Peer Community In
Ecotoxicology & Environmental
Chemistry

Effects of AMPA on *Bufo spinosus* microbiota

Marie-Agnès Coutellec *based on peer reviews by* ***Lauris Evariste***, ***Fabrice Martin-Laurent*** *and 1 anonymous reviewer*

Sabrina Tartu, Nicolas Pollet, Isabelle Clavereau, Gauthier Bouchard, Francois Brischoux (2024) Maternal body condition affects the response of larval spined toads' faecal microbiome to a widespread contaminant. bioRxiv, ver. 4, peer-reviewed and recommended by Peer Community in Ecotoxicology and Environmental Chemistry.
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The overall pollution of air, water, and soil is currently recognized as one of the five main drivers of biodiversity loss (IPBES 2019). Among chemicals, pesticides play a significant role in this global crisis, as recently re-assessed at the scale of France (Pesce et al. 2023). In this context, although parent molecules are subject to national and international regulations, based on *a priori* ecological risk assessment (*e.g.*, REACH) as well as monitoring in some environments (see *e.g.*, pesticides classified in the priority list of substances by the Water Frame Directive), pesticide metabolites are rarely considered. In the case of the widely used herbicide glyphosate, a particular concern is rising about its primary metabolite, aminomethylphosphonic acid (AMPA), due to its persistence and overlooked toxicity.

Amphibians are the most threatened class of vertebrates on earth, with two in every five species considered threatened with extinction (IUCN Red List). While this overall decline has multiple causes, the contribution of pesticides is suspected to be significant in some regions.

In this context, Tartu et al. (2024) studied the effects of AMPA on the gut microbiota of the spined toad, *Bufo spinosus*. This work complements a previous study which showed embryo mortality, oxidative stress, deformities at hatching, and delayed development (Tartu et al. 2022). Using a common garden experiment based on populations from contrasted habitats (agricultural vs woodland, same as in the previous study), the authors captured breeding pairs and collected the eggs laid in the laboratory. These were exposed to 0.4 µg/L AMPA during embryonic and larval development. Individual microbiota was analysed non-invasively, i.e., using the faeces collected in treatment vessels. Bacterial biodiversity was genetically assessed (16S rRNA). The community biomass and taxonomic structure were analysed as a function of chemical treatment, mother and father body condition (fat vs thin), as well as population of origin.

As a primary effect, AMPA reduced the microbial biomass. Furthermore, a significant interaction was detected between AMPA and mother condition on the community structure and composition. This alteration, observed in « fat » females only, was reflected through a significant decrease in Bacteroidota and a significant increase in Actinobacteriota (the latter being consistent with the ability of some species in this phylum to use AMPA as a source of phosphorus). The higher sensitivity of tadpoles from females in better condition seems counterintuitive, since better body condition is expected to be associated with higher fitness (and possibly higher ability to face chemical stress), the authors discuss this in the light of the literature (which shows that microbiome-fitness relationships are not often evidenced in natural populations), and hypothesize that these females in better conditions host a microbiota that may be more efficient, yet also more sensitive to AMPA. Not ruling out other possible factors ignored in their study, in particular genotypic effects, the authors further discuss the importance of maternally transmitted effects via the microbiota.

Altogether, the results published by Tartu et al. (2024) provide important new findings on AMPA toxicity to amphibian microbiota, and also confirm the occurrence of vertical transmission of the microbiota from mother to progeny in this vertebrate class.

References:

IPBES (2019). Global assessment report on biodiversity and ecosystem services of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services. E. S. Brondizio, J. Settele, S. Díaz, and H. T. Ngo (editors). IPBES secretariat, Bonn, Germany. 1148 pages. https://doi.org/10.5281/zenodo.3831673

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Tartu, S., Renoirt, M., Cheron, M., Gisselmann, L.-L., Catoire, S., Brischoux, F. (2022). Did decades of glyphosate use have selected for resistant amphibians in agricultural habitats? Environ. Pollut. 310, 119823. https://doi.org/10.1016/j.envpol.2022.119823

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Reviews

Evaluation round #2

Reviewed by **Fabrice Martin-Laurent**, 17 April 2024

Dear Authors,

I would like to thank you for the great job you did in producing your revision: almost all my comments have been answered and i have no additional comments to make except that the two comments you made about the 16S rRNA PCR program and about the microbial biomass could somehow be included as supplementary files. This might be meaningful for readers having some questions related to these two comments I made.

Congratulations for this nice paper.

All the best,

Fabrice ML

Reviewed by **Lauris Evariste**, 10 April 2024

The authors took into account and responded to all the comments. Thank you for considering the remarks and for this work

Evaluation round #1

DOI or URL of the preprint: `https://doi.org/10.1101/2023.12.18.572122`

Version of the preprint: 1

Authors' reply, 20 March 2024

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Decision by **Marie-Agnès Coutellec**, posted 09 March 2024, validated 09 March 2024

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

Reviewed by anonymous reviewer 1, 23 February 2024

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.

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.

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, on 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 in 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.

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).

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 led 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.

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.

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? 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.

Minor comments

Remove legally in line 34

Others instead of other in line 35

Fast instead of faster in line 35

Transformation products instead of breakdown products in line 38

Remove negative in line 45

Add 'in its ecotoxicity' after involved in line 53

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'

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

Replace predicted by hypothesized in line 93

Remove composition in line 94

Add of 'microbial communities' after 'genetic diversity' in line 144

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).

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

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

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.

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

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.

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.

Download the review

Reviewed by Lauris Evariste, 06 February 2024

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 researches 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.

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.

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.

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.

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.

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.

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 researches.