Review by Valentin Geslin, 06 Sep 2023 13:53

Overall
The manuscript is well written, clear, and precise. Assertions are well supported. The method is clear and complete with enough detail for reproducibility. Assumptions and interpretations of data are structured and well documented. Maybe a critical perspective on what could be improved in this study, as well as potential weak points or unaddressed blind spots and potential steps forward is missing at the end of the discussion.
Thank you for your interest. Regarding the point in the last part of your paragraph, about the need for a perspective critique, we have added a final perspective part, which indeed considerably improves the final message.

Title/Abstract/Introduction
The title reflects the content of the article. The summary is concise and clearly presents the main assumptions and conclusions of the study. The introduction is well detailed, it provides all the information necessary to understand the objective and the framework of the experiment. The research question is clearly presented, based on relevant literature and previous research conducted in the field.
Thank you.

Materials and Methods
Sufficient details are provided in "Materials and Methods" to allow replication of the experiment. Statistical analysis seems appropriate. However, the R script used for data analysis could be made available to allow better reproducibility of the analysis.
Thank you, the script has been added to the supplementary materials.
The experimental design seems consistent with the questions. Some remarks and questions concerning the experimental plan:

- *Have you considered the reproductive period as a potential influencing factor for AE and elimination rate (1)?*
  No, this parameter has not been considered, despite of physiological changes associated with reproduction as well as the emission of gametes could impact the bioaccumulation and elimination of metals. To avoid influence of this parameter, we only considered male gammarids with the same size (and with assumed same maturity stage).
- *What is the reason for this choice of sampling time? Where is the literature to support this choice?*
  - *"Individuals were sampled and counted at days 0.5, 1, 1.5, 2, 4, 7, 9, 11 and 14"*

  There is very little data in the literature to support this choice. We have based our decision on our knowledge of gammarids, previously assuming that the intestinal transit of gammarids was less than 48 hours. This is why we have increased the number of points acquired over this period, in order to best describe the curve used to determine metal assimilation and elimination. Today, with the data obtained, we estimate that the intestinal transit time for gammarids is around 24 hours.
- *Why have different concentrations of radiotracers been used for food contamination ? Where is the literature to support this choice?*
  - *"The leaf discs (…) were placed in (…) water contaminated with 2010, 2030 or 2000 Bq.mL-1 of 109Cd, 65Zn and 110mAg, respectively…*
  - *"Chironomids larvae were (…) exposed to 201, 203 or 200 Bq.mL-1 for 109Cd, 65Zn and 110mAg"*

  No, there's no literature to support this choice. In order to best describe the metal loss kinetics and, then, determine assimilation efficiency and elimination rates, the challenge

remained to obtain radiolabelled food as much as possible (taking into account radioprotection concerns) to accurately track radiotracers concentrations throughout the experiment. Leaf discs, as an inert material, supported high concentrations for radiolabelling whereas chironomid larvae were labelled alive. To avoid acute toxic effects of metals and acid (as a carrier solution for radioisotopes) on chironomids, we exposed them to 10-fold lower concentrations.

- *What is the reason for the difference in exposure time? Where is the literature to support this choice?*
    - *"For each metal, they were then either exposed to two radiolabeled leaf discs for 3-5 h, or to one radiolabeled thawed chironomid larva for 1h"*

  These differences were not based on the literature but on pre-experiments showing how long it takes a gammarid to eat a chironomid larva and a leaf disc (with different disc sizes). Gammarids usually eat chironomid very quickly whereas they nibble slowly on leaf discs, which are undoubtedly less appetizing for them. Whatever the food source, the aim was for the gammarid to ingest a pool of radioisotopes sufficient to follow its depuration throughout the experiment.

Results

Raw data available. Statistical results support the main conclusions. Some remarks and questions regarding the results:

- *Where are the data on mortality rate? Is there an effect on AE or elimination rate?*
  Precision is noted l.246. We had only one death, which occurred on the first day, so it is difficult to assess the impact of mortality on the parameters measured here.
- *Sampling time are not homogenous (see plot below). Measurements were more frequent during the first 100h after exposure and there are periods without sampling. Why? Where is the literature to support this choice?*
  Please see our answer above.
- *I don't understand why there are value above 100% in the percentage of remaining metal in gammarids (see plot below)?*
  Radioactivity counters are very sensitive to the position and shape of the object/organism being measured. As the radioactivity present in gammarids is measured when they are alive, and is still concentrated in the gammarid's intestine at this counting point (and not evenly distributed within the organism), it is possible that despite our best efforts some gammarids may have moved slightly, increasing the uncertainty around the measurement for these organisms (see Creswell et al., 2017, referenced in the manuscript). The 4 points concerned have been considered in the model as being at 100%, so that this does not affect the model.

Discussion

The interpretation of the data appears objective and robust. The conclusions are adequately supported by the results and literature. Maybe add what could be improved in the study, explain potential weaknesses or blind spots not covered by this experiment, and suggest potential steps forward.

A conclusions and outlook section has been added for this purpose, thank you.

References

References seem accurate and appropriate. They support the hypotheses, the results, and data interpretation. Perhaps "Material & Method" lacks a few references to support some experimental choices (choice of concentration, choice of exposure time).
In the literature, there is no standard methodology described for determining assimilation efficiency and elimination rates in organisms, but a body of papers using similar protocol plan (see papers from the IAEA-EL). This explains the absence of specific references to the literature to support our experimental plan. We calibrated the different points mainly based on previously acquired knowledge in the laboratory to 1) determine maximize the radiolabeling of food and exposed gammarids, and 2) describe as much as possible the loss kinetics considering digestive physiology of gammarids (see answers above).

Tables and figures
The tables and figures are useful, clear, and complete. They adequately illustrate the methodology and the main results. In Figure 2, maybe outline in the legend what's (a), (b), (c), (d), (e), or just remove these label from the graph.
Details have been added to the legend of Figure 2, thank you.

Bibliography
1. *McCahon CP, Pascoe D. Increased sensitivity to cadmium of the freshwater amphipod Gammarus pulex (L.) during the reproductive period. Aquat Toxicol [Internet]. 1988 Nov 1 [cited 2023 Sep 5];13(3):183–93. Available from: https://www.sciencedirect.com/science/article/pii/0166445X88900513*

Review by Davide Anselmo Luigi Vignati, 04 Mar 2024 09:05

Dear members of the managing board of PCI Ecotox Env Chem,
the contribution by Gestin et al. studies the assimilation efficiencies and elemination rates of Ag, Cd and Zn in gammarids via the trophic pathway using radio-labelling approaches. The is of interest considering that much of current knowledge and hazard/risk assessment on trace element accumulation is based on uptake or exposure via the aqueous route.
Thank you for your interest, the answers were made according to the comments.

I have three general comments and a few specific ones that are detailed below.

In the introduction, the specific aims of the paper can be stated more precisely to better explain how the present study specifically advances current knowledge. Lines 112-113 state that the aim of this study was to determine AE and elimination rates of Ag, Cd and Zn in G. fossarum. I am perfectly comfortable with the new data produced by the study, but I would appreciate more information on why this aim was chosen (see also specific comments).
Thank you, please see the answers below.

In the results and discussion section, the implications and ramifications of this research also deserve some explanations. At lines 311-312, the authors state that "...there is no general rule for predicting AE among metals and biological species". However, our ultimate objective remains precisely to find those general rules rather than go on performing experiments on a metal- and organism-specific basis. In my opinion, it would be very interesting to use the results from the present study to put forward hypotheses on how we can work toward that ultimate objective. Do we need to change methods/approaches in labelling studies so that activity in exposed organisms can be quantified in specific cellular compartments? Do we need a combination of pulse-and chase experiments with other techniques? A little bit less ambitious,

but equally important, how does the present study advances the specific knowledge on gammarids exposed to Cd, Ag and Zn?
Thank you, the aim of this document is not to determine a standard methodology, but we hope that the conclusions and perspectives section we have added at the end of the manuscript will provide some answers to this question. Regarding the last part of your question, determining assimilation efficiency and associated elimination rates is only the first step in our ambition to improve understanding of dietary metal bioaccumulation and fate in gammarids. The subsequent aim of this study is to integrate these data into toxicokinetic models, for which crucial information was lacking, such as assimilation efficiency, which will enable us to better describe the underlying mechanisms of metal bioaccumulation.

The document is sufficiently well writter and generally easy to read. Figures and tables are clear (and Figure 1 is commendable in its clarity). The authors may still want to proofread the text for a number of minor typos and minor errors in English usage such as subject in the plural and verb in the singular.
Thank you, the entire document has been revised to correct these errors.

SPECIFIC COMMENTS
Title. Please consider if "Assimilation efficiencies and elimination rates of Ag, Cd and Zn accumulated...." would not be more appropriate. We are dealing with these three specific elements. Unless some general principles for other elements can be derived from the study, I would prefer the more specific title.
The title has been changed accordingly, thank you.

Abstract. The abstract adequately reflect the content of the article. Verify if changes may be needed after adressing the general comments.

Keywords. Do we need both "trophic transfer" and "dietary pathway' in key-words? The two imply different concepts, but are practically the same within the purposes of the present article.
Indeed, in the manuscript, the concepts have not been redefined and clearly separated, the purpose of the keywords being to make the paper stand out for different searches, we have chosen to leave as it is in order to give a better visibility of our work in future keyword searches.

Introduction
Lines 59-60 and line 64. Can you please cross-check the statements in these portions of the text? Lines 59-60 state that G. fossarum is known to accumulate diet-borne metals, while line 64 states that less is known regarding the metal diet-borne assimilation and elimination. The two statements are not mutually exclusive. However, is it possible to be more specific with regard to what is actually less-known? Are available studies limited to elements other than those considered here? Do we lack clear numerical values for assimilation efficiencies and elimination rates for the metals considered in the study, despite having the corresponding values for uptake for aqueous route?
The second answer is the most appropriate, as there is a lack of data for all metals, considering the trophic pathway. Some studies have shown that metals are accumulated *via* the trophic pathway, but there is a lack of precise data to determine the importance of this. The text has been changed for greater clarity, thank you.

Line 73. Se is a metalloid. Consider using the term 'elements' instead of 'metals'.
The term has been changed, thank you.

Lines 72-77. Please verify if the references cited in this part of the text are those you actually wanted to use. I screened their abstracts, figures and conclusions. Borgmann et al. (1989) seems to be about methodological developments to optimize ecotoxicity testing in invertebrates, but does not mention exposure routes. Xu and Pascoe (1994) worked with Gammarus pulex, not with Daphnia magna. Pellet et al. (2014) is appropriate except that they could determine an AE only in the case of Cd.

We apologize for the error, as it was Borgmann et al., 2007 that is mentioned here (and not Borgmann et al., 1989, as originally noted). Regarding Xu & Pascoe 1994, this was an inaccuracy, so the reference has been replaced by Memmert, 1987. We have also rearranged the citations so that there is no misunderstanding, it was intended to understand: 1) Cd, Cu and Se for *Hyallela azteca* coming from Borgmann et al., 2007; and indeed 2) Cd only for *Gammarus pulex* coming from Pellet et al., 2014. We hope this is now clearer and thank you.

Lines 75-77. I agree that that metal handling by organisms and metal toxicity vary according to the exposure route. However, the reference cited at lines 72-75 do not fully support this statement. None of those references actually studies AE and toxicity at the same time (or I missed it). If this is the case, it would be better to say that "previous results suggest" or "may imply that"...rather than the strong statement of a causal relationship used in the original text.

The sentence has been changed accordingly, thank you.

Line 84: crosses

It has been changed, thank you.

Lines 112-113. Is it possible to have some more details on the scientific reasons for choosing these elements? Do we have a lot of information on uptake via dissolved pathway and need reliable data on the dietary pathway for comparison? Do we already have data on uptake via the dietary pathway, but data are highly uncertain and this study tries to understand why?

For zinc and cadmium, we have published data on the dissolved pathway. The data obtained here have enabled us to improve our knowledge, and to set up a toxicokinetic model of Cd accumulation by the trophic pathway, which is currently being submitted.

Lines 114-120. This part is really an excellent way to conclude the introduction. May I just ask for a little bit more information on the actual specific aims of the study? Determing AE and other parameters is a perfectly acceptable scientific objective and so is the study of the influence of food type on diet-borne accumulation. On the other hand, the introduction does not really tell us what we already know on the three elements that are considered in the present study nor on the specific influence of food type on AE of these elements for G. fossarum.

If nothing is known on the selected elements in terms of diet-borne AE, please say so in an explicit way. You are filling a knowledge gap for fundamental information, which is a worthy objective in itself. If previous but incomplete information on these elements exists, please be sure to precisely mention somwhere in the introduction what is already known on them and then explain at the end of the introduction how the present work advances current knowledge

If the introduction does not go into more detail on the specific influence of the type of diet on the AE of these elements for *G. fossarum*, this is because very little data is available on this subject. And all those we do know have been used here. This clarification has been added to the introduction, thank you.

Line 131. Could you please be more specific? Does 'regular renewal' means 'daily renewal' or other intervals?

The renewal was indeed carried out daily, the clarification was made in the text, thank you.

Lines 146-147. I am not sure to understand properly. I would say that Cd and Zn solutions were provided in a 0.1M HCl matrix. Saying that Cd and Zn were provided in their chloride form means, in my opinion, that CdCl2 and ZnCl2 were dissolved into the matrix. Analogous observations apply to Ag. Please consider if changes are necessary.

The details "CdCl2 and ZnCl2" and "AgNO3" have been added to the text for greater clarity, thank you.

Line 153-154. Can you please explain how the conversions were performed (maybe in the supporting information) or provide a reference with the details of the procedure?

This sentence has finally been removed from the manuscript, as concentrations in mass term (µg of metal per g of gammarid) were not used in the manuscript. However, if you're curious about the conversion process, we invite you to take a look at our previous article: Gestin, O., Lopes, C., Delorme, N., Garnero, L., Geffard, O., & Lacoue-Labarthe, T. (2022). Organ-specific accumulation of cadmium and zinc in *Gammarus fossarum* exposed to environmentally relevant metal concentrations. *Environmental Pollution*, *308*. https://doi.org/10.1016/j.envpol.2022.119625.

Line 157. Does this mean about 108 leaf discs for each element or 108 discs in total for the three elements?

The clarification “The leaf discs (~ 108 in all, i.e. 36 for each metal)” has been made for greater clarity, tank you.

Was there any specific reason for choosing the levels of activity used to label leaf discs? Is it possible (useful?) to provide the corresponding values in µg/L?

As explained above, the reason for the lower dose when contaminating chironomid larvae is that they are alive and the solutions containing radiotracers are acidic. Leaf discs, as an inert material, supported high concentrations for radiolabelling whereas chironomid larvae were labelled alive. To avoid acute toxic effects of metals and acid (as a carrier solution for radioisotopes) on chironomids, we exposed them to 10-fold lower concentrations.

Mass equivalent concentrations for zinc, cadmium and silver were 57.8, 0.37 and 16 $ng.L^{-1}$ respectively for leaf discs, and 5.7, 0.037 and 1.6 $ng.L^{-1}$ respectively for chironomid larvae. Allowing us to remain within environmental concentration conditions. The conversion has not been carried out in the manuscript as it does not provide any additional information, since we are not looking at toxicity.

Line 162. Please cross-check with line 139. At line 139, we say that chironomids were fed till the second larval stage. At this point, we have third instar larvae. If this is not a typo, please explain what happened while larve grew from the second to the third instar.

It was a mistake, the larvae were indeed reared to the third stage before exposure. This error has been rectified, thank you.

Line164. Same remark as line 157 for choosing activity levels.

Please refer to the answer above, thank you.

Line 167. Please specify the rinsing procedure. Was it a 5-day soaking as for leaf discs?

It's true that the previous sentence was imprecise, it has been modified as follows for greater precision: “At the end of the exposure period, the larvae were gently rinsed (i.e. rapidly soaked in 3 successive baths with clean water), then individually frozen and all counted by gamma-ray spectrometry.”, thank you.

Line 178. What procedure was used to assess which larve had eaten the most? Were the remains of leaves dried and weighed? Were pictures taken to assess the extension of the eaten surface?
Chironomid larvae were only radiolabeled being exposed to dissolved radiotracers. To determine the radioactivities they accumulated, they were all counted on the detector individually, as specified in line 168. The rate of gammarid ingestion was not defined for this experiment. Whether a gammarid had eaten or not was purely visual: if it had eaten at least one of the two discs available, it was considered to have eaten. If less than one disc was eaten, the gammarid was considered to have not eaten enough (in view of the preliminary experiments we had set up before launching the experiment concerning assimilation efficiency). Basically, gammarids that poorly fed on radiolabeled food ingested not enough radioactivity (i.e. whole-body gamma-counted alive after the pulse-chase-feeding period) allowing delineation of the loss kinetics.

Figure 1. Thank you for providing this clear overview of the experimental procedure. In the uppermost part of the figure, on the right-hand side, is it correct to indicate that gammarids were fed chironomids contaminated with 110Ag? At lines 168-169, the text says that no activity was detected in this case. Line 172 also seem to suggest that, in the end, gammarids were not fed chironomids labeled with Ag. Can you please clarify and decide if the figure needs to be modified?
Indeed, it could be confusing, the figure has been changed in this sense for better clarity, thank you.

Lines 222-223. Was a specific R package used or did the authors write the code? Please specify.
The script has been added to the supplementary materials, indicating also the R package used.

Lines 226-234. Please consider if this part of the text can be moved elsewhere in section 3. I do not have objections to the concepts put forward in the text. However, I would expect being shown the results of the present study before reading this kind of comments.
In hindsight, this sentence is more of a conclusion, so it has been removed from the beginning of the results section, thank you.

Line 236. May I suggest to modify the title of this subsection? What is described in section 3.1 is actually "data quality evaluation and selection".
The change has been made accordingly, thank you.

Section 3.2. I am missing a brief discussion of the kinetics models obtained from feeding gammarids with labeled chironomids. Modelling data for Cd and Zn are presented in Table 1, but only data for gammarids fed with alder leaves are discussed in the text.
We had a lot of data to work with, so we decided first to present the results obtained with leaves as food source and then to compare the results obtained for metals accumulated from chironomid larvae. This makes the text less heavy and less of a "data listing".

Furthermore, in the case of Cd, more than half of the gammarids fed with chirnomids had body activities below 150Bq and were excluded from the model. Excluding low-activity specimen was a sensible choice to avoid working with unreliable raw data, but please discuss if and how the exclusion of these low-activity specimen could have affected model calculations.
As explained, low initial pool of ingested radioisotopes leads to a rapid loss of gammarid whole body activities below the detection threshold of the gamma spectrometer and thus provide with too much counting uncertainties. In addition, based on *a posteriori* observation, gammarids that

showed radioactivity below 150 Bq immediately after feeding had all eaten very little (less than half a leaf disc), suggesting an altered feeding behavior, and potentially physiological disturbances hindering the right calculation of kinetic parameters for a “classic” gammarid population.

Figure 2. In the figure caption, please specify that graphs on the left refer to gammarids fed with alder leaves and graphs on the right to gammarids fed with chironomids.
The change has been made accordingly, thank you.

Table 1. Raw data in Tables S1 and S2 (and figure S1) show a large variability in the activities measured in each exposed gammarid. On the other hand, the errors on the estimates presented in table 1 rarely exceed 10%. Is it possible to comment on this?
This is because the uncertainty on the slopes of the curve is not defined by the variability of the accumulated metal quantity data (y-axis), but by the uncertainty around the time at which we switch from k_{es} to k_{el} (x-axis).

I would also be curious to see a comparison (in the supporting information) between the experimentally measured values and the corresponding modelled values to get a glimpse in the overall predictive strength of the model.
Thank you for your interest in the model, but this was not the aim of our article, and we have not carried out any such tests.

Line 295. Correct 'infinitely' to 'indefinitely'.
The change has been made accordingly, thank you.

Line 311-313. This remains indeed a big challenge. On the other hand, what can the community do to overcome this obstacle? While discussing kinetics of Ag elimination (lines 285-295), the authors correctly stressed the importance of intracellular compartimentalization to understand how gammarids handle Ag. What are the implications of these phenomena in relation to the applicability of pulse and chase experiments to determine AE? More specifically, how do the results from the present study advance knolwedge on gammarids exposed to Ag, Cd and Zn? Which questions are answered and which new ones are brought about? See also introductory comments.
A conclusion and outlook section has been added at the end of the manuscript. We hope that it corresponds to the expectations that you have formulated in this comment, thank you.

Lines 320-321. Lam and Wang (2006) and Pellet et al. (2014) do not deal with D. polymorpha. Is it appropriate to cite them here?
We're sorry, this was due to a sentence that had been changed and for which the reference had not been changed. The change has been made, thank you.

Line 327. Please define the acronym TAM (Trophically Available Metals) here. Otherwise, define it at line 330.
The change has been made, thank you.

Line 331. Do similar consideration apply to organism fed with alder leaves?
Indeed, this also applies to all other types of food. For greater clarity, the word "prey" has been changed to "food" l.324, thank you.

Lines 339-341. I am not sure that these lines support the statement at lines 336-338. An AE of 42% from algae is not higher than an AE 74% from squid. Please check and consider revision for clarity. Or am I misunderstanding the statement?
Indeed, the sentence was “a value 1.8 time lower than” and not “a value 1.8 time higher than”. The change has been made, thank you.

Lines 345-347. Was this bias not corrected by the rinse step after the labelling?
We assume that rinsing only removed metals that were weakly adsorbed, but those that were strongly adsorbed remained on the leaves.

Lines 349-354. I do not fully understand if the authors are actually recommending that TAM fractions should be determined in living food sources. If yes, what would be the methodological procedure to be followed or the methodological bottlenecks to be solved?
Furthermore, Chironomids were labelled only via the aqueous pathways, while in natural conditions metal uptake via the dietary pathway may be as important as the aqueous one. This does not invalidate the experiments. However, consider if this aspect deserves to be mentioned in the discussion as a recommendation for future studies.
In fact, determining the TAM fractions in living food sources, or dead ones for that matter, would provide a better understanding of the link between the metal concentration in food and the metal fraction assimilated by the predator, that depend on the metal form and its bioaccessibility
We agree that uptake pathway influenced metabolic speciation of metals and thus its bioaccessibility for predators. The fact that chironomids only accumulated via the dissolved pathway in our experiment was mentioned in the perspective conclusion, in order to make recommendations for future studies, thank you.

Lines 355-374. No objections on the information provided here. However, I would appreciate an explicit explanation on how the results from the present study advance current knowledge on the assimilation and elimination of Ag, Cd, Zn in gammarids and, if applicable, in invertebrates. In the same vein, based on the available results, what should be the priorities of future studies to advance knowledge in this research field?
A conclusion and outlook section has been added at the end of the manuscript. We hope that it corresponds to the expectations that you have formulated in this comment, thank you.