Report of Three 21-day Fish Endocrine Screening Assays To Complete CEFIC's Contribution to Phase-2 of the OECD Validation Program: Studies with Potassium Permanganate, *n*-Octanol, and 2-Methoxyethanol in the Fathead Minnow (*Pimephales promelas*)

Report based upon IBACON laboratory study reports.

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Phase-2 OECD 21-day Fish Screening Assay Validation Report
CEFIC Negative Test Substance Studies

Background

Under the auspices of the Validation Management Group for Ecotoxicity Testing two protocols for a fish screening assay for endocrine active compounds have been through multi-laboratory testing. The data are reported as Phase-1A and Phase-1B for each of the two protocols, differing in their protocols such as by the housing conditions of fish. The test substances in those studies tended to be potent, positive pharmaceutical compounds (e.g., 17β-estradiol and trenbolone in Phase-1A and Fadrozole, Prochloraz, and Flutamide in Phase-1B) and only a single weak positive, *t*-pentylphenol, in Phase-1B.

At the 3rd meeting of the Validation Management Group for Ecotoxicity Testing in December 2004, the Phase-1B protocol was accepted for further work. It was recommended to identify negative substance(s) and, a small number of experts proposed potassium permanganate and *n*-octanol in June 2005. Several laboratories agreed to participate, and CEFIC agreed to seek funding to support the participation of an additional laboratory.

Studies have been conducted by 4 laboratories. One laboratory has tested both substances in medaka, one laboratory has tested both substances in zebrafish, one laboratory has tested both substances in fathead minnow, and the final laboratory tested potassium permanganate in fathead minnow. These results of these studies have already been reported in the OECD Phase 2 report of the validation of the 21-d fish endocrine screening assay.

The present document includes a detailed analysis of the Phase 2 studies supported by CEFIC on potassium permanganate, *n*-octanol and 2-methoxyethanol. It should be noted that the conclusions are in full concordance with the OECD Phase 2 report of the validation of the 21-day fish endocrine screening assay. Further recommendations are proposed by CEFIC which may feed into further discussions on the Test Guideline by the appropriate group at the OECD, following the peer-review. However, these recommendations have not been examined and discussed by the VMG-eco or any other OECD group.

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SUMMARY

The three studies in this report were conducted as part of OECD program to validate a standardized OECD protocol on adult fish for the detection of endocrine active substances (i.e. estrogen, aromatase inhibitors, androgen). This work is a continuum to OECD validations studies conducted in Phase 1A and then Phase 1B for a 21-day fish endocrine screening. In June of 2005, the OECD Secretariat contacted laboratories for a commitment to investigate negative test substances. CEFIC volunteered to support a laboratory in Phase-2 for three studies to be conducted with the fathead minnow (*Pimephales promelas*).

The current studies follow the body of other studies in Phase-2. The other studies were in 3 test species: fathead minnow, medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*). The other studies completed their in-life by February in 2006 and their report was approved in January, 2007, at the VMG-eco meeting in Madrid. The last of these current studies completed in-life only in October 2006. This significant postponement was due to unforeseen delays in the approval of funding and the selection and contract of the laboratory.

The objective of the other Phase-2 studies was to test the relevance and specificity of three reproductive endpoints (spawning activity, fecundity, and gonadal histopathology) and two endocrine-related endpoints (vitellogenin (VTG) and secondary sexual characteristics (SSC)) with negative endocrine substances. The current Phase-2 studies have the same objective. Previous studies employed two negative endocrine substances selected by an expert group drawn from the VMG-eco: potassium permanganate, an inorganic oxidizer, having no known endocrine activity, and *n*-octanol, a classical organic narcotic, having no known endocrine activity. The current studies used these two substances at the previously selected nominal target concentrations of 225, 450 and 900 µg potassium permanganate/l and 0.32, 1.0, 3.2 mg *n*-octanol/l. CEFIC selected a third test substance, 2-methoxyethanol, a classical gonadal toxicant in mammalian species, known to produce gonadal histopathological lesions by non-endocrine mechanisms. For the additional negative, the nominal target concentrations selected by CEFIC for use in the current studies were 0.32, 1.0, 3.2 mg 2-methoxyethanol/l.

These studies provide support to the compliance of the OECD 21-day fish endocrine screening program with the criteria of Guidance Document 34. The studies support that interlaboratory studies have been done with the negative compounds, as the previous studies were often performed in only a single laboratory with each species. These studies also support the intention of testing representative compounds in a validation study by including additional negatives such as the 2-methoxyethanol.

The results of these studies support the overall conclusion of the OECD validation program that vitellogenin and secondary sexual characteristics can be successfully employed to screen for certain endocrine modes of actions (i.e., estrogens, androgens, and aromatase inhibitors). At concentrations absent of overt signs of toxicity, there were no false positives observed with any of the three negative test substances. The results also support the OECD validation program that certain reproductive parameters such as spawning activity and fecundity and gonadal histopathology are not specific for endocrine activity, but may be impaired by other modes of action. These conclusions are drawn based on a number of observations during these studies.

The first significant observation was that 1) mortality, symptoms of intoxication, and other sublethal effects reproduced findings in a previous Phase-2 study with permanganate in fatheads and 2) mortality, symptoms of intoxication, and other sublethal effects were clearly evident and need to be incorporated into the interpretation of the potassium permanganate and 2-methoxyethanol studies. Consistent with symptom observations, mortality at high potassium permanganate and 2-methoxyethanol concentrations achieved statistical significance versus the controls. In the case of potassium permanganate, there were symptoms of intoxication and sublethal effects at the intermediate concentration and, at the high concentration, 15 fish died on test (62.5%). All fish before death and all surviving fish showed various symptoms of intoxication and distress. In the case of 2-methoxyethanol, mortality, symptoms of intoxication, and sublethal effects began to appear at the intermediate

concentration, and were substantial at the high concentration (e.g., all male fish died). For the *n*-octanol study, there was a low level of mortalities, and symptoms of intoxication were infrequent occurrence of symptoms of intoxication with the few instances the concentration and duration of exposure. It will be noted below that many other findings related to spawning activity, fecundity, tubercle numbers and scores, and gonad histopathology follow the same concentration patterns as the intoxication symptoms and mortality. It would appear then that many of the endpoints, e.g., fecundity and gonadal histopathology, can be confounded by general, systemic toxicity and produce false positives that are not actual endocrine-specific effects.

For the reproductive endpoints, in the permanganate studies, spawning activity and fecundity appear to be associated with mortality and symptoms of intoxication as the findings were synonymous at the intermediate and high concentrations. These decreases were statistically significant versus the control and reproduced in the other Phase-2 fathead minnow study where the spawning activity was also reduced at the intermediate and high concentrations of permanganate. In the *n*-octanol studies, there was a modest increase in mortality with concentration and no major intoxication symptoms. In this study, spawning activity and fecundity was actually better in all treatment groups than in the control, and spawning activity versus control approached a statistically significance increase ($p=0.064$) for the intermediate concentration. This also reproduced the general findings in the other Phase-2 fathead minnow study. Additionally, the impact of twice per day tank cleaning due to test substance biodegradation could have impaired the reproductive behavior of the fish, although the test substance results were actually higher than the controls. In the 2-methoxyethanol studies, all males died before the end of the study at the high concentration and both males in replicate 4 of the intermediate concentration died before the end of the study. Yet, the results were so modest in the control and the low concentrations, that statistical significance was not achieved.

Cumulative egg numbers and eggs per surviving female were still not satisfactory in the current protocol even with the change in protocol design from Phase-1B. In these three studies, there were 8 instances when the cumulative egg number for a replicate was greater than 400 eggs; only one of these instances was in the 12 control replicates. The cumulative mean per replicate for the three controls was 75, 90, and 165. Immaturity of the selected fish may be one reason for the low number of eggs. This illustrates the non-ideal conditions and limited power of the protocol that are likely to occur in a large number of laboratories in practice with the current protocol specifications (including those on animal selection). Therefore, the current protocol is not sufficient to yield consistently robust reproduction in the fathead minnow.

A fundamental statistical question arose concerning the reproductive endpoints as a result of the studies. As a tank contains 2 males and 4 females, what happens to its application as a replicate when one or both males die or when one or more females die? How should the results be compared to other tanks, no longer apparent replicates, that still continue to have 2 males and 4 females and, therefore, inherently, greater chance for spawning activity and fecundity? The statistics for these current results were calculated as though any tank where mortality of one or more individuals occurred continued to be a true replicate; that is, those tanks are included.

A second protocol question also arose as two females in the permanganate study and one female in the 2-methoxyethanol study were found to have immature gonads *after* being 3 weeks on study. There was also some misidentification of immature males as females in the permanganate study due to their immaturity at the study start. This leads to the recommendations that: 1) the recommended mean age of the fathead minnow may need to be increased beyond 20 weeks (5 months) [Note: in Phase-3, one laboratory has again found the fathead minnows to be sexually immature at 20 weeks of age while still above the weight criteria of the current protocol] and 2) the use of the acclimation period to test the reproductive capacity fish received in pairs may need to be considered, if this endpoint would be pursued, so that no misassignment of sex would occur and only proven and successful breeders would be placed on test.

The VTG endpoint was successful with no changes at non-toxic doses. Absolute VTG decreases in both sexes in the potassium permanganate studies at toxic concentrations may be similar to absolute decreases in fathead minnow males in laboratory 4 in Phase-2. These decreases were not observed at the low or intermediate concentrations. The decreases occurred at the high concentration in parallel with high levels of mortality and severe symptoms of intoxication in the surviving individuals. The lack of change in VTG levels in both sexes in the *n*-octanol studies are clearly consistent with the lack of change in observed in other Phase-2 studies. There was an absolute and statistically significant increase in VTG in 2-methoxyethanol females, but no evidence of a VTG increase in males, with significant mortality and symptoms of intoxication in both sexes. Thus, there were false positives and a weight of evidence approach needs to be used in VTG interpretation.

The VTG analyses also raise certain protocol improvement and refinement considerations. The two primary protocol and interpretative questions revealed are: 1) whether immature individuals of either sex should be included or excluded from the VTG analyses as immature individuals appear to have much lower VTG levels in females and thus reduce the mean and increase the standard deviations thereby impairing the statistical sensitivity; and 2) how to take into account the appearance of high mortality and symptoms of intoxication in a group which, in concert with declines in VTG levels, strongly implies potential impairment of the individuals' ability to produce VTG in such a group and not an endocrine mediated disturbance.

The tubercle endpoint was also successful with no changes at non-toxic concentrations. The potassium permanganate and *n*-octanol groups were similar to those in the previous Phase-2 fathead minnow study. There was an absolute decline in tubercle number and a statistically significant decline in the mean tubercle score at the high concentration of the potassium permanganate study synonymous with systemic toxicity and symptoms of intoxication. There were no changes in the absolute values of the tubercle number or tubercle score at any *n*–octanol concentration and mortality and signs of intoxication were limited. However, in the 2-methoxyethanol, despite some male mortality at the intermediate concentration, and as noted below there were significant gonadal histopathological lesions in these males, there was no absolute change in the tubercle number or score at this concentration.

The appearance of mortality and symptoms of intoxication in these and other studies in Phase-1B and Phase-2 was often in parallel with gonadal histopathological changes in the groups. These included the appearance of atretic follicles in females and some evidence of testicular lesions in males. Given the obvious stress on the individuals in groups with mortality and intoxication, the impact on reproduction and the appearance of such lesions is not unsurprising. Therefore, interpretation and conclusions regarding reproductive toxicity and gonadal changes should be undertaken only when it is clear that fish are not under undue or extreme stress. This is analogous to the concept in mammalian toxicology of not exceeding a maximal tolerate dose (MTD) and not to interpret findings as evidence of specific toxicities, e.g., reproduction, or modes of action, e.g., estrogenic, when such concentrations are exceeded. This raises the need to develop an analogous maximum tolerated concentration (MTC) for ecotoxicological assays. In addition, one incidence of testis-ova was observed in the entire set of studies with a single male at the intermediate concentration of potassium permanganate. This supports the conclusion that random occurrences of testis-ova do occur, and that it is not a definitive diagnosis for endocrine activity.

A clear example of this concern is the observation of atretic oocytes. This observation was originally proposed and tested in Phase-1B. Then, these observations were retained by a meeting of histopathological experts after completion of Phase-1B as being a potential indicator of endocrine action. The rationale was based on endocrine control of successful oocyte maturation and the potential for atretic oocytes to appear when endocrine signals in the females may be impaired. However, the health status of the individual also plausibly results in the failure of oocytes to mature and the resulting appearance as atretic observations when the reproductive condition of female is impaired by systemic or other non-endocrine toxicities. In this regard, there are very clear concentration-related elevations of atretic oocytes in concert with mortality in both the potassium permanganate and 2-methoxyethanol studies (Tables 11A and 13A), and a similar modest elevation at the high concentration

of *n*-octanol (Table 12A). Therefore, these gonadal histopathological findings are clearly not specific for endocrine action, and wider experience is needed with all gonadal histopathological findings to validate that they are truly diagnostic or not for endocrine mediated modes of action.

In the 2-methoxyethanol study, testicular lesions similar to those found in mammalian studies were observed, and these lesions began to appear at a low frequency in the testes at the low test substance concentration. These lesions become more severe at the intermediate concentration. In this case, mortality and intoxication were similar to the observations at the high concentration of *n*-octanol, but the testicular lesions were clearly more frequent and severe in the case of 2-methoxyethanol. In addition, there was a concentration-related increase in fibrosis in the testes that reached severe levels and a low frequency of concentration-related appearance of general testicular degeneration. Therefore, the 2-methoxyethanol study indicates that gonadal histopathological lesions will appear with non-endocrine reproductive toxicants and are not endocrine-specific as biological plausibility would suggest. In addition, despite the testicular lesions and other effects, the protocol design was unable to provide sufficient vigor of spawning activity and fecundity in the control to provide evidence of reproductive toxicity.

In closing, these studies met the test acceptance criteria for the fish to be placed on test, met the test criteria for O_2 saturation and temperature, and the measured values of the test substances are satisfactory to accept all three studies. However, the controls in two tests did not meet the criteria for control mortality of <10%. The *n*-octanol controls encountered 12.5% mortality (3 of 24), and the 2-methoxyethanol controls had 16.7% mortality (4 of 24). The analytical recoveries with potassium permanganate and *n*-octanol are similar to other Phase-2 laboratories, except that the severe losses of *n*-octanol in another laboratory were not observed. Therefore, the studies are themselves useful to assess the relevance and the reliability of the endpoints in Phase-2 of the validation program.

The following conclusions and recommendations are offered by CEFIC. These recommendations have not yet been discussed by an OECD group and should therefore be taken as proposals, and CEFIC understands that they may or may not form the basis of further work/discussion on the future Test Guideline:

- At non-toxic concentrations, the VTG and tubercle endpoints were consistently negative as expected. The studies support the capability of these endpoints to correctly identify endocrine modes of action when other toxicities are not present.

- Given the evidence for impact of systemic toxicity and sublethal effects on several endpoints, several changes in the protocol and the interpretation of its results are recommended to resolve the issue of false positives in such circumstances:
    - Avoid concentrations with high mortality and symptoms of intoxication by refining concentration selection guidance beyond acute toxicity (LC50). The concentration selection should be informed with data from juvenile toxicity studies (as was done with *n*-octanol) or early life stage studies, or range finding studies and the consequent additional animal use are necessary.
    - Ensure that reproductive (spawning activity and fecundity endpoints) and endocrine-related (VTG and SCC endpoints) positive observations that are, in fact, due to general, systemic toxicity as evidenced by mortality and symptoms of intoxication are not interpreted as positive findings of endocrine modes of action. That is, define a MTC for the fish screen, where only findings below such an MTC would be accepted as positive for true reproductive and/or endocrine-related mode of action findings.
    - Thus, in a straightforward solution, incorporate in the interpretative section of a future Test Guideline that positive findings for possible endocrine related effects should occur in the absence of confounders for systemic toxicity and symptoms of intoxication and that particularly vulnerable endpoints such as reproduction would be recognized.

- The spawning activity and fecundity capability of the protocol, even with the increase in replicates and reduction to two males per tank is not sufficient to yield a consistently reliable and robust measurement of reproduction with the fathead minnow. This is also consistent with the results from the other fathead minnow laboratories in this and previous Phases of the validation program. Clearly, some additional work is necessary on the protocol conditions to achieve robust reproductive capacity, and the current protocol does not appear actionable for routine, large scale screening. Therefore, the previous decision of the VMG-eco to remove the spawning activity and fecundity measurements from the validation program appear to be justified on these grounds as well as the need to separate adverse effects on reproduction from endocrine mode of action screening. Further consultation with experts and statisticians is recommended for a future fish reproductive assay.

- Consultation with statisticians is also needed to understand what constitutes a statistical replicate and the use of tanks where mortality has occurred and, therefore, reproductive capacity is no longer fully equivalent among the tanks. Are there alternative methods to utilize these tanks or whether such tanks should be removed from the study altogether?

- As there is no evidence or plausibility for interaction with the estrogen receptor for either potassium permanganate or 2-methoxyethanol, the weight of evidence is that the VTG responses in these cases are not clear evidence of endocrine activity. Therefore, the current responses are judged to be false positives. In the future, VTG responses should not be interpreted automatically and in isolation without a weight of evidence approach.

- These study results with potassium permanganate would support some caution in interpreting a decline in the tubercle number and score in the presence of severe mortality and symptoms of intoxication. These study results with *n*-octanol and, particularly, 2-methoxyethanol would support the androgen specificity of this endpoint in that a clear non-endocrine testicular toxicant such as 2-methoxyethanol did not impair these endpoints even in the presence of modest mortality and intoxication.

- These studies demonstrate that:

  1) gonadal histopathology is impacted by systemic toxicity, sublethal effects, and severe stress,
  2) variable background rates of findings continue to be observed in controls making interpretation versus test concentrations difficult and raising questions about the biological relevance of some findings (that is, a high rate in controls in one study will be interpreted as healthy, normal fish while similar rates in a test concentration might be interpreted as endocrine or other effects in another study),
  3) significant work remain to identify and validate any diagnostic gonadal histopathological finding that would be specific for endocrine modes of action,
  4) there is some evidence that strong non-endocrine gonadal toxicants such as 2-methoxyethanol can be detected using gonadal histopathology, and
  5) the work and effort to conduct and clearly interpret the gonadal histopathology is not consistent with its employment in a lower tier screen where simplicity and clarity are hallmark criteria.

  However, the sensitivity of the latter finding vis a vis spawning activity and fecundity remains unknown given the inability of the protocol to consistently produced robust spawning and fecundity measurements in the fathead minnow. Collectively, these findings support the previous decision of the VMG-eco to withdraw gonadal histopathology from the protocol as an endocrine screen, and demonstrate that further work in laboratory studies on positive and negative substances is necessary for their validation.

In addition, the laboratory conducting these studies has submitted comments on the protocol and studies (Annex III).

BACKGROUND, PROTOCOL, MATERIALS, AND METHODS

Background and Objectives.

These studies follow a series of other studies in Phase-2 of the validation of the 21-day fish screening assay. Previous studies were in 3 test species: fathead minnow (*Pimephales promelas*), medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*), and these studies were only in the fathead minnow. The objective of the previous studies was to test the relevance and reproducibility of three reproductive endpoints (spawning activity, fecundity, and gonadal histopathology) and two endocrine-related endpoints (vitellogenin (VTG) and secondary sexual characteristics (SSC)). The current studies had the same objective. Previous studies employed tow negative endocrine substances: potassium permanganate and *n*-octanol. The current studies used these substances plus 2-methoxyethanol, a positive reproductive toxicant in mammals which acts via a non-endocrine mechanism, primarily on the male testes (Foster et al., 1983; Hardin and Lyon, 1984; Ku et al., 1995; Medinsky et al., 1990; Miller, 1987; Nagano et al., 1979).

Overall Design & Protocol.

The fathead minnow protocol differed from that used in Phase-1B, because little or no reproductive activity was observed when fathead minnows were used. Instead of two replicates with 5 males and 5 females per replicate (a total of 10 males and 10 females), the design was altered to four replicates with 2 males and 4 females per replicates (a total of 8 males and 16 females). This was because the fathead minnow male is highly territorial in inducing the female to spawn in a tile and then defending the eggs in the tile. This same basic design was followed by other fathead minnow laboratories in Phase-2. The protocol was the same one used by other Phase-2 fathead minnow laboratories.

Statistical Procedures.

Effects on mortality, tubercle score, number of tubercles, number of eggs, days with spawning and vitellogenin concentration were statistically analysed. Two approaches were used. First, similar to other laboratories in Phase-2 a Pairwise Mann-Whitney U-test (non-parametric test) was employed, because data did not fulfill the qualifications for parametric statistical tests, like ANOVA, i.e., homogeneity of variance and normal distribution. Differences were considered to be significant at $p < 0.05$ in all tests.

Fish Sources and Acclimation.

The test fathead minnows were obtained from Aquatic Research Organisms, 1 Lafayette Road, Hampton, NH 03843-1271, USA. The age of fish on arrival was to be 20 weeks. The fish were acclimated for 2 weeks before placement on study. The body weight and length of 10 test fish were determined at the start of the test. The mean body length and the weight of the fish at the beginning of the study were:

- Potassium permanganate. Males, body length and the weight were 5.54 ± 0.27 cm and 2.32 ± 0.24 g, respectively. Females were 4.48 ± 0.49 cm and 1.50 ± 0.21 g, respectively.
- *n*-Octanol. Males, body length and the weight were 5.79 ± 0.35 cm and 2.88 ± 0.27 g, respectively. Females were 4.31 ± 0.39 cm and 1.75 ± 0.23, respectively.
- 2-Methoxyethanol. Males, body length and the weight were 5.64 ± 0.31 cm and 2.56 ± 0.31 g, respectively. Females were 4.69 ± 0.14 cm and 1.68 ± 0.13 g, respectively.

Test Materials.

Potassium permanganate was from Sigma, Lot 14202TC345, 99.4% purity. *n*-Octanol was from Sigma, Lot 0293HC, 99.0% purity. 2-Methoxyethanol was from Sigma, Lot U16433, 99.95% purity. Vitellogenin was analysed with the fathead minnow vitellogenin ELISA Kit from Biosense Laboratories AS (Prod. No.: V01018401).

Facility, Water, and Other Experimental Conditions.

The test facility was Institut für Biologische Analytik und Consulting IBACON GmbH at Arheilger Weg 17, 64380 Rossdorf, Germany. Dechlorinated (using an activated-carbon filter) tap water was used and the fish were kept in 17-litre glass aquaria with 13.5 liter test medium. The fish loading rate was <5 g/L. There were 2 plastic spawning tiles sitting on a flat plastic base per replicate tank while on study. The tiles were observed daily, and, if eggs were found, then the tiles were removed, eggs counted, and the tiles replaced. Water temperature was 25 ± 2 °C, the lighting regime was 16 h light : 8 h dark at 540 – 1080 lux. Water pH was to be between 6.5 and 8.5 at the start of the test and then held at ± 0.5 pH units during the test. Water hardness was equivalent to 4.1 mmol/L or 410 ppm $CaCO_3$ during the studies. The fish were fed with brine shrimp (*Artemia* sp.) *ad libitum* two times per day, with at least three hours between feedings. Uneaten food and fecal material were removed from the bottom of the vessels every day by suction.

Assay Validity Criteria.

The validity criteria for the test were met in all cases: a) mortality during acclimation did not exceed the limits, b) water temperature did not differ by more than ± 1°C and was always within the temperature range 25 ± 2°C specified for fathead minnow, and c) the oxygen concentration in the test media did not fall below 60% of air saturation during the study.

However, the controls in two tests did not meet the criteria for control mortality of <10%. The *n*-octanol controls encountered 12.5% mortality (3 of 24), and the 2-methoxyethanol controls had 16.7% mortality (4 of 24).

Several factors need to be taken into consideration. First, all fish were from the same batch, and the studies were run in the sequence: a) permanganate, b) *n*-octanol, c) 2-methoxyethanol, and in each case there were no excess mortality during the acclimation or holding periods. So no batch variability is present to explain the control mortality. Second, at the low test substance concentrations in the *n*-octanol and 2-methoxyethanol studies, mortalities were 4.2% and 16.7%, respectively. Thus, the exceedence is marginal in nature, and the rates are lower and the same with the low concentrations of the test substances, respectively. Third, there was no evidence of symptoms, disease, or parasites in the fish that would contribute to observed mortality. In conclusion, the studies can still be used to inform the validation program concerning the VTG and the tubercle endpoints. There will be slightly reduced power in two studies as a result of the mortalities, reducing the possibility of false positives. Likewise, the studies can be used to inform concerning gonadal histopathology, particularly in seeking evidence for lesions induced by the test substances. Statistics are not run on this endpoint. Because one desires fish in a high-state of reproductive capacity, the marginal exceedence in mortality suggest that the reproductive measurements need to be interpreted with some caution.

RESULTS

I. Dosing and Test Substance Analyses

Procedures, analytical methods, and analytical results for the studies are described below.

a. Potassium Permanganate

The potassium permanganate stock solutions were prepared for each concentration by dissolving weighed test portions into 5 L test water by intense stirring for 5 min. The stock solutions were prepared daily. The respective stock solutions were pumped in mixing vessels (one per replicate) with constant flow rates maintained flexible-tube pumps set for each concentration. In these vessels, the stock solutions were continuously mixed with dechlorinated water using a magnetic stirrer, and the flow of dechlorinated water was adjusted by a flow controller set for each concentration. Nominal test concentrations of 0.1125, 0.225 and 0.45 mg test item/L resulted. The mixing vessels and the aquaria were connected by a tube.

The water temperatures, pH-values and dissolved oxygen concentrations of all test concentrations and the control were measured in all replicates every working day. The concentrations of manganese were determined using atomic absorbance spectroscopy. In case of some stock solutions the concentrations of potassium permanganate were analysed using photometer.

The mean concentrations of measured manganese were in the range from 79 to 107% of nominal targets. Decrease in the concentration of manganese could be observed in the last week of the test. Reduced recovery rates were not result of incorrect dosing, as can be concluded from the recovery rates of the stock solutions and samples, which were collected from the tube before entering the aquaria. The formation of insoluble MnO_2, which could be observed due to its brown colour, during the dwell time of the test item in the aquaria seemed to be responsible for the reduced recovery rates in the last week.

For the low nominal concentration of 0.1125 mg/L, mean measured values were 85% of the nominal target (0.0956 mg/L). For the intermediate nominal concentration of 0.225 mg/L, mean measured values were 84.5% of target (0.190 mg/L). For the high nominal concentration of 0.45 mg/L, mean measured values were 96.7% of target (0.435 mg/L). These values appear similar to measured percentages observed with potassium permanganate in the other fathead, medaka, and zebrafish laboratories in Phase-2.

b. *n*-Octanol

The *n*-octanol stock solutions were prepared for each concentration by dissolving weighed test portions into 11 L test water with intense stirring for 2x15 min. The stock solutions were prepared daily. The respective stock solutions were diluted and introduced in a similar manner to the potassium permanganate, and the analytical parameters and timing were similar. The concentrations of *n*-octanol were determined using gas chromatography with MS-detector.

For the low nominal concentration of 0.32 mg/L, mean measured values were 73% of the nominal target (0.23 mg/L). For the intermediate nominal concentration of 1 mg/L, mean measured values were 68% of target (0.68 mg/L). For the high nominal concentration of 3.2 mg/L, mean measured values were 58% of target (1.86 mg/L). These values appear similar to measured percentages observed with *n*-octanol in the fathead and medaka laboratories in Phase-2, and clearly higher than in the zebrafish lab (12-24%).

c. 2-Methoxyethanol

The 2-methoxyethanol stock solutions were prepared for each concentration by dissolving weighed test portions into 5 L test water with intense stirring for 5 min. The stock solutions were prepared daily. The respective stock solutions were diluted and introduced in a similar manner to the potassium permanganate, and the analytical parameters and timing were similar. The concentrations of 2-methoxyethanol were determined using gas chromatography with MS-detector.

For the low nominal concentration of 1 mg/L, mean measured values were 95% of the nominal target (0.95 mg/L). For the intermediate concentration of 10 mg/L, mean measured values were 103% of target (10.3 mg/L). For the high concentration of 100 mg/L, mean measured values were 112.5% of target (112.5 mg/L).

Dosing and Test Substance Analyses - Discussion and Conclusions

These measured values of administered test substances are satisfactory to accept all three studies. Together with the other test acceptance criteria, this indicates that the studies are themselves valid for use to assess the relevance and the reliability of the endpoints and the protocol. With potassium permanganate, this study is consistent with analytical observations made in other Phase-2 laboratories, including the formation of a precipitate at the high concentration. With *n*-octanol, this study is consistent with analytical observations made in two other Phase-2 laboratories with the observation of bacterial growth due to biodegradation. The severe *n*-octanol losses in a third lab were not observed. In the third study, 2-methoxyethanol target values were achieved based on the analyses.

II. Mortality, Intoxication, and Related Observations

During the test period all test fish were observed at least once daily for mortality and a variety of symptoms for intoxication. Dead fish were removed from the test vessel and discarded. Clearly distinguishable differences in appearance or intoxication behaviour between treated and control fish were documented daily.

a. Potassium Permanganate

No mortality or symptoms of intoxication were observed in control replicates. In the low concentration potassium permanganate replicates, one fish died on test concentration (4.2%). No intoxication no symptoms were observed on test at this concentration. In the intermediate concentration replicates, five fish died, and surviving fish routinely showed strong ventilation from day 2 forward until the end of the test. In the high concentration replicates, 15 fish died on test (62.5%). Both before death and in all surviving fish there were symptoms of intoxication and distress, i.e., strong ventilation swimming at the surface, feeding abstinence, apathy, lying on the side or back on the bottom. Mortality and symptoms of intoxication are in Table 1A; mortality differed significantly between control and highest test substance concentration for both sexes.

b. *n*-Octanol

In control replicates, one fish died on test in each of the 4 replicates (16.7%). Surviving fish showed no signs of intoxication. For the low *n*-octanol concentration, four fish died on test (16.7%), and one fish was killed during cleaning of the aquaria. A few surviving fish showed intermittent signs of intoxication after day 12, such as, strong ventilation and tumbling during swimming. For the intermediate concentration, five fish died on test (20.8%). A few surviving fish showed intermittent signs of intoxication, such as, strong ventilation, tumbling during swimming and feeding abstinence. At the high concentration, seven fish died on test. A few surviving fish showed signs of intoxication on day 13 and day 20, such as feeding abstinence and body colour light or black. Mortality and symptoms of intoxication are in Table 1B; no statistically significant differences were found between control and any test concentration.

c. 2-Methoxyethanol

In control replicates, one fish died in replicate 3 and two in replicate 1 (12.5%). None of the surviving fish showed signs of intoxication. For the low 2-methoxyethanol concentration, one fish died on test (4.2%). None of the surviving fish showed signs of intoxication until day 13. Thereafter, dark colouration was intermittently observed among some individual fish. For the intermediate concentration, seven fish died on test (29.2%). Some surviving fish showed symptoms of intoxication from day 9 until the end of the test. At the highest test concentration, 22 fish died on test (91.7%). Before expiring, fish showed various signs of intoxication, like feeding abstinence, strong ventilation, dark colouration, tumbling during swimming, swimming mainly at the water surface and apathy beginning as early as day 7. Mortality and symptoms of intoxication are shown in

Table 1C; mortality differed significantly between control and highest test substance concentration for both sexes.

Notes: Presentation of Results and Abbreviations in Tables

Replicates for each concentration are presented on a daily basis (in order to show the temporal appearance of the symptoms) as 1) "Mortality" - actually the number of dead fish / number of fish with intoxication symptoms (dead fish are added to the sum of fish with symptoms), and 2) "Symptoms" - the observed symptoms of intoxication and secondary sexual characteristics and behaviours are recorded. Abbreviations of symptoms of intoxication for which fish were observed during the study are as follows: AK: Strongly extended gills; AP: Apathy; BA: Distended abdomen ; FV: Fins clearly shortened or frayed out at the border; BC: Body colour light or black; GA: Exopthalmus; KF: Feeding abstinence; KR: Convulsions; OB: Fish mainly at the water surface; SA: Mucous secretion; SR: Fish lying on side or back on the bottom; SV: Strong ventilation; TS: Tumbling during swimming. Included are abbreviations of secondary sexual characteristics which were simultaneously recorded by the participating laboratory: VB: presence of vertical bands; KT: loss of territorial aggressiveness in males.

Table 1A. Mortality, intoxication, and related observations [a] in Fathead minnow during the 21-day screening assay exposure to potassium permanganate.

Nominal concentration [mg/L]		Replicate	Exposure Time [d] 0	1	2	3	4	5	6	7
Control	Mortality	1	0/0	0/0	0/1	0/1	0/1	0/1	0/1	0/1
		2	0/1	0/1	0/1	0/2	0/1	0/2	0/1	0/2
		3	0/0	0/0	0/1	0/1	0/2	0/2	0/2	0/2
		4	0/0	0/0	0/1	0/0	0/1	0/1	0/1	0/1
	Symptoms	1	/	/	VB	VB	VB	VB	VB	VB
		2	VB	VB	VB	VB	VB	VB	VB	VB
		3	/	/	VB	VB	VB	VB	VB	VB
		4	/	/	VB	/	VB	VB	VB	VB
0.1125	Mortality	1	0/0	0/0	0/0	0/1	0/1	0/0	0/1	1/1
		2	0/0	0/0	0/1	0/1	0/1	0/1	0/0	0/1
		3	0/1	0/1	0/1	0/2	0/1	0/1	0/1	0/1
		4	0/0	0/0	0/0	0/1	0/0	0/0	0/0	0/1
	Symptoms	1	/	/	/	VB	VB	/	VB	/
		2	/	/	VB	VB	VB	VB	/	VB
		3	VB	VB	VB	VB	VB	VB	VB	VB
		4	/	/	/	VB	/	/	/	VB
0.225	Mortality	1	0/0	0/0	0/6	0/6	1/6	1/6	1/5	1/6
		2	0/1	0/1	0/6	1/6	1/6	1/6	1/6	2/6
		3	0/0	0/0	0/6	0/6	0/6	0/6	0/4	0/6
		4	0/1	0/1	0/6	0/6	0/6	0/6	0/5	0/5
	Symptoms	1	/	/	SV	SV	SV	SV	SV	SV
		2	VB	VB	VB, SV	SV	SV	SV	SV	SV
		3	/	/	SV	SV	SV	VB, SV	SV	SV
		4	VB	VB	VB, SV	SV	SV	VB, SV	SV	VB, SV
0.45	Mortality	1	0/1	0/1	0/6	0/6	1/6	2/6	2/6	2/6
		2	0/0	0/0	0/6	0/6	2/6	2/6	3/6	3/6
		3	0/0	0/0	0/6	1/6	1/6	1/6	2/6	2/6
		4	0/1	0/1	0/6	2/6	3/6	3/6	3/6	3/6
	Symptoms	1	VB	VB	SV	VB, SV	SV	SV	SV	SV, OB, KF
		2	/	/	VB, SV	SV	SV, OB	SV, OB	SV, OB	SV, OB, KF
		3	/	/	VB, SV	VB, SV	SV, OB	SV	SV	SV, KF
		4	VB	VB	SV	SV	SV, OB	SV	SV	SV, KF

[a] Number of dead fish / number of fish with intoxication symptoms (dead fish are added to the sum of fish with symptoms), and observed symptoms of intoxication.

Table 1A continued. Mortality, intoxication, and related observations [a] in Fathead minnow during the 21-day screening to potassium permanganate.

Nominal concentration [mg/L]		Replicate	Exposure Time [d] 8	9	10	11	12	13	14
Control	Mortality	1	0/1	0/1	0/2	0/1	0/1	0/1	0/1
		2	0/2	0/2	0/1	0/2	0/2	0/1	0/2
		3	0/2	0/2	0/2	0/2	0/2	0/1	0/2
		4	0/0	0/1	0/1	0/1	0/1	0/1	0/1
	Symptoms	1	VB	VB	VB	VB	VB	VB	VB
		2	VB	VB	VB	VB	VB	VB	VB
		3	VB	VB	VB	VB	VB	VB	VB
		4	/	VB	VB	VB	VB	VB	VB
0.1125	Mortality	1	1/1	1/1	1/2	1/1	1/1	1/1	1/1
		2	0/1	0/2	0/2	0/2	0/2	0/2	0/2
		3	0/1	0/1	0/2	0/1	0/1	0/1	0/1
		4	0/0	0/0	0/1	0/1	0/1	0/1	0/1
	Symptoms	1	/	/	VB	/	/	/	/
		2	VB	VB	VB	VB	VB	VB	VB
		3	VB	VB	VB	VB	VB	VB	VB
		4	/	/	VB	VB	VB	VB	VB
0.225	Mortality	1	1/6	1/6	1/6	1/6	1/6	2/6	2/6
		2	2/6	2/6	2/6	2/6	2/6	2/6	2/6
		3	0/6	0/6	0/6	0/6	0/6	0/6	0/6
		4	0/6	0/6	0/6	0/6	0/6	1/6	1/6
	Symptoms	1	SV	SV	SV	SV, OB	SV, OB	SV	SV
		2	SV	SV	VB, SV	VB, SV, OB	SV	SV	VB, SV
		3	SV	SV	SV	VB, SV	SV	SV	VB, SV
		4	SV	SV	SV	VB, SV, OB	SV	SV	VB, SV
0.45	Mortality	1	2/6	2/6	3/6	3/6	3/6	3/6	3/6
		2	3/6	3/6	4/6	4/6	4/6	4/6	4/6
		3	2/6	2/6	2/6	3/6	3/6	3/6	3/6
		4	3/6	3/6	4/6	4/6	4/6	4/6	4/6
	Symptoms	1	SV, OB, KT, KF	SV, KT, KF	VB, SV, KF	SV, KT, KF, AP	SV, KT, KF, AP	SV, KT, AP	SV, KT
		2	SV, OB, KT, KF	SV, OB, KT, KF	SV, KT, KF, AP	SV, KT, KF, AP	SV, KT, KF, AP	SV, KT, AP	SV, KT
		3	SV, OB, KT, KF	SV, OB, KT, KF	SV, OB, KT, KF, AP	SV, KT, KF, AP	SV, KT, KF, AP	SV, KT, KF, AP	SV, KT
		4	SV, KT, KF, SR	SV, OB, KT, KF, SR	SV, KT, KF, AP, SR	SV, KT, KF, AP, SR	SV, KT, KF, AP	SV, KT, KF, AP	SV, KT

[a] Number of dead fish / number of fish with intoxication symptoms (dead fish are added to the sum of fish with symptoms), and observed symptoms of intoxication

Table 1A continued. Mortality, intoxication, and related observations [a] in Fathead minnow during the 21-day screening to potassium permanganate.

Nominal concentration [mg/L]		Replicate	Exposure Time [d] 15	16	17	18	19	20	21
Control	Mortality	1	0/2	0/1	0/2	0/2	0/1	0/2	0/2
		2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
		3	0/2	0/1	0/2	0/2	0/2	0/2	0/2
		4	0/1	0/1	0/1	0/1	0/2	0/2	0/2
	Symptoms	1	VB	VB	VB	VB	VB	VB	VB
		2	VB	VB	VB	VB	VB	VB	VB
		3	VB	VB	VB	VB	VB	VB	VB
		4	VB	VB	VB	VB	VB	VB	VB
0.1125	Mortality	1	1/1	1/1	1/2	1/2	1/2	1/2	1/2
		2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
		3	0/1	0/1	0/2	0/2	0/2	0/2	0/2
		4	0/1	0/1	0/2	0/2	0/2	0/2	0/2
	Symptoms	1	/	/	VB	VB	VB	VB	VB
		2	VB	VB	VB	VB	VB	VB	VB
		3	VB	VB	VB	VB	VB	VB	VB
		4	VB	VB	VB	VB	VB	VB	VB
0.225	Mortality	1	2/6	2/6	2/6	2/3	2/6	2/6	2/6
		2	2/6	2/6	2/6	2/3	2/6	2/6	2/6
		3	0/6	0/6	0/6	0/2	0/6	0/6	0/6
		4	1/6	1/6	1/6	1/3	1/6	1/6	1/6
	Symptoms	1	SV	VB, SV	VB, SV	VB	SV	SV	SV
		2	SV	VB, SV	VB, SV	VB	VB, SV	VB, SV	VB, SV
		3	SV	VB, SV	VB, SV	VB	VB, SV	VB, SV	VB, SV
		4	SV	VB, SV	VB, SV	VB	VB, SV	VB, SV	VB, SV
0.45	Mortality	1	3/6	3/6	3/6	3/6	3/6	3/6	3/6
		2	4/6	4/6	4/6	4/6	4/6	4/6	4/6
		3	3/6	3/6	3/6	3/6	4/6	4/6	4/6
		4	4/6	4/6	4/6	4/6	4/6	4/6	4/6
	Symptoms	1	SV, KT	VB, SV, KT	SV, KT	SV, KT	SV, KT	SV, KT	SV, KT
		2	SV, KT	SV, KT	SV, KT	SV, KT	SV, KT	SV, KT	SV, KT
		3	SV, KT	SV, KT	SV, KT	SV, KT	SV, KT	SV, KT	SV, KT
		4	SV, KT	SV, KT	SV, KT	SV, KT	SV, KT	SV, KT, TS	SV, KT, TS

[a] Number of dead fish / number of fish with intoxication symptoms (dead fish are added to the sum of fish with symptoms), and observed symptoms of intoxication.

Table 1B. Mortality, intoxication, and related observations [a] in Fathead minnow during the 21-day screening to *n*-octanol.

Nominal concentration [mg/L]		Replicate	Exposure Time [d] 0	1	2	3	4	5	6	7
Control	Mortality	1	0/0	0/0	0/0	0/2	0/2	0/2	0/2	0/2
		2	0/0	0/0	0/0	0/2	0/2	0/2	0/3	0/3
		3	0/0	0/0	0/0	0/1	0/2	0/2	0/2	0/1
		4	0/0	0/0	0/0	0/1	0/2	0/2	0/2	0/1
	Symptoms	1	/	/	/	VB	VB	VB	VB	VB
		2	/	/	/	VB	VB	VB	VB	VB
		3	/	/	/	VB	VB, GA	VB, GA	VB, GA	GA
		4	/	/	/	VB	VB	VB	VB	VB
0.32	Mortality	1	0/0	0/0	0/0	0/2	0/0	0/1	0/2	0/2
		2	0/0	0/0	0/0	0/1	0/1	0/1	0/2	0/2
		3	0/0	0/0	0/0	0/2	0/2	0/2	0/1	0/2
		4	0/0	0/0	0/0	0/2	0/2	0/1	0/1	0/0
	Symptoms	1	/	/	/	VB	VB	VB	VB	VB
		2	/	/	/	VB	VB	VB	VB	VB
		3	/	/	/	VB	VB	VB	VB	VB
		4	/	/	/	VB	VB	VB	VB	/
1.0	Mortality	1	0/0	0/0	0/0	0/0	0/2	0/0	0/1	0/3
		2	0/0	0/0	0/0	0/1	0/1	0/1	0/1	0/1
		3	0/0	0/0	0/0	0/2	0/2	0/2	0/2	0/2
		4	0/0	0/0	0/0	0/0	0/1	0/1	0/1	0/1
	Symptoms	1	/	/	/	/	VB	/	VB	VB
		2	/	/	/	VB	VB	VB	VB	VB
		3	/	/	/	VB	VB	VB	VB	VB
		4	/	/	/	/	VB	VB	VB	VB
3.2	Mortality	1	0/0	0/0	0/0	0/2	0/2	0/1	0/1	1/2
		2	0/0	0/0	0/0	0/2	1/3	1/3	2/2	2/3
		3	0/0	0/0	0/0	0/1	0/1	0/1	0/1	0/1
		4	0/0	0/0	0/0	0/1	1/2	1/2	1/1	1/2
	Symptoms	1	/	/	/	VB	VB	VB	VB	VB
		2	/	/	/	VB	VB	VB	/	VB
		3	/	/	/	VB	VB	VB	VB	VB
		4	/	/	/	VB	VB	VB	/	VB

[a] Number of dead fish / number of fish with intoxication symptoms (dead fish are added to the sum of fish with symptoms), and observed symptoms of intoxication.

Table 1B continued. Mortality, intoxication, and related observations [a] in Fathead minnow during the 21-day screening to *n*-octanol.

Nominal concentration [mg/L]		Replicate	Exposure Time [d] 8	9	10	11	12	13	14
Control	Mortality	1	0/2	0/2	1/3	1/3	1/3	1/4	1/2
		2	0/2	0/2	0/1	0/2	0/2	0/3	0/2
		3	0/3	0/3	0/2	0/2	0/1	0/2	0/1
		4	0/2	0/1	0/1	0/1	0/1	0/2	0/2
	Symptoms	1	VB	VB	VB	VB	VB	VB	VB
		2	VB	VB	VB	VB	VB	VB	VB
		3	VB, GA	VB, GA	VB, GA	VB, GA	VB, GA	VB, GA	VB, GA
		4	VB	VB	VB	VB	VB	VB, SV	VB, SV
0.32	Mortality	1	0/3	0/2	0/2	0/2	0/2	0/2	0/2
		2	0/2	1/2	1/3	2/3	2/4	2/4	2/3
		3	0/2	0/2	0/2	0/2	0/2	0/2	0/2
		4	0/0	0/1	0/2	0/1	0/2	0/1	0/2
	Symptoms	1	VB	VB	VB	VB	VB	VB	VB
		2	VB	VB	VB	VB	VB	VB	VB
		3	VB	VB	VB	VB	VB	VB	VB
		4	/	VB	VB	VB	VB, SV, TS	VB	TS
1.0	Mortality	1	0/1	0/1	0/2	0/2	0/2	1/3	1/2
		2	0/4	0/4	0/1	0/1	0/2	0/3	0/2
		3	0/1	0/1	0/1	0/2	0/2	0/3	0/1
		4	0/3	0/3	0/1	0/2	0/3	0/2	0/2
	Symptoms	1	VB	VB	VB	VB	VB, SV, TS	VB	VB
		2	VB	VB	VB	VB	VB, SV, KF	VB, SV	VB, SV
		3	VB	VB	VB	VB	VB	VB	VB
		4	VB	VB	VB	VB	VB, SV	VB	VB
3.2	Mortality	1	1/2	1/2	1/2	1/2	1/1	1/1	1/2
		2	2/3	2/4	2/2	3/3	3/3	3/3	3/3
		3	0/2	0/1	0/1	0/2	0/1	0/1	0/3
		4	1/2	1/3	1/1	2/2	3/3	3/3	3/3
	Symptoms	1	VB	VB	VB	VB	/	KF	VB
		2	VB	VB	/	/	/	KF	/
		3	VB	VB	VB	VB	VB	VB	VB
		4	VB	VB	/	/	/	KF	/

[a] Number of dead fish / number of fish with intoxication symptoms (dead fish are added to the sum of fish with symptoms), and observed symptoms of intoxication.

Table 1B continued. Mortality, intoxication, and related observations [a] in Fathead minnow during the 21-day screening to *n*-octanol.

Nominal concentration [mg/L]		Replicate	Exposure Time [d] 15	16	17	18	19	20	21
Control	Mortality	1	1/3	1/3	1/3	1/3	1/3	1/2	1/3
		2	0/1	0/1	0/2	0/2	1/2	1/1	1/2
		3	0/2	0/2	0/3	1/3	1/2	1/3	1/3
		4	1/2	1/2	1/2	1/2	1/2	1/2	1/3
	Symptoms	1	VB	VB	VB	VB	VB	VB	VB
		2	VB	VB	VB	VB	VB	/	BC
		3	VB,GA	VB,GA	VB,GA,SV	VB,GA	VB,GA	VB,GA	VB,GA
		4	VB	VB	VB	VB	VB	VB	VB
0.32	Mortality	1	0/2	0/2	0/2	0/2	0/2	0/2	0/2
		2	2/2	3/3*	3/5	3/4	3/4	3/5	3/5
		3	0/1	0/2	0/2	0/2	0/2	0/2	0/2
		4	1/2	1/1	1/2	1/2	1/2	1/1	1/2
	Symptoms	1	VB	VB	VB	VB	VB	VB	VB
		2	/	/	VB	VB	VB	VB	VB
		3	VB	VB	VB	VB	VB	VB	VB
		4	VB	/	VB, TS	VB, TS	TS	/	VB
1.0	Mortality	1	1/3	3/4	3/4	4/4	4/4	4/4	4/4
		2	0/2	0/1	0/2	0/2	1/2	1/2	1/2
		3	0/1	0/1	0/2	0/1	0/1	0/1	0/1
		4	0/0	0/0	0/2	0/2	0/2	0/2	0/1
	Symptoms	1	VB	VB	VB	/	/	/	/
		2	VB, SV	VB	VB, SV	VB, SV	VB	VB	VB
		3	VB	VB	VB	VB	VB	VB	VB
		4	/	/	VB	VB	VB	VB	VB
3.2	Mortality	1	1/2	1/1	1/3	1/2	1/3	1/3	1/3
		2	3/3	3/3	3/3	3/3	3/3	3/3	3/3
		3	0/1	0/1	0/2	0/1	0/1	0/2	0/2
		4	3/3	3/3	3/3	3/3	3/3	3/4	3/3
	Symptoms	1	VB	/	VB	VB	VB	VB, BC	VB
		2	/	/	/	/	/	/	/
		3	VB	VB	VB	VB	VB	VB, GA	VB, GA
		4	/	/	/	/	/	BC	/

[a] Number of dead fish / number of fish with intoxication symptoms (dead fish are added to the sum of fish with symptoms), and observed symptoms of intoxication.

Table 1C. Mortality, intoxication, and related observations [a] in Fathead minnow during the 21-day screening assay exposure to 2-methoxyethanol.

Nominal concentration [mg/L]		Replicate	Exposure Time [d] 0	1	2	3	4	5	6	7
Control	Mortality	1	0/1	0/2	0/1	0/2	1/2	1/2	1/2	1/3
		2	0/1	0/1	0/1	0/2	0/2	0/2	0/2	0/2
		3	0/2	0/1	0/2	0/2	0/2	0/1	0/2	0/2
		4	0/2	0/1	0/1	0/2	0/2	0/2	0/2	0/2
	Symptoms	1	VB	VB	VB	VB	VB	VB	VB	VB
		2	VB	VB	VB	VB	VB	VB	VB	VB
		3	VB	VB	VB	VB	VB	VB	VB	VB
		4	VB	VB	VB	VB	VB	VB	VB	VB
1.0	Mortality	1	0/1	0/1	0/0	0/2	0/2	0/2	1/3	1/3
		2	0/0	0/0	0/0	0/1	0/0	0/0	0/0	0/0
		3	0/2	0/1	0/2	0/2	0/2	0/2	0/2	0/2
		4	0/0	0/2	0/1	0/2	0/2	0/2	0/2	0/2
	Symptoms	1	VB	VB	/	VB	VB	VB	VB	VB
		2	/	/	/	VB	/	/	/	/
		3	VB	VB	VB	VB	VB	VB	VB	VB
		4	/	VB	VB	VB	VB	VB	VB	VB
10	Mortality	1	0/0	0/1	0/2	0/2	0/1	0/1	0/0	0/1
		2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
		3	0/1	0/2	0/1	0/2	0/2	0/1	0/2	0/2
		4	0/0	0/2	0/2	1/2	1/2	1/2	1/2	1/2
	Symptoms	1	/	VB	VB	VB	VB	VB	/	VB
		2	VB	VB	VB	VB	VB	VB	VB	VB
		3	VB	VB	VB	VB	VB	VB	VB	VB
		4	/	VB	VB	VB	VB	VB	VB	VB
100	Mortality	1	0/1	0/2	0/1	0/1	0/2	0/2	0/1	0/1
		2	0/1	0/2	0/1	0/2	1/2	1/2	1/2	1/1
		3	0/1	0/2	0/0	1/2	1/2	1/2	1/2	1/1
		4	0/0	0/1	0/0	0/1	0/2	0/1	0/2	0/2
	Symptoms	1	VB	VB	VB	VB	VB	VB	VB	VB
		2	VB	VB	VB	VB	VB,SV	VB	VB	/
		3	VB	VB	/	VB	VB	VB	VB	/
		4	/	VB	/	VB	VB	VB	VB	VB

[a] Number of dead fish / number of fish with intoxication symptoms (dead fish are added to the sum of fish with symptoms), and observed symptoms of intoxication.

Table 1C continued. Mortality, intoxication, and related observations [a] in Fathead minnow during the 21-day screening assay exposure to 2-methoxyethanol.

Nominal concentration [mg/L]		Replicate	Exposure Time [d] 8	9	10	11	12	13	14
Control	Mortality	1	1/2	2/3	2/2	2/2	2/3	2/3	2/2
		2	0/2	0/2	0/1	0/1	0/1	0/1	0/1
		3	0/2	0/1	0/1	0/1	0/1	0/1	0/2
		4	0/2	0/2	0/2	0/2	0/1	0/2	0/2
	Symptoms	1	VB	VB	/	/	VB	VB	/
		2	VB	VB	VB	VB	VB	VB	VB
		3	VB	VB	VB	VB	VB	VB	VB
		4	VB	VB	VB	VB	VB	VB	VB
1.0	Mortality	1	1/1	1/2	1/2	1/3	1/3	1/3	1/2
		2	0/0	0/0	0/0	0/0	0/0	0/1	0/1
		3	0/2	0/1	0/1	0/1	0/1	0/2	0/2
		4	0/2	0/2	0/1	0/2	0/1	0/2	0/3
	Symptoms	1	/	VB	VB	VB	VB	VB	VB
		2	/	/	/	/	/	BC,VB	BC,VB
		3	VB	VB	VB	VB	VB	BC,VB	BC,VB
		4	VB	VB	VB	VB	VB	BC,VB	VB
10	Mortality	1	0/0	0/1	0/0	0/0	0/1	0/1	0/1
		2	0/2	0/2	0/2	0/2	0/1	0/2	0/2
		3	0/0	0/1	0/0	0/2	0/3	0/3	0/0
		4	1/1	1/1	1/2	1/2	1/2	1/2	1/1
	Symptoms	1	/	SV,OB	/	/	OB	SV	SV
		2	VB	VB	VB	VB	VB	BC,VB	BC,VB
		3	/	VB	/	VB	VB	BC,VB	/
		4	/	/	VB	VB	VB	VB	/
100	Mortality	1	0/2	1/6	3/6	3/6	3/6	3/6	4/6
		2	1/1	1/6	1/6	2/6	2/6	3/6	4/6
		3	2/2	2/6	2/6	2/6	2/6	2/6	2/6
		4	1/1	0/6	0/6	1/6	1/6	3/6	3/6
	Symptoms	1	AP,BC,KF, KT,TS	BC,SV,KT, KF	SV,KF	SV,KF	SV,KF	SV,KF	SV,KF
		2	/	SV,KF	SV,OB,KF	SV,KF	SV,KF	SV,KF,TS	SV,KF,VB
		3	/	SV,KF	SV,KF	SV,KF	SV,KF	SV,KF	SV,KF,BC
		4	VB	SV,KF,KT	SV,BC,OB,KF	SV,KF,BC	SV,KF,BC	SV,KF	SV,KF

[a] Number of dead fish / number of fish with intoxication symptoms (dead fish are added to the sum of fish with symptoms), and observed symptoms of intoxication.

Table 1C continued. Mortality, intoxication, and related observations [a] in Fathead minnow during the 21-day screening assay exposure to 2-methoxyethanol.

Nominal concentration [mg/L]		Replicate	Exposure Time [d] 15	16	17	18	19	20	21
Control	Mortality	1	2/2	2/3	2/2	2/3	2/2	2/2	2/2
		2	0/1	0/1	0/1	0/1	0/1	0/1	0/1
		3	0/1	0/1	1/2	1/2	1/1	1/3	1/3
		4	0/2	0/2	0/2	0/3	0/2	0/2	0/2
	Symptoms	1	/	VB	/	VB	VB	VB	VB
		2	VB	BC	VB	VB	VB	VB	VB
		3	VB	VB	VB	VB	/	VB	VB
		4	VB	VB	VB	VB	VB	VB	VB
1.0	Mortality	1	1/3	1/3	1/3	1/2	1/3	1/3	1/3
		2	0/1	0/1	0/1	0/1	0/1	0/1	0/1
		3	0/1	0/0	0/1	0/0	0/2	0/2	0/2
		4	0/1	0/2	0/2	0/2	0/3	0/3	0/3
	Symptoms	1	VB	VB	VB	VB	VB	VB	VB
		2	BC	BC	BC	BC	BC	BC	VB
		3	VB	/	VB	/	VB	VB	VB,GA
		4	VB	BC,VB	BC,VB	BC,VB	VB	VB	VB,GA
10	Mortality	1	0/1	1/2	1/2	1/2	1/1	1/3	1/3
		2	0/2	0/2	0/2	0/2	1/3	1/3	1/3
		3	0/1	0/2	0/3	0/3	0/1	0/3	0/3
		4	1/2	1/2	1/3	1/2	3/4	3/5	5/5
	Symptoms	1	SV,TS	BC	BC,KT	BC	/	GA,BC	GA,BC
		2	VB,BC	VB	VB	VB	VB	VB	VB
		3	VB	VB	VB,SV	VB,SV	SV,GA	GA, SV	GA, SV
		4	VB	VB	VB,BC	VB	VB	BC	/
100	Mortality	1	4/6	5/6	5/6	5/6	5/6	5/6	5/6
		2	5/6	5/6	6/6	6/6	6/6	6/6	6/6
		3	3/6	4/6	5/6	5/6	6/6	6/6	6/6
		4	4/6	4/6	4/6	4/6	5/6	5/6	5/6
	Symptoms	1	SV,KF	SV,KF	SV,KF	SV,KF	SV,KF	SV,KF	SV,KF
		2	SV,KF	TS,SV	/	/	/	/	/
		3	SV,KF,TS	SV,KF	SV,KF	SV,KF	/	/	/
		4	SV,KF	SV,KF	SV,KF	SV,KF	SV,KF	SV,KF	SV,KF

[a] Number of dead fish / number of fish with intoxication symptoms (dead fish are added to the sum of fish with symptoms), and observed symptoms of intoxication.

Mortality, Intoxication, and Related Observations - Discussion and Conclusions

Only one laboratory encountered significant mortality in Phase-1B, and that included the controls. In the related US EPA program, only one laboratory study with methoxychlor has shown clear concentration-related mortality (i.e., methoxychlor (Battelle, 2003)). Therefore, the instances of concentration-related mortality deserve careful examination. These events were not specific to this laboratory. For comparison, mortalities in the laboratory 1 study with fathead minnow were low while the mortalities in the laboratory 4 study were 63% at a nominal concentration of 0.45 mg/L permanganate and 29% at a nominal concentration of 3.2 mg *n*-octanol/L. The latter compares favourably to 63% and 24% in the current studies, respectively. All medaka also died at a higher concentration of 0.9 mg/L potassium permanganate. So, the mortality effects appear generally reproducible. Regarding the symptoms of intoxication, these were not consistently included in the protocol or previously reported, so while common to most aquatic assays with fish, these are new explorations for the needs of the current protocol.

Mortality and symptoms of intoxication and other possible sublethal effects need to be incorporated into the interpretation of the potassium permanganate and 2-methoxyethanol studies. Mortality and symptoms of intoxication were related to concentration and to the duration of exposure at a given concentration. Symptoms of intoxication were frequent and related to concentration and to the duration of exposure at a given concentration. In the case of potassium permanganate, the symptom of intoxication at the intermediate concentration was strong ventilation. This symptom was uniformly observed among all fish and occurred almost from the inception of the study (day 2). In the case of 2-methoxyethanol, there were a variety of symptoms of intoxication at the intermediate concentration that were more sporadic and less frequent. Consistent with symptom observations, mortality at the high concentrations of potassium permanganate and 2-methoxyethanol achieved statistical significance versus the controls. For the *n*-octanol study, there was a low level of mortalities, and symptoms of intoxication were infrequent occurrence of symptoms of intoxication with the few instances the concentration and duration of exposure.

In subsequent sections, many other findings related to spawning activity, fecundity, tubercle numbers and scores, and gonad histopathology follow the same concentration patterns as the intoxication symptoms and mortality. Thus, many of the endpoints appear to be confounded by general, systemic toxicity. Under the current protocol, the simple statistical appearance is a *de facto* positive, and these data show such results are false positives.

Given the evidence for impact of systemic toxicity on several endpoints, to resolve the issue of false positives several changes in the protocol and the interpretation of its results are recommended:

- Avoid concentrations with high mortality and symptoms of intoxication by refining concentration selection guidance beyond acute toxicity (LC50). The concentration selection should be informed with data from juvenile toxicity studies (as was done with *n*-octanol) or early life stage studies, or range finding studies and the consequent additional animal use are necessary.
- Ensure that reproductive (spawning activity and fecundity endpoints) and endocrine-related (VTG and SCC endpoints) observations that are confounded or caused by general, systemic toxicity as evidenced by mortality and symptoms of intoxication are discounted and not interpreted as positive findings of endocrine modes of action. That is, define a maximum tolerated concentration (MTC) for the fish screen, where only findings below such an MTC would be accepted as positive for true reproductive and/or endocrine-related mode of action findings.
- Thus, in a straightforward solution, incorporate in the interpretative section of a future Test Guideline that positive findings for possible endocrine related effects should occur in the absence of confounders for systemic toxicity and symptoms of intoxication and that particularly vulnerable endpoints such as reproduction would be recognized.

III. Spawning Status and Fecundity

Spawning status and fecundity were both measured during the assay. For spawning status, the laboratory recorded the presence of eggs (yes/no answer) in treated and control replicates on a daily basis. The data are presented as the total number of days that eggs were observed in each of the four replicates per group / the total number of days on test and then as the mean number of days that eggs were observed per group, e.g., 12 / 21. For fecundity, the spawning tiles with eggs were removed from the replicate tanks each day and the eggs counted. The fecundity data are presented in two ways: a) the cumulative number of eggs in each replicate for the entire test period and as a mean of the cumulative eggs per group for the entire test period and b) as the mean number of eggs per female that survives the test. There is currently no means to take into account for the death of one or both males in the replicates.

a. Potassium Permanganate

The spawning data were variable, and the spawning activity even in some control replicates was low. For example, under optimal conditions, the fathead spawning cycle is approximately 4-5 days. With two males and four females per replicate, robust spawning activity would be expected to yield an observation of eggs on most days on test, e.g. 15-19 days out of 21, and with multiple spawning and average clutch sizes of 40-80 eggs a cumulative number of eggs can exceed 2000 per replicate on test, and the mean number of eggs per female can exceed 300. In this study, eggs were observed in two control replicates on 7 of 21 days and, in the other two replicates, on only 1 of 21 days. Cumulative numbers of eggs were 147 and 170 in the former replicates and only 3 and 38 in the latter replicates.

The spawning status and fecundity data for potassium permanganate are presented in Table 2. Spawning activity and mean number of eggs per surviving female were significantly reduced at both the intermediate and high concentrations of the test substance.

Spawning, measured by mean days of egg production per replicate, occurred on 4 of 21 days in the control replicates. No spawning days were recorded for the intermediate concentration and, only one spawning day was recorded for the high concentration, resulting in mean values of 0 and 0.25, respectively. As a result, even with the limited spawning in the control, the spawning status decreased significantly in the intermediate and high concentrations ($p=0.014$ and 0.029, respectively). Cumulative egg numbers were 358 in the control and 290 in the low concentration, but 0 and 1 in the intermediate and high concentrations, respectively. The number of eggs per female was 23.4 in the control, followed by 19.7 eggs per female at the low concentration and only 0 and 0.5 eggs per female at the intermediate and high concentrations, respectively. Therefore, both cumulative egg numbers and number of eggs per female decreased significantly at the intermediate and high concentrations.

Additionally, as noted, the fish may not have been completely mature. This impacted the distribution of males and females in several replicates, increasing the number of males and decreasing the females. Based on gonadal histopathology, these included replicate 3 in the controls; replicates 2, 3, and 4 at the low concentration; replicate 3 at the intermediate concentration, and, due to mortalities, any misdistribution at the high concentration could not be determined. Fortunately, this allows the control and the intermediate concentrations to be compared where 3 out of 4 replicates had the correct distribution.

b. *n*-Octanol

The spawning status and fecundity data for *n*-octanol are presented in Table 3. Significant differences were observed, but, surprisingly, the intermediate test concentration had a *greater* value for the mean number of eggs per surviving female than the control.

The spawning data were again variable, and the spawning activity even in some control replicates was again low. Spawning as measured by egg production in any replicate occurred on 2.75 of 21 days (mean value) in the control replicates, 5.25 in the low concentration, 7 in the intermediate concentration, and 5.25 in the high concentration. The minimum values in a given replicate were 1, 1, 3 and 3, respectively. Thus, in this study, the

absolute values for all test substance concentrations were greater than the control. The difference between the control and the intermediate concentrations, meaning that spawning activity was potentially greater than the controls, approached statistical significance (p=0.064).

In the case of the mean cumulative number of eggs, the respective values were 74.8, 457.3, 700, and 184 per female, meaning that the test concentrations were 2-9 fold greater than the controls. The respective mean number of eggs per surviving female (minimum value among replicates) were 27.9 (1.33), 248.7 (5.33), 208.9 (104.5), and 186.4 (13). Taking into account the variability apparent from the minimums and reduced statistical power, only the intermediate concentration value was significantly greater than controls (p=0.029).

c. 2-Methoxyethanol

The spawning status and fecundity data for 2-methoxyethanol are presented in Table 4. No significant differences were observed between the control and any test concentration.

The spawning data were again variable, and the spawning activity even in some control replicates was again low. The respective spawning activity values for the groups were 3.75, 1.5, 2, and 1.75 days out of 21 days on test. Two replicates from the low concentration and one replicate from the high concentration had no observed spawning during the test. As a result, it was the low concentration which had the lowest statistical p value (0.157) versus the control. Maximums and minimum replicate values for cumulative egg numbers varied widely: 538 to 4 for the control, 172 to 0 for the low concentration, 108 to 6 for the intermediate concentration, and 74 to 0 for the high concentration, and no significant differences were observed. The mean number of eggs per surviving female for the respective concentrations were 40, 10.9, 18.1, and 21.1 with no significant differences observed (the low concentration *p*-value was again the lowest – again, 0.157).

Table 2. Overall spawning status and fecundity parameters of Fathead minnow during the 21-day screening assay exposure to potassium permanganate.

		Nominal concentration [mg/L]			
	Replicate	Control	0.1125	0.225	0.45
No. of days with spawning "yes" / observed days	1	7/21	8/21	0/21	1/21
	2	1/21	0/21	0/21	0/21
	3	7/21	1/21	0/21	0/21
	4	1/21	1/21	0/21	0/21
Mean No. of days with spawning "yes" / observed days		4/21	2.5/21	0/21	0.25/21
Cumulative number of eggs	1	170	265	0	4
	2	38	0	0	0
	3	147	20	0	0
	4	3	5	0	0
Mean number of eggs		89.5	72.5	0	1
Number of eggs per female survived	1	42.5	66.3	0	2
	2	12.7	0	0	0
	3	36.8	10	0	0
	4	1.5	2.5	0	0
Mean number of eggs per female survived		23.4	19.7	0	0.5

Table 3. Overall spawning status and fecundity parameters of Fathead minnow during the 21-day screening assay exposure to *n*-octanol.

		Nominal concentration [mg/L]			
	Replicate	Control	0.32	1.0	0.32
No. of days with spawning "yes" / observed days	1	1/21	5/21	3/21	3/21
	2	5/21	9/21	6/21	4/21
	3	2/21	6/21	6/21	4/21
	4	3/21	1/21	13/21	10/21
Mean No. of days with spawning "yes" / observed days		2.75/21	5.25/21	7/21	5.25/21
Cumulative number of eggs	1	4	487	126	204
	2	67	715	492	39
	3	122	611	418	78
	4	106	16	1764	415
Mean number of eggs		74.75	457.25	700	184
Number of eggs per female survived	1	1.33	121.75	126	102
	2	16.75	715	164	13
	3	40.67	152.75	104.5	19.5
	4	53	5.33	441	207.5
Mean number of eggs per female survived		27.94	248.71	208.88	186.38

Spawning Activity and Fecundity – Discussion and Conclusions

In Phase-1B, the design of five males and five females in two replicate tanks was clearly unsatisfactory to address the spawning activity and fecundity of the fathead minnow. The current design was changed to four replicate tanks with two tiles using 2 males and 4 females per tank, thus providing 8 opportunities per day to observe an egg clutch. This should not be infrequent with a total of 16 females and a spawning cycle in healthy fathead minnows of 4-5 days, if the territorial issues with the males are resolved by this design.

In the permanganate studies misidentification of immature males may have confounded the spawning and fecundity to some degree in the controls and the intermediate concentration where one replicate in each case had too many males. The low permanganate concentration, on the other hand, had the proper distribution in only one of four replicates. The best observations were 13 days in replicate 4 of the low concentration of *n*-octanol, 0 days in replicate 4 at the high concentration of *n*-octanol, 9 days in replicate 2 at the low concentration of *n*-octanol, and 8 days in replicate 1 of the low concentration of potassium permanganate [Note: here there appear to have been the correct number of 2 males and 4 females]. Therefore, the use of two active males per tank is still not consistent for vigorous spawning activity, and the current design appears to be insensitive and erratic in many cases. For the spawning activity in the two other fathead studies in Phase-1B, the control observations were 4 and 2.75, so these studies appear comparable with the previous studies in this respect and to have reproduced those results. The consistent low values lead to questions about the power of this endpoint, and the

need for improved acceptance criteria (because the current fish met both the age and weight criteria) if this endpoint would be pursued in the future. In addition, twice per day tank cleaning due to test substance biodegradation may have stressed the fish in the *n*-octanol study.

Table 4. Overall spawning status and fecundity parameters of Fathead minnow during the 21-day screening assay exposure to 2-methoxyethanol.

		Nominal concentration [mg/L]			
	Replicate	Control	1.0	10	100
No. of days with spawning "yes" / observed days	1	4/21	1/21	1/21	0/21
	2	7/21	0/21	5/21	1/21
	3	2/21	0/21	1/21	4/21
	4	2/21	5/21	1/21	2/21
Mean No. of days with spawning "yes" / observed days		3.75/21	1.5/21	2/21	1.75/21
Cumulative number of eggs	1	8	2	15	0
	2	538	0	108	1
	3	87	0	6	41
	4	4	172	39	74
Mean number of eggs		159.25	43.5	42	29
Number of eggs per female survived	1	2.67	0.67	5	0
	2	134.5	0	27	0.25
	3	21.75	0	1.5	10.25
	4	1	43	39	74
Mean number of eggs per female survived		39.98	10.92	18.13	21.13

Cumulative egg numbers and eggs per surviving female were still not satisfactory even with the change in protocol design form Phase-1B. There were 8 instances when the cumulative egg number for a replicate was greater than 400 eggs; only one of these instances was in the 12 control replicates. The cumulative mean per replicate for the three controls was 75, 90, and 165. This reinforces the non-ideal conditions and limited power. Mean number of eggs produced per female on study in the controls was 23, 28, and 40; this also reinforces the non-ideal conditions and limited power. The fecundity data for the other 3 fathead minnow Phase-2 studies were not provided in two cases, and then presented graphically for cumulative egg numbers in the third case. The eggs per female results were not provided for any of the 3 studies in the final report. The estimate for the single permanganate study is a mean of about 700 eggs per replicate in the controls, which was clearly more robust than the current studies.

In the Phase-2 fathead minnow studies, spawning activity was reduced at the intermediate and high permanganate concentration and not by *n*-octanol exposure as with these studies. This reduction in spawning

activity with permanganate was observed consistent with mortality in medaka, but also occurred in zebrafish without evidence for increased mortality. In laboratory 1, fathead minnow fecundity was also erratic, cumulative egg numbers were highest at 0.9 mg/L followed by the intermediate concentration, the control, and with the low concentration having only about 50% of the eggs as the high concentration. Medaka fecundity was reduced at 0.225 mg/L and higher, and zebrafish fecundity at 0.45 mg/L and higher.

A fundamental statistical question arose as a result of the studies. As a tank contains 2 males and 4 females, what happens to its application as a true replicate when one or both males die or when one or more females die? How should the results be compared to other tanks, no longer full and true replicates, that still continue to have 2 males and 4 females and, therefore, inherently, greater chance for spawning activity and fecundity? These current results are presented as though any such with tank mortality in the individuals continued to be a replicate, but discussions with experts and statisticians are underway to assess alternative methods or whether such tanks should be removed from the study altogether.

Several questions arise from these studies and are not completely answered:

- In the permanganate studies, spawning activity and fecundity appear to be associated with mortality and symptoms of intoxication which were evident at the intermediate and high concentrations. In the intermediate and high concentrations, only one spawning event, a clutch of just 4 eggs was observed. This resulted in the achievement of statistically significant decreases versus the control.
- In the *n*-octanol studies, there was a modest increase in mortality with concentration and no major intoxication symptoms and spawning activity and fecundity was actually better in all treatment groups than in the control. As a result, spawning activity versus control approached statistical significance ($p=0.064$) for the intermediate concentration.
- In the 2-methoxyethanol studies, all males died before the end of the study at the high concentration and both males in replicate 4 of the intermediate concentration died before the end of the study. Yet, the results were so modest in the control and the low concentrations, that statistical significance was not achieved and, in fact, differences were modest.
- Two females in the permanganate study and one female in the 2-methoxyethanol study were found to have immature gonads *after* being 3 weeks on study. There was also some misidentification of males as females in the permanganate study. Thus, it would appear that the fish were not fully mature and reproductively capable in all cases at the beginning of the study. This leads to the recommendations that:

    - The recommended mean age of the fathead minnow may need to be increased beyond 20 weeks (5 months). [Note: in Phase-3, one laboratory has again found the fathead minnows to be sexually immature at 20 weeks of age.]
    - The use of the acclimation period to test the reproductive capacity fish received in pairs may need to be considered if this endpoint would be pursued so that only proven and successful breeders would be placed on test.

In conclusion, the spawning activity and fecundity capability of the protocol, even with the increase in replicates and reduction to two males per tank is not sufficient to yield a consistently reliable and robust measurement of reproduction. This is also consistent with the results from the other fathead minnow laboratories in this and previous Phases of the validation program. Therefore, the previous decision of the VMG-eco to remove the spawning activity and fecundity measurements from the validation program appear to be justified.

IV. Vitellogenin

VTG is a lipoprotein complex that is normally produced by the sexually active female in the liver, released into circulation, taken up by the developing oocyte, and processed to become part of the egg lipid and protein

nutrients. VTG production is under estrogen control, where the mRNA transcription is under positive control by the ER-estradiol receptor-ligand complex. VTG production in the sexually active male is minimal. Therefore, the presence of elevated levels of VTG in the male is a sensitive indicator of estrogen exposure. Reduction in female VTG levels is considered an indicator of antiestrogens or aromatase exposure. VTG is measured by sampling serum at necropsy and analyzing using a specific, monoclonal antibody ELISA kit. Due to some kit to kit and laboratory variations, a standardization curve is necessary for an analysis. There is typically a high variability of VTG values (high SD's), but true responses are sensitive and dramatic, and, thus, high enough to easily reach statistical significance.

a. Potassium Permanganate

Previous work cited in the Phase-2 report show that potassium permanganate does not activate an estrogen sensitive cell transcription reporter system or interfere with the binding of the native estradiol ligand to the medaka estrogen receptor.

The potassium permanganate study was the first VTG analysis performed in this laboratory. The circulating levels of VTG in male fish decreased in absolute value at the high concentration and differed significantly with the control (p-value = 0.043). There was also an absolute decrease in female circulating VTG at the high concentration, but this did not achieve statistical significance. Several females were observed to be immature when gonad histopathology was performed, and their VTG levels were typically 0-100 ng/L. These values remained in the analyses and are one source for the high SD values and limited statistical sensitivity. Several values were outside the confidence levels of the standard curve, and these values have been removed prior to statistical analyses. The data are presented in Table 5.

Table 5. Circulating VTG analyses for male and female fathead minnows exposed to potassium permanganate.

Group	Nominal KMnO4 (mg/L)	Circulating VTG (ng/ml)			
		Male		Female	
		Mean Individual	Mean Replicate	Mean Individual	Mean Replicate
Control	0	147.3	181.1	$1{,}070 +10^3$	$870 +10^3$
Low	0.1125	296.1	296.1	$1{,}269 +10^3$	$1{,}490 +10^3$
Intermediate	0.225	113.5	125.3	$2{,}089 +10^3$	$2{,}142 +10^3$
High	0.45	4.3	4.3*	$83 +10^3$	$83 +10^3$

* Statistically significant change in replicates; NA – not applicable.

b. *n*-Octanol

Previous work cited in the Phase-2 report show that *n*-octanol does not activate an estrogen sensitive cell transcription reporter system or bind to the medaka estrogen receptor.

The circulating levels of VTG in the male groups and the female groups did not differ significantly between the control and any of the test concentrations. There were variations in the absolute values between the groups, but these absolute changes appear random and are not concentration related, e.g., the male values are a minimum at the intermediate concentration and the minimum female values occur in the low and the high concentrations. No issues with female immaturity or outlying data were encountered in this study. The data are presented in Table 6.

Table 6. Circulating VTG analyses for male and female fathead minnows exposed to *n*-octanol.

Group	Nominal *n*-octanol (mg/L)	Circulating VTG (ng/ml) Male Mean Individual	Male Mean Replicate	Female Mean Individual	Female Mean Replicate
Control	0	169.1	165.	1,899 $+10^3$	1,801 $+10^3$
Low	0.32	164.4	164.4	901 $+10^3$	1,008 $+10^3$
Intermediate	1	26.4	23.1	1,474 $+10^3$	1,389 $+10^3$
High	3.2	887.0	1305.9	845 $+10^3$	785 $+10^3$

c. 2-Methoxyethanol

2-Methoxyethanol has not been tested for ER binding, but with a shorter overall length and presence of the ether oxygen vis a vis the negative *n*-octanol, binding is highly implausible.

The mean circulating levels of VTG in the male low and intermediate test concentrations did not differ in absolute values or differ significantly with the control. No males survived in the high concentration. In contrast, the absolute values of females appeared to increase in a concentration-responsive manner. The analysis of the increase at the intermediate concentration deserves particular attention. One individual was immature. If that individual was removed, this group achieved statistical significance (p=0.043). With only 4 surviving females at the high concentration, there were insufficient numbers to achieve significance despite the higher absolute values should that individual have been included. The data are presented in Table 7.

Table 7. Circulating VTG analyses for male and female fathead minnows exposed to 2-methoxyethanol.

Group	Nominal 2-Methoxyethanol (mg/L)	Circulating VTG (ng/ml) Male Mean Individual	Male Mean Replicate	Female Mean Individual	Female Mean Replicate
Control	0	225.3	191.5	1,287 $+10^3$	1,301 $+10^3$
Low	1	214.3	214.3	2,286 $+10^3$	2,172 $+10^3$
Intermediate	10	273.3	273.3	3,690 $+10^3$	4,417 $+10^3$
Removing immature	10	NA	NA	4,055 $+10^3$*	4,730 $+10^3$
High	100	- -	- -	5,058 $+10^3$	5,058 $+10^3$

* Statistically significant change, NA – not applicable.

Discussion and Conclusions – Vitellogenin Analyses

The VTG analyses should be interpreted in context with other parameters in this study and with other Phase-2 studies. The two primary protocol and interpretative questions revealed are:

- whether immature individuals of either sex should be included in the VTG analyses as immature individuals appear to have much lower VTG levels in females and thereby reducing the mean and increasing the standard deviations so to impair the statistical sensitivity; and
- how to take into account the appearance of high mortality and symptoms of intoxication in a group which, in concert with declines in VTG levels, strongly implies potential impairment of the individuals' ability to produce VTG at such a concentration and not an endocrine mediated disturbance.

The lack of change in VTG levels in both sexes in the *n*-octanol studies are clearly consistent with the lack of change in observed in other Phase-2 studies. There was no significant mortality or symptoms of intoxication. Further, no females in this study were observed to be immature when the gonads were examined at necropsy.

The absolute VTG decreases in both sexes in the potassium permanganate studies may be similar to absolute decreases in fathead minnow males in laboratory 4 in Phase-2. These decreases were not observed at the low or

intermediate concentrations. The decreases occurred at the high concentration in parallel with high levels of mortality and severe symptoms of intoxication in the surviving individuals. There no evidence for normal spawning activity at the intermediate or high concentrations. There was some evidence for concentration related gonadal histopathological lesions. This generally raises questions about hepatotoxicity that could impair baseline VTG production in males and normal production in females as well as whether levels of estrogen may have dropped in females thereby reducing VTG production in the liver. Therefore, the VTG decreases would typically not be judged as clear evidence for an endocrine action, such as anti-estrogenicity or aromatase inhibition, by potassium permanganate, and the statistically significant VTG decrease would be discounted. However, the basis for this argument is placed in some question by the following 2-methoxyethanol data.

The absolute and statistically significant increase in VTG in 2-methoxyethanol females must likewise be critically examined. Foremost, there was no evidence of a VTG increase in males, which the Phase-1B data clearly indicate are the more responsive sex to both a strong and a weak estrogenic substance. However, this VTG increase is occurring at concentration levels with this substance where mortality and symptoms of intoxication were even more severe than with potassium permanganate above. These absolute VTG increases in these 2-methoxyethanol data bring into question the interpretation that severe toxicity might lead to sufficient liver or sex steroid impairment to explain the above VTG decrease with potassium permanganate.

In both cases, there is no evidence or plausibility for interaction with the estrogen receptor. Therefore, the weight of evidence is that the VTG responses here are not clear evidence of endocrine activity and that in the future VTG response should not be so interpreted without a weight of evidence approach. That is, the current responses are judged to be false positives.

V. Secondary Sexual Characteristics

The primary observation for secondary sexual characteristics (SCC) in the fathead minnow is based on the presence and size of tubercles in the normal sexually active male and their diminution in inactive males and their absence in the normal female. The tubercles are counted per individual, and the size of the tubercles on the individual are scored to generate a semi-quantitative value to represent the reproductive status of that male. The rating scores for the tubercle size are: 1=present, 2=enlarged, 3=pronounced. Representative photographs have been introduced in the OECD protocol. The individual values are converted into an overall mean tubercle number and score per replicate and mean tubercle number and score per concentration.

A secondary observation is the presence in the fathead minnow of vertical colouration stripes in the sexually active male and their absence or diminution in the non-spawning male and their absence in the normal female.

a. Potassium Permanganate

Potassium permanganate exposure resulted in decreases in the absolute values of both the tubercle number and score at the high concentration relative to the others. The decrease in tubercle number at the high concentration did not achieve statistical significance ($p=0.086$), but the decrease in the tubercle score at the high concentration did achieve statistical significance ($p=0.043$). Without further interpretation or integration with other data, this finding would signal a positive response of the test substance, potassium permanganate, as a potential endocrine active compound. The data are presented in Table 8.
The daily observations of vertical bands were also decreased in male fish of the high concentration. In addition, the typical territorial behaviour of fathead minnow males was not observed at the high concentration after the first week through the end of the test (mortality Table 1A).

b. *n*-Octanol

There was no absolute or statistically significant change in tubercle number between control and any of the test concentrations. In fact, the control mean of 16.9 tubercles was less than any of the test concentrations. This appears to have been due entirely to observations in replicate 4 in the control were the value was 6.7. The mean

tubercle scores also showed no absolute or statistically significant change in the number of tubercles between control and any of the test concentrations. Again, replicate 4 in the controls had an outlying low value of 14.3. The data are presented in Table 9. The daily observations of vertical bands were not changed during the test, and no change in the typical territorial behaviour of fathead minnow males was observed (mortality Table 1B).

c. 2-Methoxyethanol

There was no absolute or statistically significant change in the mean number of tubercles or mean score of tubercles at the low concentration. There was no absolute change in the mean number of tubercles or mean score of tubercles at the intermediate concentration. Because of the high level of mortalities, there were insufficient numbers of individuals for confidence in the statistics, if this had been needed. There were no surviving males at the high concentration. The data are presented in Table 10.

The daily observations of vertical bands indicated that the frequency of their occurrence began to diminish about day 16, and there were a concurrent instances of mortality and the appearance of feeding abstinence. However, no change in the typical territorial behaviour of fathead minnow males was noted (mortality Table 1C).

Table 8. Tubercle numbers and scores for Fathead minnow males during the 21-day screening assay exposure to potassium permanganate.

		Nominal $KMnO_4$ concentration (mg/L)			
	Replicate	Control	0.1125	0.225	0.45
Mean number of tubercles per replicate	1	20.5	18	17	16
	2	18.5	21	8.5	15
	3	18	17.75	18.5	0
	4	13.25	13.75	21.5	0
Mean number of tubercles per concentration		17.6 ± 3.1	17.6 ± 3.0	16.4 ± 5.6	7.8 ± 9.0
Mean tubercle score per replicate	1	37	28	26	21
	2	36	33.75	11	15
	3	35.5	27.75	30.25	0
	4	21	20.25	39	0
Mean tubercle score per concentration		32.4 ± 7.6	27.4 ± 5.5	26.6 ± 11.7	9.0 ± 10.7*

* Statistically significant decrease

Table 9. Tubercle numbers and scores for Fathead minnow males during the 21-day screening assay exposure to *n*-octanol.

		Nominal *n*-octanol concentration (mg/L)			
	Replicate	Control	0.32	1	3.2
Mean number of tubercles per replicate	1	20	18	21	25
	2	19	19	16	21
	3	22	21.5	24	21
	4	6.7	23	21.5	26
Mean number of tubercles per concentration		16.9 ± 6.9	20.4 ± 2.3	20.6 ± 3.4	23.3 ± 2.6
Mean tubercle score per replicate	1	37	36	46	52
	2	34	34.5	27	38
	3	48	41	43.5	36.5
	4	14.3	40.5	38.5	28
Mean tubercle score per concentration		33.3 ± 14	38 ± 3.2	38.8 ± 8.4	38.6 ± 9.9

Table 10. Tubercle numbers and scores for Fathead minnow males during the 21-day screening assay exposure to 2-methoxyethanol.

		Nominal 2-methoxyethanol concentration (mg/L)			
	Replicate	Control	1	10	100
Mean number of tubercles per replicate	1	23	20.5	21	- - [a]
	2	23.5	22.5	17.5	- -
	3	17	22	20	- -
	4	21	18.5	- -	- -
Mean number of tubercles per concentration		16.9 ± 6.9	20.4 ± 2.3	20.6 ± 3.4	- -
Mean tubercle score per replicate	1	40	37.5	34	- -
	2	39.9	34	35.5	- -
	3	37	38.5	35.5	- -
	4	43	36	- -	- -
Mean tubercle score per concentration		33.3 ± 14	38 ± 3.2	38.8 ± 8.4	- -

[a] No surviving males in the replicate or concentration.

Secondary Sexual Characteristics – Discussion and Conclusions

The findings in the potassium permanganate and *n*-octanol groups are similar to those in the previous Phase-2 fathead minnow study. There was an absolute decline in tubercle number and a statistically significant decline in the mean tubercle score at the high potassium permanganate concentration synonymous with systemic toxicity and symptoms of intoxication. There were no changes in the absolute values of the tubercle number or tubercle score at any –octanol concentration and mortality and signs of intoxication were limited. However, in the 2-methoxyethanol, despite some male mortality at the intermediate concentration, and as noted below there were significant gonadal histopathological lesions in these males, there was no absolute change in the tubercle number or score at this concentration.

These study results would support some caution in interpreting a decline in the tubercle number and score in the presence of severe mortality and symptoms of intoxication. At first, the androgen sensitivity of this endpoint might be questioned in that a clear non-endocrine testicular toxicant did not impair these endpoints even in the presence of modest mortality and intoxication. However, the action of 2-methoxyethanol is primarily on sperm production in mammals and impacts on the androgen production by Leydig cells would not be expected although considerable fibrosis was observed as noted in the following section.

VI. Gonadal Histopathology

The purpose of histopathological analyses of the gonads of fathead minnow *(Pimephales promelas)* was to identify any concentration dependent-effects in the gonads of fathead minnow and to separate by interpretation, if possible, endocrine-related from other toxic-related effects. This demand for specificity introduces considerable difficulty and uncertainty in the interpretation of the observation. To date, no information about the test substances, potassium permanganate, *n*-octanol, or 2-methoxyethanol suggests any endocrine action. However, 2-methoxyethanol is a known testicular toxicant in mammals by non-endocrine mechanisms and results in clear histopathological lesions in the testes. Thus, these negative compounds present the opportunity to test the reliability and specificity of the gonadal observations and to extend the study of the frequency of background observations in the controls. This is because the background occurrence and frequency of such observations in controls was rather variable in Phase 1B. Identifications and scoring used for the histopathology are in Annex I.

a. Potassium Permanganate

Many of histopathological observations after exposure to *n*-octanol were modest and could be considered to result from variability within a fish population. This implies the need to account for modest changes and variability as possibly normal findings in the interpretation of the data in the 21-day fish screening assay.

The changes attributed to potassium permanganate exposure were three: (1) general increase of fibrotic tissue in the gonads of both sexes; (2) increased incidence of testicular degeneration and intraluminal histolytic cells in the testes; (3) decreased maturity in females. These observations were concentration-dependent and more obvious in males. The observations were attributed to general toxic stress and not to any endocrine-related mechanism.

The potassium permanganate observations are summarized in Table 11A for females and Table 11B for males.

Table 11A: Histopathological findings in female fathead minnows exposed to potassium permanganate. Results are expressed n % of fish examined in each group. Numbers in brackets indicate the absolute number of fish.

			Control				0.1125 mg/L PP			0.225 mg/L PP			0.450 mg/L PP			
	Code	Grade	1	2	3	4	1	2	3	1	2	3	1	2	3	4
	F01	Perinucleolar oocytes increased	8													
	F02	Cortical alveolar oocytes decreased	8													
FEMALE	F05	Oocyte atresis, immature á	8	8						22	11		25	50		
	F06	Oocyte atresis, mature á	38				30	20		11	11					
	F08	Asychr. develop. left & right gonad						10								
	F09	Proteinaceous fluid, intravascular	38	8			50			44						
	F10	Proteinaceous fluid, interstitial		8			10	40		22	11					
	F11	Intersitial fibrosis	15		8	8	50		10	22		11	25		25	25
	F12	Postovulatory follicles á	23	15			40	10			33					
	F17	Oocyte membrane folding	8					10		11	33					
	F18	Egg debris oviduct	8	8			20		20		11					
	F19	Empty cysts	23				20			33						
	Stage of maturation	Stage 0	15										50			
		Stage 1	8										25			
		Stage 2									11		25			
		Stage 3	23				60			22						
		Stage 4	54				40			67						
	MT	missing tissue (% /n of exp. group)	8/1										25/1			
	% Females/exp. group (n total/n investigated)		54 (14/13)				43 (10/10)			40 (8/8)			44 (5/4)			

In light of the past interest in testis-ova and whether this is a diagnostic indication of endocrine disruption, it should be noted that one incidence in the entire set of studies was recorded with a single male at the intermediate potassium permanganate concentration.

Details of the gonadal histopathological findings in the fish exposed to potassium permanganate are in Annex II.

b. *n*-Octanol Results

Many of histopathological observations after exposure to *n*-octanol were modest and could be considered to result from variability within a fish population. This implies the need to account for modest changes and variability as possibly normal findings in the interpretation of the data in the 21-day fish screening assay.

The observations attributed to *n*-octanol exposure were three. (1) concentration-dependent hyperplasia of the ovarian wall in females; (2) several observations related to a reduced egg quality in females such as increased occurrence of egg debris in the gonoduct and atresia of mature and immature oocytes; (3) decreased maturity in males of the highest concentration paired with an increased number spermatids. These observations were concentration-dependent and more obvious in female. The observations were attributed to general toxic stress and not to any endocrine-related mechanism.

Table 11B: Histopathological findings in male fathead minnows exposed to potassium permanganate. Results are expressed in % of fish examined in each group. Numbers in brackets indicate the absolute number of fish.

			Control			0.1125 mg/L PP			0.225 mg/L PP			0.450 mg/L PP		
	Code	Grade	1	2	3	1	2	3	1	2	3	1	2	3
MALE	M01	Spermatogonia increased		10							10			
	M02	Spermatocytes decreased		10						10				
		Spermatids decreased	10								10			
	M03	Testis-ova								10				
	M05	Testicular degeneration								10		33	33	
	M07	Asynchronous development, gonad		10		8								
	M08	Asynchr. Develop. left & right gonad	10				8							
	M09	Proteinaceous fluid, intravascular							10					
	M11	Intersitial fibrosis	30			38	15		30	50	10			100
	M16	Histolytic cells, intraluminal				15			10	10		67		33
	M18	Fibrotic cysts					23	15	30	10	10	33		33
	MT	missing tissue (% /n of exp. group)										33/1		
	Stage of maturation	Stage 0												
		Stage 1		10			15			20				
		Stage 2		70			69			20				
		Stage 3		20			15			50			100	
		Stage 4								10				
	MT	missing tissue (% /n of exp. group)											33/1	
	% Males/exp. group (n total/n investigated)			46 (10)			57 (13)			60 (10)			56 (4/3)	

The *n*-octanol observations are summarized in Table 12A for females and Table 12B for males.

Table 12A: Histopathological findings in female fathead minnows exposed to *n*-octanol. Results are expressed in % of fish examined in each group. Numbers in brackets indicate the absolute number of fish.

			Control				0.32 mg/L Octanol			1 mg/L Octanol				3.2 mg/L Octanol			
	Code	Grade	1	2	3	4	1	2	3	1	2	3	4	1	2	3	4
FEMALE	F01	Cortical alveolar oocytes ↑	7				17										
		Early vitellogenic oocytes ↑	14				17										
		Late vitellogenic oocytes ↑	7							8							
		Mature/spawning oocytes ↑		7													
	F02	Early vitellogenic oocytes ↓	7				8										
	F05	Oocyte atresis, immature ↑	7				8		8	17				18			
	F06	Oocyte atresis, mature ↑	29	29			25	17		8		8	8	18	36	18	
	F07	Asynchronous development, gonad	7														
	F08	Asychr. develop. left & right gonad						17									
	F09	Proteinaceous fluid, intravascular	14				17	17			17	8		18			
	F10	Proteinaceous fluid, interstitial	36	21			17	8		25	25				18		
	F11	Intersitial fibrosis	7	21	7	7	25	25		17	17				9	27	18
	F12	Postovulatory follicles ↑		36						8				45	27		
	F17	Oocyte membrane folding	29				17							9			
	F18	Egg debris oviduct	29	7	7		33	17		25	8				27	9	9
	F19	Hyperplasia of ovarian wall tissue					8	8		8	8			36	9	9	
	F20	altered structure of mature oocytes												9		9	18
	F21	Fibosis in kidney									8			9	9	9	
	Stage of maturation	Stage 1						1									
		Stage 2		21				25			33				27		
		Stage 3		64				54			50				64		
		Stage 4		14				13			17				9		
	% Females/exp. group (n total)			70 (14)				57 (12)			60 (12)				73 (11)		

Table 12B: Histopathological findings in male fathead minnows exposed to *n*-octanol. Results are expressed in % of fish examined in each group. Numbers in brackets indicate the absolute number of fish.

			Control			0.32 mg/L Octanol				1 mg/L Octanol			3.2 mg/L Octanol		
	Code	Grade	1	2	3	1	2	3	4	1	2	3	1	2	3
MALE	M01	Spermatids ↑					13			14			17	51	
	M02	Spermatogonia ↓					13								
	M11	Intersitial fibrosis			17	25	25		13	14			17		
	M12	Sertoli cell hypertrophy				25				14					
	M18	Fibrotic cysts		17		13	13	13		28		14			
	M19	infertile cell clusters intraluminal					13								
	Stage of maturation	Stage 0													
		Stage 1													
		Stage 2		17			50				29			67	
		Stage 3		83			50				71			33	
		Stage 4													
	% Males/exp. group (n total)			30 (6)			40 (8)				37 (7)			83 (6)	

Details of the gonadal histopathological findings in the fish exposed to *n*-octanol are provided in Annex II.

c. 2-Methoxyethanol Results

Exposure to 2-Methoxyethanol caused specific effects that can be interpreted as a consequence of endocrine action. The overall changes in fish exposed to 2-Methoxyethanol are: (1) Concentration-dependent increase of fibrosis in both sexes and other degenerative parameters in males like histolytic cells in the lumen of the seminiferous tubules, (2) Phenomena related to a reduced egg quality like increased abundance of egg debris in the gonoduct and atresia of mature and immature oocytes and (3) Concentration-dependent decrease of maturity in males paired with a decreased number of pachytene spermatocytes, oligospermia and proliferation of Sertoli cells after exposure to 2-Methoxyethanol. Changes after exposure to 2-Methoxyethanol indicate a concentration-dependent action for most parameters, which is more obvious in male fish. Although effects like increased fibrosis, oligospermia, testicular degeneration, reduction of pachytene spermatocytes in males and increased oocyte atresia as well as the decrease of early developmental oocytes can possibly impair reproduction, most effects can be interpreted as adaptive. Additionally, a toxic component can be considered as well, since in the highest concentration no males and only two females survived.

However, it should be noted, that control fish of the present experiment displayed a very poor health status, which might well have taken influence on the appearance of 2-Methoxyethanol-related changes of gonadal histology.

The 2-methoxyethanol observations are summarized in Table 13A for females and Table 13B for males.

Details of the gonadal histopathological findings in the fish exposed to 2-methoxyethanol are in Annex II.

Gonadal Histopathology - Discussion and Conclusions

The use of gonadal histopathology in the protocol is confronted by several issues: 1) the background presence and variation in fish populations of histopathological findings that must be distinguished from reproductive toxicants and general, 2) the potentially subtle nature of endocrine-mediated changes in contrast to the more severe lesions such as with 2-methoxyethanol, and 3) the need to distinguish these from lesions from non-endocrine modes of action from the compounds and even systemic toxicity.

Table 13A: Histopathological findings in female fathead minnows exposed to 2-methoxyethanol. Results are expressed in % of fish examined in each group. Numbers in brackets indicate the absolute number of fish.

			Control				1.0 mg/L Methoxyethanol				10 mg/L Methoxyethanol				100 mg/L Methoxyethanol			
	Code	Grade	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
FEMALE	F01	Perinucleolar follicle ↑													50			
	F02	Perinucleolar follicle ↓													50			
		Cortical alveolar oocytes ↓															100	
		Early vitellogenic oocytes ↓															100	
	F05	Oocyte atresis, immature ↑			7		7	7	7	7	9	9					50	
	F06	Oocyte atresis, mature ↑		20	20	13	13	13	13	7	18	18	18	18			50	50
	F07	Asynchronous development, gonad					7											
	F08	Asychr. develop. left & right gonad		7					7			9						
	F09	Proteinaceous fluid, intravascular	27	7			20	7	7			9			50	50		
	F10	Proteinaceous fluid, interstitial	13	33	7		7	7			18	9					100	
	F11	Intersitial fibrosis	27	27	13	7	13	33	20	13	27	36	18	18	50	50		
	F12	Postovulatory follicles á	20	13								9						
	F17	Oocyte membrane folding	7				40	7			9							
	F18	Egg debris in oviduct	13	13	7		13	20	7			9	9				50	
	F19	Hyperplasia of ovarian wall tissue	13	7														
	F20	Fibrotic cysts	7				7	13			9							
	F21	Fibrosis around oocytes	13		13						9		18					
	F22	Atrophy of follicle cells															100	
	Stage of maturation	Stage 0									18							
		Stage 1							3				9					
		Stage 2			13								5					
		Stage 3			37				77				36					
		Stage 4			50				20				32				100	
	% Females/exp. group (n total)				71 (15)				66(15)				65(11)				100(2)	

Table 13B: Histopathological findings in male fathead minnows exposed to 2-methoxyethanol. Results are expressed in % of fish examined in each group. Numbers in brackets indicate the absolute number of fish.

			Control			1 mg/L Methoxyethanol				10 mg/L Methoxyerthanol			
	Code	Grade	1	2	3	1	2	3	4	1	2	3	4
MALE	M01	Spermatogonia ↑	33			13		13					17
		Leptotene Spermatocytes ↑					13						
		Zygotene Spermatocytes ↑				13	38					17	
		Intersitial (Leydig-) cells ↑	17			25				17		17	
	M02	Spermatogonia ↓									17		
		Pachytene Spermatocytes ↓					13				33	17	
		Spermatozoa ↓					13				33	17	
	M05	Testicular degeneration					13			17	17	17	
	M06	Asynchr. Development Spermatocyst				38				33			
	M08	Asynchr. Development left & right gonad	17										
	M11	Intersitial fibrosis	50			38	25	13	13	17	33	17	17
	M12	Sertoli cell hypertrophy				25	13			33	33	17	
	M16	Histolytical cells			17	25	38	25		17	17	50	
	M18	Fibrotic cysts			17	13	38	13			17	33	
	M19	Loosely packed spermatocysts					25				33		
	Stages of maturaiton	Stage 1											
		Stage 2		75			50				33		
		Stage 3		25			50				67		
		Stage 4											
	% Males/exp. group (n total)			29(6)			35(8)				35(6)		

The first two issues are interrelated. Most of the histopathologic findings that have been associated with EDCs in fish are considered to be changes or amplifications of "normal", physiologic findings rather than frank lesions (e.g., changes in overall oocyte atresia, increased postovulatory follicles, etc. (Tyler and Sumpter, 1996)). This presents some difficulty in working with baseline variations within and, particularly, among studies. That is, based upon these studies as well as those in other laboratories in Phase-1B and Phase-2, findings of subtle differences in controls and between test concentrations within a study are expected to be common. Any test substance-related effects must then be distinguished from normal variation. Second, findings that may occur in a test concentrations in one study may appear in the control group of another study, leading to questions if the findings in the first study are actually biologically relevant and should not be interpreted as a positive finding.

Collectively, the three studies show a host of observations. Many histopathological observations are of low frequency and lack concentration-related patterns. The degree of variation between groups ranges from minimal through what might be taken as a significant and reportable finding if it were to occur only in the high concentration. This suggests that a general record of observations within and among laboratories is needed to allow histopathologists with judgements of what constitutes a background finding and normal variation rather than a substance-related finding.

In light of the past interest in testis-ova and whether this is a diagnostic indication of endocrine disruption, it should be noted that one incidence of testis-ova was observed in the entire set of studies with a single male at the intermediate potassium permanganate concentration. This supports that low rates of random occurrences of testis-ova do occur, and that isolated observations of testis-ova are not a definitive diagnosis for endocrine activity.

The appearance of mortality and symptoms of intoxication in these and other studies in Phase-1B and Phase-2 was often in parallel with gonadal histopathological changes in the groups. These included the appearance of atretic follicles in females and some evidence of testicular lesions in males. Given the obvious stress on the individuals in concentrations with mortality and intoxication, the impact on reproduction and the appearance of such lesions is not unsurprising. Therefore, interpretation and conclusions regarding reproductive toxicity and gonadal changes should be undertaken only when it is clear that fish are not under undue or extreme stress. This is analogous to the concept in mammalian toxicology of not exceeding a maximal tolerate (MTD) and not to interpret findings when such concentrations are exceeded. This is another example of the need to develop a maximum tolerated concentration concept (MTC) for ecotoxicity studies.

A clear example of this concern is atretic oocytes. This observation was originally proposed and tested in Phase-1B. Then, these observations were retained by a meeting of histopathological experts after completion of Phase-1B as being a potential indicator of endocrine action. The rationale was based on endocrine control of successful oocyte maturation and the potential for atretic oocytes to appear when endocrine signals in the females may be impaired. However, the health status of the individual also plausibly results in the failure of oocytes to mature and the resulting appearance as atretic observations when the reproductive condition of female is impaired by systemic or other non-endocrine toxicities. In this regard, there are very clear concentration-related elevations of atretic oocytes in concert with mortality in both the potassium permanganate and 2-methoxyethanol studies (Tables 11A and 13A), and a similar modest elevation at the high concentration of *n*-octanol (Table 12A). Therefore, these gonadal histopathological findings are clearly not specific for endocrine action, and wider experience is needed with all gonadal histopathological findings to validate that they are truly diagnostic or not for endocrine mediated modes of action.

In the 2-methoxyethanol study, testicular lesions in the sperm production in the tubules similar to those found in mammalian studies were absent or appeared at a low frequency in the testes at the low test substance concentration (1 mg/L). These lesions then become obvious and severe at the 10-fold higher intermediate concentration (10 mg/L). In this case, the rates of mortality and intoxication were similar to the observations at the high concentration of *n*-octanol, but the testicular lesions were clearly more frequent and severe in the case of 2-methoxyethanol. In addition, there was a concentration-related increase in fibrosis in the testes that reached severe levels and a low frequency of concentration-related appearance of general testicular degeneration (Table 13B). Therefore, the 2-methoxyethanol study shows a consistency with mammalian findings and indicates that gonadal histopathological lesions will appear with non-endocrine reproductive toxicants. This finding is consistent with biological plausibility from mammalian organisms that many histopathological effects in the gonads of both sexes are not endocrine-specific. Further, it is worrisome that despite the testicular lesions and other effects, the protocol design was unable to provide sufficient vigor of spawning activity in the control to provide evidence of reproductive toxicity, although it is noteworthy that fertilization was not measured in this and other studies

These studies demonstrate that 1) gonadal histopathology is impacted by systemic toxicity and severe stress, 2) background rates of findings continue to be observed in controls making interpretation versus test concnetrations difficult and raising questions about the biological relevance of some findings, 3) significant work remain to identify and validate any diagnostic gonadal histopathological finding for endocrine modes of action, 4) there is some evidence that strong non-endocrine gonadal toxicants such as 2-methoxyethanol can be detected using gonadal histopathology, and 5) the work and effort to conduct and clearly interpret the gonadal histopathology is not consistent with its employment in a lower tier screen. However, the sensitivity of the latter finding vis a vis spawning activity and fecundity remains unknown given the inability of the protocol to consistently produced robust spawning and fecundity measurements in the fathead minnow.

Collectively, these findings support the previous decision of the VMG-eco to withdraw gonadal histopathology from the guideline as an endocrine screen, given the variability among the studies and groups as well as the production of effects from the non-endocrine mediated 2-methoxyethanol.

OVERALL DISCUSSION AND CONCLUSIONS

The three studies in this report were conducted as part of OECD program to validate a standardized OECD protocol on adult fish for the detection of endocrine active substances (i.e. estrogen, aromatase inhibitors, androgen). This work is a continuum to OECD validations studies conducted in Phase 1A and then Phase 1B for a 21-day fish endocrine screening. In June of 2005, the OECD Secretariat contacted laboratories for a commitment to investigate negative test substances. CEFIC volunteered to support a laboratory in Phase-2 for three studies to be conducted with the fathead minnow (*Pimephales promelas*).

The current studies follow the body of other studies in Phase-2. The other studies were in 3 test species: fathead minnow, medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*). The other studies completed their in-life by February in 2006 and their report was approved in January, 2007, at the VMG-eco meeting in Madrid. The last of these current studies completed in-life only in October 2006. This significant postponement was due to unforeseen delays in the approval of funding and the selection and contract of the laboratory.

The objective of the other Phase-2 studies was to test the relevance and specificity of three reproductive endpoints (spawning activity, fecundity, and gonadal histopathology) and two endocrine-related endpoints (vitellogenin (VTG) and secondary sexual characteristics (SSC)) with negative endocrine substances. The current Phase-2 studies have the same objective. Previous studies employed two negative endocrine substances selected by an expert group drawn from the VMG-eco: potassium permanganate, an inorganic oxidizer, having no known endocrine activity, and *n*-octanol, a classical organic narcotic, having no known endocrine activity. The current studies used these two substances at the previously selected nominal target concentrations of 225, 450 and 900 µg potassium permanganate/l and 0.32, 1.0, 3.2 mg *n*-octanol/l. CEFIC selected a third test substance, 2-methoxyethanol, a classical gonadal toxicant in mammalian species, known to produce gonadal histopathological lesions by non-endocrine mechanisms. For the additional negative, the nominal target concentrations selected by CEFIC for use in the current studies were 0.32, 1.0, 3.2 mg 2-methoxyethanol/l.

These studies provide support to the compliance of the OECD 21-day fish endocrine screening program with the criteria of Guidance Document 34. The studies support that interlaboratory studies have been done with the negative compounds, as the previous studies were often performed in only a single laboratory with each species. These studies also support the intention of testing representative compounds in a validation study by including additional negatives such as the 2-methoxyethanol.

The results of these studies support the overall conclusion of the OECD validation program that vitellogenin and secondary sexual characteristics can be successfully employed to screen for certain endocrine modes of actions (i.e., estrogens, androgens, and aromatase inhibitors). At concentrations absent overt signs of toxicity, there were no false positives observed with any of the three negative test substances. The results also support the OECD validation program that certain reproductive parameters such as spawning activity and fecundity and gonadal histopathology are not specific for endocrine activity, but may be impaired by other modes of action. These conclusions are drawn based on a number of observations during these studies.

The first significant observation was that mortality, symptoms of intoxication, and other sublethal effects were clearly evident and need to be incorporated into the interpretation of the potassium permanganate and 2-methoxyethanol studies. For comparison, mortalities in the laboratory 4 study with fathead minnow were 63% at a nominal concentration of 0.45 mg/L and 29% at a nominal concentration of 3.2 mg *n*-octanol/L. This compares favourably to 63% and 24% in the current studies, respectively. In the case of potassium permanganate, the symptom of intoxication at the intermediate concentration was strong ventilation. This symptom was uniformly observed among all fish and occurred almost from the inception of the study (day 2). At the high concentration, 15 fish died on test. All fish before death and all surviving fish showed various symptoms of intoxication and distress, i.e. strong ventilation swimming mainly near the water surface, feeding

abstinence, apathy, lying on the side or back on the bottom. In the case of 2-methoxyethanol, there were a variety of symptoms of intoxication at the intermediate concentration that were more sporadic and less frequent. Consistent with symptom observations, mortality at the high concentrations of potassium permanganate and 2-methoxyethanol achieved statistical significance versus the controls. For the *n*-octanol study, there was a low level of mortalities, and symptoms of intoxication were infrequent occurrence of symptoms of intoxication with the few instances the concentration and duration of exposure. It will be noted below that many other findings related to spawning activity, fecundity, tubercle numbers and scores, and gonad histopathology follow the same concentration patterns as the intoxication symptoms and mortality. It would appear then that many of the endpoints are then confounded by general, systemic toxicity and are not endocrine-specific effects.

For the reproductive endpoints, in the permanganate studies, spawning activity and fecundity appear to be associated with mortality and symptoms of intoxication as the findings were synonymous at the intermediate and high concentrations. At the high and intermediate concentrations, only one spawning event, a clutch of just 4 eggs was observed. These decreases were statistically significant decreases versus the control and reproduced the other Phase-2 fathead minnow study where the spawning activity was also reduced at the intermediate and high concentration of permanganate. In the *n*-octanol studies, there was a modest increase in mortality with concentration and no major intoxication symptoms and spawning activity and fecundity was actually better in all treatment concentrations than in the control. As a result, spawning activity versus control approached statistical significance ($p=0.064$) for the intermediate nominal concentration. This also reproduced the general findings in the other Phase-2 fathead minnow study. Additionally, the impact of twice per day tank cleaning due to test substance biodegradation may have impaired the reproductive behavior of the fish. In the 2-methoxyethanol studies, all males died before the end of the study at the high concentration and both males in replicate 4 of the intermediate concentration died before the end of the study. Yet, the results were so modest in the control and the low nominal concentration, that statistical significance was not achieved.

Cumulative egg numbers and eggs per surviving female were still not satisfactory even with the change in protocol design form Phase-1B. In these three studies, there were 8 instances when the cumulative egg number for a replicate was greater than 400 eggs; only one of these instances was in the 12 control replicates. The cumulative mean per replicate for the three controls was 75, 90, and 165. This illustrates the non-ideal conditions and limited power of the protocol that are likely to occur in a large number of laboratories in practice. Therefore, the current protocol is not sufficient to yield consistently robust reproduction in the fathead minnow.

A fundamental statistical question arose concerning the reproductive endpoints as a result of the studies. As a tank contains 2 males and 4 females, what happens to its application as a replicate when one or both males die or when one or more females die? How should the results be compared to other tanks, no longer apparent replicates, that still continue to have 2 males and 4 females and, therefore, inherently, greater chance for spawning activity and fecundity? The statistics for these current results were calculated as though any tank where mortality of one or more individuals occurred continued to be a true replicate; that is, those tanks are included.

In addition, two females in the permanganate study and one female in the 2-methoxyethanol study were found to have immature gonads *after* being 3 weeks on study. There was also some misidentification of males as females in the permanganate study. While limited to one replicate in the control and the intermediate concentrations in the permanganate studies, 3 replicates were impacted at the low permanganate concentration. Thus, it would appear that the fish were not fully mature and reproductively capable in all cases at the beginning of the study. This leads to the recommendations that: 1) the recommended mean age of the fathead minnow may need to be increased beyond 20 weeks (5 months) [Note: in Phase-3, one laboratory has again found the fathead minnows to be sexually immature at 20 weeks of age while still above the weight criteria of the current protocol] and 2) the use of the acclimation period to test the reproductive capacity fish received in pairs may need to be considered, if this endpoint would be pursued, so that no misassignment of sex would occur and only proven and successful breeders would be placed on test.

The absolute VTG decreases in both sexes in the potassium permanganate studies may be similar to absolute decreases in fathead minnow males in laboratory 4 in Phase-2. These decreases were not observed at the low or intermediate concentrations. The decreases occurred at the high concentration in parallel with high levels of mortality and severe symptoms of intoxication in the surviving individuals. The lack of change in VTG levels in both sexes in the *n*-octanol studies are clearly consistent with the lack of change in observed in other Phase-2 studies. There was an absolute and statistically significant increase in VTG in 2-methoxyethanol females, but no evidence of a VTG increase in males, with significant mortality and symptoms of intoxication in both sexes. Thus, there were false positives and a weight of evidence approach needs to be used in VTG interpretation.

The VTG analyses also raise certain protocol improvement and refinement considerations. The two primary protocol and interpretative questions revealed are: 1) whether immature individuals of either sex should be included or excluded from the VTG analyses as immature individuals appear to have much lower VTG levels in females and thus impact the reduce mean and increase the standard deviations thereby impairing the statistical sensitivity; and 2) how to take into account the appearance of high mortality and symptoms of intoxication at a concentration which, in concert with declines in VTG levels, strongly implies potential impairment of the individuals' ability to produce VTG at such a concentration and is not evidence of an endocrine mediated disturbance.

The findings in the potassium permanganate and *n*-octanol concentrations are similar to those in the previous Phase-2 fathead minnow study. There was an absolute decline in tubercle number and a statistically significant decline in the mean tubercle score at the high concentration of the potassium permanganate study synonymous with systemic toxicity and symptoms of intoxication. There were no changes in the absolute values of the tubercle number or tubercle score at any –octanol concentration and mortality and signs of intoxication were limited. However, in the 2-methoxyethanol, despite some male mortality at the intermediate concentration, and as noted below there were significant gonadal histopathological lesions in these males, there was no absolute change in the tubercle number or score at this concentration.

The appearance of mortality and symptoms of intoxication in these and other studies in Phase-1B and Phase-2 was often in parallel with gonadal histopathological changes at these concentrations. These included the appearance of atretic follicles in females and some evidence of testicular lesions in males. Given the obvious stress on the individuals at concentrations with mortality and intoxication, the impact on reproduction and the appearance of such lesions indicating a loss of reproductive condition and capacity is not surprising. Therefore, interpretation and conclusions regarding reproductive toxicity and gonadal changes should be undertaken only when it is clear that fish are not under undue or extreme stress. This is analogous to the concept in mammalian toxicology of not exceeding a maximal tolerate concentration (MTC) and not to interpret findings when such concentrations are exceeded as evidence of the toxicity under investigation (e.g., reproduction) or mode of action of interest (e.g., estrogenicity). In addition, one incidence of testis-ova was observed in the entire set of studies with a single male at the intermediate potassium permanganate concentration. This supports the conclusion that random occurrences of testis-ova do occur, and that rare instances are not a definitive diagnosis for endocrine activity.

A clear example of this concern is the observation of atretic oocytes. This observation was originally proposed and tested in Phase-1B. Then, these observations were retained by a meeting of histopathological experts after completion of Phase-1B as being a potential indicator of endocrine action. The rationale was based on endocrine control of successful oocyte maturation and the potential for atretic oocytes to appear when endocrine signals in the females may be impaired. However, the health status of the individual also plausibly results in the failure of oocytes to mature and the resulting appearance as atretic observations when the reproductive condition of female is impaired by systemic or other non-endocrine toxicities. In this regard, there are very clear concentration-related elevations of atretic oocytes in concert with mortality in both the potassium permanganate and 2-methoxyethanol studies (Tables 11A and 13A), and a similar modest elevation at the high concentration

of *n*-octanol (Table 12A). Therefore, these gonadal histopathological findings are clearly not specific for endocrine action, and wider experience is needed with all gonadal histopathological findings to validate that they are truly diagnostic or not for endocrine mediated modes of action.

In the 2-methoxyethanol study, testicular lesions similar to those found in mammalian studies began to appear at a low frequency in the testes at the low test substance concentration. These lesions become more severe at the intermediate concentration. In this case, mortality and intoxication were similar to the observations at the high concentration of *n*-octanol, but the testicular lesions were clearly more frequent and severe in the case of 2-methoxyethanol. In addition, there was a concentration-related increase in fibrosis in the testes that reached severe levels and a low frequency of concentration-related appearance of general testicular degeneration. Therefore, the 2-methoxyethanol study indicates that gonadal histopathological lesions will appear with non-endocrine reproductive toxicants and are not endocrine-specific as biological plausibility would suggest. In addition, despite the testicular lesions and other effects, the protocol design was unable to provide sufficient vigor of spawning activity and fecundity in the control to provide evidence of reproductive toxicity.

In closing, these studies met the test acceptance criteria for the fish to be placed on test, met the test criteria for O_2 saturation and temperature, and the measured values of the test substances are satisfactory to accept all three studies. However, the controls in two tests did not meet the criteria for control mortality of <10%. The *n*-octanol controls encountered 12.5% mortality (3 of 24), and the 2-methoxyethanol controls had 16.7% mortality (4 of 24). The analytical recoveries with potassium permanganate and *n*-octanol are similar to other Phase-2 laboratories, except that the severe losses of *n*-octanol in another laboratory were not observed. Therefore, the studies are themselves valid for use to assess the relevance and the reliability of the endpoints in Phase-2 of the validation program.

The following conclusions and recommendations are offered by CEFIC. These recommendations have not yet been discussed by an OECD group and should therefore be taken as proposals, and CEFIC understands that they may or may not form the basis of further work/discussion on the future Test Guideline:

- At non-toxic concentrations, the VTG and tubercle endpoints were consistently negative as expected. The studies support the capability of these endpoints to correctly identify endocrine modes of action when other toxicities are not present.

- Given the evidence for impact of systemic toxicity and sublethal effects on several endpoints, several changes in the protocol and the interpretation of its results are recommended to resolve the issue of false positives in such circumstances:
    - Avoid concentrations with high mortality and symptoms of intoxication by refining concentration selection guidance beyond acute toxicity (LC50). The concentration selection should be informed with data from juvenile toxicity studies (as was done with *n*-octanol) or early life stage studies, or range finding studies and the consequent additional animal use are necessary.
    - Ensure that reproductive (spawning activity and fecundity endpoints) and endocrine-related (VTG and SCC endpoints) observations that are confounded or caused by general, systemic toxicity as evidenced by mortality and symptoms of intoxication are discounted and not interpreted as positive findings of endocrine modes of action. That is, define a MTC for the fish screen, where only findings below such an MTC would be accepted as positive for true reproductive and/or endocrine-related mode of action findings.
    - Thus, in a straightforward solution, incorporate in the interpretative section of a future Test Guideline that positive findings for possible endocrine related effects should occur in the absence of confounders for systemic toxicity and symptoms of intoxication and that particularly vulnerable endpoints such as reproduction would be recognized.

- The spawning activity and fecundity capability of the protocol, even with the increase in replicates and reduction to two males per tank is not sufficient to yield a consistently reliable and robust measurement of reproduction in all cases. This finding is also consistent with the results from the other fathead minnow laboratories in this and previous Phases of the validation program. Clearly, some additional work is necessary on the protocol conditions to achieve robust reproductive capacity, and the current protocol does not appear actionable for routine, large scale screening. The logistical needs of a reproductive protocol appear to be more rigorous and difficult. Therefore, the previous decision of the VMG-eco to remove the spawning activity and fecundity measurements from the validation program appear to be justified on these grounds as well as the need to separate adverse effects on reproduction from endocrine mode of action screening. Further consultation with experts and statisticians is recommended for a future fish reproductive assay.

- Consultation with statisticians is also needed to understand what constitutes a statistical replicate and the use of tanks where mortality has occurred and, therefore, reproductive capacity is no longer fully equivalent among the tanks. Are there alternative methods to utilize these tanks or whether such tanks should be removed from the study altogether?

- As there is no evidence or plausibility for interaction with the estrogen receptor for either potassium permanganate or 2-methoxyethanol, the weight of evidence is that the VTG responses in these cases are not clear evidence of endocrine activity. That is, the current responses are judged to be false positives. In the future Test Guideline, VTG responses should not be interpreted automatically and in isolation without a weight of evidence approach.

- These study results with potassium permanganate would support some caution in interpreting a decline in the tubercle number and score in the presence of severe mortality and symptoms of intoxication. On the other hand, these study results with *n*-octanol and, particularly, 2-methoxyethanol would support the androgen specificity of this endpoint in that a clear non-endocrine testicular toxicant such as 2-methoxyethanol did not impair these endpoints even in the presence of modest mortality and intoxication.

- These studies demonstrate that:
  1) gonadal histopathology is impacted by systemic toxicity, sublethal effects, and severe stress,
  2) variable background rates of findings continue to be observed in controls making interpretation versus test concentrations difficult and raising questions about the biological relevance of some findings (that is, a high rate in controls in one study will be interpreted as healthy, normal fish while similar rates in a test concentration might be interpreted as endocrine or other effects in another study),
  3) significant work remain to identify and validate any diagnostic gonadal histopathological finding that would be specific for endocrine modes of action,
  4) there is some evidence that strong non-endocrine gonadal toxicants such as 2-methoxyethanol can be detected using gonadal histopathology, and
  5) the work and effort to conduct and clearly interpret the gonadal histopathology is not consistent with its employment in a lower tier screen where simplicity and clarity are hallmark criteria.

  However, the sensitivity of the latter finding vis a vis spawning activity and fecundity remains unknown given the inability of the protocol to consistently produced robust spawning and fecundity measurements in the fathead minnow. Collectively, these findings support the previous decision of the VMG-eco to withdraw gonadal histopathology from the protocol for endocrine screening, and demonstrate the further work in laboratory studies of positive and negative substances is necessary for their validation.

In addition, the laboratory conducting these studies has submitted comments on the protocol and studies (Annex III).

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ANNEX I
DESCRITPION OF GONADAL HISTOPATHOLOGY METHODS

Detailed Materials and Methods for Histopathology Procedures

1. Tissue Processing

After anesthetization with 0.3 % MS222, blood was collected immediately as recommended by the OECD vitellogenin protocol for validation Phase 1B (see also: method for measurement of vitellogenin). After blood collection, carcasses were opened and the terminal intestine was severed and retracted prior to removal of the gonads. Gonads were prefixed *in situ* for at least 60 seconds with Davidson´s fixative, then excised and stored in Davidson´s fixative at 4° C until further use. After dehydration in a graded series of ethanol (3 x 70 % ethanol, 1 x 80%, 1 x 90 % and 1 x 96 % for 20 minutes each) and then isopropanol (2 x 100 % isopropanol for 30 minutes each) and clearing in methylbenzoate (2 x overnight, 1 x 3 hours), specimens were incubated in paraffin two times overnight and afterwards embedded in paraffin.

2. Embedding, Microtomy and Staining

3-5 µm thick longitudinal serial sections of the gonads were prepared, including one or more microscope slides (at least five sections per slide) from the periphery and the centre of the gonad, respectively. Sections were stained with H&E staining (Romeis, 1989); cover-slipped and labeled according to the histology and histopathology guidelines for validation Phase 1B.

3. Histopathological Analysis

In a first trial, sections were used for sex determination and for rough screening for obvious morphological alterations like testis-ova or hermaphroditism. In a second trial, slides were screened for maturation stages and low-magnification effects like asymmetry of gonadal development, oocyte atresia, fibrosis or proteinaceous fluid. In the last trial, slides were analyzed at high magnification for possible membrane folding, asynchronous development of cells and any other alterations.

4. Staging of fish gonadal maturation (Grading)

To simplify the process of histopathological analysis and to create a system more transparent than describing every variation in size of the different cell populations, fish were categorized into stages of maturation. Therefore, only changes that do not fit into the staging system were described separately by means of the abbreviation code supplied by the OECD protocol (see below).

Stage 0
Stage 0 is the entirely immature phase. The ovary consists of oogonia to perinucleolar oocytes. In the testis only spermatogonia, spermatocytes and spermatids can be detected. Spermatozoa are completely missing.

Stage 1
Stage 1 describes the early vitellogenic/spermatogenic phase. In the ovary, the vast majority of germ cells are pre-vitellogenic follicles, predominantly perinucleolar. In the testis, immature phases predominate, but spermatozoa may also be observed.

Stage 2
Stage 2 is the time when germ cells are in the mid vitellogenic/spermatogenic phase. In the ovary, at least half of the follicles observed are early to mid-vitellogenic. In the testis, spermatocytes, spermatids and spermatozoa are present in roughly equal portions.

Stage 3
Stage 3 is the late vitellogenic/spermatogenic phase, where the majority of the ovary consists of late vitellogenic follicles. All stages may be observed in the testes, however; mature sperm predominate.

Stage 4
At stage 4, the majority of follicles in the ovary are late vitellogenic and mature/spawning follicles. Follicles are larger than in stage 3. In the testis, only loose connective tissue with some remnant sperms can be observed.

Additionally, the severity of alterations was recorded within a grading system from 1 – 4 according the OECD histology and histopathology protocol for phase 1B validation:

Grade 1 (minimal)
Discrete change example: 0 to 2 occurrences per microscopic field, or 1 to 2 occurrences per tissue section.
Spatial change example: the change occupies a miniscule area of either a specific tissue type or the entire tissue section.
Global change example: the least perceptible alteration relative to control animals or prior experience.

Grade 2 (mild)
Discrete change example: 3 to 5 occurrences per microscopic field or tissue section.
Spatial change example: the change occupies a larger area than Grade 1, but still less than or equal to 25% of either a specific tissue type or the tissue section.
Global change example: the alteration is easily appreciated, but still not dramatic.

Grade 3 (moderate)
Discrete change example: 6 to 8 occurrences per microscopic field or tissue section.
Spatial change example: the change occupies more than 25% but less than or equal to 50% of either a specific tissue type or the entire tissue section.
Global change example: the alteration is dramatic, but a more pronounced alteration can be envisioned.

Grade 4 (severe)
Discrete change example: 9 or more occurrences per microscopic field or tissue section.
Spatial change example: the change occupies more than 50% of either a specific tissue type or the entire tissue section.
Global change example: essentially, the most pronounced alteration.

ANNEX II
GONADAL HISTOPATHOLOGICAL OBSERVATIONS WITH THREE TEST SUBSTANCES

The detailed results of the histopathological findings for the fish gonads are presented individual sections for each test substance (potassium permanganates, *n*-octanol, and 2-methoxyethanol) with subsections for each sex arranged by increasing concentration.

a. Potassium Permanganate

Females– Detailed Results for Potassium Permanganate

Controls

Since no ovarian tissue could be obtained from one female, it was excluded from the calculations of percentage for this group. The female control had a relatively high rate of interstitial fibrosis. Fibrosis was minimal in 2 individuals, moderate in 1 individual, and in 1 individual 70 % of the gonadal tissue was fibrotic tissue. The majority of females in the control were fully mature and either at late developmental stage (stage 3, 23 %) or late developmental/hydrated state (stage 4, 54 %). On the other hand, two individuals (15 %) were undeveloped (stage 0, 15%) and one female (8 %) was in early developmental state (stage 1, 8%). Compared to the female fish of the exposure groups in this test, the females of the control showed the highest variability in maturation.

0.1125 mg/L Potassium Permanganate

In the low concentration, no fish was less developed than late vitellogenic or late vitellogenic/hydrated stage. As a result, no observations for atresia of immature follicles could be made. There were observations of slight to moderate increases in atresia of mature oocytes, interstitial proteinaceous fluid, postovulatory follicles and egg debris in the oviduct. One individual showed an asynchronous development between the left and right gonad with one gonad being stage 4 and the right gonad stage 3. In another individual, there was a mild increase of oocytes showing unusual membrane folding. In summary, the most obvious change was a mild to moderate increase of interstitial fibrosis.

0.225 mg/L Potassium Permanganate

In the intermediate concentration, with one exception, all females were in late developmental state. In contrast to the low concentration, the maturation of majority of females (67 %) was stage 4 and, therefore, further developed. In contrast to the lowest concentration, interstitial fibrosis was not elevated. In the intermediate concentration, there was a minimal to mild increase in the occurrence of membrane folding in late vitellogenic mature oocytes.

0.450 mg/L Potassium Permanganate

Since ovarian tissue could not be obtained from one female, it was excluded from the calculations of percentage for this group. The number of immature fish was markedly elevated in the high concentration (stage 0, 50 %). No individuals in stage 3 or 4 were observed. Histopathologic analyses showed in all females a minimal to moderate increase of interstitial fibrosis. Absent fully mature fish, observations for alterations like atresia of mature oocytes, increased postovulatory follicles or egg debris in the oviduct could not be made.

Males– Detailed Results for Potassium Permanganate

Controls

In the control, 70 % were mid spermatogenic (stage 2) and 20 % were further developed and in late spermatogenetic state (stage 3). One individual was immature (stage 1), showing a mild increase of spermatogonia as well as a decrease of spermatocytes and spermatids. Apart from a minimal fibrosis in 30 % of fish, and one individual showing an asynchronous development in one gonad, no further aberrations could be detected.

0.1125 mg/L Potassium Permanganate
In the low concentration, the maturation grade of the males was comparable to the controls. The only marked change was an increase in minimal to mild fibrosis. Fibrosis appeared in two forms: the first was a diffuse interstitial fibrosis mainly located in the periphery and between the spermatocytes, concerning 40 % (minimal) and 16 % (mild) of exposed group, respectively. The other form were concentrations of fibrotic tissue that often had a cyst-like appearance ("fibrotic cysts") in 23 (minimal) and 15 % (mild) of cases. A minimal occurrence of intraluminal histolytic cells was observed in two individuals (8 %).

0.225 mg/L Potassium Permanganate
In the intermediate concentration, number of late spermatogenic fish was slightly versus the control. One individual at maturation stage 4 showed a testis-ova consisting of a large portion of moderately degenerated testicular tissue in which a row of perinucleolar oocytes was embedded. In this individual, the testicular part showed a moderate increase of spermatogonia, while spermatocytes and spermatids were reduced. Compared to the control and low concentration, fibrosis was even more abundant in a concentration responsive manner. Interstitial fibrosis was observed: 30 % (minimal), 50 % (mild) and 10 % (moderate). Fibrotic cysts in severity grades from 1 - 3 (30, 10, 10 %, respectively, for grades) were observed in half of the males. A minimal to mild increase in the number of intraluminal histolytic cells was observed in two individuals.

0.450 mg/L Potassium Permanganate
In the high concentration, males were late spermatogenic. The concentration dependent increase in moderate interstitial fibrosis was confirmed, affecting 100 % of individuals. Fibrotic cysts were observed in 2 of 3 surviving individuals. Likewise, in two individuals, intraluminal histolytic cells were minimally to moderately elevated.

b. n-Octanol

Females – Detailed Results for *n*-Octanol

Controls
The maturity of the controls was somewhat variable. The majority of females in the control were fully mature and in late developmental stage (stage 3, 64%) or late developmental-hydrated state (stage 4, 14%). On the other hand, three individuals were in mid-developmental state (stage 2, 21%). There were frequent observations connected to the reproductive cycle of fish like atresia of mature oocytes, interstitial proteinaceous fluid, postovulatory follicles and egg debris in the oviduct. These observations were minimal to moderate. The primary finding in the female control was a relatively high abundance of interstitial fibrosis. Fibrosis was minimal in one individual, fibrotic tissue increased mildly in 3 individuals, the fibrotic increase was moderate in 1 individual, and there was a severe increase of fibrosis with approximately 50 to 70% of the gonad consisting of fibrotic tissue in 1 final individual.

0.32 mg/L n-Octanol
In low concentration, only slight alterations compared to the controls could be detected. The number of stage 3 females was slightly lower when compared to the control. One individual was early vitellogenic (stage 1) showing a moderate increase of immature atretic follicles. The amount of cyst-like structures filled with proteinaceous fluid (intravascular proteinaceous fluid) increased minimally, and 3gg debris was slightly more abundant. Another observation was a minimal to mild increase in the overall thickness of the ovarian wall especially in the gonoduct of two individuals. The overall number of fish showing a minimal to mild interstitial fibrosis increased from 42 to 50%. In contrast to the controls, a moderate or severe interstitial fibrosis could not be observed in this group, no elevated number of postovulatory follicles were observed at this concentration.

1 mg/L n-Octanol
In the intermediate concentration, the only conspicuous observation was a minimally increased atresia of immature oocytes in 17% of individuals. Although the number of atretic mature follicles was lower as in the

control, the severity of effects increased to moderate in 1 individual and to severe atresia of mature oocytes in 1 individual. Consistent with the low concentration, there was a minimal to mild increase in the overall thickness of the ovarian wall. The number of females with interstitial fibrosis was 34%, which was again somewhat even lower than in the control (overall 42%).

3.2 mg/L n-Octanol
In the high concentration, the number of fully mature fish (stage 4) was slightly decreased. The frequency of the presence of egg debris in the oviduct was (with an overall amount of 45% of fish) was greater than control, but the severity was elevated: 27% mild, 9% moderate and 9% severe. Post-ovulatory follicles could be observed in 45% (minimal) and 27% (mild) of the high concentration individuals. Another distinct effect was the high abundance of atresia of mature oocytes with an overall frequency of 72%. The frequency and severity of the interstitial fibrosis increased versus the controls (18% displayed a severe fibrosis with 50 to 70% of the ovary consisting of solid fibrotic tissue). There was an obvious alteration in the appearance of mature oocytes as they were usually filled with granulous eosinophilic yolk droplets. However, in most cases, the oocytes were atretic, where the yolk droplets were less eosinophilic with haematoxylin-stained violet spots scattered within the yolk. Cells of the zona radiata, the layer between the chorion and the vitelline envelope, were highly atretic. There was a high frequency of a thickened ovarian wall: minimal in 36% of fish and mild or moderate in 9%.

Males – Detailed Results for *n*-Octanol
Controls
In the control, 17% of males were mid spermatogenic (stage 2) with the remaining 83% had developed further into the late spermatogenetic stage (stage 3). Testicular fibrosis was observed in two forms: 1) a moderate diffuse interstitial fibrosis, mainly located in the periphery and between the spermatocyts in one individual and 2) an accumulations of fibrotic tissue that often had a cyst-like appearance (“fibrotic cysts”) in another individual (minimal severity**)**. Apart from these findings, no further aberrations were observed in the control.

0.32 mg/L n-Octanol
In the low concentration, maturity was decreased with 50% of males were mid spermatogenic (stage 2) and 50 late spermatogenetic (stage 3). The ratio of the different sperm cell populations was altered in two individuals. One stage 2 male showed a minimal decrease of spermatogonia, and one stage 3 male spermatids were elevated. A marked increase in fibrosis was observed. Interstitial fibrosis rose from 17% in the control to 63%. In one male, 60% of the testis consisted fibrotic tissue. Additionally, this individual showed a large number of fibrotic cysts.

1 mg/L n-Octanol
In the intermediate concentration, effects observed in male fish were less conspicuous when compared to the low concentration. The number of late spermatogenic fish was slightly decreased (71% vs. 83% in controls). A minimal number of fibrotic cysts were observed in 28% of males. One male showed a moderate increase in fibrotic cysts that differed in structure from all others and was attributed to parasitic action.

3.2 mg/L n-Octanol
In the high concentration, the maturity in a majority of males exposed to 3.2 mg/L *n*-octanol was decreased with 67% mid-spermatogenic (stage 2). These stage 2 males showed a minimal to mild increase of spermatids.

c. 2-Methoxyethanol
Females– Detailed Results for 2-Methoxyethanol

Controls
The female control revealed a high abundance of interstitial fibrosis (overall 74%). Fibrosis was minimal and mild in four individuals (27%), moderate in one individual, and severely increased with approximately 50 to 70% of the gonad consisting of fibrotic tissue in another individual. Fibrosis in this study was increased relative

to the potassium permanganate and *n*-octanol studies. It is noteworthy that fish from the same batch were used in all three studies. In the present study, fibrosis was no longer only diffusely scattered as noted in the other two studies, but formed cyst-like structures as well. The majority of females in the control were fully mature and in late developmental stage (stage 3, 37%) or late developmental/hydrated state (stage 4, 50%). Two individuals were in mid-developmental state (stage 2, 14%). Many phenomena connected to the reproductive cycle of fish were frequently observed like a mild to severe atresia of mature oocytes, interstitial proteinaceous fluid (overall 53%), intravascular proteinaceous fluid (overall 34%), minimal to mild postovulatory follicles (20% and 13%) and egg debris in the oviduct (overall 33%). The high abundance, especially of fibrosis and other effects as well as the severity of the observed alterations, lead to the consequence that the female control fish can display severe disorders that makes their use as reference group for histopathological analysis difficult.

1 mg/L 2-Methoxyethanol

In the low concentration, interstitial fibrosis findings were similar in the controls (74% to 79%). Atresia of mature oocytes decreased slightly, while atresia of immature oocytes increased in number (overall from 7 to 28%) as well as in severity. The frequency of oocyte debris in the oviduct was slightly higher than in the controls, but no elevation in the number of postovulatory follicles was observed. Stage 3 females (40%) were more abundant compared to controls, while the number of fully mature stage 4 females dropped to 20%. One individual showed asynchronous development of the left and right gonad, with the one being stage 2 and the other stage 3. The number of females with interstitial proteinaceous fluid decreased from 53% in the controls to 14%. Additionally, the abundance of individuals showing in-foldings of oocyte membranes increased from 7% in the controls to 47%.

10 mg/L 2-Methoxyethanol

In the intermediate concentration, female maturation was highly variable with a clear shift in maturation ratio towards less developed stages. The majority of fish were still late vitellogenic (stage 3,) or mature (stage 4) (32% and 36%, respectively). However, 3 females were completely immature and one was early vitellogenic (stage 1). In one female, the left and right ovary were asynchronous; one being stage 2 and the other stage 4. Almost every fish showed fibrosis in different grades. Fibrosis was minimal in four (27%), mild in five (33%), moderate in three (20%) and severe in two individuals (13%). This concentration-dependent increase of fibrosis occurred in both diffuse as well as cystic forms. Atresia of mature oocyte also increased in a concentration-dependent manner. The number of females with atretic immature oocytes was elevated versus the control, but similar to the low concentration. The number of females with egg debris in the oviduct decreased markedly from 33% in the control to 18%. Additionally, interstitial proteinaceous fluid as well as cyst like structures with proteinaceous fluid inside (intravascular proteinaceous fluid) were less abundant. Only one individual showed a minimal increase in oocyte membrane folding.

100 mg/L 2-Methoxyethanol

In the high concentration, only two female fish survived. Due to the small number of samples, results should be interpreted with caution. Both fish were fully mature and in stage 4. Females showed a minimal to moderate fibrosis as well as a minimal to mild abundance of interstitial as well as intravascular proteinaceous fluid. One female showed egg debris in the oviduct, and moderate to severe atresia of mature oocytes were observed in both females. However, the appearance of the atresia differed from other groups in that the oocytes were more well-formed oocytes, losing their inner structure by thinning or depletion of the chorion and/or the zona radiata, the layer between the chorion and the vitelline envelope. Both females displayed a high abundance of atrophic follicle cells surrounding the oocytes. Cortical alveolar as well as early vitellogenic oocytes were markedly decreased, and in one female the number of perinucleolar follicles as well. In contrast, the number of perinucleolar follicles increased minimally in the other female.

Males– Detailed Results for 2-Methoxyethanol

Controls
In control males, 75% of the individuals were mid spermatogenic (stage 2), and 25 % were further developed and in late spermatogenetic state (stage 3). Asynchronous development of the left and right gonad (stage 2 and 3) could be detected in one male. As with the other studies, fibrosis manifested in two forms: 1) a minimal diffuse interstitial fibrosis, mainly located in the periphery and between the spermatocytes in 50 % of control males, and 2) a moderate concentration of fibrotic tissue that often had a cyst-like appearance ("fibrotic cysts") in one individual. Another male showed dense cell clusters within the lumen of many seminiferous tubules, characterized as a beginning fibrosis. Additionally, minimal variations in the number of spermatogonia was observed in two males, and one male showed a minimal increase of interstitial ("Leydig-") cells. Apart from these findings, no relevant findings were observed.

1 mg/L 2-Methoxyethanol
In the low concentration, half of the males were mid and late spermatogenic, respectively. Spermatogonia increased minimally and moderately in one individual each. The major finding was a marked increase in fibrosis. The overall amount of males showing an interstitial fibrosis rose from control levels (50 %) to 89 % in the present exposure group. In one male, interstitial fibrosis was severely elevated with approximately 60 % of the testis consisting of fibrotic tissue. The abundance of fibrotic cysts was 47 % higher than controls, ranging from minimal to moderate in severity. In 88 % of males, the lumen of the seminiferous tubules, which usually are densely packed with sperms, small free areas could be detected. Higher magnification usually revealed one or a few bigger cells that were characterized as histolytic cells. Apart from a severe fibroses, one of these males also showed minimal signs of testicular degeneration.

As 2-methoxyethanol appears to act in mammals by interrupting early spermatogenesis, a careful review of the findings in the tubules of the testes is in order. The spermatocyte analyses showed modest changes at the low concentration. There were minimal to mild alterations in the ratio of the different maturation stages of spermatocytes (leptotene, zygotene and pachytene): One male (13 %) showed a mild increase of leptotene spermatocytes. A minimal to mild increase of zygotene spermatocytes could be detected in one and three (38 %) individuals, respectively. The number of pachytene spermatocytes as well as of spermatozoa decreased mildly in one male. Spermatocysts revealed an overall decreased cell density in 25 % of fish. Additionally, 38 % of males showed a minimal abundance of asynchronous developed spermatocytes, mainly containing pachytene spermatocytes and spermatids. Sertoli cells increased minimally and mildly in two (25 %) and one (13 %) individuals, respectively.

10 mg/L 2-Methoxyethanol
In the intermediate concentration, the major findings were: 1) the frequency of interstitial fibrosis was comparable with the low concentration; fibrotic cysts were less frequent, but, when present, were more severe; 2) increased numbers of histolytic cells in the lumen of the seminiferous tubules were observed in 84 % of males. The severity was a minimal to mild in one individual each, and of moderate severity in almost half the males; 3) three individuals presented with a minimal to moderate increase of testicular degeneration.

In the intermediate concentration, the number of late spermatogenic fish was 42 % higher, and no obvious increase in early spermatocytes was observed. 33% and 17 % of males showed a mild to moderate decrease in pachytene spermatocytes. The density of spermatozoa decreased mildly to moderately in nearly half of the males. As in the low concentration, asynchronously developed spermatocytes could be detected in the present exposure group. Other findings were that Sertoli cells increased minimally and mildly in 33 % of individuals and moderately in one (17 %), which is more than two times higher when compared to the low concentration. Two fish (33 %) revealed a moderate number of spermatocytes with low cell density.

100 mg/L 2-Methoxyethanol
In this concentration, no samples could be taken since no male fish survived

ANNEX III.
IBACON LABORATORY REPORT ON PROTOCOL AND METHODS

Discussion Statement on the Evaluation Studies of the Fish Screening Assay for Endocrine Active Substances Performed at IBACON

Screening studies for endocrine Effects of Potassium Permanganate, Octanol and 2-Methoxyethanol to Fathead Minnow (*Pimephales promelas*) in a 21 Day Flow-throughTest

(Study based on the Proposal for Phase 1B of the validation of the fish screening assay for endocrine active substances, Revision 2 dated 15 April 2004)

Dr. Ralf Petto and Dr. Katja Schneider, March 01, 2007

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Discussion Statement

In the three tests performed at IBACON, potassium permanganate, *n*-octanol and 2-methoxyethanol were tested as endocrine negative substances to evaluate the fish screening assay for endocrine disrupters. However, there are some critical points, which should be discussed here.

Potassium permanganate

First point, potassium permanganate raised issues due to the chemical characteristics, e.g. the formation of insoluble MnO_2, of this test substance. It strongly degraded to pyrolusit during the dwell time of the test item in the aquaria, which could be observed due to its brown colour. This effect was strongest in the highest test concentration of nominal 0.45 mg test item/L, where more than half of the fish died during the test. This indicates, that the high amount of pyrolusit in this test concentration could be responsible for the **mortality** of the test fish, e.g. due to damaging the gills.

The second point to discuss was the **maturity stage** of the test fish. At the start of the test the fish were seven months old and reached the weight of 1.5 ± 20% for adult females and 2.5 ± 20% for adult male fish recommended in the guideline. Nevertheless, it was difficult to differentiate between the sexes by secondary sexual characteristics. Eventually, the histomorphological analysis showed that the sex of the test fish was incorrectly evaluated at the start of the test, which made it difficult to evaluate the results. Therefore, the size and age of the fish (approximately 20±2 weeks) recommended in the draft guideline is not consistently appropriate for the sexual maturity of fathead minnows. It should be far more important to observe the actual reproductive status of the fish batch.

Octanol

In contrast to potassium permanganate, **octanol was metabolised** rapidly by bacteria, which made it difficult to keep the concentration level of the test substance constant. Furthermore, due to the strong bacterial growth, the test system (mixing vessel and aquaria) needed to be cleaned twice a day. As a consequence the fish may have been stressed and this may have influenced the health of the fish and, more importantly, the reproductive behaviour in the control as well as the test substance concnetrations.

Methoxyethanol

First point, the recommended **test concentrations were too high** and this resulted in mortality in the test concentration of nominal 10 and very high mortality in the 100 mg test item/L. As a consequence, the analysis of the toxic effect of 2-methoxyethanol to the gonads of the test fish was limited to two concentrations.

The second critical point was may be the **fitness of the fish**. At the start of the test, the fish were nine month old. Spawning was observed in the culture and the mortality of the fish batch was <5%. In addition, only the fittest fish were selected for this test. However, despite of this effort, three fish died in the control and gonadal fibrosis occurred also in the control fish. Despite these observed effects and the control mortality above the validity criteria, we regard the tests as valid for the purpose of the study, as only one fish died in the lowest test concentration.

To conclude, we recommend to organize a discussion meeting with all laboratories in order to discuss the difficulties of interpreting the data from the tested substances, potential amendments to the study protocol, and improved concentration selection criteria for highest concentrations to be tested (mortality and intoxication issues).