Author’s response to reviews

Title: Assessment of xenoestrogenic exposure by a biomarker approach: application of the E-Screen bioassay to determine estrogenic response of serum extracts

Authors:

Dr Thomas H Rasmussen (thoj@health.sdu.dk)
Flemming Nielsen (fnielsen@health.sdu.dk)
Helle R Andersen (hrandersen@health.sdu.dk)
Jesper B Nielsen (jbnielsen@health.sdu.dk)
Pal Weihe (pal@ahs.fo)
Prof Philippe Grandjean (pgrandjean@health.sdu.dk)

Version: 2 Date: 28 Jul 2003

PDF covering letter
Dear Editor,

Please find attached the revised manuscript Manuscript ID: 1046355491605743 by Thomas Hoj Rasmussen et al.

We are very pleased that the referees find our results interesting, and we appreciate the constructive criticism from the reviewers. Corrections have been made in accordance with the reviewers’ comments, and we have compiled a point by point response to the reviewers’ comments, along with a summary of the changes we have made.

We hope that this revised version is suitable for publication in Environmental Health.

Point by point response to reviewers comments

Reviewer #1 (David Sherr, Boston University School of Public Health)

On page 2, we have indicated that the whale blubber is contaminated with persistent halogenated contaminants.

On page 11, last paragraph, “more reasons” have been changed to “many reasons”.

On Figure 1, the symbol representing data points for cells treated with the test compound alone has been changed from diamonds to circles. The x-axis has been moved to make the results representing zero activity easier to see.

Reviewer #2 (Marieta Fernandez, University of Granada, Granada, Spain)

We agree that the manuscript needed clarification and hope that this has been achieved.
The wordings “the integrated estrogenic activity” and "the integrated and functional xenoestrogenic response" have been rephrased to “the combined functional estrogenic response” throughout the manuscript.

The sentence in the abstract "recovery of added lipophilic toxicants was acceptable" has been deleted.

The phrase “\(p,p'\)-DDE was found to be a potent xenoestrogen” has been changed to “\(p,p'\) -DDE induced cell proliferation”.

Concerning our choice of study population: our HPLC separation protocol was not developed specifically for PCB-containing samples, but rather as a “universal” separation method. However, our detailed knowledge of the retention time of an extensive number of compounds (including alkyl phenols, phthalates and carbamate pesticides) allows us to customize the HPLC separation to populations with specific exposures, such as the Faroese. In this manuscript, we present our results of the serum estrogenicity of a cohort of PCB exposed women. Apart from their exogenous exposure, these women were pregnant and thus had high levels of endogenous estrogens. Thus, our separation protocol was customized to accommodate these particular conditions. We examined this cohort because our study is linked to ongoing studies of a birth cohort of the children of these women. We chose to focus on the serum estrogenicity in this study, but the HPLC separation may in principle be followed by a range of different effect monitoring assays depending on the focus of the research.

Concerning the comparison between Faroese and the Danish populations: We agree with the reviewers’ comment about referring to the Danish group as “unexposed”. Because the Danish diet does not include whale blubber, the exposure to persistent organohalogen compounds with potential estrogenic effects is much lower than the Faroese. Thus, we have changed the wording to “Although we did not have exposure information for these women, the absence of whale blubber from the diet and the low-level exposures to persistent organohalogen compounds in Scandinavia suggest that they be considered controls for comparison with the Faroese cohort.”

Concerning the selection of compounds: The compounds examined were not arbitrarily selected. Among the PCB congeners included in this study are PCB138, PCB153 and PCB180. These three major congeners comprise about 50% of the total amount of PCBs in human samples. Additionally, we examined three hydroxylated PCB metabolites, OH-PCB107, OH-PCB146 and OH-PCB187, which constitute the three most abundant OH-PCBs in human serum. The remaining persistent environmental pollutants examined in this study have been determined to be present in serum samples from the Faroese cohort. We agree with the reviewer that the included compounds do not represent all organohalogen compounds potentially present in serum, however the concentration of these persistent compounds show substantial collinearity, thus the pesticide residues and persistent industrial chemicals included in our article are indicative of the overall body burden of persistent organohalogen compounds.

Methoxychlor and dieldrin have been included in Table 2.

We agree that the description of additive and antagonistic effects needed clarification, and we have elaborated on this topic in the Discussion section.

Concerning our choice of E2 concentrations for establishment of standard curves and for examination of mixture effects: in every experiment we included a dose-response curve for E2 to monitor the behavior of our cells in regard to sensitivity (the lowest concentration inducing cell
proliferation significantly higher than that of solvent-treated cells) as well as responsiveness (the maximum proliferative response achieved). In all experiments, the sensitivity was 1 pM, whereas the maximum response was achieved upon treatment with 1 nM E2, hence we used concentrations of E2 from 1 pM to 10 nM. Mixture effects were, however, examined only at a E2 concentration of 10 pM, since this concentration induced one half of the maximal proliferative response. Thus, this approach reveals important information about mechanistic interactions, because it allows detection of enhancing effects (i.e., additivity or synergism) as well as anti-estrogenic effects in one single test.

We agree that the figure legends needed clarification, and corrections have been made, e.g., the HPLC retention times of fractions s4 and s8 have been included.

Concerning the exclusion of subfraction s4 and s8: The decision to exclude the two subfractions were not based on the two samples alone, but the detailed analysis presented was performed only on two samples. A number of results from pilot samples had indicated that pregnancy-related endogenous estrogens eluted in Fraction-1. A prior study of the estrogenic response of Fraction-1 (in which s4 and s8 had not been removed) of samples from another cohort of pregnant women gave very high estrogenic responses in all samples. Some of these samples were re-examined after exclusion of s4 and s8, and consequently gave low responses. Thus, the presence of endogenous estrogens in our Fraction-1 gave a high background noise, and thus hampered the detection of low-potency xenoestrogens. Further, we examined the estrogenic response of Fr-1 (without s4 and s8), subfraction s4 as well as s8 of samples from non-pregnant women and pregnant women at different gestational age (12th – 38th week). These results indicated that in contrast to the estrogenic response of Fraction-1, the estrogenic response of both s4 and s8 increased with gestational age.

Reviewer #3 (Leon Bradlow)

We agree that the description of the serum extraction protocol in the Methods section needed clarification, and changes have been made.

Concerning the potential influence of lipids on HPLC retention times: to control and adjust time windows for collection of the HPLC fractions, we used a standard mixture diluted in n-heptane:ethyl acetate (9:1). We agree with the reviewer that to get accurate GC retention times it is important to use delipidated serum samples. However, in HPLC using normal phase chromatography the lipids present in the serum samples do not influence the retention times. To verify this, all of our recovery experiments are made in non-delipidated serum supplemented with standards, and the resulting retention times are identical with the retentions times of standards supplemented to pure solvent.

Concerning the potential influence of summer/winter diet on plasma levels: Although pilot whale is caught only during the summer, frozen and cured meat and blubber are available all year. As example, blood-mercury concentrations do not change seasonally.

As proposed by the reviewer, we have addressed the point about the relative low potency of individual compounds in the Discussion section.

Concerning the endogenous estrogens present in subfractions s4: we were unable to identify the endogenously produced estrogenic substance that eluted in subfraction s4. Although fatty acid esters of estrone or estradiol may be present in serum, their potential occurrence in subfraction s4 could not be documented.
It is possible that a lipoidal estrogen, e.i., a fatty acid ester of estrone or estradiol may account for the estrogenic activity because these substances elutes in Fraction-1 because of their none polar nature.

We thank for the useful comments that have helped to improve our paper, and we have made corrections and changes as suggested by the reviewers. We hope that this revised version is suitable for publication in Environmental Health.

Yours sincerely

Thomas Høj Rasmussen