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The PhD thesis entitled "UPTAKE, ACCUMULATION AND ELIMINATION OF ENDOCRINE DISRUPTING PHENOLIC COMPOUNDS IN SELECTED WATERBIRDS FROM THE SOUTHERN BALTIC" submitted by Karina Bodziach at the Department of Chemical Oceanography and Marine Geology, University of Gdańsk. The thesis was supervised by Assoc. Prof. Marta Staniszewska with auxiliary supervision of Asst. Prof. Iga Nehring. The thesis comprises 131 pages and includes an introductory review including both the summary and conclusion (in Polish and English) and four scientific papers with the contribution statement of authors. Karina Bodziach is the first author of all these papers and three of them have already been published in Science of the Total Environment (IF=9.8, 2022), and the fourth one was submitted to the same journal. The declared contribution of K. Badzioch ranged from 50 to 55% thus, it can be concluded, that the candidate has predominantly executed the work independently in all the papers.

The thesis begins with a table of contents and abstract, including rationale, aim hypotheses, a description and objectives of the study, description of collected material and chemical analysis and finally conclusion on the overall findings of the thesis work. In the conclusion, the candidate refers to the published papers and submitted papers. Further, the thesis includes the 3 already published papers accompanied by supplementary material and finally, the fourth, submitted manuscript.

Evaluation of each main section of the thesis, including, e.g., objectives, hypotheses, methods, results, discussions, perspectives.

The introduction to the thesis is relatively short, however, comprehensive enough to demonstrate the candidate's overview and understanding of the thesis subject and the work that has been done. In the rationale of the study, the theoretical and empirical bases for this work are relevant and sound. Based on literature data, the author presents the toxicity and mechanistic pathways in which the studied endocrine disruptors (bisphenol A (BPA), 4-tert-octylphenol (4-t-OP) and 4-nonylphenol (4-NP)) potentially affect organisms. Subsequently, the sources and fate of the pollutants in the environment are described. Moreover, information about current legislation (both national and European) concerning the use of the compounds is given, followed by the rationale for using birds as

bioindicators of pollution in the Baltic. Next, the novelty of this work and basic study design are explained. Finally, a short description of the four papers is given. All these are presented well in the context of the thesis aims. However, more information about processes and fate of contaminants in the atmosphere could have been provided, as this seems to be important for the thesis subject (see paper 1).

The overall aim of the work was to investigate the main sites of accumulation of phenol derivatives in the Baltic bird species, their concentrations, and the potential of using birds for environmental biomonitoring. Due to scarce information, the research problem is novel and interesting, and the work is filling existing scientific knowledge gaps related to these endocrine disruptors. In the chapter 'aims of the study' clear hypotheses and objectives are formulated. The aims of the study could be presented more concisely with no references to the other studies.

The coherence of the thesis is further reinforced by the integration of individual papers. The topics of the papers create a cohesive narrative that builds upon the findings and insights from each paper. This interconnection of topics explored across the papers demonstrates a good level of coherence, providing a compelling narrative and flow from one investigation to the next, contributing to the overall strength of this PhD.

In the following chapter (Collected material and chemical analysis), the studied bird species were shortly presented.

The methods, theoretical and empirical bases for this work are relevant and sound. Analytical/laboratory methods include dissection of the tissues/organs and their preparation (freeze-drying, homogenization), ultrasonic extraction and SPE/liquid purification, and final contaminants determination with HPLC-FD. Additional and relevant analyses (stable isotope ratios) were done in another laboratory. The techniques seem to be adequately applied and relevant for answering the scientific questions raised, however, not very broad and diverse. The method details are mostly clearly described when needed; sometimes, instead of providing detailed protocols, reference is made to the original method development work. The summary (Conclusions) of the major findings is both well-organized and written.

The data analysis predominantly employs basic statistics, which, nevertheless, proves sufficient for this type of research. The results are presented comprehensively, and their interpretation reflects the candidate's sound knowledge within the field. In some parts results seem to be too overgeneralized, thus the thesis would benefit from a slightly more critical result interpretation and discussion.

The first three papers have already undergone peer review and have been published. However, as part of my role in providing criticism, I will pose some questions to facilitate the discussion.

Paper 1.
In this study, BPA, 4-t-OP and 4-NP were analyzed in the intestine, lungs and blood of by-caught species of aquatic birds from the Southern Baltic. The tissue/organ and species differences in the concentrations were explained based on the spatial distribution of the birds, their trophic position (based on stable isotopes of C and N enrichment), and the major intake pathways (respiratory and diet - gastrointestinal tract). The paper presents a new approach for estimating possible exposure pathways to phenolic compounds by analyzing concentration in the lungs and intestines, which is an interesting part of the thesis.

I would like to discuss with the candidate the toxicokinetics of the studied compounds in more depth and explore its consequences for explaining the results. Can concentration in the lungs and intestine directly estimate respiratory and dietary intakes? For example in rats intravenously injected with 4-n-NP (that is with no respiratory exposure), the order of the accumulation tissue of 4-n-NP was as follows: kidney > liver > brain > lung > spleen > GI-tract > heart > adipose > muscle (in male rats) and liver > lung > brain > kidney > spleen > GI-tract > adipose > heart > muscle (in female rats). Thus, it seems that 4-n-NP with no respiratory exposure still accumulates in high concentrations in the lungs. Moreover, in other studies using microsomes prepared from rat intestinal tissue it was found that while NP was glucuronidated within the gut wall, this form remained for a long time in the intestinal wall, probably due to the long side-chain of NP which may impair transport. How could these findings affect the interpretation of the data?

The lipid content was not analyzed in the samples and the results are presented on the dry weight base. How does it affect the results and its interpretation?

In addition, I have a few minor questions:

Chapter 3. What anatomical part of the intestine was collected? The blood was collected from the hearts of dead birds. Was it possible to estimate the volume of coagulated blood during sampling, considering that these results were calculated per mL?

p. 5. Chapter 4.4. "On the contrary, in the long-tailed ducks from the Pomeranian Bay, the correlation was reversed". I am not sure what kind of correlation is meant here.

Chapter 5.1. "These results are consistent with the reports of the General Inspectorate of Environmental Protection in Poland" – do the reports contain data about phenolic compounds?

Fig 2. What it means 25% - 75% on the graph legend?

Throughout the thesis, there is a slight confusion in the use of terminology regarding bioaccumulation versus biomagnification. To address this, please provide clear definitions for both terms.

Chapter 5.2.2. In the intestines of the studied birds, the concentrations of 4-NP increased with the trophic level occupied by the birds (Fig. 5), and the differences between the individual species were statistically significant (Kruskal Wallis test; $p < 0.05$). Which differences were statistically significant? Based on the graph, it looks like only for GO SL the 4-NP increase with TL.

What does the candidate mean by 'poor literature' on the presence of phenol derivatives in the atmosphere?

5.3.1. Risk assessment: in this chapter, the risk assessment seems to be a bit speculatory, and solely based on the correlations analysis only. For example: 'correlations seem to confirm the previously formulated theses that 4-t-OP enters a bird organism mainly through the respiratory tract, while 4-NP does so through the gastrointestinal tract.' There are few correlations, and they are notably weak between the lungs and blood. Moreover, the observation that all these correlations are negative raises questions that have not been addressed—what does this negative correlation mean?

Can you elaborate more on the following topic, based on the applied methodology?: 'In this study, the assayed concentrations of phenol derivatives in the blood of birds constitute their free fraction, i.e. not bound to plasma proteins'.

In addition, have you considered the phenomenon that some xenobiotics were shown to substitute hormones/vitamins in blood transport proteins? This results in increased hormone excretion via glomerular filtration, and thus might lead to endocrine disruption effects.

Paper 2
In the second paper, the organ/tissue distribution of phenolic compounds was investigated, which is a continuation of the research conducted in the previous study. This is a logical extension of the first paper.

Here, I would appreciate more detailed explanations in the discussion section to further clarify certain points.

Several internal processes, including absorption, distribution, metabolism, and excretion, influence the distribution of contaminants among tissue/organs. The equilibrium distribution of hydrophobic pollutants among tissues is determined by the fugacity capacities and this is largely dependent on the lipophilicity of the contaminants. Consequently, the equilibrium distribution of POPs can often be approximated by differences in lipid contents between the tissues. However, it seems that no lipid content was measured in the samples. Wouldn't lipid content be a good variable in explaining the distribution of pollutants?

In addition, more discussion about toxicokinetics, including the metabolism of these compounds in other organisms could add to the paper's significance and maybe help in the explanation of results. E.g. in pigs, only 77% of the oral dose of BPA was absorbed and underwent an extensive first-pass glucuronidation either in the gut (44%) or in the liver (53%), thus accounting for the low systemic bioavailability of BPA (0.50%). Upon ingestion of BPA in adult humans, the biologically active parent molecule undergoes rapid first-pass metabolism to BPA glucuronide, which is biologically inert and rapidly cleared in the urine. In rats, up to 80% of an oral dose of NP is rapidly absorbed, the remainder being excreted unchanged in faeces. Alkyl chain hydroxylation (OH-NP) constituted the major metabolism pathway representing 43.7 or 62.2% (depending on the mass transition used for quantification) of the NP dose excreted in urine (humans). Could these findings help in the interpretation and discussion of this study?

In the introduction, based on the provided reference, the sentence ('**All of the studied bird species** are characterized by a biogeographically declining population and are under species protection (BirdLife International, 2021') seems to be not valid:

Goosander *Mergus merganser*: **least concern**: the population is not believed to be decreasing sufficiently rapidly to approach the thresholds under the population trend criterion

Razorbill *Alca torda*: **The population trend appears to be increasing**, and hence the species does not approach the thresholds for Vulnerable under the population trend criterion

Only long-tailed duck: apparent severe decline detected in the wintering population in the Baltic Sea between the early 1990s and late 2000s.
Can the candidate comment on this?

Materials and methods:
How was the body condition estimated? It would be good to provide more details about this here.

The calibration range for the compound analysis is not very extensive. Were all the results within the calibration range?

The liver/muscle index was used for Hg. Is it valid for phenolic compounds?

Chapter 5.2.2. The concentrations between blood and brain did not correlate for long-tailed ducks and elsewhere where $p>0.05$, means no correlation assuming statistical significance at this level. As non-parametric statistic was applied, did the candidate try data transformation to meet parametric statistic assumptions?

For BMF calculation, only mussels (which species?) were used for the calculation of BMFs for long-tailed ducks and herring for razorbill. Do we know about diet and its variability in these bird species? What kind of problem do we encounter when BMFs are calculated only for bird muscles? In addition, 4-t-OP seems not to biomagnify.

In conclusion, it is noted that BMF might be underestimated; I request further elaboration on this point. Additionally, there is a lack of discussion and comparison of values to the literature regarding BPA. Please refer to paper 4, chapter 4.1, where the concentration of BPA in different trophic levels suggests a lack of biomagnification. I observe an inconsistency between the discussions in different papers.

Can you distinguish between parent compounds and metabolites with the applied methodology?

Graphs – the model is significant, but it is not clear which groups are different.

Avoid statements about the correlation, when in fact there was a lack of correlation, that is $p>0.05$: for example chapter 5.2.1. 4-t-OP and BPA in liver: $p=0.1$.

In my opinion, there is no evidence (no comparison with toxicity data) to claim: 'Therefore, potential changes in livers and kidneys caused by high accumulation of the tested xenobiotics in these tissues should be expected, as they have the potential to do so even at environmentally relevant concentrations.'

Conclusions:
Could you explain how 'the respiratory exposure limited the distribution of these compounds mainly to the kidneys and muscles.'?

'Ability to adapt to pollutants' – please elaborate.

As we do not have laboratory data for all the organisms, it would be beneficial to seek data from model organisms or related species. See: 'effects of exposure to phenol derivatives in seabirds are unknow'.

Paper 3
In the third study, the effectiveness of excreting phenol derivatives in birds through claws and remiges is examined. The study also performs a preliminary assessment of the utility of these

epidermal products for environmental biomonitoring and estimating bird exposure levels. The paper is commendable for its self-critical analysis, exploration of future research directions, and caution in the data interpretation.

The study determined the free (unconjugated) forms of the compounds; however, the method employed for this determination needs clarification.

While the provided information about molting is helpful, additional details regarding the birds' diet would enhance the overall understanding.

In this study 'it was assumed that the average concentrations in all the tissues examined so far...constitute 100 % of the contamination with these compounds in birds'. However, plumage constitutes only few % of body weight, while muscles, e.g. the supracoracoideus and the pectorals together make up about 25–40% of the bird's full body weight.

So, can we accurately estimate the 'large proportion of these compounds that are eliminated' without accounting for variations in organ/tissue mass, and consequently, the organ/weight proportion of the total body burden for each compound?

No standard method for washing exists and previously it was found that the washing does not remove all the external contaminants. However, washing should work for claws better than feathers (feather structure), so, interestingly, the extreme values are visible in claws, not feathers.

There were no significant positive correlations between feathers/claws and blood. Can the feathers be used for biomonitoring of phenolic compounds?

Discussion:
What is a justification for this statement in the discussion: 'It seems, therefore, that xenobiotics deposited in the tissues of long-tailed ducks while wintering in the Baltic Sea, could have been remobilized from fat reserves', when there is no evidence ($p=0.41$) for higher 4-t-OP values in remiges in long-tailed ducks compared to other species?

Similarly, later 'This suggests that phenol derivatives accumulated in long-tailed duck feathers originate not only from the place where the birds changed the feathers, but also from the xenobiotic pollution of the Baltic Sea, as a result of their remobilisation from tissues during the feather replacement process.'

What kind of disturbance it is meant here: 'the relationship of phenol derivatives concentrations between tissues and feathers could have been disturbed by the passage of several months from the time of their replacement to the time of collection and analysis'

Claws growth and monitoring of pollutants – 'claws of migratory species may not be a reliable indicator of phenols contamination in a particular place': can the candidate suggest some solutions for using claws for biomonitoring?

'Moreover, the obtained correlations (Table 2) indicate that phenol derivatives are removed from almost all tissues studied so far'. It seems that there are not too many significant correlations in Table 2; The negative correlations support the elimination hypothesis, however, some correlations are positive. How this could be interpreted?

It is mentioned that 'claws ... contributed the most to elimination'. However, what is the weight of the claws in relation to the total body weight of the organism? Are not the major elimination pathways via urine and feces for these contaminants?

Overall, this paper is commendable for its self-critical analysis, delineation of future research directions, and exercise of caution in data interpretation.

Paper 4
In this manuscript, concentrations of phenol derivatives in the gonads of seabirds were determined and the authors examined age, sex and region effects on the contaminant bioaccumulation. The introduction to this draft is smoothly written, indicating progress in the writing skills of the candidate. Some comments on the manuscript:

Wang et al. 2021: This is a review and the authors did not do their own studies on the migration of these compounds from plastics ingested by birds and accumulated in their bodies. The question of the role of plastic particles as vectors for plastic additives and exposure in birds is largely unresolved based on the current literature.

'The comparison of mean ranks in three groups showed that only the concentrations of 4-NP differed statistically significantly from the concentrations of BPA' – were the concentrations of 3 different chemicals compared in all gonads? What is the reasoning for a such comparison?

What has been done with <LOQ data during the statistic?

Can we conclude that mature birds were characterized by higher concentrations of BPA and 4-t-OP compared to immature ones when these differences were not statistically significant and the median BPA and 4-t-OP concentrations in mature and immature specimens were at the same level?

What has been done with the extreme values (females) for statistics?

I recommend exercising caution when interpreting data for male/female differences, given the absence of statistical significance.

Discussion

4.1. Quite a lot of discussion is based on previous papers; not so much about the results from this study concerning accumulation in gonads.

'Considering the organs that would be the most sensitive to the effect of endocrine disrupting phenol derivatives' which organs are meant here?

Line 351. Recalculate units for comparison purposes. It is difficult to compare the concentrations from the literature to the results of this study (concentration in gonads). Did the other authors measure the concentrations of compounds in gonads, that could be used for toxicity estimation?

Line 354. 'Feminization was also observed in Japanese quail embryos to which a dose of 200 ng/egg of 4-NP and 20 ng/egg of BPA was administered for 14 days'. In that study, the authors applied a single *in-ovo* injection, not 14 days of administration.

Line 364: There is no evidence for 'Thus, the current pollution of the Baltic Sea environment with endocrine disrupting compounds indicates that adverse effects of the exposure of the reproductive and endocrine systems of long-tailed ducks are likely to occur', based on the data from this study, as no gonadal toxicity threshold levels were provided.

Chapter 4.3. Quite a speculative narrative about the population of long-tailed ducks and the EDC; in addition, the decline of the Baltic wintering population would suggest that the breeding 'clean areas' does not promote their reproduction.

Line 420. 'The indirect effect may reach females'. however, the females have lower concentrations than males.

Line 434. It is difficult to conclude that 'gonads are one of the most sensitive organs to the endocrine disrupting compounds' based on the discussion: see my point in line 364.

Conclusion

In summary, despite the above-mentioned comments, I highly appreciate Karina Bodziach's doctoral dissertation. The thesis is a comprehensive and well-structured investigation of the bioaccumulation and effects of endocrine-disrupting phenolic compounds in waterbirds from the Baltic Sea. Karina Bodziach's thesis makes a valuable contribution to the understanding of the uptake, accumulation, and elimination of endocrine-disrupting phenolic compounds in waterbirds. The coherence of the thesis is evident in the integration of individual papers, creating a cohesive narrative that builds upon the findings from each investigation. Notably, three of these papers have already been published in the prestigious journal *Science of the Total Environment*, while the fourth one is under consideration for publication. This interconnection between the studies contributes to a compelling overall story and strengthens the thesis.

Therefore, based on the evaluation of Karina Bodziach's doctoral dissertation, I confirm that the thesis meets the requirements for doctoral theses, as specified in Article 13 of the Act of March 14, 2003, on academic degrees and academic titles, and degrees and titles in the field of art (Journal of Laws No. 65/2003, item 565, as amended). Therefore, I submit a request to the Discipline Council for Earth and Environmental Sciences, University of Gdańsk, Poland, to recognize that Karina Bodziach's dissertation meets the requirements for doctoral theses and to allow the doctoral candidate to proceed to further stages of the doctoral process.

Considering the high quality of the published works that constitute the core of the doctoral dissertation and the contribution to knowledge on the accumulation of phenolic compounds in birds of the Baltic Sea, I formally propose to distinguish Karina Bodziach's doctoral dissertation with an appropriate award.

Tomasz Maciej Ciesielski