues for contamination during the trapping that all animals included in our study experienced.

In case the reviewer asks about transfer in the field, some information has been provided in a previous study where both soils and earthworms (which are part of the diet of several of the small mammals studied) were analysed for residues of GLY, AMPA and GLUF. The results of this study, Pelosi et al. (2022), are already discussed in the discussion. The rest of the interpretation is based on literature. In fact, it is likely that diet is one pathway of exposure and this is discussed in the MS.

c. *Pesticides analysis: hair decontamination, despite it could be a very interesting issue, I am not sure if decontamination with sodium dodecyl sulfate solution and with methanol can be a good procedure for compounds such as glyphosate, which as addressed in the introduction, it has singular physico-chemical properties, characterised by the fact that it is a particularly hydrophilic polar compound. In fact, the hair decontamination procedure used from Duca et al (2014) focuses on different chemical classes, but does not include the glyphosate. Perhaps decontamination by washing with water/formic acid or methanol/formic acid might be more appropriate. In this regard, have you been able to analyse whether you found differences between before and after washing?*

- We thank the reviewer for raising this relevant question regarding sample decontamination. The authors acknowledge that in the article used as a reference for the description of the washing procedure, glyphosate, AMPA and glufosinate were not included. Indeed, the heavy procedure described in Duca et al, 2014, including various contamination processes and comparing different solvent efficacy cannot be reproduced for each new compound tested in hair. Nevertheless, this procedure was precisely developed to be as "universal" as possible, and was tested on a large panel of chemicals with different physicochemical properties, some of them being rather hydrophilic (ex: 0.3 g/l for carbofuran, 0.5 g/l for imidacloprid) although less than glyphosate, which represents an extreme situation. For this purpose, the two steps (SDS and methanol) were found to be the best compromise to remove both lipophilic and hydrophilic chemicals from hair surface. It also has to be kept in mind that SDS solution is an aqueous solution (5% SDS / 95% water), and is therefore well adapted to remove hydrophilic compounds such as GLU, GLY and AMPA. To make this fact clearer for the reader, this information has been added in the manuscript (Supplementary information: Annex 1. Text A2: Reagents and procedures used for the chemical analyses of glyphosate, AMPA and glufosinate). Note that in other studies where hair samples were washed prior to analyses of GLY or GLY and AMPA, Hooper et al. (2022) decontaminated hair of bats using ultrapure (MilliQ) water and subsequently isopropanol while Alvarez et al. (2022) used dichloromethane twice to wash human hair samples. This has been added in the text (Supplementary information: Text A2)

Alvarez J, Etting I, Larabi IA. 2022. Glyphosate and aminomethylphosphonic acid in human hair quantified by an LC-MS/MS method. Biomedical Chromatography 36; doi:10.1002/bmc.5391.

Hooper SE, Amelon SK, Lin C-H. 2022. Development of an LC-MS/MS Method for Non-Invasive Biomonitoring of Neonicotinoid and Systemic Herbicide Pesticide Residues in Bat Hair. Toxics 10:73; doi:10.3390/toxics10020073.

Possibly, different options such as the ones suggested by the reviewer (water/formic acid or methanol/formic acid) might provide relevant results for the removal of hydrophilic chemicals. This would of course require more extensive experimental work that goes beyond the scope of the present study.

Eventually, although considering the worst case scenario of an incomplete efficiency of the washing procedure, the presence of GLU, GLY and AMPA would still indicate exposure of the animals, and therefore not compromise the conclusions of this article.

d. In general, in the analysis section, more detail on the procedure followed is needed.

i. On which analytical method is it based?

ii. Internal standard concentration

iii. Calibration curve

iv. Mobile phases

- The analytical procedure used in the present study has been developed specifically and is fully detailed. The additional information requested by the reviewer regarding internal standard, calibration curve and mobile phase have been added to the revised manuscript (Supplementary information: Annex 1. Text A2: Reagents and procedures used for the chemical analyses of glyphosate, AMPA and glufosinate and Lines 257-299).

e. Limit of detection/quantification: This is maybe one of the most important comment to address, because the number of samples detected as positive depends on it. The authors set the LOD as the lowest detected value, but it is not a formal way of calculating it, indeed it is not a calculation per se. In the case of LOQ, they do not indicate how they calculate it. In view of the extensive literature on this subject (see e.g. Armbruster and Pry 2008, Wenzl et al. 2016), I think that a pre-established methodology should be used. Furthermore, in the Table 1, for glufosinate, LOQ appears to be lower than LOD, which is conceptually impossible. Please check this carefully!

We understand the questions raised by the reviewer and would like to give more details on the approach used in the present work. The methodology used here for the determination of the LOD has actually been used in all the studies conducted in our laboratory and results from a deep reflection including, among other aspects, requirements specific to the field of medico legal analytical toxicology. A key aspect is that LOD has to be determined experimentally (measured), and cannot be calculated from samples supplemented at higher concentration levels. Indeed, the past approaches based on signal/noise ratio (ex: S/N = 3 for LOD) are no more accepted in most of contexts, as LOD values extrapolated from higher concentration levels do not guarantee that the real LOD concentration could actually be detected. Moreover, it also has to be considered that with tandem mass spectrometry, flat baseline is often observed around the peak corresponding to the target analyte, making irrelevant any approach based on background noise, as suggested in the literature suggested by the reviewer.

In that regard, the most pragmatic approach is to determine the lowest concentration level that can actually be detected in a sample. Experimentally, this can be conducted in two manners.
First, by analysing blank samples supplemented with increasing amount of the target chemical until it is detected. Nevertheless, this implies that 1) blank material (preferably) is available, and 2) a sufficient amount of homogenous material is available to prepare the supplemented samples. Ideally, a specific LOD should even be determined for each sample (each subject) if considering the inter-individual variability in the matrix effect. This approach is unfortunately not realistic considering the limited amount of sample collected from wild animals such as small mammals.
The second approach consists in considering that the lowest detected concentration in a set of samples corresponds to the limit of detection (literally the lowest concentration level that can be surely detected). If the number of samples is sufficient, this value will mechanically tend to the “ideal” lowest detectable level of concentration. The 2 approaches described here will normally give the same LOD, since both are based on real measurements. The second approach can possibly slightly overestimate LOD, which has no consequence on results interpretation (contrary to LOD underestimation). It has to be noted that the first approach, conducted as step-by-step increased concentration (and not purely continuous) can also lead to slight LOD overestimation. These two approaches, based on experimental measurements, remain preferable than LOD extrapolated from higher concentration values.

LOQ was determined as the lowest concentration level for which variability and accuracy was below 25%. This information was missing in the original manuscript and has been added to the revised version. Since field samples are not available in sufficient amount to conduct method validation, it has to be conducted on blank samples from laboratory animals supplemented with standards. Eventually, a LOQ can be determined at a certain level (ex: 1 mg/mg), while the lowest detected values in the field samples (used as LOD) is higher (ex: 2 pg/mg). This explains that in rare cases, LOD is higher than LOQ, even it could be considered conceptually impossible if the approaches used to determine LOQ and LOD are not considered. We however fully understand that such situation can be confusing, and modified the tables and the text accordingly in the revised manuscript. Since we present lowest detected values instead of formal LOD, we have changed the wording to make it clearer: we indicated "Minimum" and therefore it is understandable that the lowest value can quantified and was higher than the LOQ

To follow as far as possible the recommendations of the reviewer, we have also:

- explicitly described our approach toward the LOD and the inclusion of lowest detected values together with literature references (Lines 287-299 and Supplementary information: Annex 1. Text A2).
- clarified that we used all detected values, including values below the LOQ together with a literature reference that was already cited in the first draft of the MS about the relevance of using values below the LOQ (Lines 293-299).

f. Data analysis: This is another confusing subsection. In order to achieve greater clarity, the statistical analyses could be described in the same order used later to explain the results.

g. In general, were the interactions spp/farming and/or spp/habitat included in those models performed?

- We have deeply reworked this section to answers the comments of the 3 reviewers. See Lines 343-415.

3. Results: Due to this study is based on a sampling design including conventional/organic farming and two different type of habitat. I think that a table showing the concentrations detected and the number of detection frequencies at least in supplementary information would be appreciated.

- This information was already provided in Sup Mat figures. We did not detail the values in the main table were detection frequency and concentration are shown because there were no significant differences (therefore the values would be more or less the same). To follow the reviewer's recommendation, we have moved some figures from Sup Mat to the main text (new Figure 6).

4. Discussion: Should focus on the essentials and be shortened, it is too long and difficult to follow.

- More sub-sections have been added to ease the reading. We have deleted some sentences in order to focus on the most important issues.

Other comments

Line 147: please change M. By Microtus due to this is the first time it is appointed.

- Done

Lines 164-170: please rewrite these lines, or move the sentence "A total of.." before "Individual samples of ", to avoid misinterpretation.

- Done

Lines 340-341: The sentence mixes results of previous work with the present work, please rewrite for clarity.

- We conducted the "PING" research programme which resulted in several publications, the present MS and the publication cited in the MS Pelosi et al. (2022). For more clarity, the sentence has been modified (see lines 470-471).

Lines 343-345: Given that in the previous and subsequent sentences the authors are giving values for detection rates, I think it would make more sense to give these also from the work cited.

- We have rephrased to clearly indicate that GLY was detected in all of the three samples analysed (so rate is 100%) (Line 495).

Line 364: Please, specify which compound you are referring to.

- Done

Lines 376-379: This could be due to differences in the tissue analysed in each study.

- According to this comment, the sentence has been modified as follows (lines 551-555): "However, AMPA was detected in two voles only at 130 and <160 pg/mg (LD of 100 pg/mg) (Newton et al. 1984), which is far lower than in our study. This may be due to the fact that the lowest detected value in our work was much lower (i.e. 0.240 pg/mg) and that different tissues were used to measure residues."

Line 477: Please, include the year of the reference cited.

- Done.

References

Armbruster DA, Pry T. 2008. Limit of blank, limit of detection and limit of quantitation. Clin Biochem Rev 29:S49-52.

Wenzl, T., Johannes, H., Schaechtele, A., Robouch, P. and Stroka, J., Guidance Document on the Estimation of LOD and LOQ for Measurements in the Field of Contaminants in Feed and Food, EUR 28099 EN, Publications Office of the European Union, Luxembourg, 2016, ISBN 978-92-79-61768-3, doi:10.2787/8931, JRC102946.