Evaluation report of the doctoral dissertation presented by Msc Alan Puckowski entitled: "Analytics, sportion and ecotoxicology evaluation of mixtures of selected veterinary pharmaceutics in the aquatic environment"

Overall recommendation

The presented thesis is of quality and in accordance with the requirements for a PhD.

Analysis of the Content

The thesis introduction has been, in general, well prepared and the scientific topic compressively revised, at times with fair enthusiasm. In particular, the candidate presented well the environmental problems provoked by the emergent pollutants under analysis- fluoroquinolones and benzimidazoles. The potential broad risks of these toxicants were carefully revised. Importantly, many of the limitations and knowledge gaps in the established literature on the research topic were presented with great detail. This facilitated the presentation of the selected experimental approach designed to overcome some of the technical limitations found in current literature. The research problem was well explained as well as the questions to be investigative.

The research methods were well chosen and their appropriateness was described in a convincingly way. Collectively, they constitute already a sept forward to the most conventional used methods. The candidate identified limitations/inconsistencies in literature and proposed adequate experimental means to overcome such, namely i) ecotoxicological test battery comprising several aquatic organisms; ii) focussing the effect of both single compounds and their mixtures (validate or not through well-known prediction models), iii) and considering both the compound's solubility/stability using a robust analytical method as well as iv) preliminarily their potential to be carrier by micro-plastics.

Naturally, the generated datasets are of quality and collectively they present knowledge that goes beyond that of the state-of-the-art, including interesting findings on the toxic effects of mixtures of toxicants or of prolonged exposure times, and the preliminary analysis of the threats associated to micro-plastic as carriers of the toxicants. All these aspects are extremely relevant and timely, and were discussed also in a convincingly way. The ability to use, evaluate and in part refute existing opinions in the field has been made clear, despite of a few speculative statements which may have been further elaborated.

The conclusions of the thesis synthesise with precision the core results and point out some of the open questions to be addressed in the near future, comprising in general aspects which have been discussed before (*e.g.* degradation intermediates, fate mechanism and mode of action). The prediction methods for the toxicity of mixtures and the reasons why there are deviations from the observed experimental data could have been further develop. Means to specifically address some of these open questions have not been discussed. Thought this is reasonable since the already develop methods will be used to address unresolved aspects, new methods are required to analyse in detail for

example the mode of action of the toxicants. The last likely require expertise on biology that is lacking.

Technical aspects

The thesis language is in general of good quality and the style of writing is often personal, enthusiastic and in some moments presents a tutorial style that is elegant. The layout (chapters) of the thesis is well structured and the flow of the information is rational, regardless that sometimes the use of results in the experimental chapters is confounding (*e.g.* to explain the toxicity prediction models). The references were used in a consistent way and were timely. The figures and tables (including schematics) are functional and reflect well the acquired data.

List of questions

1. What is the production level or usage levels of benzimidazoles, worldwide?
2. How often the selected drugs co-exist in the environment?
3. To understand the toxicity of mixtures of compounds at a real scenario why you have focus on drugs belonging to the same class? Can you elaborate on this?
4. The toxicants may be processed by the gut microbiota of non-target organisms; any idea of potential metabolites that will be generated?
5. Do you expect that sportion of the drugs to the outermost cellular structures of the microbiota to play an important role?
6. You stated that antimicrobial are usually non-biodegradable (page 18); can you elaborate further?
7. In the recent study by Daughton CG (Sci Total Environ. 2018 619-620:748-764) they propose the concept "Sewage Chemical-Information Mining"; can you link this to your thesis?
8. Bioavailability is more complex and expensive to analyse than the total amount (pag. 22); can you elaborate on this?
9. Do you consider that the same MoA will always translate in a concentration addition effect?
10. If compounds possessing distinct MoAs ultimately led to baseline toxicity, will you always get a combine CA+IA model?
11. Why have you choose *Lema* for the prolonged toxicity test?
12. Why you have not analysed the bio-accumulated fraction of the toxicants? How would this impact your datasets?
13. The biomass could affect the bioavailability of the toxicants differently from biodegradation; can you elaborate how you could have addressed this?
14. The selected positive controls are not always from the same class of the toxicants; any reason; *e.g.* $K_2Cr_2O_7$ (page 50); could a better one be used?
15. All standardised assays used mineral medium; to move towards a real scenario how would you complement/alter your assays?
16. Why specifically only mixtures of drugs belonging to the same class were considered; do they usually co-exist in the environment?
17. The preliminary dataset with the micro-plastics could have considered the analysis of their surface area and topography; can you discuss how these factors could impact their potential as carriers=
18. Page 66; reference – 143 – refers to the MoA of FEN; can you explain such MoA; how the MoA is usually disclosed?

19. The increased toxicity of the mixtures compared to the single compounds was less significant than anticipated; the best model was the CA and not the IA. Can you elaborate how to improve the prediction models?
20. No synergetic effects were observed and the compounds were stable under the test conditions. Why the CA model results in sub-estimation?
21. Can the presence of inert (dead) biomass affect the stability and bioavailability of the compounds? How would you access this?
22. What would you do different right now?
23. Choose the scientific question that you would like to pursuit further
24. What is the true impact of your thesis; in the broad scientific community?
25. Do you expect your thesis to influence legislators and/or colleagues?

Overall impression

In summary, I was overall very well impressed by the present thesis, namely by the clarity and significance of the problem under study, the ability of the experimental strategy to produce datasets that go beyond that of current knowledge and practices; and the discussion of the strengths and weakness of the collected datasets which point out to what is still missing. The quality of the research undertaken is emphasised by the resulting co-authored publications. To think creatively out of the box and move out of a comfort zone (*e.g.* exploit non-conventional eco-toxicological assays) could help to generate scientific data that questions the established knowledge – that would be my recommendation for future.

Sincerely,

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