Revision round #1

Decision for round #1: ***Revision needed***

Dear Dr Tartu,

Thank you for submitting your manuscript entitled « Maternal body condition affects the response of the gut microbiome to a widespread contaminant in larval spined toads » to PCI Ecotoxicology. It has been now reviewed by two reviewers, and as you will see, both are positive about the study. As recommender of your manuscript, I also found the work reported of high interest and fully relevant to the scope of the journal. Reviewers also raised a number of detailed and constructive suggestions and comments that I wish you to consider before your manuscript can be accepted for publication.

I look forward to your revision.

Sincerely,

Marie-Agnès Coutellec

by ***Marie-Agnès Coutellec****, 09 Mar 2024 10:51*

Manuscript: **https://doi.org/10.1101/2023.12.18.572122**
version: 1

Authors: We are very thankful for the recommender's and reviewers' positive comments and constructive suggestions. We have taken into account all the comments raised by the two reviewers and we hope our manuscript is now suitable for the readers of PCI Ecotoxicology.

Review by anonymous reviewer 1, 23 Feb 2024 12:42

Review of the manuscript entitled 'Maternal body condition affects the response of the gut microbiome to a widespread contaminant in larval spined toads' submitted by Tartu et al. for publication in PCI in Ecotoxicology.

General comments

The main goal of this study was to assess the effect of AMPA the main transformation product of glyphosate on the gut microbiome of spined toad tadpoles. The approach employed by the authors consist in catching tadpoles from forest or agricultural sites and keeping them in the lab until oviposition occurred. 240 tadpoles were incubated in the Lab and some were exposed to AMPA at environmental concentration, 0.4µg.L-1. Growth and development of tadpoles were monitored over time. On two subsamples of 120 tadpoles each, the feces were collected (and merged by 3, 40 samples per treatment (control and AMPA)) to study the microbial communities. Data was analysed to assess the effect of AMPA considering the body conditions of the parents (male vs female) and the site of origin of the parents (forest vs agricultural). The study demonstrates that AMPA had a significant effect on the composition of the microbial community of the feces and that this was influenced by mother body conditions. Changes in the

composition of the microbial community of the feces were significant but the size of the effect was rather low: OTUs belonging to two phylum (Bacteroidetes and Actinobacteriota) were found to be responsible for the changes observed.

Overall, the paper is of interest, but it has to be improved to better put the emphasis on the main objectives of the study: assessment of the ecotoxicological effect of AMPA on tadpoles growth, development and feces microbial community. The title has to be changed: it is not about the gut microbiome but the microbiome of feces of larval spined toads. The discussion has to be shortened and to make it less speculative.

Authors: As suggested by reviewer 1, we have now modified the title as follows: "Maternal body condition affects the response of larval spined toads' faecal microbiome to a widespread contaminant". We have now significantly shortened the discussion and made it less speculative as detailed below in the specific comments.

Major comments

The origin of AMPA can be better described in the introduction by adding information on the enzyme involved in its formation: glyphosate is transformed by the enzyme GOX A (glyphosate oxidase) to AMPA.

Authors: We agree and added the following sentence in the revised version of the manuscript l. 39-42: "Although AMPA has two primary sources, phosphonate and glyphosate degradation, through the lysis of the C-P bond and action of the enzyme glyphosate oxidoreductase, respectively (Jaworska et al., 2002; Zhan et al., 2018), its origin in surface water and groundwater is mainly linked to the latter (Carles et al., 2019; Struger et al., 2015).»

The gut microbiome was studied by analyzing the bacterial communities found in tadpoles' feces: although the microbial communities in the feces are often used as a proxy of the gut microbiota as it has the advantage of being noninvasive, it is still debated if microbial communities of the feces are truly reflecting the ones of the gut microbiota (see https://doi.org/10.3389/fcimb.2020.00151). In addition, feces were collected after 4 to 6 days at the bottom of the aquarium. Therefore, one can hypothesize that feces have been potentially contaminated by the microbes found in the water coming from different origin. To me these two points have to be exposed in the paper: the second point is less important than the first one as we may expect that the abundance and diversity of the microbial communities in the feces are higher than those in the water.

Concerning the first point it has to be mentioned that the choice has been made to use a noninvasive method to get a proxy of the gut microbiome of tadpole in order to be able to release them after the experiment done.

Authors: We agree this information was missing in the previous version of the manuscript, we therefore added this sentence l. 148-150: "Although there are some controversies about using faecal microbiome to reflect gut microbiome comprehensively (Tang et al., 2020), we still privileged this non-invasive sampling method to release the toadlets upon metamorphosis."

For the second point, we also analysed bacteria in tap water to control for external bacterial input, as mentioned in the 'spike control' section: "We obtained 19,270 and 28,848 reads in the

water samples; and 26,579 and 38,633 reads in the kit samples, from which respectively 98.25; 98.73; 99.77; and 99.71% were identified as the spiked bacteria *Imtechella* and *Allobacillus*."

As put forward by reviewer 1, we added the following sentence to inform the readers that the abundance and diversity of the microbial communities in the faeces are higher than those in the water l. 217-219: "Therefore, the bacterial inputs from the dechlorinated tap water to faecal samples are negligible; spiked bacteria only represented between 0.002-0.06% in faecal samples."

It is mentioned that the feces from three siblings tadpoles receiving the same treatment were pooled to increase the genetic diversity (of microbial gut microbial communities I guess?). This statement is not pretty obvious though for different reasons mainly because the environment of the siblings was the same and also because the origin of their genetic diversity (same parents) was rather limited. To me it is better to justify this statement for technical reasons (less samples to be analyzed and higher quantity of feces obtained for DNA extraction).

Authors: yes, the pooling was to increase genetic diversity of microbial communities. We modified the sentence referring to the pooling as follows: "To increase the quantity of faeces per sample and the genetic diversity of microbial communities, we pooled the faeces from three sibling tadpoles receiving the same treatment (control or AMPA) in one tube. We thus obtained 40 pools (**Figure 1**)".

The 16S rRNA amplicons were generated by PCR carried out on DNA extracted from the feces: the PCR program is a bit strange with a two-step PCR one for 10 cycles at 57°C as melting temperature and another one at 65°C as melting temperature for 25 cycles. Why this choice was made? The first part of the PCR is carried out below the theoretical melting temperature of the primers: this may lead to the amplification of 16S rRNA amplicon with mismatch and create artificially diversity in the sequences amplifies latter on in the second part of the PCR.

Authors: Our primers include a synthetic region upstream of the specific part, to perform a two-step PCR, and therefore the theoretical melting temperature is relatively high in comparison to classical PCR primers that are more in the Tm=60°C range. The specific primers we use here are BACT27F and BACT1391R, and their theoretical annealing temperature computed for a 400 nM primer concentration and the LongAmp Taq (NEB https://tmcalculator.neb.com) is 60°C. We performed a comparison of several polymerase for 16S metabarcoding and decided to use the tiAMplus polymerase (https://eurx.com.pl/product/ek2930/) and these optimized PCR conditions with an annealing of 57°C for the initial cycles.

The two-step PCR strategy in which the second PCR is used to attach synthetic index sequences enables to pool PCR products before a so-called multiplex sequencing (PCR-barcoding amplicons, https://community.nanoporetech.com/docs/prepare/library_prep_protocols/pcr-barcoding-96-amplicons-sqk-lsk110/v/pbac96_9114_v110_revl_10nov2020/pcr-barcoding-amplicons-cdna?devices=minion). Therefore, the specificity of the amplification in the initial cycles is dependent on the melting temperature of the specific part. This explains why we use an annealing temperature of 57°C for the first ten cycles. After these ten cycles, most amplification will rely on a perfect match over the whole length of the primers, and we use an increased annealing temperature of 65°C. Our results on mock community control and on the spike control show that we do not suffer from an artificial diversity due to PCR, actually the diversity we observe is in line with published reports on similar microbiomes.

The estimation of the microbial biomass in the feces by monitoring the spiked-in bacteria (inverse relationship) is not the classical tool used: usually as molecular-based approach (a range of other methods are available to quantify microbial biomass) 16S rDNA is quantified by qPCR and expressed in number of sequences per ng of DNA or per g of sample analyzed (here tadpole feces). As this method is not often used it is rather difficult to compare to other studies. This proxy is difficult to relate to the microbial biomass. I would suggest to remove it from the paper as it does not give much information or to present it a different way as the method used does not allow to assess properly the microbial biomass of feces.

Authors: Although we agree that this tool may not be as classic as the qPCR approach, we decided to use the Zymobiomics spike-in control because we found it exactly fit for the purpose of calibration of ratios of absolute abundances to compare biomass across samples as described in the commercial protocol (https://zymoresearch.eu/collections/zymobiomics-microbial-community-standards/products/zymobiomics-spike-in-control-i-high-microbial-load) and also in the scientific literature :

Jones MB, Highlander SK, Anderson EL, Li W, Dayrit M, Klitgord N, Fabani MM, Seguritan V, Green J, Pride DT, Yooseph S, Biggs W, Nelson KE, Venter JC. Library preparation methodology can influence genomic and functional predictions in human microbiome research. Proc Natl Acad Sci U S A. 2015 112:14024-9. doi: 10.1073/pnas.1519288112.

Stämmler F, Gläsner J, Hiergeist A, Holler E, Weber D, Oefner PJ, et al. Adjusting microbiome profiles for differences in microbial load by spike-in bacteria. Microbiome. 2016;4(1):28. https://doi.org/10.1186/s40168-016-0175-0.

Tkacz A, Hortala M, Poole PS. Absolute quantitation of microbiota abundance in environmental samples. Microbiome. 2018 Jun 19;6(1):110. doi: 10.1186/s40168-018-0491-7).

Maybe this explanation lacked in the method section, we therefore modified the sentence l.174-175: "We added 1 µL of the Zymobiomics Spike-in control I (High microbial Load D6320) to each sample as *in situ* positive control (Galla et al., 2023) » and added l. 230-232: "We also analyzed the spike-in fraction (D6320) out of the total bacterial abundance to estimate bacterial biomass *in situ* (Jones et al., 2015; Stämmler et al., 2016; Tkacz et al., 2018)."

Our result on the controls underscores the effectiveness of this approach: as expected and pointed in our answer on a previous comment, we indeed observed a very low abundance of bacteria in tap water (spiked-in bacteria represented 98.25% and 98.73% of the total abundance). We are confident this analysis provides a robust estimation of the bacterial biomass in a sample; we would therefore prefer to keep it throughout the manuscript.

A missing information is the fate of AMPA during the growth of spined toad *Bufo spinosus* tadpoles: was AMPA detected and quantified in the water of the aquarium?

Authors: Yes, we quantified AMPA in aquarium water. We added the following sentence l. 127-128: "An independent accredited analytical laboratory confirmed AMPA concentration in water (QUALYSE, Champdeniers-Saint-Denis, France)".

As it is stated in the manuscript several microbes are able to degrade AMPA. So one can hypothesize that overtime AMPA concentration decreased, changing the scenario of exposure.

Authors: We actually spiked the aquarium water every time the water was changed (on a weekly basis). Although there might be some fluctuations of AMPA concentration within a week, these fluctuations are smoothed over the entire exposure period. To make it clearer to the readers we modified our sentence l. 142-144 as follows: "Water was changed weekly and 0.4 µg L^{-1} AMPA was added to the AMPA group tanks after each water change".

Minor comments

Remove legally in line 34

Authors: "legally" has been removed

Others instead of other in line 35

Authors: this has been corrected

Fast instead of faster in line 35

Authors: this has been corrected

Transformation products instead of breakdown products in line 38

Authors: this has been corrected

Remove negative in line 45

Authors: "negative" has been removed

Add 'in its ecotoxicity' after involved in line 53

Authors: we added "in its ecotoxicity"

Cut the sentence in line 60 and start with 'A dysbiosis consisting in a modification in the composition and function of the gut microbiota in response to a stress'

Authors: the sentence has been modified accordingly

'Considering the widespread presence of AMPA' instead of 'considering the higher presence of AMPA' in line 71

Authors: the sentence has been modified accordingly

Replace predicted by hypothesized in line 93

Authors: this has been modified

Remove composition in line 94

Authors: "composition" has been removed

Add of ‘microbial communities’ after ‘genetic diversity’ in line 144

Authors: the sentence has been modified accordingly

Add information on the amount of DNA extracted from the feces (mean value ± standard error). Similarly add some information on the amount of amplicon obtained after processing them for MinION sequencing (in ng/µL of DNA) to give an idea on how much you need to reach the 150 ng loaded on the flow cell (information provided in line 179).

Authors: We evaluated DNA concentration by spectrophotometry (Nanodrop) after extraction and obtained a mean concentration of 63.6 ± 49.6 ng/µL (median 54.0 ng/µL, minimum 9.7 ng/µL, maximum 219.8 ng/µL). This equated in terms of DNA quantity to 3180 ± 2479 ng DNA per faeces sample (median 2697 ng, minimum 485 ng, maximum 10990 ng). We also quantified DNA concentration using a Qubit Broad Range kit and obtained values corresponding to 82 ± 20 % (N=24 measures) of the ones determined using the Nanodrop spectrophotometer. After the first round of PCR (16S), we obtained a mean quantity of 1.82 ± 0.59 µg of pooled PCR products after purification. We used 100 ng of each PCR product for a second round of PCR (multiplexing) and finally obtained 3.4 ± 1.3 µg of purified product. We pooled all products in an equimolar way, using 170 ng of each barcoded PCR product and used 1 µg of the pool as input for the DNA repair step of the SQK-LSK-109 kit for sequencing library construction.

As suggested, we added in the revised manuscript the amount of DNA extracted from faeces, l. 180-182: “We controlled the quality and quantity of extracted DNA (3180 ± 2479 ng DNA per faeces sample) using spectrophotometry (Nanodrop) and fluorometry (Qubit).”

And l. 192-195: “After the first round of PCR (16S), we obtained a mean quantity of 1.82 ± 0.59 µg of pooled PCR products after purification. We used 100 ng of each PCR product for a second round of PCR (multiplexing) and finally obtained 3.4 ± 1.3 µg of purified product.”

In the figure 5 line 313 the r2 of the regression should be added on the graphs shown in the panels A and B.

Authors: We have added the conditional r2 (which considers the variance of both the fixed and random effects) in the legend of figure 5: “Bacteroidota (A) and Actinobacteriota (B) abundances (number of reads) were differently associated with maternal body condition as inferred by their scaled mass index (SMI) according to AMPA exposure (A, conditional r^2 = 0.23 and B, conditional r^2 = 0.46).

In line 340, change or remove gut microbiota biomass (see my suggestion in major comment section).

Authors: As put forward in the general comments section, we have strong evidence that spike-in material represents a good proxy of *in situ* bacterial biomass and would prefer to keep the discussion around these analyses as is.

In line 446 the term agrochemicals is not appropriate: AMPA is a transformation product of glyphosate. AMPA is not an agrochemical: glyphosate is one such.

Authors: we agree and modified our section title by: Effects of agrochemicals transformation products according to gut microbiota composition

In line 448 change or remove gut microbiota biomass (see my suggestion in major comment section).

Authors: As put forward in the general comments section, we have strong evidence that spike-in material represents a good proxy of *in situ* bacterial biomass and would prefer to keep the discussion around these analyses as is.

The two paragraphs from line 477 to line 495 contains elements that are too speculative at this stage. I would prefer a discussion on the ecological relevance of the observations made here: only two phyla affected by AMPA and in different ways.

Authors: We have now shortened these paragraphs, made them more informative and put forward the ecological relevance of a decrease of Bacteroidota in the gut. The revised version reads as follows:

"For instance, Sphingobacteriales can produce sphingolipids that regulate the immune system and lipid metabolism (An et al., 2011; Bai et al., 2023; Olsen and Jantzen, 2001). Flavobacteriales, on the other hand, play several roles in various metabolic pathways, including vitamins, amino-acid and fatty acid biosynthesis (Rosas-Pérez et al., 2014; Yang et al., 2017; Zhou et al., 2022). Flavobacteriales can thus bear positive effects on the host growth and development (Pan et al., 2023). At the genus level, *Cloacibacterium* sp. can degrade cellulose and may have a critical role in transforming plant-derived complex dietary carbohydrates into essential short-chained fatty acids (SCFA) for herbivore organisms such as spined toad tadpoles (Flint et al., 2012; Fujimori, 2021; Hu et al., 2021; Martens et al., 2011; Zhang et al., 2018). Therefore, a decrease in Bacteroidota could disrupt nutrient intakes, leading to a delayed development length, as observed in agricultural AMPA-exposed tadpoles from the present study (Tartu et al., 2022). In the crucian carp (*Carassius auratus*), for instance, glyphosate exposure resulted in a dysbiosis of Bacteroidota at the phylum level, and Bacteroidota abundance was negatively correlated with different metrics of growth performance (condition factor, fat ratio and specific growth rate) (Yan et al., 2022)."

Line 496: as the effect of AMPA was not affected by the origin of the parents (forest vs agricultural sites) this last paragraph of the discussion is speculative and might be removed or shorten drastically.

Authors: We agree that the effects of AMPA on faecal microbiota were not affected by the origin of parents, however several other fitness metrics (such as deformity rate upon hatching and development duration) monitored in the same individuals were affected by AMPA and the origin of the parents. And therefore, find it relevant to underline that AMPA might affect differently some traits (deformity rate at GS25 in forest individuals), faecal microbiota at GS37 regardless the habitat (interestingly at this stage the effects of AMPA according to origin were not very strong), total development duration length in agricultural individuals. We believe it is important to underline the fact that although agricultural populations have been exposed over a long period of time, they do not seem to show adaptation, during some stages the effects of AMPA are even only observed in agricultural tadpoles.

We have modified the paragraph as follows to make it clearer to the reader that although effects of AMPA on faecal microbiota did not depend on parents' habitat, other fitness traits did:

"We have previously reported that AMPA exposure was associated with a higher deformity rate upon hatching, especially in individuals from AMPA-free forest habitats, and increased development length in AMPA-exposed individuals from agricultural sites (Tartu et al., 2022). While embryonic stages may be more sensitive to AMPA exposure in forest individuals (AMPA-preserved population), they might be more resilient to a gut microbiome dysbiosis as no further effects on fitness were observed at metamorphosis (Tartu et al., 2022). In contrast, agricultural individuals (AMPA-exposed population) could be more resistant during embryonic stages. However, gut microbiome dysbiosis could still result in a longer development duration (Tartu et al., 2022). These findings again underline the part that may play the host genotype in shaping the consequences of gut microbiota dysbiosis. Yet, we have to remain cautious as we only followed the exposed individuals until metamorphosis and deleterious effects could appear later in life, as early-life microbiota composition shapes fitness trajectories in amphibians (Knutie et al., 2017; Warne et al., 2019). In addition, there is alarming evidence of the disappearance of breeding spined toads in agricultural habitats (Renoirt et al., in press), which could be a long-term effect of early-life exposure to toxicants."

Review by Lauris Evariste, 06 Feb 2024 16:44

The article from Tartu and collaborators deals with the relationship between maternal body condition and the responses of the tadpole's gut microbiota to AMPA exposure, which is the main degradation metabolite of the herbicide glyphosate. The article is well written and present a well conducted gut microbiota study. The authors monitored the gut microbiota composition using the feces produced which constitutes a good way to perform analysis over time in a non-invasive way. The data provided here are relevant as they point out the need to monitor such parameters in genitors for further studies and probably associate sampling of parental gut microbiota. I only added some comments that, I hope, would help to improve the reading of the study.

Introduction

The introduction section is well written and provide relevant information related to the topic of the research conducted. I would only suggest to add some information that would help the reader to better understand some points.

Line 45: While the molecule is highly frequently detected in the environment, it might be interesting to indicate the range of environmental concentrations of AMPA earlier than in the material and method section. Then, are the controversial effects associated to AMPA exposure found in the literature are associated to relevant exposure concentration/scenarios. This might help the reader to understand the choices of the tested concentrations in this study.

Authors: We agree and have modified the paragraph in the introduction as follows l.43-47: "Importantly, AMPA is detected much more frequently (20-50% more detected) and is more persistent in the environment (half-life 2-8 times longer) than glyphosate, with concentrations in surface water generally ranging between 0.2 and 5 µg L^{-1} (Duke, 2020; Grandcoin et al., 2017; Kolpin et al., 2006; Maggi et al., 2020; Ojelade et al., 2022). »

Line 65: To justify that studies dealing with the effects towards the gut microbiota were mainly carried out using glyphosate. It might be relevant to mention that the glyphosate targets the

enzyme EPSP synthase, providing its "specific" herbicide action. However, this enzyme is also found in bacteria, which can lead to deleterious effects, especially in host associated bacteria which homeostasis relies on.

Authors: Thank you for this suggestion, we have now added the following sentence l.71-77 in the revised version of our manuscript: "Because of its mode of action which is to inhibit the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) of the shikimate pathway, a metabolic pathway specific to plants but also microorganisms (Herrmann and Weaver, 1999), several studies have tested the effects of glyphosate exposure on vertebrates and invertebrates gut microbiota composition (Blot et al., 2019; Cuzziol Boccioni et al., 2023; Ding et al., 2021; Fréville et al., 2022; Iori et al., 2020; Lehman et al., 2023; Lozano et al., 2018; Mesnage et al., 2021; Motta et al., 2018; Owagboriaye et al., 2021; Puigbò et al., 2022; Ruuskanen et al., 2020; Walsh et al., 2023). »

Line 89: I think it would be wise to provide the information about gut microbiota transmission before the hypothesis involving gut microbiota dysbiosis. Stating first that previously observed effects altering development and inducing oxidative stress or mortality might be associated to gut microbiota dysbiosis seems like a small shortcut.

Authors: We agree, the previous version of the paragraph was much of a shortcut. We modified this by adding arguments on the fact delayed development could be related to gut microbiome dysbiosis l.98-100: "The composition of gut microbiota is tightly associated with growth rate and metamorphosis in anuran species (Emerson and Woodley, 2024; Lv et al., 2023; Park et al., 2023)".

We have also moved the information about gut microbiota transmission upper in the manuscript, in the paragraph describing the gut microbiome, l. 60-63: "The gut microbiota composition depends on both horizontal (e.g. habitat, diet, conspecifics) and vertical transmission (e.g. parents) in vertebrates (Comizzoli et al., 2021; Moeller et al., 2018; Murphy et al., 2023; Robinson et al., 2019; Scalvenzi et al., 2020). »

Material and Methods

Do the procedures used for capture and release of the animals as well as exposure protocols required submission to ethical committee for regulatory validation? If so, please indicate agreement numbers or any relevant information.

Authors: The requested information appears in the 'Ethics statement' section, still we added the agreement numbers as recommended l. 144-146: "Ethics committees approved this study (permits APAFIS#13477–2018032614077834 and DREAL/2020D/8041)."

Results

Figure 2: It might be interesting for illustration purpose to represent the (non)observed effects on beta diversity based on the Unweighted and weighted unifrac distances through the presentation of PCOA results for example.

Authors: As suggested, we added an illustration of these effects. However, we rather represented boxplots of the calculated unweighed and weighed Unifrac distances according to

treatment, as the effect is visually more obvious using this representation than with a PCoA. We added this as Figure S2 in supporting information.

While the exposure to the AMPA affected the gut microbiota of exposed tadpoles, is there any related effects observed in the host organism such as altered growth or other endpoint? If such data are available, even without apparent effects, it might be valuable for the article to indicate them.

Authors: We actually did observe significant effects of AMPA on increased deformity rate and development duration on the exposed tadpoles, however these results have been published in a companion study using the same individuals. References to these results are mentioned in the introduction l.94-98: "In addition, in a companion study using the same individuals as the present one, we reported that environmentally relevant concentrations of AMPA (0.4 µg/L) led to increased deformity rate upon hatching and increased development length, with effects depending on the habitat of origin of the parents (agricultural *versus* forest, Tartu et al., 2022). »

And tried to make it clearer in the discussion as well l. 506-508 : "Therefore, a decrease in Bacteroidota could disrupt nutrient intakes, leading to a delayed development length, as observed in agricultural AMPA-exposed tadpoles from the present study (Tartu et al., 2022)."

And in the last paragraph: "We have previously reported that AMPA exposure was associated with a higher deformity rate upon hatching, especially in individuals from AMPA-free forest habitats, and increased development length in AMPA-exposed individuals from agricultural sites (Tartu et al., 2022). While embryonic stages may be more sensitive to AMPA exposure in forest individuals (AMPA-preserved population), they might be more resilient to a gut microbiome dysbiosis as no further effects on fitness were observed at metamorphosis (Tartu et al., 2022). In contrast, agricultural individuals (AMPA-exposed population) could be more resistant during embryonic stages. However, gut microbiome dysbiosis could still result in a longer development duration (Tartu et al., 2022). These findings again underline the part that may play the host genotype in shaping the consequences of gut microbiota dysbiosis."

Discussion

The authors provided some discussion sections that are welcome such as the possible vertical transmission of the gut microbiota that might be involved in the observed effects. While an extended part of the discussion focusses on the gut microbiome, the authors successfully mentioned other possible mechanisms involved in the observed effects which is appreciable.

In the last discussion section, it would be interesting to discuss the obtained results regarding the works performed by Knutie and collaborators (which is not cited in this work), demonstrating that early alteration of the tadpole's gut microbiota might lead to detrimental physiological effects at adulthood. Thus, the fact that early gut microbiota alteration might compromise frog homeostasis after metamorphosis would provide wider perspectives in amphibian protection research.

Authors: We totally agree with this statement, and have added the following sentence at the end of our last paragraph: "Yet, we have to remain cautious as we only followed the exposed individuals until metamorphosis and deleterious effects could appear later in life, as early-life microbiota composition shapes fitness trajectories in amphibians (Knutie et al., 2017; Warne et al., 2019). In addition, there is alarming evidence of the disappearance of breeding spined toads

in agricultural habitats (Renoirt et al., 2024), which could be a long-term effect of early-life exposure to toxicants."