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REVISED DRAFT REPORT

PHASE 1B OF THE VALIDATIONOF THE 21-DAY FISH ASSAY FOR THE DETECTION OF ENDOCRINE ACTIVE SUBSTANCES

February 2005

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SUMMARY

i) This report provides the results from an OECD inter-laboratory study conducted in 2004 to examine the relevance and reproducibility of a standardized OECD protocol for the 21-day fish assay. The work carried out under Phase 1B follows initial work conducted in Phase 1A where the protocol transferability had been evaluated. Phase 1B study was primarily aimed at experimentally establishing the relevance of the assay for the detection of weakly active substances acting as estrogen, anti-androgen and aromatase inhibitors, when conducted in a choice of three generally used fish species. The second objective of Phase 1B was to check the reproducibility of the assay by comparing test results obtained in a variety of laboratories in geographical diverse locations, using the same fish species, the same test substances, the same measurement method for vitellogenin and the same procedures for gonadal histology. A combination of three fish species and three test substances were repeatedly used throughout fourteen laboratories to allow a sufficient number of repeats (3 in average) for inter-laboratory comparison of test results.

ii) The protocol for the 21-day fish assay for the detection of endocrine active substances can be summarised by the following description: reproductively active male and female fish were housed in groups of 5 males and 5 females and exposed to test chemical for 21 days. Three core endpoints as indicators of endocrine disrupter activity were measured, namely: i) gross morphology (i.e., secondary sexual characteristics), ii) vitellogenin (VTG) levels, and iii) gonadal histology. Additionally the spawning status was daily checked in all groups. Three fish species, i.e., fathead minnow (*Pimephales promelas*), medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) were used as test fish. Test chemicals were 4-*tert*-pentylphenol (100, 320 and 1000 µg/l) as a weak estrogen, flutamide (100, 500, 1000 µg/l) as an anti-androgen, and prochloraz (20, 100, 300 µg/l) as an aromatase inhibitor. 17β-estradiol (100 ng/l) and fadrozole (100 µg/l) were used as positive controls.

iii) Fourteen laboratories in 7 countries participated in this Phase 1B of the validation work. Participation was on a voluntary basis and laboratories committed their own resources and time in this work. A total of 31 studies were planned to ensure a minimum of three repetitions per fish species per substance. On one occasion, an invalid study reduced the number of repetitions to 2 (fathead minnow, 4tPP).

iii) Measured concentrations of the test substances and positive controls in the 31 studies showed that 19 studies remained within the range 80%-120% of nominlal values; one study was above 120% nominal concentration; 11 studies were below 80% nominal values. One study (not included in the 31 studies) using the anti-androgen flutamide with fathead minnow was invalid because of high mortality in the control group. Test results from that study are not presented in this report.

iv) 4-*tert*-Penthyphenol exposure induced dose-dependent VTG synthesis in males of three fish species, indicating that this protocol can detect estrogenic effect of weak estrogen. Secondary sex characteristics in males and spawning status were inhibited in fathead minnow. Prochloraz exposure decreased VTG levels dose-dependently in females of three fish species simultaneous with spawning cessation. These findings suggest that these responses follow a cascade triggered by aromatase inhibition. Consistency across studies was generally good, and where it was not it could be explained by e.g. low measured concentration of the test substance. Gonadal histopathology was particularly useful in zebrafish and fathead minnow for investigating effects caused by flutamide, since exposure to this mammalian anti-androgen caused no clear alteration in other core endpoints except in the spawning status at highest concentration in fathead minnow studies.

vi) One laboratory measured the concentrations of persistent organic pollutants (POPs) in the fish food

(e.g., brine shrimp). The results indicated that the food contained the estrogenic chemical, *o,p'*-DDT, although these levels were quite low.

vi) An extensive draft guidance document on gonadal histotechniques and histopathology was developed and available to the participating laboratories. Following Phase 1B, pathologists reviewed results yielded and agreed to streamline histological evaluation for a future standard test by reducing exposure-related diagnoses to a minimum of four per male and four per female.

vii) Overall, the results yielded in Phase 1B demonstrate that the protocol for the 21-day fish assay using three fish species is able to detect endocrine disrupting responses of weak estrogens such as 4-*tert*-penthyphenol, and of aromatase inhibitors such as prochloraz through vitellogenin measurement and observation of the spawning status. Concerning the anti-androgen, whereas the applicability of secondary sex characteristics and vitellogenin measurement might be limited in detecting a treatment-related response, histological changes in the gonads, especially in fathead minnow and zebrafish were notable and useful in characterising the anti-androgen mode of action. Phase 1B also demonstrated that following the protocol, using reproductively active fish and keeping test concentrations close to nominal values, then reproducibility across laboratories is good. However, it also showed that the sex ratio 5:5 is sub-optimal and creates territoriality among males, thus disturbing normal spawning in the group.

1- INTRODUCTION

1. The need to develop and validate a fish assay able to detect endocrine active substances originates from the concerns that environmental levels of chemicals may be causing adverse effects in both humans and wildlife due to the interaction of these chemicals with the endocrine system. Several cases were reported where the exposures of exogenous chemicals have resulted in effects in wildlife, and in particular in fish (1) (2) (3). In 1997, OECD countries advised that existing test methods were insufficient to identify such substances and characterise their effects. As part of the OECD Test Guidelines Programme a *Special Activity on the Testing and Assessment of Endocrine Disrupters* was initiated to revise existing, and develop new OECD Test Guidelines for the screening and testing of potential endocrine disrupters. A Task Force on Endocrine Disrupters Testing and Assessment (EDTA) was subsequently established to provide a focal point within OECD to consider and recommend priorities for the development of testing methods for endocrine disrupters.

2. Two Fish Expert Consultation were organised in London in 1998 and in Tokyo in 2000 to review the state-of-the-knowledge and science in the area of fish screening and fish testing in relation to endocrine disrupters. The outcome of these Expert Consultations was a recommendation on three promising core endpoints that should be part of the future fish screening assay. These endpoints were the vitellogenin level, the gross morphology (including secondary sex characteristics and the gonado-somatic index) and gonad histology. In 2001, during the First Meeting of the Validation Management Group for Ecotoxicity Testing (VMG-eco) proposals for candidate protocols were made. On that basis, a Fish Drafting Group was established to agree a common OECD protocol and to prepare a proposal to the VMG-eco for validation of the test method. One constraint for the protocol was the need to check its applicability to the three fish species commonly used in OECD countries for regulatory testing: fathead minnow (*Pimephales promelas*), medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*). Following an agreement on the protocol, a phased approach to it validation was taken. Phase 1A, summarised hereafter, aimed at verifying the transferability of the assay from one laboratory to another. Phase 1B was intended to evaluate the relevance of the assay and its reproducibility. It is the subject of this report.

3. The fish screening assay fits into the OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupters, discussed and agreed at the sixth Meeting of the EDTA Task Force. This framework identifies approaches, assays and long-term tests of increasing biological complexity, meant to gather information on potential endocrine disrupters. Each of the tools added to the framework will require validation to ensure its relevance and reliability, the two main validation principles. The OECD draft Guidance Document 34 provides definitions, principles and concrete examples of validation, applied in different areas of hazard assessment.

4. The 21-day fish assay validated in Phase 1A and Phase 1B is intended for the detection of individual chemicals acting as estrogen, androgen, aromatase inhibitor or anti-androgen. Reproductively active adults males and females are exposed together, but then analyzed separately. The assay is based on the principle that certain sex characteristics and protein are under the control of endogenous hormones, and that exposure to exogenous substances in the water can induce these characteristics in the opposite sex or reduce their normal occurrence in individuals where they are usually observed. The aim of the validation was to develop a robust, relevant and reliable test method for the detection of chemicals mentioned above. It is also the purpose of the validation to understand and define the area of application of the assay and any limitation to its use.

2- OUTCOME OF PHASE 1A

5. Following discussions in 2002 on the protocol for the Fish Screening Assay for the detection of endocrine active substances, and out of concern for resource optimization and animal welfare, Phase 1 was split into Phase 1A and Phase 1B.

6. Phase 1A was indeed considered to be a feasibility study; adult fish of both sexes were exposed to two known chemicals (an estrogen and an androgen) during 21 days. Males and females were kept in separate tanks to prevent spawning. Three core endpoints, recommended by two Expert Consultations and approved by the Validation Management Group for Ecotoxicity Testing (VMG-eco) were measured in the assay: vitellogenin (VTG) level, gross morphology (including secondary sex characteristics and gonado-somatic index (GSI)) and gonad histology. Measurements were analyzed separately for males and females. This first step was limited and conducted in four laboratories, each using at least two of the three possible fish species (fathead minnow, medaka and zebrafish) to ensure that repeats could be compared across laboratories. Each laboratory used the two relatively strong test substances: 17-β estradiol (estrogen) and 17-β trenbolone (androgen) with each fish species. Males and females fish were analyzed separately. A total of 20 tests were performed in 2002.

7. The Fish Drafting Group of the Validation Management Group for Ecotoxicity Testing met in October 2003 to discuss the outcome of Phase 1A and to plan Phase 1B.

8. It was concluded from Phase 1A studies that the fish screening assay was reliable for the detection of the strong estrogenic compound 17-β estradiol and the strong androgenic compound 17-β trenbolone in all three species, showing the potential relevance of this assay. Analysis of male VTG was valid for strong estrogen and measurement of female VTG and secondary sex characteristics in medaka and fathead minnow was sensitive to androgenic exposure. The GSI was poorly responded both to 17-β estradiol and 17-β trenbolone, and variability among responses was high. Concerning the evaluation of gonadal histology, the outcome of Phase 1A showed that, despite exposure-related responses, female fish showed pathological symptoms (e.g. oocyte atresia) attributable to their non-spawning situation. Therefore, to improve the fitness of fish and the biological relevance of the assay, fish experts recommended holding males and females in the same test chamber, thus allowing fish to spawn. A detailed guidance document was prepared after Phase 1A for the harmonized evaluation of the histopathology. A revised draft Report of Phase 1A was made available for comments and approval of the VMG-eco in December 2003.

9. An action plan including detailed standard operating procedures, test substances and concentrations, and timelines for the work was submitted to the VMG-eco for approval at the end of 2003. After agreement of the VMG-eco on the plan for Phase 1B, laboratories starting scheduling their experiments: experimental work ran from March until September 2004. Fourteen laboratories from 7 countries and industry (Denmark, Germany, Japan, the Netherlands, Switzerland, the United Kingdom and the United States) took part in Phase 1B.

3- OBJECTIVES OF PHASE 1B

10. The objective of Phase 1B was primarily to experimentally establish the relevance of the assay for the detection of weakly active substances acting as estrogen, anti-androgen and aromatase inhibitor, when conducted in a choice of three generally used fish species. The second objective of Phase 1B was to check the reproducibility of the assay by comparing test results obtained by a variety of laboratories in geographical diverse locations. Here is a summary of key goal of Phase 1B:

- Obtain additional information on the relevance of assay endpoints and in particular their ability to respond weakly active substances with diverse modes of action in the context of this assay.

- Collect a set of data for three weakly active substances;

- Obtain additional information on possible differences in species sensitivity to a weak estrogen, a mammalian anti-androgen weakly active in fish, and a weak aromatase inhibitor;

- Check that the protocol contains enough details to enable laboratories to conduct the assay in a reproducible manner;

- Check the reproducibility of test results in laboratories located in diverse geographical areas, and with diverse levels of experience in conducting this type of assay.

4- ORGANISATION OF PHASE 1B

4.1 Introduction

11. For Phase 1B, a technical lead laboratory for each species was designated (LAB 1 for medaka, LAB 7 for fathead minnow and LAB 12 for zebrafish). Each technical lead laboratory was responsible for making standard operation procedures for secondary sex characteristics and vitellogenin measurements in each fish species and for answering questions from other laboratories using the same species. Each participating laboratory, 3 to 4 for each species (see **Annex 3** for laboratories contact details), was asked to conduct experimental work in compliance with the agreed protocol and to submit a study plan for its participation in Phase 1B to the overall Lead laboratory, LAB 1, with a copy to the OECD Secretariat, including a schedule of work.

12. The main change in the design between Phase 1A and Phase 1B was in holding adult males and females of a narrowly defined age and in spawning conditions, in the same tank. It was considered that these natural conditions might improve the biological relevance of the assay, and also improve the response to chemical exposure by removing potential confounders noted in Phase 1A. Endpoints remained the same as in Phase 1A, except the GSI which was dropped, and the qualitative assessment of spawning (yes/no answer) was included as a new observation. Following recommendations from the EDTA Task Force at its seventh Meeting to use weakly active compounds to appraise the sensitivity of the protocol, the Fish Drafting Group of the VMG-eco made proposals on test substances, already known from the published literature as weakly active on the fish endocrine system.

13. Detailed standard operating procedures was drafted for participating laboratories to follow; with respect to gross morphology, experts advised to provide participating laboratories with a detailed guidance for the measurement of secondary sex characteristics in fathead minnow and in medaka. These standard operating procedures were prepared by LAB 1 and LAB 4 and made available in appendix 6 of the Phase 1B protocol. The development of an extensive draft guidance document for gonad hitotechniques and hitopathology was coordinated by LAB 4; it was available within appendix 6 of the protocol.

4.2 Overview of the test method

14. The experimental work was conducted according to the protocol prepared for Phase 1B of the validation of the Fish Screening Assay for Endocrine Active Substances (**Annex 1** to this report). A summary of noteworthy aspects of the protocol is provided below.

15. The protocol was designed to detect endocrine active chemicals in sexually dimorphic fish. The assay was initiated with fish sampled from populations that were in spawning condition. The assay was conducted using three chemical exposure concentrations for each test substance, as well as a water control. The use of a solvent carrier was not needed in principle. However, when solvent was used, a solvent control was added. One concentration of a defined positive control substance was included for both 4-*tert*-pentylphenol and prochloraz studies. Two vessels (replicates) per treatment were used (each vessel containing 5 males and 5 females). The exposure was conducted for 21 days, at the end of which fish were sampled. Daily qualitative observations of the spawning status in each test vessel were recorded (yes/no). A spawning substrate was placed in the test chamber for the fathead minnow and zebrafish to enable fish to spawn in normal conditions; eggs were removed daily from the test chamber.

16. After humane killing of the 20 fish (10 males and 10 females) per treatment level, blood samples were collected for determination of vitellogenin (note - liver was sampled for VTG analysis in medaka). Secondary sex characteristics in fathead minnow and medaka were quantitatively evaluated. For gonad histology in medaka and zebrafish, animals were fixed and embedded directly, whereas for fathead minnow gonads were excised from the body cavity, fixed and embedded. The concept for this fish assay is derived from work on the fathead minnow *(Pimephales promelas)* (4)(5)(6)(7), the Japanese medaka (*Oryzias latipes*) (8) (9) (10) (11) (12) and the zebrafish (*Danio rerio*) (13) (14) (15) (16).

4.3 Test fish

17. Fathead minnow (*Pimephales promelas*), medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) were used in the Phase 1B validation work. These are commonly used species for regulatory work in OECD member countries. The fish strain was not defined by the protocol. For medaka, LAB 1, LAB 2, Lab 3, LAB 4, but not LAB 6, used the orange-red strain.

18. The exposure phase was started with sexually dimorphic adult fish from a laboratory supply of reproductively mature animals (namely, with clear secondary sexual characteristics visible) and actively spawning. It was recommended that fathead minnow be approximately 20 (± 2) weeks of age (assuming they had been cultured at 25 ± 2°C throughout their lifespan), medaka be approximately 16 (± 2) weeks of age (assuming they had been cultured at 25 ± 2°C throughout their lifespan) and zebrafish be approximately 15 (± 2) weeks of age (assuming they had been cultured at 25 ± 2°C throughout their lifespan). The age of the test fish is listed in Table 1. Several studies deviated from the protocol because of the delay in receiving test chemicals or ELISA kits for VTG measurement.

Table 1: Age of the test fish used in each laboratory.

Laboratory's name	Age of the test fish (months)		
	4-tert pentylphenol	**Prochloraz**	**Flutamide**
	Medaka		
LAB 1	4	6	
LAB 3	4 (17 weeks)		4 ½ (18 weeks)
LAB 2	4	4 ½	
LAB 5	4		4
LAB 4		4	4
LAB 6		5 ½ (22 weeks)	5 ½ (22 weeks)
	Fathead minnow		
LAB 7	approximately 4.5		6
LAB 8	4.5	5.5	
LAB 9	6	6	
LAB 10			5 ½
LAB 11		12	Invalid study
LAB 4		4.5	4.5
	Zebrafish		
LAB 12	3	6	3
LAB 13	5-6	5-6	
LAB 14	2.5		3
LAB 6		ca. 4 (17 weeks)	ca. 4 (17 weeks)

19. Following a 48-hour settling-in period, mortalities were recorded and the following criteria applied:

- mortalities of greater than 10% of population in seven days: reject the entire batch;
- mortalities of between 5% and 10% of population: acclimation for seven additional days; if more than 5% mortality during second seven days, reject the entire batch;
- mortalities of less than 5% of population in seven days: accept the batch.

20. The animals did not receive any treatment for disease in the 2-week acclimation period preceeding the test, or during the exposure period. In a sub-sample of the whole batch, animals were individually weighed at the start of the test. If possible, it was recommended to remain within a range of 20% of the arithmetic mean weight.

4.4 Test chemicals and concentrations

21. The Fish Drafting Group followed the recommendation from the EDTA Task Force to use weak substances. Three chemicals representing different modes of action were proposed:

Table 2: Test substances used in Phase 1B.

Test substance	CAS number	Lot no., purity	Supplier	Mode of action
4-*tert*-pentylphenol	80-46-6	13625LB, 99.9 %	Sigma Aldrich, US	Estrogen
prochloraz	67747-09-5	2226X, 99.5 %	Sigma Aldrich, US	Aromatase inhibitor
flutamide	1331-84-7	073K1004, >99 %	Sigma Aldrich, US	Anti-androgen

Positive controls substances were used for the estrogenic and aromatase inhibitor modes of action:

Table 3: Positive controls used in Phase 1B.

Positive control	CAS number	Lot no., purity	Supplier	Mode of action
17β-estradiol	50-28-2	103K1117, 100 %	Sigma Aldrich, US	Estrogen
fadrozole	102676-47-1	-	Novartis Pharma, Switzerland	Aromatase inhibitor

22. All substances except fadrozoke were managed through a Central Chemical Repository located in the US: Battelle Marine Sciences Laboratory (WA, USA). The chemical repository allowed a central coordination of the delivery of chemicals to the participating laboratories, with the assurance that chemicals were coming from a unique lot. Fadozole was provided by Novartis Pharma, Switzerland.

23. The choice of test substances and test concentrations was extensively discussed experts of the Fish Drafting Group in October 2003. The selection of 4-*tert*-pentylphenol as a weak estrogen agonist is based on work done in Japan on medaka (17) and in Europe on fathead minnow (20). Concentrations recommended are in line with those used in the published literature for the work on medaka, and one order of magnitude higher compared to the work on fathead minnow. The spacing of concentrations follows a factor 3.2, which enables to cover an order of magnitude when 3 concentrations are used in this type of test. The selection of one concentration of 17β-estradiol to serve as a positive control for estrogenic effects was based on Phase 1A and the top concentration used in Phase 1A was selected.

24. Prochloraz was used as an aromatase inhibitor (interfering with the conversion of testosterone into 17-β estradiol in females); it has not been used very often in the past in fish and required a range-finding study to be conducted prior to Phase 1B to identify suitable concentrations. The range-finding study started with literature review to identify the water solubility of prochloraz, followed by a search in the AQUIRE database for the LC_{50}. One laboratory in the United States carried out a 7-day study on fathead minnow - one tank per concentration, containing 4 females and two males- taking 3 concentrations, spread on a log-based 10 series. The top concentration was 1/3 $LC_{50.}$ Fish were sampled at termination of the study and blood collected for vitellogenin measurement, to ensure that the dose-response curve was covered in the range of concentrations used. Fadrozole was used as a positive control substance for the aromatase inhibition mode of action. Previous work documented endpoints response in fish and the concentration to be utilize to elicit a response (18) (19).

25. Flutamide, a known mammalian anti-androgen, also used in work on fathead minnow in the United Kingdom and in the United States on fathead minnow, was selected in Phase 1B to represent the

anti-androgen mode of action. It is known as a relatively weak substance in fish for this specific mode of action. The three concentrations of flutamide used in Phase 1B are in line with those from the existing literature (18)(40).

26. Concentrations of the test substances in Phase 1B were as follows:

- 4-*tert*-pentylphenol: 100, 320 and 1000µg/l (+ water control);
- 17β-estradiol: 100 ng/l to be used as positive control;
- Flutamide: 100, 500, 1000µg/l, highest as positive control (+ water control);
- Prochloraz: 20, 100, 300µg/l (+ water control)
- Fadrozole: 100µg/l to be used as positive control;

4.5 Participating laboratories

27. Following a call for participation in Phase 1B, fourteen laboratories in 7 countries (Denmark, Germany, Japan, the Netherlands, Switzerland, United States, United Kingdom) expressed interest. The contact details of each participating laboratory are provided in **Annex 3**.

Table 4: Participating laboratories.

Laboratory's name	**Test substance**		
	4-tert pentylphenol	Prochloraz	Flutamide
	Medaka		
LAB 1	X	X	
LAB 3	X		X
LAB 2	X	X	
LAB 5	X		X
LAB 4		X	X
LAB 6		X	X
	Fathead minnow		
LAB 7	X		X
LAB 8	X	X	
LAB 9	X	X	
LAB 10			X
LAB 11			X
LAB 4		X	X
	Zebrafish		
LAB 12	X	X	X
LAB 13	X	X	
LAB 14	X		X
LAB 6		X	X

28. The distribution of species and test substances was meant to ensure a minimum of three repetitions for inter-laboratory comparisons. Among the problems and changes from the initial plan:

- LAB 9 using fathead minnow encountered problems with the ELISA kit for vitellogenin measurements from the prochloraz study, therefore no VTG results from LAB 9 are presented in the report.
- In LAB 9, fathead minnow in the 4tPP study suffered from an overdose in the last day of the experiment in the mid-dose and a lack of test substance delivery in the high dose group in the last 15hours of the study; vitellogenin measurements for the 4tPP study from LAB 9 are not presented in this report either.
- LAB 10 only performed the flutamide study on feathead minnow and not the 4tPP study, due to a lack of time.
- LAB 11 had very high mortalities in the flutamide study on fathead minnow, making test results invalid.

4.6 Time schedule

29. The experimental work started in March 2004 after preliminary approval of this proposal by the VMG-eco. All in-life parts of Phase 1B were completed in September 2004. The overall lead laboratory was responsible for the collection of test results, previously checked for accuracy by the laboratories themselves. The overall lead laboratory performed the statistical analysis and prepared the present report in September-October 2004.

30. A preliminary version of the present report was presented to the Validation Management Group for ecotoxicity testing (VMG-eco) at its meeting in December 2004. The preliminary version only gave a partial view of the test results because the evaluations of gonadal histopathology were not yet included. Additionally, measures of the reproducibility of the assay across laboratories, such as coefficients of variation intra- and inter-laboratory, were not part of the report. The current version, dated February 2005, is inclusive of all data made available by the participating laboratories.

4.7 Preparation of test solutions

31. Test solutions of the selected concentrations were prepared by dilution of a stock solution. The stock solution was prepared by simply mixing or agitating the test substance in the dilution water by using mechanical means (e.g. stirring or ultrasonication). Saturation columns (solubility columns) were used for achieving a suitable concentrated stock solution in some laboratories. Detailed standard operating procedures for each substance were used (Appendix 9 in **Annex 1**). Acetone was used as a solvent and solvent control was set in LAB 12 for the flutamide experiment in zebrafish. LAB 12 encountered difficulties in achieving the solubility of 4tert-pentylphenol in the stock solution without solvent; this possibly explains the low measured concentrations of 4tPP in LAB 12 (see Table 7).

4.8 Analytical methods for determination of test concentration

Table 5: Test substances and analytical methods.

Test substance (TS) or Positive control (PC)	Analytical method for determination of test concentration	References
4-tert-pentylphenol	HPLC	(17) (20) (21)
Prochloraz	HPLC	Appendix 9 in **Annex 1**
17β-estradiol	LC-MS	(12) (22)
Flutamide	HPLC	(23) (24)
PC for aromatase inhibition	HPLC	(23) (25)

32. Analytical chemistry results for each substance and laboratory are presented in Section 5.1. Analysis was performed on a weekly basis in each tank and reported in the spreadsheet.

4.9 Test conditions

33. A flow-through test system was used. Such a system continually dispenses and dilutes a stock solution of the test substance (e.g. metering pump, proportional diluter, saturator system) in order to deliver a series of concentrations to the test chambers. The flow rates of stock solutions and dilution water were checked at intervals and were not supposed to vary by more than 10% throughout the 21 days of the test.

Feeding

34. Further to the recommended food diet and ration, LAB 1 measured the presence of contaminants such as persistent organic pollutants (POPs) in food. Results are presented in ***Section 5.5***, Table 47.

4.10 Test acceptance criteria

35. For the test results to be acceptable, the following conditions applied:

- mortality in the control(s) did not exceed 10 per cent at the end of the exposure period, and signs of disease visible in less than 10 per cent of control animals during the course of the test.
- dissolved oxygen concentration was at least 60 per cent of the air saturation value (ASV) throughout the exposure period;
- water temperature did not differ by more than ± 1 °C between test vessels at any one time during the exposure period and was maintained within a range of 2°C within the temperature ranges specified for the test species.

36. In LAB 11, the fathead minnow study using the aromatase inhibitor turned out to be invalid because of high mortality rates. Test results from that study are not presented in this report.

4.11 Endpoints studied

37. Three core endpoints as indicators of endocrine activity of the test substances were observed during the course of the test or measured at termination of the test, namely:

i) gross morphology (e.g., secondary sexual characteristics such as nuptial tubercles on the head in fathead minnow and papillary processes on the anal fin in medaka were counted according to the standard operating procedures available, Appendix 6 of the protocol),
ii) vitellogenin levels,
iii) gonadal histology.

38. Additionally, participating laboratories were requested to record daily the presence of eggs (yes/no answer) in treated and control groups. The data is presented in a temporal way in this report. Knowledge of the spawning status was intended as a check on the reproductive maturity of the fish in test in the control tank.

39. On the one hand, complete cessation of spawning in a group can be a useful piece of information when interpreting the gonadal histopathology; especially when temporally reported, it gives an indication of when the fish stopped spawning and may assist in interpreting some of the pathological findings in females. On the other hand, there are also limitations to interpretation of variations in the spawning status of fish, as reported in Phase 1B:

- Unless there is complete cessation, it is not possible to have information of the spawning status of individual females to aid the gonadal histopathology evaluations;
- A qualitative recording (yes/no) does not reflect variation in the quantity of eggs spawned and only cessation can make a difference for further interpretation.

40. Daily egg counts were left as an optional endpoint; LAB 12, LAB 13 and LAB 14 – all using the zebrafish- counted fish eggs daily in a quantitative way (LAB 12 and LAB 14) or semi-quantitative way (LAB 13). All egg counts are reported in this document in graph format.

41. Retrospectively, laboratories who counted eggs were asked to indicate the time spent daily on this operation for the whole 21-day study (control and 3 concentrations). Responses varied between ¾ hour and 1hour ½ to 2 hours/day.

4.12 Other observations

Survival

42. Fish were examined daily during the test period and any external abnormalities (such as hemorrhage, discoloration) noted. Mortality was recorded and the dead fish was removed as soon as possible.

Behavior

43. Any abnormal behavior (relative to controls) was noted; this included signs of general toxicity including hyperventilation, uncoordinated swimming, loss of equilibrium, and atypical quiescence or feeding.

4.13 Data collection

44. Participating laboratories recorded the raw experimental data from their Phase 1B on standardized Excel spreadsheets (**Annex 2**) developed specifically for the validation study. A workbook (collection of Excel spreadsheets for each endpoint) was prepared for each species. In addition to raw data, means and standard deviations were calculated. All completed data sheets with laboratory results are available from the Lead laboratory and from the OECD Secretariat.

4.14 Vitellogenin measurement and supply of kits

45. VTG is a phospholipoglycoprotein precursor to egg yolk protein that normally occurs in sexually-active females of all oviparous species; the production of VTG is controlled by interaction of estrogens with the estrogen receptor. Significantly, males maintain the capacity to produce VTG in response to stimulation with estrogen receptor agonists; as such, induction of VTG in males and immature females has been successfully exploited as a biomarker specific for estrogenic compounds in a variety of OECD fish species. Inhibition of VTG in mature females also appears to be a biomarker of exposure to aromatase inhibitor, where the conversion of endogenous androgen to estrogen is blocked, thus stopping estrogen receptor activation, itself controlling VTG production.

46. With regard to vitellogenin measurement, a number of comparative studies were conducted in parallel with Phase 1A in OECD member countries: United States (24), France and Japan (27). The outcome of each study was reviewed by the Fish Drafting Group at its meeting in October 2003. The important conclusions relevant to the Validation of the Fish Screening Assay were that:

i) homologous ELISA methods should be used, i.e. using species-specific antibodies and VTG standard;

ii) the method should have demonstrated that it can detect levels as low as few ng/ml plasma (alternatively liver for medaka), this is to ensure that the assay can differentiate between control males and males producing low levels of VTG after induction by a weak estrogenic compound for example; and finally

iii) in the validation exercise the same method should be used by all laboratories using the same fish species.

47. However, an important note should be made here: the choice of a particular method to be used in laboratories in Phase 1B for each species does not imply that it is the only valid method; it only means that from results gleaned in the comparative studies conducted in the United States, in France and in Japan, the method selected has demonstrated to be sensitive enough for the intended purpose and that experts felt confident enough to use it in the validation exercise.

48. After the Fish Drafting Group meeting in October 2003, LAB 1 in Japan, LAB 4 in the US, and LAB 12 in Germany selected the methods used by laboratories working on medaka, fathead minnow and zebrafish respectively. Standard operating procedures (SOPs) for the sampling procedure (blood or liver collection, pre-treatment and detailed specifications of the method used) have been added as appendices to the Phase 1B protocol (Appendix 6). The measurement of VTG was based upon validated homologous ELISA methods. VTG measurement kits, produced by EnBioTec for VTG measurements in fathead minnow and medaka were centrally distributed by Amersham/GE Health Science in the United States and Europe at a negotiated rate to the participating laboratories.In zebrafish, VTG measurement was performeed according to the method published by Holbech (28).

Fathead minnow:

- Fathead Minnow Vitellogenin ELISA system (EnBioTec Laboratories, Tokyo, Japan) using monoclonal antibodies;
- ELISA method used at US EPA MED, described by Korte *et al*, 2000 (26), using polyclonal antibodies;

Medaka:

- Medaka Vitellogenin ELISA system (EnBioTec Laboratories Co., Ltd., Tokyo, Japan) using monoclonal antibodies (38), and
- Medaka Vitellogenin (VTG) ELISA kit (TRANS GENIC INC., Kumamoto, Japan) using polyclonal antibodies (39);

The above two kits were selected because the sensitivity and reproducibility of these two kits are comparable, and a good correlation exists between these two kits when a common VTG standard is used (27). Therefore, it was decided to use Battelle VTG standard for the standard curve;

Zebrafish:

- BCA Protein Assay Reagent Kit (Pierce, Rockford, USA) for the measurement of total protein concentration in zebrafish samples;
- ELISA method used at Odenske University, described by Holbech's method (Holbech *et al*, 2001) (28).

49. VTG standard proteins for each of the three species were prepared centrally in Battelle Marine Science Laboratory in the United States, and delivered to all participating labs for the purpose of Phase 1B. In all VTG assays using medaka and zebrafish the Battelle standard was used for the standard curve in order quantify the specimen. The VTG standard contained in the kit was not basically used. VTG kit and VTG standard protein used in each laboratory are listed in Table 6. Standard operating procedures for blood/liver sampling and pre-treatment, for the purification of vitellogenin, for the use of BCA Protein Assay Reagent kit and for the use of the VTG ELISA kits were available to the laboratories.

Table 6: VTG kit and VTG standard protein used in each laboratory.

Laboratory's name	VTG kit / VTG protein for standard curve		
	4-tert pentylphenol	Prochloraz	Flutamide
	Medaka		
LAB 1	TG-MK[1] / Battelle-VTG standard		
LAB 3	TG-MK / Battelle-VTG standard		
LAB 2	EnBio-MK[2] / Battelle-VTG standard		
LAB 5	EnBio-MK / Battelle-VTG standard		
LAB 4	EnBio-MK / Battelle-VTG standard		
LAB 6	EnBio-MK / Battelle-VTG standard		
	Fathead minnow		
LAB 7	EnBio-FHM[3] / Battelle-VTG standard		
LAB 8	EnBio-FHM / Battelle-VTG standard and VTG standard contained in the kit		
LAB 9	EnBio-FHM / Battelle-VTG standard		
LAB 10	EnBio-FHM / Battelle-VTG standard		
LAB 11	EnBio-FHM / VTG standard contained in the kit		
LAB 4	EnBio-FHM / Battelle-VTG standard		
	Zebrafish		
LAB 12	Pierce-ZF[4] / Battelle-VTG standard		
LAB 13	Pierce-ZF / Battelle-VTG standard		
LAB 14	Pierce-ZF / Battelle-VTG standard		
LAB 6	Pierce-ZF / Battelle-VTG standard		

1) : Medaka VTG ELISA kit (TRANS GENIC INC., Kumamoto, Japan)
2) : Medaka VTG ELISA system (EnBioTec Laboratories Co., Ltd., Tokyo, Japan)
3) : Fathead Minnow VTG ELISA system (EnBioTec Laboratories, Tokyo, Japan)
4) : BCA Protein Assay Reagent Kit (Pierce, Rockford, USA) for the measurement of total protein concentration in zebrafish samples

4.15 Guidance for gonadal histopathology

50. The experience from Phase 1A had shown that an insufficient level of guidance and standardization of procedure was available to participants to evaluate gonad samples in a harmonized and comparable way. Further work was warranted to develop a consensus guidance document for use in Phase 1B. A group of experienced fish pathologists met after Phase 1A in October 2003, to identify areas where they could contribute further advice regarding the histological procedures and the pathological evaluation.

51. Considerable efforts were made to draft a comprehensive document, with all necessary standard operating procedures, illustrated with many annotated photos (e.g., dissection procedures, histological slides), providing the diagnostic terminology and a consensus severity scoring system to be applied in Phase 1B. The guidance document was available as Appendix 6 to the Phase 1B protocol. In the future, it will become a stand alone document.

52. Following Phase 1B, pathologists met and reviewed their findings. They explained that none of them was blind to the tratment of fish when they first evaluated the slides. In their opinion, for studies such as Phase 1B, blind reading is not appropriate for the initial evaluation of sections because it increases the chances that subtle exposure-related effects will be missed (i.e., blind reading primarily guards against false positive results at the expense of false negative results). Conversely, blind reading may be entirely appropriate for future routine assays because the possible results to a set of previously established histologic findings have already been limited (i.e., we are proceeding under the assumption that there are no new types of fndings to be discovered). Pathologists recommended that there should be initial knowledge of the control group because some of the diagnoses are expressed as relative increase or decrease of a particular cell type. In Phase 1B, pathologists confirmed that they were blind to the reproductive cycle status of fish.

53. In order to streamline diagnoses that are directly relevant to treatment, a short list of primary diagnoses was prepared by pathologists' post-Phase 1B evaluations. These primary diagnoses, when found in the study experiments in a consistent manner through the laboratories have been marked by dark cells throughout the tables in the following results' sections.

Table 7: Primary gonad histopathological diagnoses

No.	Males:	Females:
1	Increased proportion of spermatogonia	Increased oocyte atresia
2	Presence of testis-ova	Perifollicular cell hyperplasia/hypertrophy
3	Increased testicular degeneration	Decreased vitellogenesis
4	Interstitial (Leydig) cell hyperplasia/hypertrophy	Gonadal staging (based on improved staging criteria)

4.16 Statistical analysis: procedure followed

54. Statistics were conducted in accordance with the protocol (**Annex 4**).The statistical analysis was performed on secondary sex characteristics of fathead minnow and medaka, and vitellogenin of three fish species. The spawning status and the gonad histology were excluded because these data are qualitative or semi-quantitative. Statistical analysis was conducted on the basis of individuals rather than unit of replication (tanks) because two replicates were not sufficient for statistics. The statistical analysis of optional data (e.g., egg numbers) has not been performed. A two-side test was performed because the initial hypothesis was that either an increase or a decrease in endpoints measured could be considered as damageable outcomes.

55. If a solvent control was used for a test, homogeneity of variances between the control and solvent control groups was checked by Levene's test, and then either Student's *t*-test (parametric data) or Mann-Whitney *U* test (non-parametric data) were used before data analysis to determine whether differences exist between the control and solvent control groups. Where necessary, data were log-transformed for normalization and to reduce variance heterogeneity. If no difference was found, these groups were pooled for subsequent analysis. If differences were found, the control group without solvent was excluded from the subsequent analyses.

56. The experimental data was checked for homogeneity of variances across treatments by Levene's test. When no homogeneity was observed in the data, a log-transformation was performed and the transformed data was checked for homogeneity of variances across treatments again. When the assumptions were met (with or without transformation), the data were subjected to one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. When no homogeneity was observed even in the transformed data, the nonparametric Kruskal-Wallis test was used, followed by the Mann-Whitney *U* test with Bonferroni's adjustment. Differences were conclusively determined in Dunnett's multiple comparison test or Mann-Whitney *U* test with Bonferroni's adjustment. VTG values lower than the determination limit were transformed to half the value of the determination limit for each analysis. Statistical analysis between positive control and control groups was performed by either Student's *t*-test (parametric data) or Mann-Whitney *U* test (non-parametric data) after Levene's test. Where necessary, data were log-transformed.

57. Differences were considered to be significant at $p < 0.05$ in all tests; however, Bonferroni's *p* value was used in Mann-Whitney *U* test. All statistical analyses were performed by JMP ver.4.05J produced by SAS Institute Japan.

5. RESULTS

5.1 Analytical chemistry

5.1.1 Analytical chemistry of 4-tert-pentylphenol studies

58. Mean concentrations measured in both replicates at weekly intervals (3 weekly measurements in the 21-day studies) are reported in tables below for each test substance.

Table 8: The means of measured 4-*tert*-pentylphenol and positive control concentrations in the test solutions.

Means of measured concentrations (% nominal) in tank 1 and 2					
Nominal values		100 µg/L	320 µg/L	1,000 µg/L	PC[1] (100 ng/L)
LAB 1	Medaka	91.0(91)	284(89)	882(88)	102(102)
LAB 3		98.6(99)	314(98)	937(94)	103(103)
LAB 2		86.8(87)	287(90)	959(96)	105(105)
LAB 5		85.5 (86)	272 (90)	857 (85)	78 (78)
LAB 7	Fathead minnow	80.1(80)	270(85)	862(86)	90.6(91)
LAB 8		85.7(86)	298(93)	887(89)	No data
LAB 9		81 (81)	277 (80)	820 (82)	No data
LAB 12	Zebrafish	90.0(90)	294(92)	787(79)	No data
LAB 13		76.8(77)	229(72)	721(72)	No data
LAB 14		22.6(23)	69.5(22)	473(47)	No data

[1] Positive control (17β-estradiol)
Dark cells: low measured concentrations

59. The means of measured 4-*tert*-pentylphenol and positive control concentrations in the test solutions during the exposure period indicate that the nominal concentrations of these chemicals remained consistent and within the 80%-120% range throughout the exposure period in most of the studies. LAB 13 was slightly below the 80%-120% range; LAB 14 (see shaded cells) was very much below the expected range (23%, 22% and 47% respectively). LAB 14 explained that it had great difficulties preparing the stock solution without using solvent; the low measured concentration may therefore be due to test substance not sufficiently dissolved in the stock solution.

5.1.2 Analytical chemistry of prochloraz studies

60. The means measured concentrations of prochloraz and of the positive control remained consistent throughout the exposure period in most of the studies. The actual concentrations in a few laboratories tended to vary from the nominal concentrations.

Table 9: The means of measured prochloraz and positive control concentrations in the test solutions.

Means of measured concentrations (% nominal) in tank 1 and 2					
Nominal values		20 µg/L	100 µg/L	300 µg/L	PC[1] (100 µg/L)
LAB 1	Medaka	17.9(89)	92.5(92)	279(93)	94.5(95)
LAB 2		20.4(102)	94.6(95)	284(95)	111(111)
LAB 4		22.9(115)	99.5(100)	296(99)	99.4(99)
LAB 6		6.73(34)	54.0(54)	217(72)	16.7(17)
LAB 8	Fathead minnow	19.8(99)	97.5(98)	299(100)	No data
LAB 9		21.2 (105)	108 (108)	341 (114)	93 (93)
LAB 11		15.3(76)	68.9(69)	275(92)	97.1(97)
LAB 4		24.1(120)	121(121)	382(127)	105(105)
LAB 12	Zebrafish	14.7(73)	67.1(67)	166(55)	135(135)
LAB 13		19.0(95)*	82.7(83)	194(65)	41.6(42)
LAB 6		6.73(34)	54.0(54)	217(72)	16.7(17)

*: data of week 3 was excluded from mean calculation (tank 1: 415 µg/L, tank 2: 112 µg/L).
[1] Positive control (Fadrozole)
Dark cells: low measured concentrations

61. In particular, LAB 6 had very low measured concentrations for all the treatments, respectively 34%, 54% and 72% of nominal values for both the medaka and fathead minnow studies. LAB 12 and LAB 13 had low measured concentrations for the high dose group (55% and 65% of nominal values respectively).

5.1.3 Analytical chemistry of flutamide studies

62. The means of measured flutamide concentrations in the test solutions during the exposure period indicate that the nominal concentrations of these chemicals remained consistent throughout the exposure period in most of the studies.

Table 10: The means of measured flutamide concentrations in the test solutions.

Means of measured concentrations (% nominal) in tank 1 and 2				
Nominal values		100 µg/L	500 µg/L	1,000 µg/L
LAB 3	Medaka	95.5(95)	518(104)	1,060(106)
LAB 5		94.9 (95)	434.5 (87)	880.7 (88)
LAB 4		97.4(97)	501(100)	996(100)
LAB 6		55.8(56)	221(44)	552(55)
LAB 7	Fathead minnow	68.7(69)	354(71)	754(75)
LAB 10		83.5 (83)	445 (89)	875 (87)
LAB 11		Invalid because of high mortality rates		
LAB 4		88.8(89)	464(93)	940(94)
LAB 12	Zebrafish	76.6(77)	250(50)	788(79)
LAB 14		74.7(75)	397(79)	730(73)
LAB 6		55.8(56)	221(44)	552(55)

Dark cells: low measured concentrations

63. However, LAB 6 had very low measured concentrations for both the medaka and the zebrafish studies: 54%, 44% and 55% of nominal values respectively.

5.2 4-tert-pentylphenol studies

5.2.1 4-tert-pentylphenol studies with medaka

Mortality

64. Mortalities in the 4-*tert*-pentylphenol groups and positive control were ≤ 5% during the exposure period, except in LAB 5, where mortality in the treated groups was relatively high.

Table 11: Mortality (%) in the 4-*tert*-pentylphenol studies with medaka at the end of exposure.

Nominal values	Cont.	100 µg/L	320 µg/L	1,000 µg/L	PC[1] (100 ng/L)
LAB 1	0	0	0	0	0
LAB 3	0	0	0	0	0
LAB 2	0	0	0	5	0
LAB 5	0	0	20	20	35

[1]: PC= positive control (17β-estrdiol)

Spawning status

65. All control groups spawned well, except in LAB 2. No variation in spawning could be observed between the control groups and the 4-*tert*-pentylphenol treated groups and positive control in LAB 1. A dose-dependent decrease in spawning was observed in at least two of the studies (LAB 3 and LAB 5).

Table 12a: Spawning status in the 4-*tert*-pentylphenol studies with medaka during the exposure period.

No. of days with spawning "yes" (average of tank 1 and 2) / observed days					
Nominal values	Cont.	100 µg/L	320 µg/L	1,000 µg/L	PC[1] (100 ng/L)
LAB 1	21/21	21/21	21/21	21/21	21/21
LAB 2	14.5/21	21/21	18/21	16.5/21	19/21
LAB 3	20/21	19/21	20.5/21	13/21	21/21
LAB 5	21/21	19/21	15/21	11/21	13.5/21

[1]: PC= positive control (17β-estradiol)

66. A temporal recording of the spawning status indicates that when spawning tended to decrease, it was towards the second half of the 21-day study.

Table 12 b: daily recording of the spawning status in the 4-*tert*-pentylphenol studies with medaka.

LAB 1																						
C		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
L		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
M		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
H		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
LAB 2																						
C		½	½	1	1	½	1	1	1	1	1	1	0	0	1	½	½	½	1	½	½	½
L		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
M		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	½	½	0
H		½	½	1	1	1	1	1	1	1	1	1	1	½	½	½	½	½	½	½	½	½
LAB 3																						
C		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	½	1
L		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	½	1	1	½	1
M		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	½	1	1	1	1
H		1	1	1	1	1	1	1	1	1	1	0	1	0	0	½	½	0	½	½	0	0
LAB 5																						
C	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
L	½	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	½	½	0	½	1
M	½	1	1	1	1	1	1	1	1	1	1	1	½	½	½	½	½	0	½	0	0	½
H	1	1	1	1	1	1	1	1	1	½	1	½	0	0	0	0	0	0	0	0	0	0

1: spawning observed in both replicate tanks
½ : spawning observed in one of the replicate tanks
0: no spawning observed in any of the two replicate tanks.

67. LAB 1 recorded, as optional data, the number of eggs spawned daily by medaka under 4tPP treatment (Figure 1a), and the fertility of eggs (Figure 1b). No dose-dependent response was observed for any of the two parameters. Such information was not collected by the other laboratories.

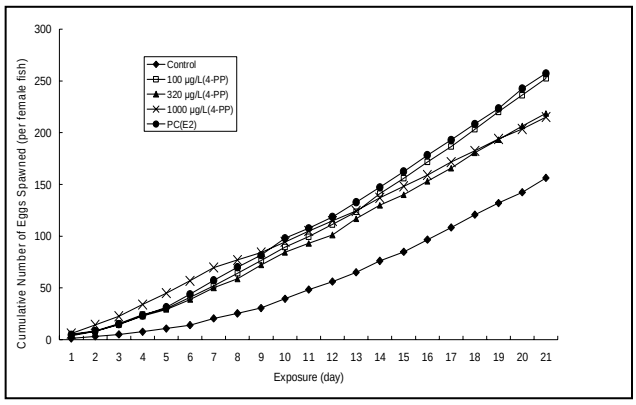


Fig. 1a: The cumulative number of eggs spawned in medaka 4-*tert*-pentylphenol.
(Optional data of LAB 1)

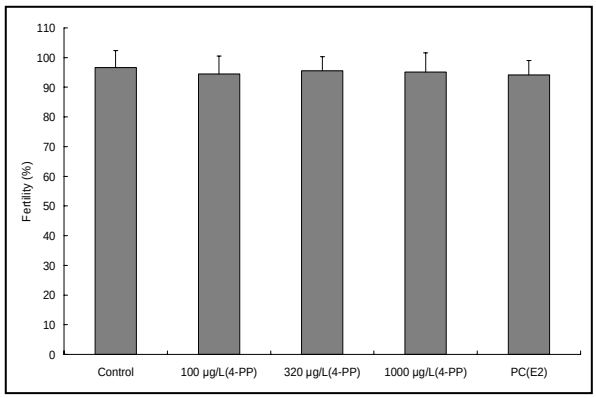


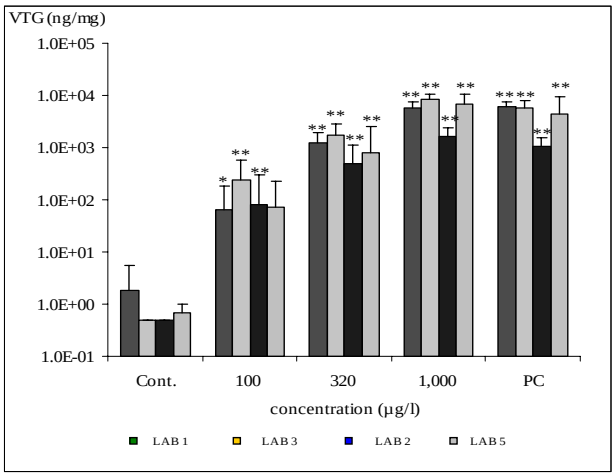
Fig. 1b: The means of fertility in medaka exposed to 4-*tert*-pentylphenol.
(Optional data of LAB 1)

Vitellogenin

68. In male and female medaka exposed to 4-*tert*-pentylphenol, hepatic VTG was induced in a concentration-dependent manner in all studies. Significant VTG increases compared to control were found in all exposed groups in both sexes, except in LAB 2 in females at 100 µg/l and in LAB 5 in females at all treatment levels -only the positive control was statistically significant.

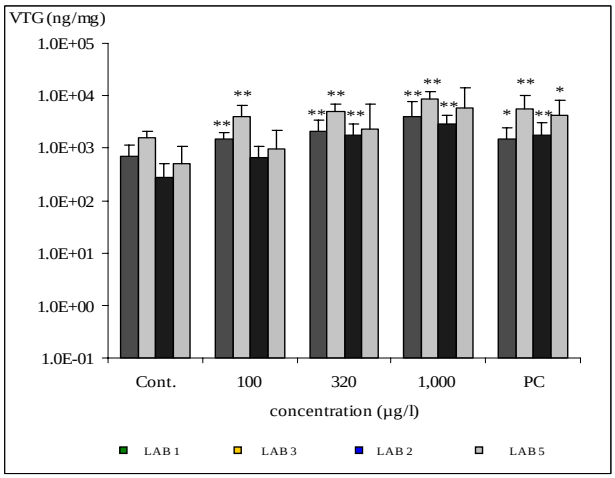
69. In LAB 5 in males, although Figure 2a seems to indicate a large increase of VTG in males at the lowest concentration, it is not statistically significant because only one animal had a high VTG level compared to the others.

70. In LAB 5 in females, statistical significance was not achieved despite a dose-dependent increase of VTG, probably due to a high variability of measurements within each group, combined with a reduced sample size because of mortality. Evaluation of variability within and across laboratories is provided in Section 6.



Significant level *:5% **:1%

Fig. 2a: VTG in male medaka exposed to 4-*tert*-pentylphenol.



Significant level *:5% **:1%

Fig. 2b: VTG in female medaka exposed to 4-*tert*-pentylphenol.

Secondary sex characteristics

71. Secondary sex characteristics in medaka appear in males as papillary processes on the anal fin. They normally respond to androgenic stimulation and can be quantitatively evaluated.

72. No significant difference in the number of joint plate with papillary processes was found in male medaka exposed to 4-tert-pentylphenol compared to control, except in LAB 3 at 1000µg/L. In females, no papillary process was observed in any of the treatment groups, nor in the positive control.

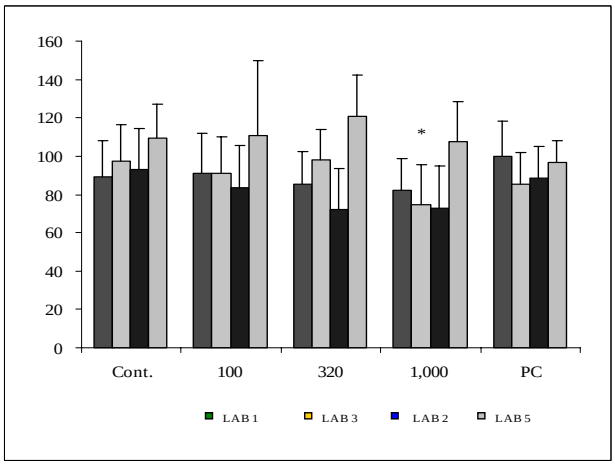


Fig. 3: Number of joint plate with papillary processes in male medaka exposed to 4-*tert*-pentylphenol.

73. In a study by Seki *et al*. (17), papillary processes in male medaka responded to estrogenic exposure to 4tPP in a full life-cycle experiment. At 224 µg/l, males exposed from fertilisation until 101 days post hatch, showed a significant decrease of papillary processes. In Phase 1B, it may be that the assay is either too short to see a response on the secondary sex characteristics, or not including exposure during the life-stage that is most sensitive.

Gonad histology

Table 13: Staging data for males and females medaka exposed to 4-tert-pentylphenol

Dose	MDK-Male							
	LAB 1		LAB 2		LAB 3		LAB 5	
	Median of staging	N	Median of staging	N	Median of staging	N	Median of staging	N
C	2	10	2	10	3	10	2	9
L	1	10	2	10	2	10	2	8
M	1	10	2	10	2	10	2	9
H	2	10	2	10	2	9	3	7
P	1	10	2	7	2	10	3	6
Dose	MDK-Female							
	LAB 1		LAB 2		LAB 3		LAB 5	
	Median of staging	N	Median of staging	N	Median of staging	N	Median of staging	N
C	2	9	3	10	0.5	10	2	9
L	2	10	3	10	1	10	2	7
M	2	10	2.5	10	1.5	10	2	7
H	2	10	1	10	0.5	10	1	7
P	2	10	3	10	1.5	10	1	7

74. There was no clear dose-related change in staging in either male or female medaka exposed to 4-tert-pentylphenol.

Table 14 a: Histopathological findings in male medaka exposed to 4-tert-pentylphenol.

Diagnosis	**Dose**	**LAB 1**			**LAB 3**			**LAB 2**			**LAB 5**		
		Average of Grade	**N**	**Obser.**	**Average of Grade**	**N**	**Obser.**	**Average of Grade**	**N**	**Obser.**	**Average of Grade**	**N**	**Obser.**
Increased cells - ICS	C												
	L										1	1	8
	M										1	1	9
	H												
	P												
Increased cells - SPA	C										2	2	9
	L										3	1	8
	M										2.7	3	9
	H	1	1	10	1.8	4	10				2.7	3	7
	P				1.5	4	7				2.5	4	6
Increased cells - SPZ	C										1.5	2	9
	L										1	1	8
	M										1	2	9
	H										1.3	6	7
	P										1.7	6	6
Decreased cells - SPA	C												
	L												
	M				1	1	10						
	H				1.5	2	10						
	P												
Decreased cells - SPC	C												
	L												
	M												
	H				1.8	4	10						
	P	3	1	10	1.8	4	7						
Decreased cells - SPT	C												
	L												
	M												
	H				1.8	4	10						
	P	3	1	10	1.8	4	7						
Decreased cells - SPZ	C												
	L												
	M										2	1	9

	H				1	1	10						
	P	3	1	10	1.8	4	7						
Testis-ova	C												
	L												
	M												
	H	1	1	10	1	2	10						
	P	3.3	6	10	2.8	4	7	1	1	10			
Testicular degeneration	C				1	1	10						
	L				1	2	10				2	2	8
	M				1	5	10				1.7	3	9
	H	1.4	7	10	1.9	9	10				1.8	6	7
	P	1.5	8	10	2	9	7				1.8	4	6
Asynchronous development, gonad	C												
	L												
	M										1	1	9
	H										1	1	7
	P										1.5	2	6
Asynchronous development, spermatocyte	P										2	1	6
Proteinaceous fluid(intravascular)	C				1	3	10				1	2	9
	L												
	M				1	3	10				1	2	9
	H	2	2	10	1.9	10	10				1.5	4	7
	P	1.5	2	10	1.8	10	7						
Proteinaceous fluid(interstitial)	C												
	L												
	M												
	H				1.7	7	10						
	P	1.5	2	10	1.1	9	7						
Interstitial fibrosis	C							2.5	2	10			
	L							2.3	3	10			
	M	1.5	2	10	1	1	10	2	1	10			
	H	1.8	5	10	2	4	10	3.3	3	9			
	P	1.9	7	10	1.7	6	7	3	4	10	1	5	6
Granulomatous inflammation	C										2	1	9
	L												
	M												
	H												
	P										1	1	6
histiocytic cells(intraluminal)	C												
	L	1	1	10	1.5	2	10			10			
	M				1.3	4	10			10	1	1	9
	H	1.3	6	10	1.2	5	10			9	1	1	7
	P	1.9	7	10	2.1	9	7			10			

C= control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.
ICS: interstitial cells; SPA: spermatogonia; SPZ: spermatozoa; SPC: spermatocytes; SPT: spermatids.
Dark cells: primary diagnoses identified

75. Pathologists from LAB 1, LAB 3 and LAB 5 identified treatment-related changes in the proportion of germ cells: an increase of spermatogonia at the high dose and positive control (dark cells). This was accompanied by a decreased proportion of spermatocytes, spermatids and spermatozoa in LAB 1 and LAB 3. Testicular degeneration was reported several timesin treated groups in LAB 1, LAB 3 and LAB 5. The presence of testis ova was found in LAB1, LAB 2 and LAB 3. Dose-dependent increases were more pronounced by the number of animals affected than by the severity of diagnosis reported.

76. Other findings included a dose-dependent increase of intravascular and/or interstitial proteinaceous fluid, a dose-dependent increase of histiocytic cells (intraluminar).

Table 14 b: Histopathological findings in female medaka exposed to 4-tert-pentylphenol.

Diagnosis	Dose	LAB 1			LAB 3			LAB 2			LAB 5		
		Average of Grade	N	Obser.	Average of Grade	N	Obser.	Average of Grade	N	Obser.	Average of Grade	N	Obser.
Increased cells - OOG	C												
	L												
	M												
	H										2	1	7
	P												
Decreased cells	H							2	1				
Oocyte atresia, increased, immature	C				1	1	9	1	4	10	2	2	9
	L				2.3	4	10	1	1	10	1	2	7
	M	1	2	10	2.3	4	10	1.7	3	10	1.3	6	7
	H	1.67	3	10	2.6	8	10	1	3	10	1.6	7	7
	P	1	1	10	2.3	4	10				1.3	4	7
Oocyte atresia, increased, mature	C										2	1	9
	L	2	1	10	1	2	10						
	M	1	2	10				1	1	10			
	H	1	2	10	1	3	10	2.3	3	10	1.3	3	7
	P				2	1	10				1	1	7
Oocyte atresia, increased, late atretic											3	2	9
											1	1	7
											1	1	7
											1	1	7
											1	1	7
Proteinaceous fluid, interstitial	C												
	L	1.5	2	10	2	1	10						
	M	2	2	10	1.8	5	10	2	2	10	1.6	5	7
	H	1.8	4	10	2.4	8	10	1.5	2	10	1.8	4	7
	P	2	2	10	1.5	4	10	1	2	10	1.4	5	7
Interstitial fibrosis	C							2	1	10			
	L												
	M												
	H												
	P												
Granulomatous inflammation	C										2.3	6	9
	L										1.5	2	7
	M										2	5	7
	H										1	1	7
	P										2	3	7
Macrophage aggregates, increased	C												
	L				1	1	10						
	M				1.7	3	10						
	H				2.3	3	10						
	P												
Oocyte membrane folding	C												
	L												
	M												
	H							2	1	10			
	P												
Egg debris, oviduct	C												
	L				1	2	10						
	M				1	1	10						
	H				1	1	10						
	P				1	1	10						

C= control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.
OOG: oogonia;
Dark cells: primary diagnoses identified

77. Females medaka exposed to 4-tert-pentyphenol showed an increase of immature and mature oocyte atresia, reported in all four laboratories. Interstitial proteinaceous fluid also increased dose-dependently in all four laboratories.

5.2.2 4-tert-pentylphenol studies with fathead minnow

Mortality

78. Mortalities in the 4-*tert*-pentylphenol groups and positive control were ≤ 5% in LAB 7, although those increased dose-dependently in 4-*tert*-pentylphenol exposure conducted at LAB 8. In LAB 9, mortalities in the medium concentration were 100% on the last day of the experiment because fish suffered from over-dosage of the test substance – VTG data and secondary sex characteristics for LAB 9 are therefore not reported in this section.

Table 15: Mortality in the 4-*tert*-pentylphenol studies with fathead minnow at the end of exposure.

Laboratories	Nominal concentrations				
	Cont.	100 µg/L	320 µg/L	1,000 µg/L	PC[1] (100 ng/L)
LAB 7	0	0	0	5	5
LAB 8	0	0	15	45	0
LAB 9	0	10	100	30	5

[1]: PC= positive control (17β-estradiol)

Spawning status

79. The control groups did not spawn very well throughout the study, especially in LAB 8 and LAB 9. Fathead minnow are territorial and the group-spawning conditions proposed in Phase 1B were sub-optimal for fathead minnow. Therefore a different male to female sex ratio (e.g. 2:4) may be more appropriate.

80. Spawning was reduced in a concentration-dependent manner in all studies; especially no eggs were produced in the highest concentration of 4-*tert*-pentylphenol in all three studies.

Table 16a: Spawning status of fathead minnow exposed to 4-*tert*-pentylphenol.

No. of days with spawning "yes" (average of tank 1 and 2) / observed days					
Laboratories	Nominal concentrations				
	Cont.	100 µg/L	320 µg/L	1,000 µg/L	PC[1] (100 ng/L)
LAB 7	17.5/21	14.5/21	13/21	0/21	15/21
LAB 8	4.5/21	3/21	0.5/21	0/21	2/21
LAB 9	7/21	6/21	1/21	0/21	3.5/21

[1]: PC= positive control (17β-estrdiol)

Table 16b: Daily recording of the spawning status iof fathead minnow exposed to 4-*tert*-pentylphenol.

LAB 7																							
C	0	½	1	½	1	1	1	1	½	1	1	1	½	½	1	1	1	½	1	1	1	1	
L	0	½	½	½	½	½	1	½	1	½	1	½	1	1	1	½	1	½	½	½	1	½	
M	0	1	½	1	1	0	½	½	1	½	½	½	½	1	½	½	½	½	1	½	½	½	
H	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
LAB 8																							
C	0	1	½	0	0	½	1/2	0	½	0	0	0	0	0	0	½	1/2	0	0	½	0	0	
L	0	½	0	0	0	½	0	0	½	½	0	½	0	0	0	0	0	0	0	0	0	½	
M	0	0	0	0	0	0	0	0	0	0	0	½	0	0	0	0	0	0	0	0	0	0	
H	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
LAB 9																							
C		½	½	½	1	0	0	½	0	½	½	½	0	0	½	0	½	½	½	0	½	0	
L		0	0	½	0	½	1	½	½	½	½	0	0	½	0	½	0	0	0	½	0	½	
M		0	0	½	½	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
H		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

1: spawning observed in both replicate tanks
½ : spawning observed in one of the replicate tanks
0: no spawning observed in any of the two replicate tanks.

Vitellogenin

81. Due to a problem in LAB 9 with the test substance dispenser in the last hours of the experiment, resulting in either mortalities on day 20 of the experiment (mid-dose group) , or in non-exposure in the last 15 hours (high dose group), VTG results do not appear in the following graphs.

82. In male fathead minnow exposed to 4-*tert*-pentylphenol serum VTG was induced in a dose dependent manner at ≥ 320 µg/L, resulting in significant differences at this and higher concentrations. In females, VTG levels also increased in the 4-*tert*-pentylphenol treatment groups and positive control.

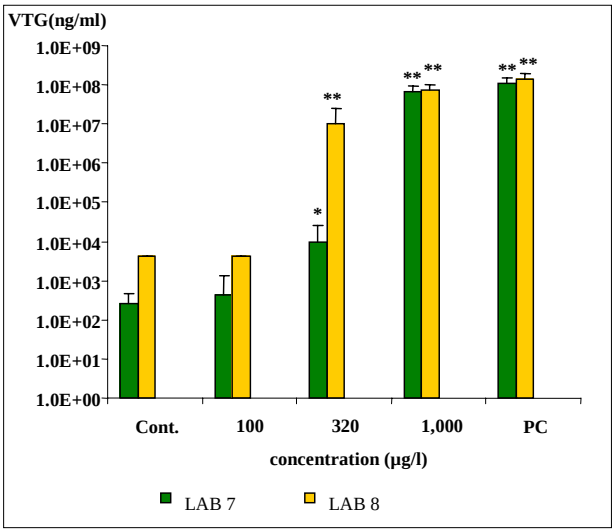


Fig. 4a: VTG in male fathead minnow exposed to 4-*tert*-pentylphenol.

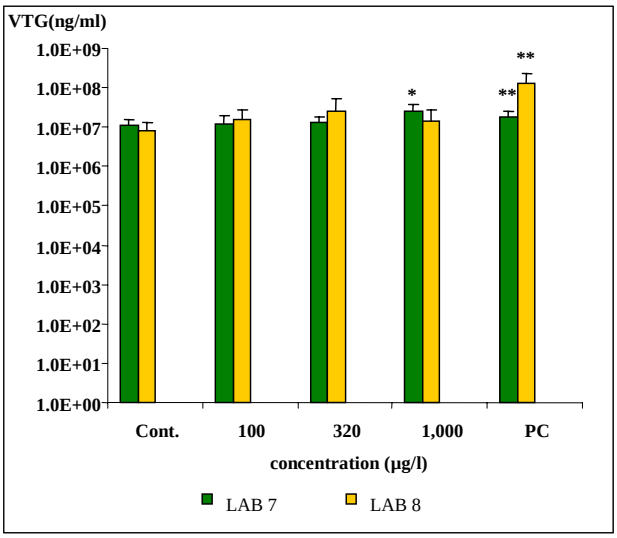


Fig. 4b: VTG in female fathead minnow exposed to 4-*tert*-pentylphenol.

Secondary sex characteristics

83. In male fathead minnow the total score of nuptial tubercles decreased with increasing 4-*tert*-pentylphenol concentration, resulting in significant differences at 1,000µg/L in all three studies. No nuptial tubercle was observed in any of the treatment group or positive control in females.

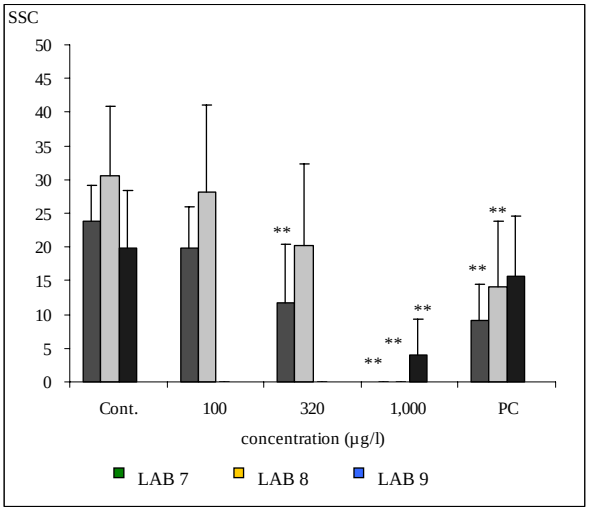


Fig. 5: Total score of the nuptial tubercles in male fathead minnow exposed to 4-*tert*-pentylphenol.

Gonad histology

Table 17: Staging data for males and females fathead minnow exposed to 4-tert-pentylphenol

Dose	FHM-Male					
	LAB 7		LAB 8		LAB 9	
	Median of staging	N	Median of staging	N	Median of staging	N
C	2	10	2	10	3	10
L	2	10	2.5	10	-	
M	2	10	2	7	-	
H	3	2*	2	1	3	9
P	2	9	2	10	2	9
Dose	FHM-Female					
	LAB 7		LAB 8		LAB 9	
	Median of staging	N	Median of staging	N	Median of staging	N
C	3	10	3	10	3	10
L	2.5	10	3	10	-	
M	2	10	3	8	-	
H	2	10	4	6	3	7
P	3	10	3	10	4	10

*7 additional fish were severely affected and could not be staged

Table 18a: Histopathological findings in male fathead minnow following 4-tert-pentylphenol exposure.

Diagnosis	Dose	LAB 8			LAB 7			LAB 9		
		Aver. Grade	N	Obser.	Aver. Grade	N	Obser.	Aver. Grade	N	Obser.
Increased cells – SPA	C	1	2	10				1	1	10
	L	1	1	10						
	M	2	2	7						
	H	2.8	5	1	3.3	7	2	2.3	7	9
	P	1.7	7	10				1.7	6	9
Increased cells – SPZ	H				4	6	H			
Decreased cells - SPC	C									
	L									
	M	2.8	4	7						
	H	3.6	5	1	3.4	7	2	2.25	4	9
	P	2.3	7	10				2	2	9
Decreased cells - SPT	C									
	L									
	M	3.4	5	7						
	H	3.8	5	1	3.3	7	2	2.3	4	9
	P	3.5	8	10				2	2	9
Decreased cells - SPZ	C	2	2	10				3	1	10
	L									
	M	2.8	5	7						
	H	2.6	5	1						
	P	2.9	7	10						
Testis-ova	C									
	L									
	M	3.0	1	7						
	H									
	P									
Testicular degeneration	C							1.5	2	10
	L									
	M	1	1	7						
	H				4	7	2	1.3	6	9
	P	1.3	4	10				1	2	9
Asynchronous development, spermatocyst	C									
	L									
	M									
	H							2	1	9
	P									
Asynchronous development, gonad	C									
	L									
	M									
	H									
	P							2	1	9
Proteinaceous fluid, intravascular	H							1.3	4	9
	P	1.5	2	10				1.4	9	9
Proteinaceous fluid, interstitial	C	1.5	2	10						
	L	1.1	7	10						
	M	1.6	7	7						
	H	2.4	5	1						
	P	3.4	10	10						
Interstitial fibrosis								2	2	9
Granulomatous inflammation	C									
	L									
	M									
	H							1	1	9
	P							1	1	9
Histiocytic cells, intraluminal	C							1.8	4	10
	L									
	M									
	H							2.5	6	9
	P							1	1	9

Retained peritoneal attachments	C			1	3	10
	L					
	M					
	H			1.2	6	9
	P			1.4	5	9

C= control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.
ICS: interstitial cells; SPA: spermatogonia; SPZ: spermatozoa; SPC: spermatocytes; SPT: spermatids.
Dark cells: primary diagnoses identified

84. In male fathead minnows exposed to 4-tert-pentylphenol, an increase in the proportion of spermatogonia was reported in all three laboratories. A decrease in the proportion of spermatocytes and spermatids was also reported in all three laboratories. A decreased proportion of spermatozoa was found in one study only.

85. A dose-dependent increase of animals with testicular degeneration was identified in all three studies. One study also identified an increase of interstitial proteinaceous fluid.

Table 18b: Histopathological findings in female fathead minnow following 4-tert-pentylphenol exposure.

Diagnosis	Dose	LAB 8			LAB 7			LAB 9		
		Aver. Grade	N	Obser.	Aver. Grade	N	Obser	Aver. Grade	N	Obser
Increased cells - LVO	L	3	1							
Increased cells - MSO	C									
	L	2	2	10						
	M									
	H									
	P	3	1	10						
Decreased cells - LVO	C	1.5	2	10						
	L	1.5	2	10						
	M									
	H									
	P	2	1	10						
Oocyte atresia, increased, immature	C							2	2	10
	L									
	M									
	H				3	2	10	1.5	4	7
	P							2	2	10
Oocyte atresia, increased, mature	C	1	1	10						
	L	1.5	4	10						
	M	1	2	8						
	H	2.3	3	6				1	1	7
	P	1.5	2	10						
Asynchronous development, gonad	P							1	1	
Proteinaceous fluid, intravascular	C									
	L									
	M									
	H							2	1	7
	P							1.5	8	10
Proteinaceous fluid, interstitial	C							2	1	10
	L	2	2	10						
	M	1	2	8						
	H	2	2	6				1	2	7
	P	2.2	10	10				2	6	10
Post-ovulatory follicles, increased	C	1.5	4	10						
	L	1.6	5	10						
	M	1.3	7	8						
	H	1	1	6						
	P	2	6	10						
Hepatocyte basophilia, decreased	H	2	3							

Granulomatous inflammation	C	1	1	10						
	L				1	2	10			
	M				1	1	10			
	H				3	4	10			
	P	1.5	2	10	1	5	10			
Oocyte membrane folding	C	1.5	2	10						
	L	1.3	3	10						
	M	1	2	8						
	H									
	P	1.2	5	10						
Egg debris, oviduct	C	2.8	4	10						
	L	2.4	5	10						
	M	2.2	6	8						
	H	1	1	6	4	1	10			
	P	2.3	6	10						
Ovarian cyst	C							1.5	2	10
	L									
	M									
	H							1	1	7
	P							1,3	4	10
Oocyte atresia, late, atretic	C							3	1	10
	L									
	M									
	H							3	1	7
	P									

C= control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.
MSO: mature/spawning oocytes; LVO: late vitellogenic oocytes;
Dark cells: primary diagnoses identified.

86. Histopathological findings in female fathead minnows were less consistent across laboratories than findings in males. Oocyte atresia (mature or immature), including oocyte membrane folding was identified in all studies, but also in control animals.

87. Egg debris in the oviduct and increased post-ovulatory follicles were found in a number of animals in LAB 8, including in the control group.

88. As described earlier in the section, the spawning status in control groups of fathead minnow in these studies was not good; especially in LAB 8 and LAB 9. This may explain that some histopathological findings were also reported in control animals.

5.2.3 4-tert-pentylphenol studies with zebrafish

Mortality

89. Mortalities in the 4-*tert*-pentylphenol groups and positive control in all studies were ≤ 10% during the exposure period.

Table 19: Mortality (%) in the 4-*tert*-pentylphenol studies with zebrafish at the end of exposure.

Laboratories	Nominal concentrations					
	Cont.	SC[1]	100 µg/L	320 µg/L	1,000 µg/L	PC[2] (100 ng/L)
LAB 12	5	0	5	0	10	0
LAB 13	0	-	0	0	0	0
LAB 14	0	-	0	5	5	5

[1] : SC=solvent control (acetone); [2]: PC= positive control

Spawning status

90. Control groups spawned regularly throughout the 21-day experiment in all three studies. No clear decrease of spawning could be observed following chemical treatment in any of the three studies.

Table 20a: Spawning status in the 4-*tert*-pentylphenol studies with zebrafish during the exposure period.

No. of days with spawning "yes" (average of tank 1 and 2) / observed days						
Laboratories	Nominal concentrations					
	Cont.	SC[1]	100 µg/L	320 µg/L	1,000 µg/L	PC[2] (100 ng/L)
LAB 12	19.5/21	-	20/21	19/21	16.5/21	20.5/21
LAB 13	6/6(tank 1) 6/6(tank 2)	-	7/7(tank 1) 4/6(tank 2)	7/7(tank 1) 3/6(tank 2)	7/7(tank 1) 3/6(tank 2)	7/7(tank 1) 5/6(tank 2)
LAB 14	18/21	-	18/21	19.5/21	18/21	No data

[1] : SC=solvent control (acetone); [2]: PC= positive control

Table 20b: Daily recording of spawning status of zebrafish exposed to 4-*tert*-pentylphenol.

LAB 12																							
C		1	½	1	1	1	1	1	½	1	1	1	1	1	1	1	1	1	1	1	1	½	
L		½	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	½	1	
M		1	½	1	½	1	1	1	1	1	1	11	1	1	1	1	1	1	1	1	½	½	
H		½	1	½	½	1	1	½	½	1	1	1	½	1	1	1	1	½	½	1	½	½	
LAB 13																							
C							1			1			1			1			1			1	
L					1	1		1	0		1	1		1	0		1	1		1	1	1	
M					1	1		1	0		1	1		1	0		1	0		1	1	1	
H					1	1		1	0		1	1		1	1	1	0		1	0	1		
LAB 14																							
C	1	1	½	1	½	1	1	1	½	1	1	1	½	1	1	1	1	1	1	1	1	0	
L	1	1	1	1	½	1	1	½	½	1	½	1	1	1	½	1	1	½	1	1	1	1	
M	1	1	1	½	1	1	1	1	1	1	1	1	1	1	1	1	½	1	1	1	1	½	
H	1	1	1	1	1	1	1	1	½	1	1	1	½	1	1	1	1	½	1	0	1	½	

1: spawning observed in both replicate tanks

½ : spawning observed in one of the replicate tanks

0: no spawning observed in any of the two replicate tanks.

91. LAB 12, LAB 13 and LAB 14 collected optional data on egg counts. Cumulative numbers of eggs are reported in Figures 6b, 6c and 6e. In LAB 12 and LAB 14, there was no treatment-related response and the tanks where females produced fewer eggs were not necessarily those with treated animals. In LAB 13 (Figure 6e), there was a dose-dependent decrease of spawning, although statistical significance could not be reached, except in the positive control, because of high variability.

92. Fertility was not affected by 4tPP treatment in LAB 12 (Figure 6a). In LAB 13 (Figs 6d), although there was a decreasing trend in fertility, statistical significance could not be reached, except in the positive control, because of high variability.

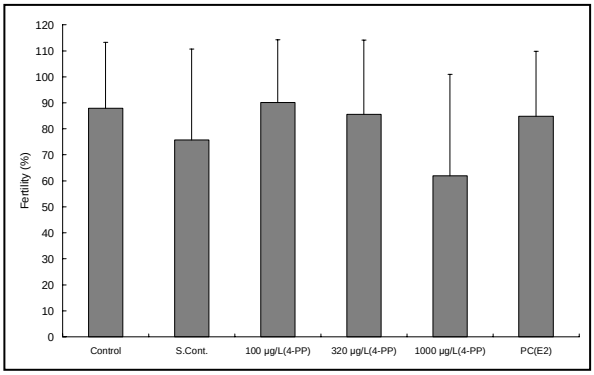


Fig. 6a: The means of fertility in zebrafish exposed to 4-*tert*-pentylphenol.
(Optional data of LAB 12)

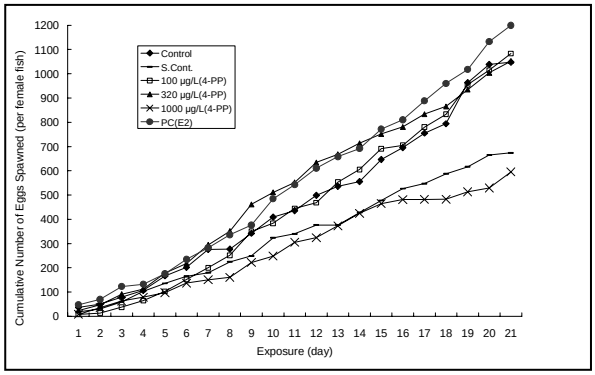


Fig. 6b: The cumulative number of eggs spawned in zebrafish exposed to 4-*tert*-pentylphenol.
(Optional data of LAB 12)

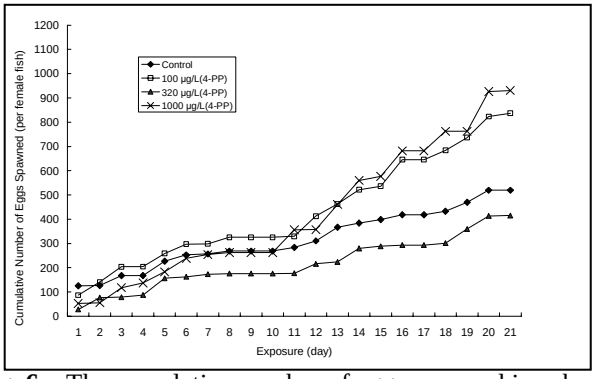


Fig. 6c: The cumulative number of eggs spawned in zebrafish exposed to 4-*tert*-pentylphenol.
(Optional data of LAB 14)

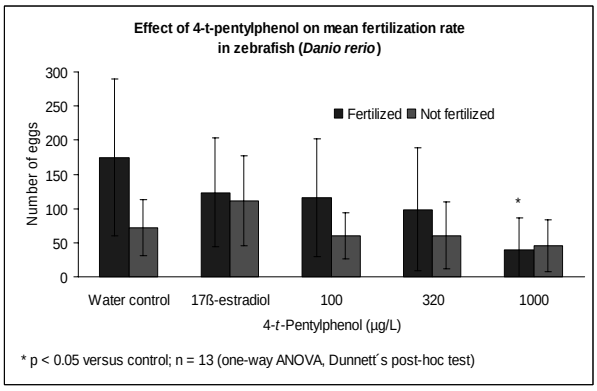


Fig. 6d: Effect of 4-*tert*-pentylphenol on mean fertilization rate in zebrafish (*Danio rerio*).
(Optional data of LAB 13)

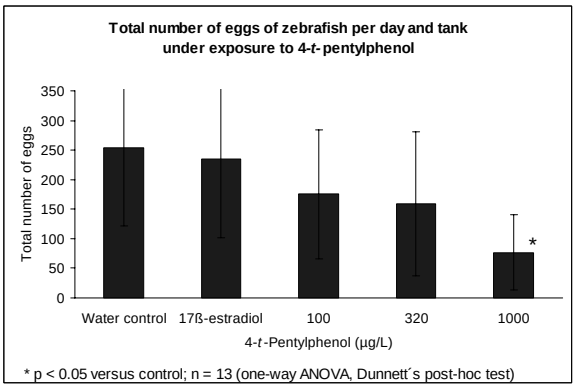


Fig. 6e: Total number of eggs of zebrafish per day and tank under exposure to 4-*tert*-pentylphenol
(Optional data of LAB 13)

Vitellogenin

93. In male zebrafish (Figure 7a) serum VTG was induced with increasing 4-*tert*-pentylphenol concentration higher than 320µg/L. Although VTG levels increased in females, it was less pronounced than in males, due to high background level in control animals.

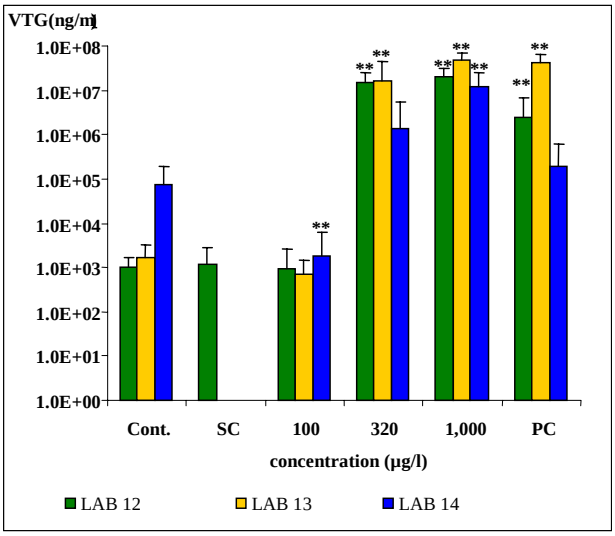


Fig. 7a: VTG in male zebrafish exposed to 4-*tert*-pentylphenol.

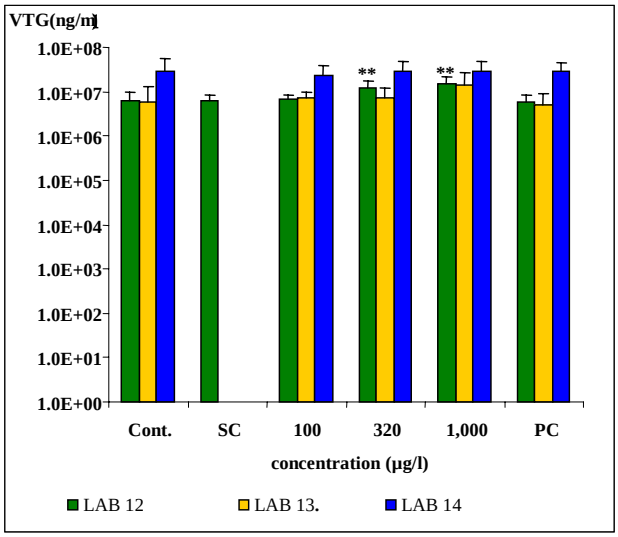


Fig. 7b: VTG in female zebrafish exposed to 4-*tert*-pentylphenol.

94. It is worth noting that LAB 14 had very low measured concentrations of the test substance: 23%, 22% and 47% of nominal values respectively. The highest measured concentration was 473 µg/l and induced a significant increase of VTG in male zebrafish in LAB 14. These low concentrations combined with a relatively high VTG value in control prevented the detection of statistically significant increase of VTG in at 320µg/l in LAB 14 in male zebrafish exposed to 4-tert-pentylphenol.

Gonad histology

Table 21: Staging data for males and females zebrafish exposed to 4-tert-pentylphenol.

Dose	ZBR-Male					
	LAB 12		LAB 13		LAB 14	
	Median of staging	N	Median of staging	N	Median of staging	N
C	2	8	2	9	2	9
L	1.5	4	2	11	2	8
M	2	5	1	12	2	9
H	1	6	2	11	1.5	10
P	1.5	7	1	10	2	7
Dose	ZBR-Female					
	LAB 12		LAB 13		LAB 14	
	Median of staging	N	Median of staging	N	Median of staging	N
C	2	9	3	10	2	9
L	2	8	2	9	3	9
M	2	9	3	10	2	9
H	2	9	2.5	7	3	8
P	2	9	2	8	2	8

Table 22a: Histopathological findings in male zebrafish exposed to 4-tert-pentylphenol.

Diagnosis	Dose	LAB 12			LAB 13			LAB 14		
		Average of Grade	N	Obser.	Average of Grade	N	Obser	Average of Grade	N	Obser.
Increased cells - ICS	C									
	L									
	M									
	H							1	2	10
	P							1	5	7
Increased cells - SPA	C									
	L				2	2	11	1	1	8
	M	1.7	3	5	1.9	7	12	1	2	9
	H	2	1	6	2.9	7	11	1	4	10
	P	2	1	7	2	6	10	1	4	7
Increased cells - SPC	H	1	1	6						
Decreased cells - SPT	P	3	1	7						
Decreased cells - SPZ	C									
	L				1.3	3	11			
	M				1.9	8	12			
	H				2.7	9	11			
	P				2.1	9	10			
Testis-ova	C									
	L									
	M									
	H	1	2	6						
	P	3	1	7						
Testicular degeneration	H				3	1	11			
Asynchronous development, spermatocyst	H							1	2	10
Proteinaceous fluid, intravascular	M	1	4	5						
	H	1.5	6	6						
Interstitial fibrosis	C									
	L				1	3	11			
	M				1.8	5	12			
	H				3.3	3	11	1	1	10
	P				2	2	10			
Sertoli cell hypertrophy	C									
	L									
	M	1.3	4	5						
	H	2	4	6						
	P	1.8	4	7						

C= control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.
ICS: interstitial cells; SPA: spermatogonia; SPZ: spermatozoa; SPC: spermatocytes; SPT: spermatids.
Dark cells: primary diagnoses identified

95. In males zebrafish expose to 4-tert-pentylphenol, an increase in the proportion of spermatogonia was reported in all studies. A dose-dependent decrease of spermatozoa was reported by LAB 13.

96. Other findings were not consistently reported in all studies.

Table 22b: Histopathological findings in female zebrafish exposed to 4-tert-pentylphenol.

Diagnosis	Dose	LAB 12			LAB 13			LAB 14		
		Average of Grade	N	Obser.	Average of Grade	N	Obser.	Average of Grade	N	Obser.
Increased cells - PNO	C									
	L				3	1	9			
	M				2	3	10			
	H									
	P				1	1	8			
Increased cells - MSO	M				2.5	2	10			
Increased cells - PFC	C							1.3	3	9
	P							3	1	7
Decreased cells-EVO	H				2	1	7			
Oocyte atresia, increased, immature	M				2	1	10			
Oocyte atresia, increased, mature	C	1	3	9				1	3	9
	L	1.5	4	8				1	1	8
	M	1.6	7	9				1.3	3	9
	H	1.8	9	9				1	1	10
	P	1.8	4	9						
Proteinaceous fluid, intravascular	C			9						
	L	1	2	8						
	M	1	6	9						
	H	1.3	6	9						
	P	1	3	9						
Proteinaceous fluid, interstitial	C							1.2	5	9
	L							1	1	8
	M							1	4	9
	H							1	2	10
	P							2.3	3	7
Interstitial fibrosis	C									
	L				1.8	5	9	1	1	8
	M				2	4	10			
	H				1.6	5	7			
	P				1.5	2	8			
Post-ovulatory follicles, increased	C	2	1	9						
	L	2	1	8						
	M				2	1	10			9
	H									
	P				1.5	2	8			7
Egg debris, oviduct	C	1.7	6	9						
	L	1.8	6	8						
	M	1.8	5	9						
	H	2	3	9						
	P	2.3	6	9						

C= control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.
PNO: perinucleolar oocytes; MSO: mature/spawning oocytes; EVO: early vitellogenic oocytes; PFC: perifollicular oocytes;
Dark cells: primary diagnoses identified.

97. No clear treatment-related finding was consistently reported in female zebrafish exposed to 4-tert-pentylphenol. For several findings, they equally occurred in control females too (e.g. egg debris in oviduct, proteinaceous fluid, mature oocyte atresia, increased perifollicular cells).

5.3 Prochloraz studies

5.3.1 Prochloraz study with medaka

Mortality

98. Mortalities in the prochloraz groups and positive control in all studies were ≤ 10% during the exposure period.

Table 23: Mortality (%) in the prochloraz studies with medaka at the end of exposure.

	Nominal concentrations				
Laboratories	Cont.	20 µg/L	100 µg/L	300 µg/L	PC[1] (100 µg/L)
LAB 1	0	0	0	5	0
LAB 2	0	0	0	5	10
LAB 4	0	0	0	0	5
LAB 6	0	0	0	0	0

Spawning status

99. Spawning in all of the control groups was regular and continuous throughout the 21-day study. In the medium (100 µg/L) and high-dose (300 µg/L) groups, females progressively ceased to spawn.

Table 24a: Spawning status in the prochloraz studies with medaka during the exposure period.

No. of days with spawning "yes" (average of tank 1 and 2) / observed days					
	Nominal concentrations				
Laboratories	Cont.	20 µg/L	100 µg/L	300 µg/L	PC[1] (100 µg/L)
LAB 1	21/21	21/21	12/21	3/21	8.5/21
LAB 2	18.5/21	20.5/21	5/21	2/21	1.5/21
LAB 4	21/21	21/21	11/21	2/21	1.5/21
LAB 6	19/20	15.5/20	13/20	1/20	1/20

[1:]: PC= Positive control

Table 24b: Daily recording of spawning status in the prochloraz studies with medaka exposed to prochloraz.

LAB 1																							
C		1	1	1	1	1	1	1	1	1	1	1	1	1	1		1	1	1	1	1	1	
L		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
M		1	1	½	½	1	½	0	1	1	1	0	1	½	½	1	½	0	0	½	0	½	
H		1	1	½	0	0	½	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
LAB 2																							
C		½	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	½	½	½	
L		1	½	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
M		1	½	1	0	½	½	1	½	0	0	0	0	0	0	0	0	0	0	0	0	0	
H		1	½	0	0	0	0	0	0	½	0	0	0	0	0	0	0	0	0	0	0	0	
LAB 4																							
C		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
L		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
M		½	½	1	½	0	½	1	1	½	1	0	½	1	0	½	½	0	0	0	0	½	
H		0	0	1	½	0	0	0	½	0	0	0	0	0	0	0	0	0	0	0	0	0	
LAB 6																							
C	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		
L	0	0	0	½	½	½	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1		
M	0	0	½	0	½	½	½	1	1	½	0	½	½	1	1	1	1	1	½	1	1		
H	0	0	½	½	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		

1: spawning observed in both replicate tanks

½ : spawning observed in one of the replicate tanks

0: no spawning observed in any of the two replicate tanks.

100. As optional data, LAB 1 recorded daily the number of eggs spawned and the fertility. Fertility was not clearly affected by chemical treatment.

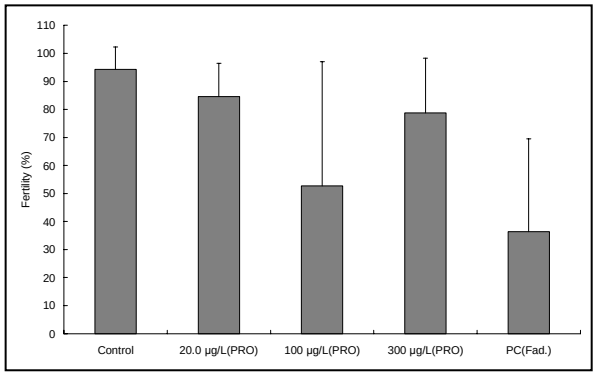


Fig. 8a: The means of fertility in medaka exposed to prochloraz. (Optional data of LAB 1)

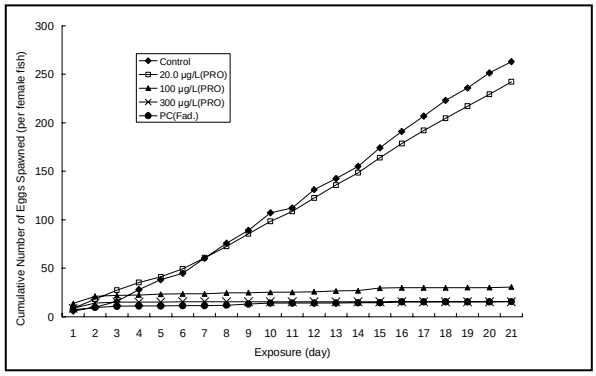


Fig. 8b: Cumulative number of eggs spawned in medaka exposed to prochloraz. (Optional data of LAB 1)

Vitellogenin

101. In male medaka exposed to prochloraz no consistent dose-dependent response could be observed on VTG levels across laboratories, although several significant differences were found. In female medaka VTG concentrations decreased dose-dependently in all studies.

102. Measured concentrations in LAB 6 were only 34%, 54% and 72% of nominal values whereas other laboratories maintained concentrations close to 100% niminal values. These low concentrations in LAB 6 could explain the lack of statistically significant deacrease of VTG in females at 20 µg/L and 100 µg/L prochloraz.

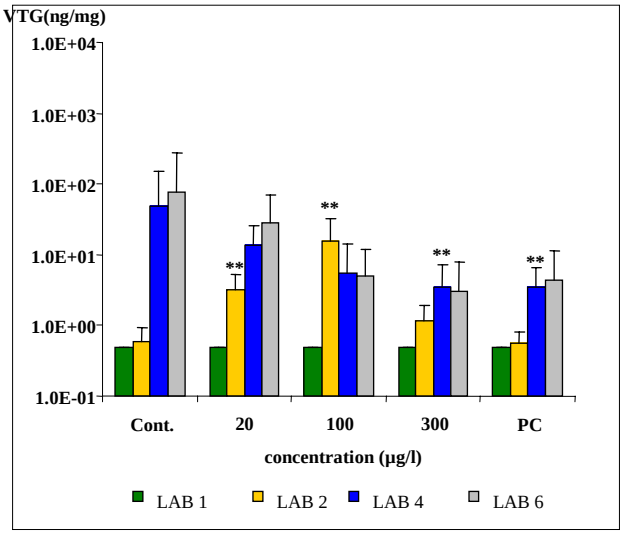


Fig. 9a: VTG in male medaka exposed to prochloraz.

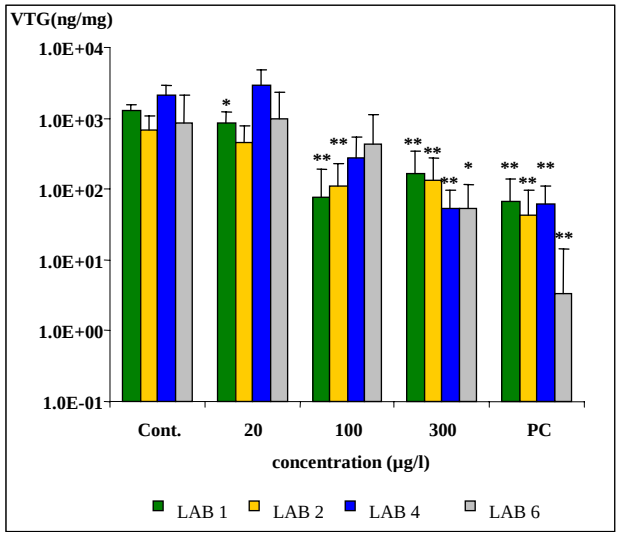


Fig. 9b: VTG in female medaka exposed to prochloraz.

103. Decrease in vitellogenin level in females and cessation of spawning are consistent with the aromatase inhibition mode of action, where conversion of testesterone and keto-testosterone to 17beta-estradiol is inhibited, thus preventing vitellogenin production and susequent egg production.

104. It is worth noting that for low VTG values (Figure 9a), absolute numbers varied between LAB 1 and LAB 3 (Japanese laboratories) and LAB 4 and LAB 6 (US and European laboratories).

Secondary sex characteristics

105. The number of papillary process in male medaka did not respond to prochloraz exposure, or to fadrozole exposure in any of the studies. No papillary process was observed in any of the treatment groups or positive control in females. This is consistent with findings from the literature on fathead minnow exposed to aromatse inhibitor treatment (18)(19).

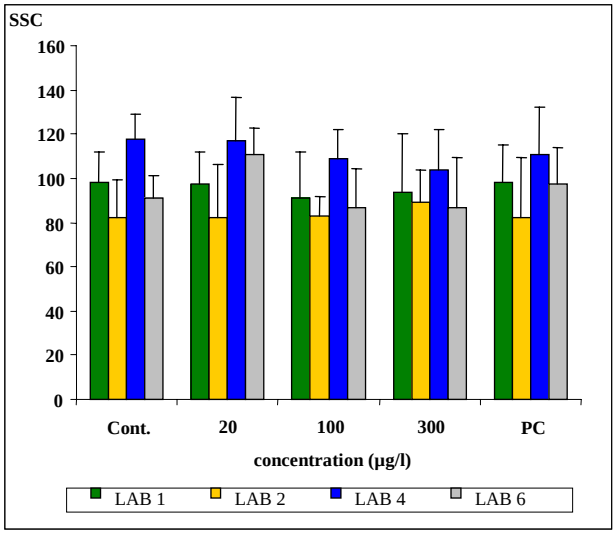


Fig. 10: Number of the joint plate with papillary processes in male medaka exposed to prochloraz.

Gonad histology

Table 25: Staging data for males and females medaka exposed to prochloraz.

Dose	MDK-Male					
	LAB 1		LAB 2		LAB 4	
	Median of staging	N	Median of staging	N	Median of staging	N
C	2	10	2	10	2	10
L	2	10	2	10	2	9
M	2	10	2	10	2	10
H	3	10	2	10	3	9
P	2	10	2	10	3	10

Dose	MDK-Female					
	LAB 1		LAB 2		LAB 4	
	Median of staging	N	Median of staging	N	Median of staging	N
C	2	10	2	10	2	10
L	2	10	1.5	10	2	10
M	1	10	0	10	1	10
H	1	9	0	10	1	10
P	1	10	0	10	1	9

Table 26 a: Histopathological findings for male medaka exposed to prochloraz.

Diagnosis	Dose	LAB 1			LAB 2			LAB 4			LAB 6		
		Average of Grade	N	Obs.	Average of Grade	N	Obs.	Average of Grade	N	Obs.	Average of Grade	N	Obs.
Increased cells - ICS	C												
	L							1	2	9			
	M							1	6	10	2.5	2	10
	H							1.6	9	9	1.7	4	10
	P							1	3	10			
Increased cells - SPZ	C							1	2	10			
	L							1.3	3	9			
	M							1.3	4	10			
	H							1.7	9	9	2	1	10
	P							1.3	6	10			
Decreased cells- SPA	M										2	1	10
	H										2	1	10
Decreased cells- SPZ	L										2	1	10
	M										1.3	3	10
Testis-ova	C										3	1	10
	L										2.6	3	10
	M										2.3	3	10
	H										1.5	2	10
Testicular degeneration	L										4	2	10
Sertoli cell hypertrophy	M										3	1	10
	H										2	1	10
Hepatocyte basopholia, incvreased	M										3	2	10
Interstitial fibrosis	C				2	1	10						
	L				2	1	10						
	M												
	H												
	P	2	1	10	2	2	10						
histiocytic cells(intralumi nal)	C	1	2	10									
	L	1	2	10				1	1	9			
	M	2	1	10									
	H												
	P							1	1	10			

C= control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.
ICS: interstitial cells; SPA: spermatogonia; SPZ: spermatozoa; SPC: spermatocytes; SPT: spermatids.
Dark cells: primary diagnoses identified

106. Increased Leydig cells and increase proportion of spermatozoa were reported in LAB 4 and LAB 6. Other findings identified in treated animals included testis-ova, Sertoli cell hypertrophy, interstitial fibrosis and histiocytic cells (intra-luminal). However, they were not broadly identified across laboratories and included control animals occasionally.

Table 26b: Histopathological findings for female medaka exposed to prochloraz

Diagnosis	Dose	LAB 1			LAB 2			LAB 4			LAB 6		
		Average of Grade	N	Obs.	Average of Grade	N	Obs.	Average of Grade	N	Obs.	Average of Grade	N	Obs.
Increased cells - PFC	C										1	1	10
	L							1	1	10			
	M							1.4	9	10	1.5	2	10
	H							2	4	10	2.5	10	10
	P							2.1	9	9	2	10	10
Decreased cells - PNO	M							1	3	10			
	H							1	3	10			
	P							1.4	8	9			
Oocyte atresia, increased, immature	C	2	3	10	1	1	10						
	L	2.3	4	10	1	2	10						
	M	3.6	10	10	1	2	10	1.9	9	10	2	1	10
	H	3.4	9	9	1.7	6	10	2.6	10	10	2	10	10
	P	3.7	9	10	1.7	8	10	2	7	9	3.3	10	10
Oocyte atresia, increased, mature	C	1	1	10							1	2	10
	L	2	1	10	1	1	10						
	M	1.6	9	10	1	2	10				2	3	10
	H	1	3	9									
	P	1.7	3	10									
Interstitial fibrosis	C												
	L												
	M				2	1	10						
	H	2	1	9	2	2	10						
	P				1.5	2	10						
Granulomatous inflammation	M							1.1	7	10			
	H							1.8	9	10			
	P							1.3	7	9			
Follicles hypertrophy	L				2	1	10						
	M				2	1	10						
	P				2	1	10						
Lack of vitellogenesis	M										3.3	3	10
	H										3.7	10	10
	P										3.8	10	10
Decreased vitellogenesis	M							3.3	10	10			
	H							4	10	10			
	P							4	9	9			
Increased hypertrophy-PFC	L							1.1	7	10			
	M							2	9	10			
	H							2.3	4	10			
	P							1.9	9	9			

C= control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.
PNO: perinucleolar oocytes; PFC: perifollicular cells;
Dark cells: primary diagnoses identified.

107. Treatment-related findings in females medaka exposed to prochloraz were several and consistently reported across studies. They included:

- Decreased gonad stage,
- Increased perifollicular cells and follicle hypertrophy,
- Immature and mature oocyte atresia,
- Decrease vitellogenesis (decrease yolk formation),

108. Findings increased dose-dependently in severity and in number of animals affected.

5.3.2 Prochloraz studies with fathead minnow

Mortality

109. Mortalities in the prochloraz groups and positive control in the two submitted studies were ≤ 10% during the exposure period.

Table 27: Mortality in the prochloraz studies with fathead minnow at the end of exposure.

	Nominal concentrations				
Laboratories	Cont.	20 µg/L	100 µg/L	300 µg/L	PC[1] (100 µg/L)
LAB 4	5	0	0	0	5
LAB 8	0	0	5	0	0
LAB 9	0	0	0	0	0
LAB 11	5	0	10	0	0

[1]: PC=positive control

Spawning status

110. Control groups of fathead minnows did not spawn well throughout any of the four studies. Territorial behaviour may be the cause of this problem. For this reason, no clear cessation of spawning due to the aromatase inhibitor could be detected. Only in LAB 9 a dose-dependent decrease was visible.

Table 28a: Spawning status in the prochloraz studies with fathead minnow during the exposure period.

No. of days with spawning "yes" (average of tank 1 and 2) / observed days					
	Nominal concentrations				
Laboratories	Cont.	20 µg/L	100 µg/L	300 µg/L	PC[1] (100 µg/L)
LAB 8	0.5/21	2/21	3/21	0.5/21	1.5/21
LAB 9	7.5/21	4.5/21	4.5/21	0/21	-
LAB 11	0.5/5	0/5	0/5	0/5	0.5/5
LAB 4	1.5/21	3/21	0.5/21	0.5/21	1/21

[1]: PC=positive control

Table 28b: Spawning status in the prochloraz studies with fathead minnow during the exposure period.

LAB 4																						
C	½	0	0	0	0	½	½	0	0	0	0	0	0	0	0	½	0	0	0	0	0	0
L	0	0	0	0	0	0	0	½	0	0	0	0	0	0	1	0	0	0	½	0	0	½
M	1/2	0	0	0	0	0	1/2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H	½	½	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LAB 8																						
C	0	0	0	½	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L	0	0	0	0	1	0	0	0	½	0	0	0	0	0	0	0	0	0	0	0	0	½
M	0	0	0	½	½	1	½	0	0	0	0	0	0	0	0	0	½	0	0	0	0	0
H	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LAB 9																						
C		0	½	½	1	½	0	½	½	1	0	0	0	½	½	½	1	½	0	0	0	0
L		0	½	0	½	½	½	0	0	½	0	0	½	0	0	0	0	½	0	½	0	½
M		1	0	0	0	½	½	1	0	0	0	0	½	0	0	0	½	0	0	0	0	½
H		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

1: spawning observed in both replicate tanks

½ : spawning observed in one of the replicate tanks

0: no spawning observed in any of the two replicate tanks.

Vitellogenin

111. In male fathead minnow exposed to prochloraz no dose-dependent effect was observed. In female fish VTG concentrations decreased dose-dependently in all studies with prochloraz, resulting in significant difference in the highest treatment group. This is consistent with previous findings on the fathead minnow (18)(19) with fadrozole. Aromatase inhibitors block the conversion of testosterone and ket-testosterone to 17beta-estradiol in females, thereby preventing VTG production which is under estrogen control.

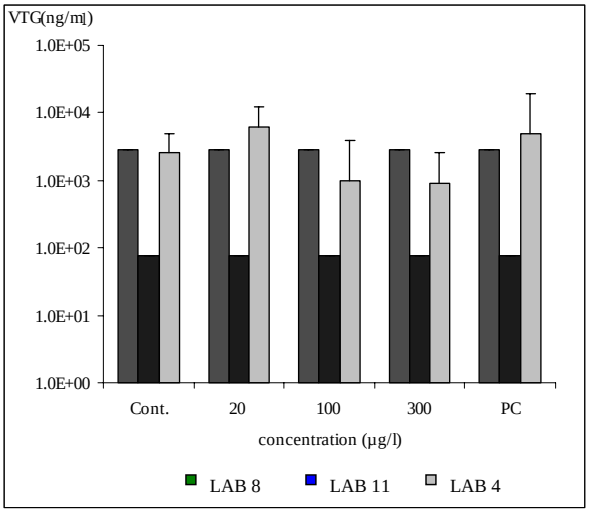


Fig. 11a: VTG in male fathead minnow exposed to prochloraz.

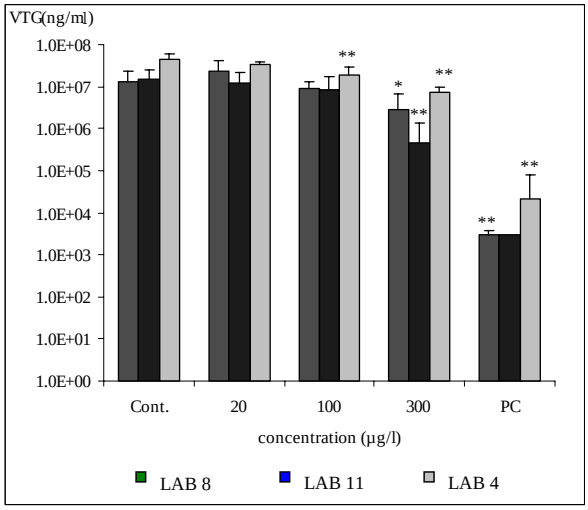


Fig. 11b: VTG in female fathead minnow exposed to prochloraz.

112. In the positive control female group, statistical significance was not achieved in LAB 11 because calculations were made only on one fish; there were problems in determining precisely VTG concentrations in other animals.

Secondary sex characteristics

113. In male fathead minnow exposed to prochloraz no clear response could be observed on the number of nuptial tubercles. This is consistent with previous findings from the literature (18)(19), where alterations in male secondary sex characteristics were not demonstrated following fadrozole exposure. This is because nuptial tubercles are under androgen control and there is no induction of androgen following aromatase inhibition . In females exposed to prochloraz, appearance of one tubercle was found occasionally in two studies.

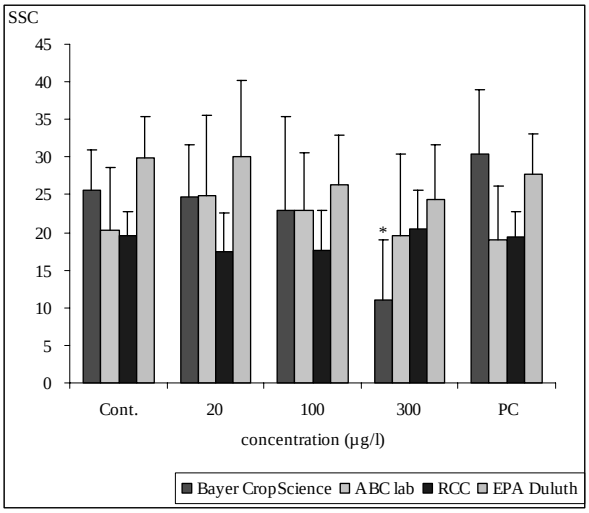


Fig. 12a: Total score of the nuptial tubercles in male fathead minnow exposed to prochloraz.

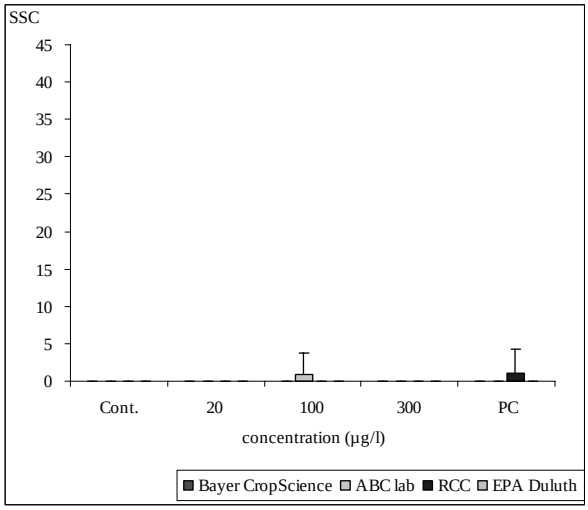


Fig. 12b: Total score of the nuptial tubercles in female fathead minnow exposed to prochloraz.

Gonad histology

Table 29: Staging data for males and females fathead minnow exposed to prochloraz

Dose	FHM-Male							
	LAB 8		LAB 9		LAB 11		LAB 4	
	Median of staging	N	Median of staging	N	Median of staging	N	Median of staging	N
C	2	10	3	9	2	10	2.5	10
L	2	9	3	10	2.5	10	3	10
M	2	9	3	11	3	10	3	10
H	2	9	3	10	2.5	10	3	10
P	3	10	3	10	2	9	3	10
Dose	FHM-Female							
	LAB 8		LAB 9		LAB 11		LAB 4	
	Median of staging	N	Median of staging	N	Median of staging	N	Median of staging	N
C	3	10	3	10	3	9	3	9
L	3	10	3	10	3	10	3	9
M	3	9	3	9	4	9	3	10
H	3	10	2	10	3	10	3	10
P	2	10	2	10	4	7	2	9

Table 30a: Histopathological findings in males fathead minnow exposed to prochloraz.

Diagnosis	Dose	LAB 11			LAB 8			LAB 4			LAB 9		
		Average of Grade	N	Obs.	Average of Grade	N	Obs,	Average of Grade	N	Obs.	Average of Grade	N	Obs.
Increased cells - ICS	C												
	L										1	4	10
	M	1	4	10							2	1	11
	H	1	3	10				1	3	10	1	1	10
	P	1.8	5	9				1.7	6	10	1.6	5	10
Increased cells - SPA	C				1.3	3	10	2	1	10	1	1	9
	L				1.5	2	9	2	1	10	1	2	10
	M				2	1	9	1	2	10	1	1	11
	H				1	1	9	1.5	4	10	1	3	10
Increased cells - SPC	C				2	1	10						
Increased cells - SPT	L				1.7	3	9						
	M				2.3	3	9						
	H				1.7	3	9						
	P				2	2	10						
Increased cells - SPZ	C							1	1	10			
	L				1	1	9						
	M				1.8	5	9				1	1	11
	H				1.5	2	9	1	2	10			
	P				1.4	7	10	1	6	10			
Decreased cells - SPC	C				2	1	10						
	L				2	2	9						
	M										1	1	11
	H										1	1	10
	P												
Decreased cells - SPT	C				3	1	10				1	1	9
	L				2	1	9						
	M				1	1	9				1	1	11
Decreased cells - SPZ	C				2	4	10						
	L				1	1	9						
	M				3	1	9						
	H							1	1	10	1	1	10
	P				2	1	10						
Testis-ova	C	-	1	10									
	L	-	3	10									
	M	-	2	10									
	H	-	4	9									
Testicular degeneration	C				1.5	2	10				1	1	9
	L				1	2	9	2	1	10	1	2	10
	M				1	1	9	2	1	10	1	4	11
	H				1.3	3	9				1	2	10
	P				1	2	10						
Asynchronous development, gonad	C												
	L							1	2	10	1	2	10
	M							1	3	10			
	H							1	3	10	1.3	3	10
	P							1.3	3	10	1	3	10
Proteinaceous fluid, intravascular	H				1	2	9						
Proteinaceous fluid, interstitial	C				1.5	2	10						
	L				1.5	2	9						
	M				2	6	9						
	H				1.8	9	9						
	P				1.8	6	10						
Sertoli cell hypertrophy	P	1.3	4	9									
Granulomatous	C							1.5	2	10			

inflammation	L							1	1	10
	M			1	2	10		1	1	11
	H			1	1	10				
	P			1.5	2	10				
Histiocytic cells, intraluminal	C							1	1	9
	L			1	1	10		1.5	2	10
	M			1.3	4	10		1.4	5	11
	H			3	1	10		1	2	10
	P			2	1	10		1	1	10
Mineralisation	C			1.2	5	10		1	3	9
	L			1	3	10		1	3	10
	M			1.3	3	10		1	2	11
	H			1	5	10		1	1	10
	P			1	2	10		1	2	10

C= control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.
ICS: interstitial cells; SPA: spermatogonia; SPZ: spermatozoa; SPC: spermatocytes; SPT: spermatids.
Dark cells: primary diagnoses identified

114. A treatment-related increase of Leydig cells was identified across studies, except in LAB 8. Three studies reported an increased proportion of spermatozoa.

115. Although an increase of the proportion of spermatogonia was found in all studies, it also occurred in control animals; therefore it is unlikely to be meaningful as a result of aromatase inhibitor treatment. Other findings did not appear to be treatment-related or were reported in single study.

Table 30b: Histopathological findings in females fathead minnow exposed to prochloraz.

Diagnosis	Dose	LAB 11			LAB 8			LAB 4			LAB 9		
		Average of Grade	N	Obs.	Average of Grade	N	Obs.	Average of Grade	N	Obs.	Average of Grade	N	Obs.
Increased cells - LVO	M				2	5	9						
	H				2.5	6	10						
Increased cells - EVO	H				2.5	2	10						
	P				3.1	9	10						
Decreased cells - LVO	L				2.5	2	10						
	P				2	1	10						
Oocyte atresia, increased, immature	C							2	2	9	2.5	2	10
	L							1.5	2	9	1.5	2	10
	M							2.3	6	10	2.5	4	9
	H				2	2	10	1.8	8	10	2.4	8	10
	P				1.8	6	10	2.3	8	9	2.2	9	10
Oocyte atresia, increased, mature	C				1	1	10						
	L												
	M	1	2	9	1.7	3	9						
	H	1.8	4	10	2.3	9	10						
	P				1.8	8	10						
Proteinaceous fluid, interstitial	C				1	4	10						
	L				1	1	10						
	H				1.6	5	10						
	P				1.6	7	10						
Post-ovulatory follicles, increased	C				1.9	9	10						
	L				2.4	7	10	1	1	9			
	M				1.5	4	9						
	H				1	7	10						
	P				2.7	10	10						
Granulomatous inflammation	C				1	1	10	1	3	9	1	4	10
	L							1	1	9	1.5	2	10
	M				1.5	2	9				1	1	9
	H										1	1	10
	P										1	1	10
Oocyte membrane folding	C				1	6	10						
	L				1.3	4	10						
	M				1.6	5	9						
	H				1.6	5	10						

	P		2.8	8	10				2.2	6	10
Egg debris, oviduct	C		2	10	10						
	L		2	6	10						
	M		2	3	9						
	H		2.5	2	10						
	P		1	2	10						
Decreased vitellogenesis	H								1.5	2	10
	P					1.2	4	9	1.6	8	10
Ovarian cyst	C								1	1	10
	L								1	2	10
	M								1	1	9
	H					1	1	10			
	P								1	2	10
Ovarian atresia, increased, late atretic	L					1.5	2	9			
	H								2	1	10

C= control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.
LVO: late vitellogenic oocytes; EVO: early vitellogenic oocytes;
Dark cells: primary diagnoses identified.

116. All four studies reported oocyte atresia (mature or immature). The number of females affected increased dose-dependently but control females were also affected. This is likely to be due to the bad spawning achieved in control groups. Decreased vitellogenesis (or decreased yolk formation) was identified in two studies in the high dose group and/or positive control group.

5.3.3 Prochloraz studies with zebrafish

Mortality

117. No fish died during the exposure period in two studies; 5-20 % mortalities in prochloraz treatment groups were found in LAB 6 (measured concentrations in LAB 6 were 34%, 54% and 72% of nominal).

Table 31: Mortality (%) in the prochloraz studies with zebrafish at the end of exposure.

	Nominal concentrations				
Laboratories	Cont.	20 µg/L	100 µg/L	300 µg/L	PC (100 µg/L)
LAB 12	0	0	0	0	0
LAB 13	0	0	0	0	0
LAB 6	5	10	10	20	0

Spawning status

118. Spawning was not checked strictly everyday of the 21-day study in LAB 12 and LAB 13, however, spawning was regular throughout the study in the control groups. LAB 6 did not provide information on the spawning status of fish.
Prochloraz caused a decrease of egg production at the top-dose and positive control of the study conducted in LAB 12. The study in LAB 13 did not indicate any clear effect (this is visible from Tables 27a and 27b, and also from Figure 13d). The spawning status in the positive control was inhibited in both studies.

Table 32a: Spawning status in the prochloraz studies with zebrafish during the exposure period.

No. of days with spawning "yes" (average of tank 1 and 2) / observed days					
	Nominal concentrations				
Laboratories	Cont.	20 µg/L	100 µg/L	300 µg/L	PC (100 µg/L)
LAB 12	14.5/15	14.5/15	13.5/15	10.5/15	10/15
LAB 13	5/6	6/6	5/6	6/6	2.5/6
LAB 6	No data available				

Table 32b: Daily recording of spawning status of zebrafish exposed to prochloraz.

LAB 12																						
C	1	1			1	1	1	1	1			1	1	1	1	1			1	1	1	
L	1	1			1	1	1	1	1			1	1	1	1	½			1	1	1	
M	1	½			1	1	½	1	1			1	1	1	1	1			1	1	½	
H	1	1			1	1	0	½	1			1	1	½	1	½			½	½	0	
LAB 13																						
C					1			1			½			1			½			1		
L			1	1		1	1		1	1		1	1		1	1		1	1			
M			0	1		1	1		1	1		1	1		1	1		1	1			
H			1	1		1	1		1	1		1	1		1	1		1	1			

1: spawning observed in both replicate tanks
½ : spawning observed in one of the replicate tanks
0: no spawning observed in any of the two replicate tanks.

119. It is worth noting again that measured concentrations of prochloraz in both LAB 12 and LAB 13 were low: 73%, 67%, 55%, and 95%, 83%, 65% respectively for each study. This may explain that the cessation of spawning was not marked, especially at the top-dose.

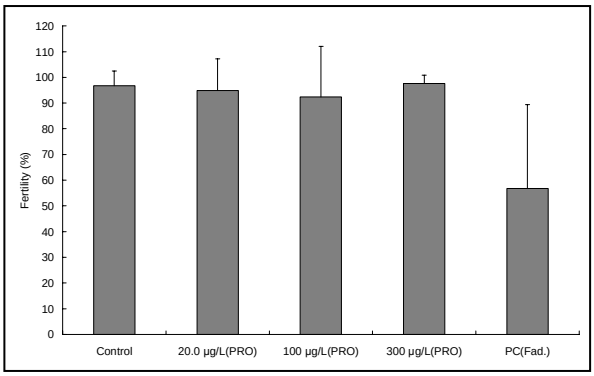


Fig. 13a: The means of fertility in zebrafish exposed to prochloraz.
(Optional data of LAB 12)

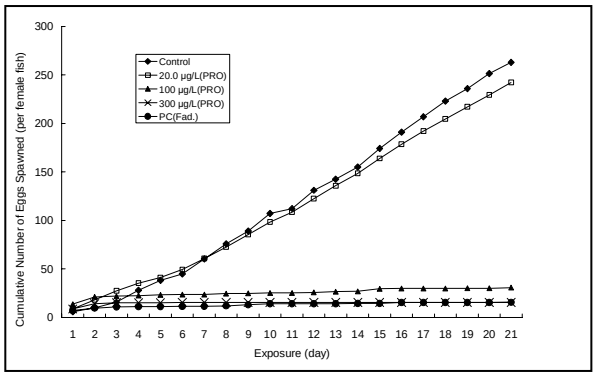


Fig. 13b: The cumulative number of eggs spawned in zebrafish exposed to prochloraz.
(Optional data of LAB 12)

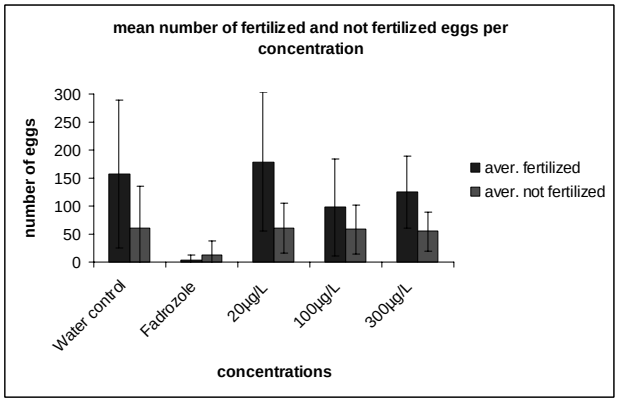


Fig. 13c: Effect of prochloraz on mean fertilization rate in zebrafish.
(Optional data of LAB 13)

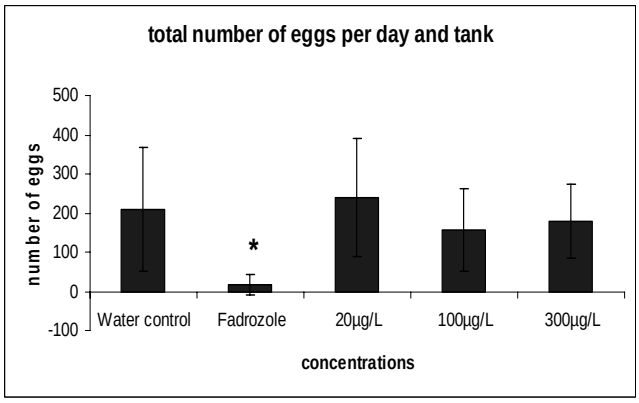


Fig. 13d: Total number of eggs of zebrafish per day and tank under exposure to prochloraz.
(Optional data of LAB 13)

120. These figures are about optional data colelcted in LAB 12 and LAB 13. It confirms that the decrease in egg production was visible from the number of days with spawning as well as the number of eggs spawned (LAB 12 for mid- and top-concentrations and positive control and LAB 13 for positive control). When there was no clear decrease in the number of days with spawning (LAB 13 for prochloraz), the decrease in the total number of eggs was not obvious either.

121. Fertility was not affected in any of the two studies (Figures 13a and 13c).

Vitellogenin

122. In male zebrafish exposed to prochloraz no significant difference could be observed, although high VTG level was detected in the control of one laboratory.

123. Vitellogenin levels in females zebrafish decreased dose-dependently. At 100µg/l, 2/3 laboratories detected a significant decrease (LAB 12 and LAB 13). In LAB 6, low measured concentrations, (34%,

54% and 72% of nominal values), combined with a relatively lower mean VTG level in the control group compared to other studies, may have posed a problem for a significant detection of decrease VTG at 100µg/l.

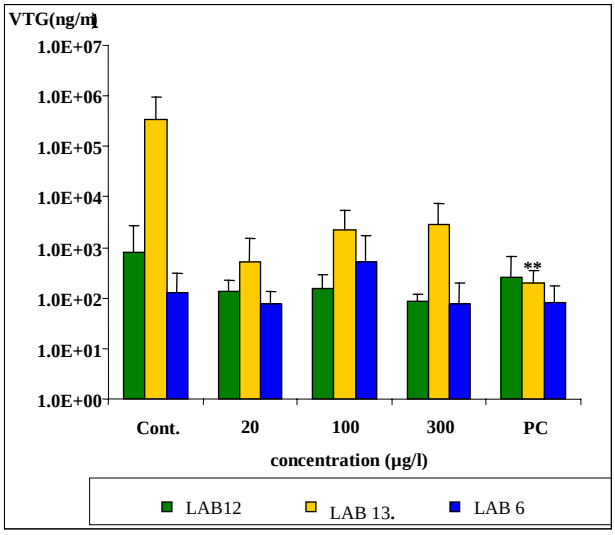


Fig. 14a: VTG in male zebrafish exposed to prochloraz.

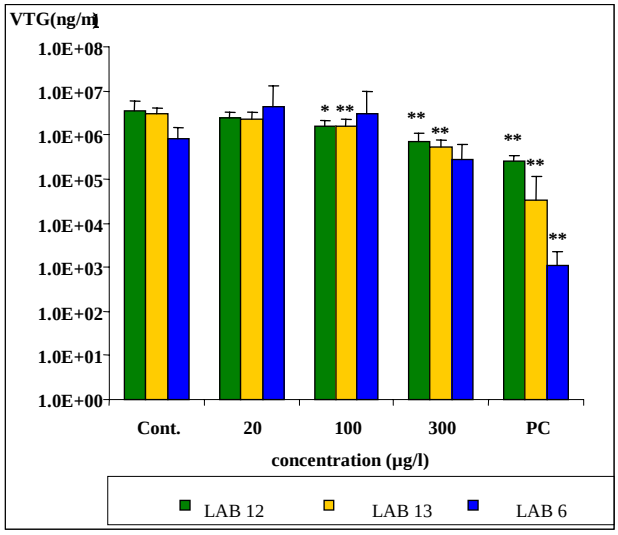


Fig. 14b: VTG in female zebrafish exposed to prochloraz.

Gonad histology

Table 33: Staging data for males and females zebrafish exposed to prochloraz

Dose	ZBR-Male			
	LAB 12		LAB 13	
	Median of staging	N	Median of staging	N
C	1	10	2	9
L	2	10	2	8
M	2	10	2	8
H	2.5	10	2	7
P	2	10	2.5	10
Dose	ZBR-Female			
	LAB 12		LAB 13	
	Median of staging	N	Median of staging	N
C	2	10	2	11
L	2	10	3	9
M	2	10	2	9
H	2	9	3	10
P	1	8	2	10

Table 34a: Histopathological findings in male zebrafish exposed to prochloraz

Diagnosis	Dose	LAB 12			LAB 13		
		Average of Grade	N	Obs.	Average of Grade	N	Obs.
Increased cells - ICS	H	1	3	10			
	P	1	2	10			
Increased cells - SPA	L				3	2	8
	M				2	3	8
	P				2	2	10
Increased cells - SPC	L				2.6	7	8
	M				2.7	7	8
	H				2.9	8	7
	P				2.7	6	10
Increased cells - SPZ	H				2	1	7
	P				1.9	7	10
Decreased cells - SPA	L				2.7	3	8
	M				2.8	4	8
	P				4	3	10
Interstitial fibrosis	L				2.5	8	8
	M				3.1	8	8
	H				3.5	8	7
	P				2.5	10	10

C= control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.
ICS: interstitial cells; SPA: spermatogonia; SPZ: spermatozoa; SPC: spermatocytes; SPT: spermatids.
Dark cells: primary diagnoses identified

124. In males zebrafish exposed to prochloraz, consistency of findings between LAB 12 and LAB 13 was not good. Results in LAB 13 suggest a treatment-related increase of spermatocytes and a decrease in the proportion of spermatogonia.

Table 34b: Histopathological findings in female zebrafish exposed to prochloraz

Diagnosis	Dose	LAB 12			LAB 13		
		Average of grade	N	Obs.	Average of grade	N	Obs.
Increased cells - PNO	M				2	3	9
Increased cells - LVO	M				3	1	9
	H				3	1	10
	P				3.1	8	10
Increased cells - EVO	P				3.2	5	10
Increased cells - MSO	H				2.5	2	10
Decreased cells - LVO	P				4	1	10
Decreased cells - PNO	P				3.6	7	10
Decreased cells - MSO	P				3.8	8	10
Oocyte atresia, increased, immature	L				1	1	9
	M				1	1	9
	P				2	6	10
Oocyte atresia, increased, mature	C	2	1	10			
	L	1.8	4	10			
	P	1.6	7	8			
Interstitial fibrosis	L				2	7	9
	M				2.7	6	9
	H				2	1	10
Post-ovulatory follicles, increased	C	1.3	3	10			
	L	1.0	2	10	2	1	9
	M			10	2.5	2	9
Hepatocyte basophilia, decreased	P	3.1	8	8			
Oocyte membrane folding	L				2	3	9
	M				2	6	9
	H				1.5	4	10
	P	2.2	9	8	2.4	10	10

Egg debris, oviduct	C	2.5	8	10	
	L	2.8	6	10	
	M	2.3	4	10	
	H	2	2	9	

C= control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.
PNO: perinuclea oocytes; EVO: early vitellogenic oocytes; LVO: lates vitellogenic oocytes; MSO: mature/spawning oocytes;
Dark cells: primary diagnoses identified

125. Mature and immature oocyte atresia, including oocyte membrane folding, was identified in both studies in LAB 12 and LAB 13.

5.4 Flutamide studies

5.4.1 Flutamide Study with medaka

Mortality

126. Mortalities in the flutamide treatment groups in the three submitted studies were ≤ 10% during the exposure period.

Table 35: Mortality (%) in the flutamide studies with medaka at the end of exposure.

Nominal concentrations				
Laboratories	Cont.	100 µg/L	500 µg/L	1,000 µg/L
LAB 3	5	5	0	0
LAB 5	0	35	45	10
LAB 4	0	0	0	0
LAB 6	0	5	5	0

Spawning status

127. Control groups spawned regularly throughout the 21-day study. Flutamide caused inhibition of egg production in a concentration-dependent manner in one study (LAB 3), whereas no clear treatment-related response could be observed in the other three studies.

Table 36a: Spawning status in the flutamide studies with medaka during the exposure period.

No. of days with spawning "yes" (average of tank 1 and 2) / observed days				
Laboratories	Nominal concentrations			
	Cont.	100 µg/L	500 µg/L	1,000 µg/L
LAB 3	15/21	14/21	11.5/21	8.5/21
LAB 5	21/21	13/21	13.5/21	21/21
LAB 4	21/21	20.5/21	21/21	21/21
LAB 6	19/20	17/20	18/20	18.5/20

Table 36b: Daily recording of spawning status in medaka exposed to flutamide.

LAB 4																							
C		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
L		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
M		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
H		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
LAB 3																							
C		1	1	1	1	1	1	1	½	½	½	½	1	1	0	1	½	½	0	1	½	½	
L		1	1	1	1	1	1	1	1	1	1	½	½	0	0	½	½	0	0	½	1	½	
M		1	1	1	1	1	1	1	1	1	½	0	½	½	0	0	0	0	0	½	0	½	
H		1	1	½	1	½	½	½	½	0	0	0	½	1	0	½	½	0	0	0	0	0	
LAB 5																							
C	½	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
L	1	1	1	1	1	1	1	1	1	1	1	½	?	½	½	0	0	0	0	½	½	½	
M	1	1	1	1	1	1	1	1	1	1	½	½	½	½	½	0	0	0	½	½	½	½	
H	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	

1: spawning observed in both replicate tanks
½ : spawning observed in one of the replicate tanks
0: no spawning observed in any of the two replicate tanks.

Vitellogenin

128. One study (LAB 4) out of four detected a significant increase of vitellogenin in both males and females. No dose-related trend in VTG response could be observed in any of the three other studies. The analytical chemistry showed measured concentrations close to nominal ones, except in LAB 6 (56%, 44% and 55% of nominal values).

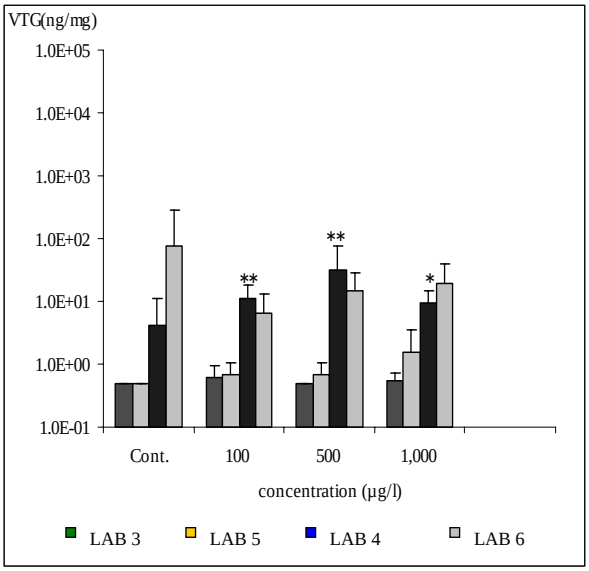


Fig. 15a: VTG in male medaka exposed to flutamide.

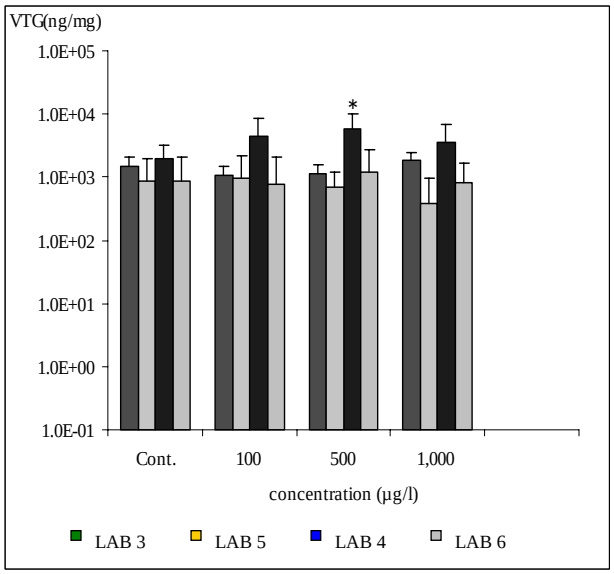


Fig. 15b: VTG in female medaka exposed to flutamide.

Secondary sex characteristics

129. No treatment-related response could be found on male medaka exposed to flutamide. No papillary process was observed in any treatment group or positive control in females.

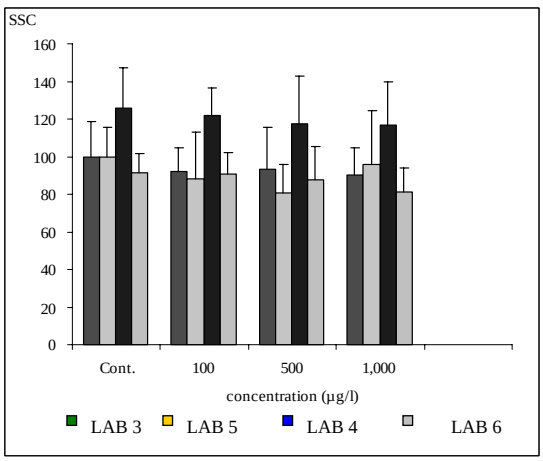


Fig. 16: Number of the joint plate with papillary processes in male medaka exposed to flutamide.

Gonad histology

Table 37: Staging data for males and females medaka exposed to fluamide

Dose	MDK-Male					
	LAB 3		LAB 5		LAB 4	
	Median of staging	N	Median of staging	N	Median of staging	N
C	2	10	2	9	1.5	10
L	2	9	2.5	6	2	10
M	2	10	2.5	6	2	9
H	3	10	2.5	8	2	10
Dose	**MDK-Female**					
	LAB 3		LAB 5		LAB 4	
	Median of staging	N	Median of staging	N	Median of staging	N
C	2	9	1.5	10	2	10
L	2	10	1	7	2	10
M	2	10	2	6	2	10
H	1	10	1	9	2	9

Table 38a: histopathological findings in male medaka exposed to flutamide

Diagnosis	**Dose**	**LAB 3**			**LAB 5**			**LAB 4**			**LAB 6**		
		Average of Grade	**N**	**Obs.**	**Average of Grade**	**N**	**Obs.**	**Average of Grade**	**N**	**Obs.**	**Average of Grade**	**N**	**Obs.**
Increased cells-ICS	C				1	1	9	1	1	10			
	L							1	1	10			
	M							1	2	10	3	2	8
	H				1	2	8	1	3	10	3	1	9
Increased cells-SPA	C				3	1	9						
	L	1	1	9	2.5	2	6	3	1	10			
	M				3.5	3	6						
	H				1	3	8						
Increased cells-SPZ	C				1.5	4	9	1	1	10			
	L				1.4	5	6	1	1	10			
	M				1	3	6	1	2	10			
	H				1.4	5	8	1.7	3	10			
Decreased cells-SPA	M				3	1	6				2	2	8
	H										2	1	9
Testis-ova	C							1	1	10			
	L							3	1	10			
	H							1	1	10	2	1	9
Testicular degeneration	C	1	1	10	2	4	9	1	1	10			
	L	2	1	9	2	1	6	1	2	10			
	M	1	1	10	1.7	3	6	1	1	10			
	H	2	1	10	2	3	8						
Asynchronous dev. spermatocyst	L				1	1	6						
	M				1	1	6						
Asynchronous dev. gonad	M				2	1	6	1	1	9			
	H				1	1	8						
Interstitial fibrosis	H				2	1	8						
Sertoli cell hypertrophy	M										2	2	8
	H										2.3	3	9
Granulomatous inflammation	L				1	1	6						
	H				2	1	8						
Histiocytic cells, intraluminal	L				1	1	6	1	1	10			
	H				1.5	2	8						

C= control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.
ICS: interstitial cells; SPA: spermatogonia; SPZ: spermatozoa; SPC: spermatocytes; SPT: spermatids.

Dark cells: primary diagnoses identified

130. Increased Leydig cells, increased proportion of spermatogonia, testicular degeneration and testis-ova were reported, however consistency across studies was not good, and control male were affected too, thereby making the evaluation of treatment-related response more difficult.

Table 38b: histopathological findings in female medaka exposed to flutamide.

Diagnosis	Dose	LAB 3			LAB 5			LAB 4			LAB 6		
		Average of Grade	N	Obs.	Average of Grade	N	Obs.	Average of Grade	N	Obs.	Average of Grade	N	Obs.
Increased cells-OOG	H				1	1	8						
Oocyte atresia, increased, immature	C	1.3	3	9	1.2	6	10						
	L	1.3	6	10	2.3	3	7						
	M	1.6	7	10	1	1	5						
	H	1.9	8	10	1.6	5	8	2	1	10			
Oocyte atresia, increased, mature	C										1	2	2
	L	1	1	10							1	1	1
	M										1	1	1
	H				1	1	8				2	2	2
Proteinaceous fluid	C						8				3	1	1
	M										3	2	2
Post-ovulatory follicle, increased	H				1	1	8						
Nephropathy	H				1	1	8						
Granulomatous inflammation	C				1	3	10						
	L				1.7	3	7						
	M				2	1	5						
	H				1	4	8						
Macrophage aggregates, increased	C	1	1	9									
	L	2	1	10									
	M	1.7	3	10									
	H	1	1	10									
Egg debris, oviduct	H	2.7	3	10									
Oocyte atresia, late, atretic	C				1	2	10						
	L				1	2	7						
	H				1.6	3	8						
Post-ovulatory follicle, decreased	L							x	6	10			
	M							x	4	9			
	H							x	6	10			

C= control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.
OOG: oogonia;
Dark cells: primary diagnoses identified

131. There was no clear treatment-related response in ovaries of females medaka exposed to the anti-androgen. Where findings identified as primary diagnoses were reported (e.g. oocyte atresia), they affected control animals in equal proportion as treated animals.

5.4.2 Flutamide studies with fathead minnow

Mortality

132. Mortalities in the flutamide treatment groups in two submitted studies were ≤ 5% during the exposure period.

Table 39: Mortality (%) in the flutamide studies with fathead minnow at the end of exposure.

Laboratories	Nominal concentrations			
	Cont.	100 µg/L	500 µg/L	1,000 µg/L
LAB 7	0	0	0	5
LAB 10	0	0	0	5
LAB 11	Invalid because of high mortality rates			
LAB 4	0	0	0	0

Spawning status

133. Control fish did not spawn well throughout the 21-day study, most likely because of territorial behaviour, which disturbed animals. Despite low spawning in the control groups, flutamide exposure inhibited eggs production at the highest concentration in all studies. This is consistent with previous findings (40) reported in the literature.

Table 40a: Spawning status in the flutamide studies with fathead minnow during the exposure period.

No. of days with spawning "yes" (average of tank 1 and 2) / observed days				
Laboratories	Nominal concentrations			
	Cont.	100 µg/L	500 µg/L	1,000 µg/L
LAB 7	15.5/21	13.5/21	15.5/21	6/21
LAB 10	5/21	1/21	2.5/21	2.5/21
LAB 4	5.5/21	2.5/21	0/21	0/21

Table 40b: Daily recording of spawning status of fathead minnow exposed to flutamide.

LAB 7																							
C	0	½	½	1	½	½	½	½	1	½	½	1	1	½	½	1	1	1	½	1	1	1	
L	1	½	0	½	1	½	½	1	1	0	½	1	1	1	1	1	½	½	1	0	½	½	
M	0	1	½	1	1	1	1	1	1	0	½	1	1	0	½	1	1	½	½	0	1	1	
H	0	0	0	0	0	½	0	½	0	½	½	½	½	½	0	0	1	0	0	0	1	½	
LAB 4																							
C	1	½	0	0	½	0	0	0	½	1	1	0	0	½	0	0	½	½	½	0	0	0	
L	½	0	0	0	0	½	1	½	0	0	0	0	0	0	0	0	½	0	0	0	0	0	
M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
H	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
LAB 10																							
C	1	0	1	0	½	½	0	0	0	½	0	0	0	½	1	½	½	0	0	0	0	0	
L	1	0	½	0	0	0	0	0	0	0	0	0	0	0	½	0	0	0	0	0	0	0	
M	½	0	½	0	½	0	0	0	0	0	½	0	0	0	0	0	0	½	0	0	0	0	
H	1	0	½	0	0	½	0	0	0	0	½	0	0	0	0	½	0	½	0	0	0	0	

1: spawning observed in both replicate tanks
½ : spawning observed in one of the replicate tanks
0: no spawning observed in any of the two replicate tanks.

Vitellogenin

134. No dose-dependent effect could be found in either male or female fathead minnows exposed to flutamide conducted in two submitted studies. Only one study reported a significant increase of VTG in females at the mid-concentration, but because it was not reproduced at the top-dose, it can hardly be taken as a really meaningful result. All measured concentrations were within the 80%-120% range.

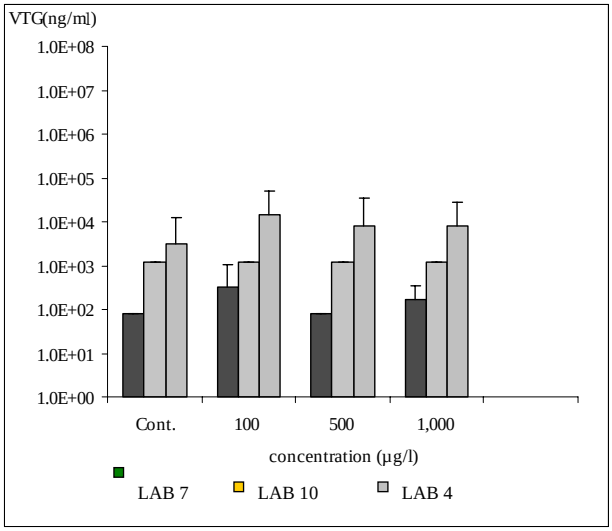


Fig. 17a: VTG in male fathead minnow exposed to flutamide.

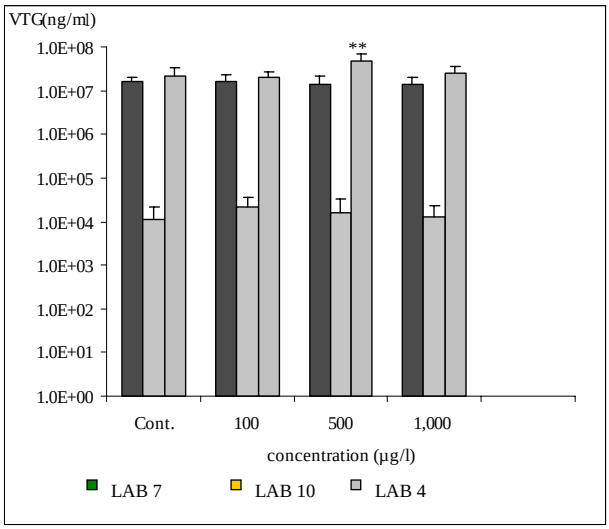


Fig. 17b: VTG in female fathead minnow exposed to flutamide.

Secondary sex characteristics

135. In male fathead minnow exposed to flutamide total score of nuptial tubercles significantly decreased at 1,000 µg/L in one study, although the other two studies showed no significant difference. Nuptial tubercles were not observed in any of the treated females.

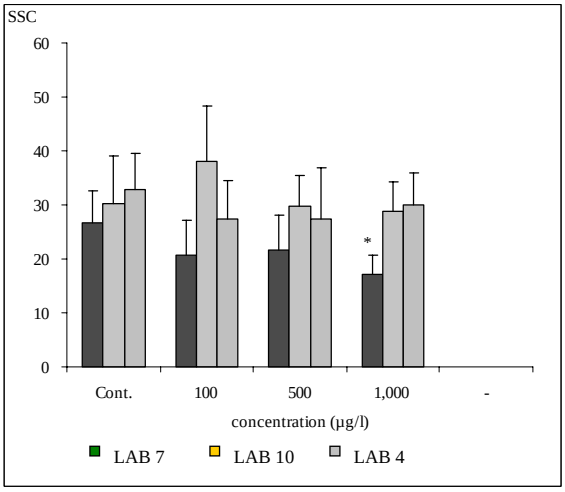


Fig. 18: Total score of the nuptial tubercles in male fathead minnow exposed to flutamide.

136. Although secondary sex characteristics in fathead minnow are under the control of androgens, one possible reason for not always observing an anti-androgen type of response (e.g. decrease of nuptial tubercles number) was explained by Jensen *et al*. (40) due to the maintainance of peripheral androgen levels by the males via feedback systems. Another study (18) reported a reduction in the number of nuptial tubercles in males fathead minnows at 1000µg/l, and also noted that the inhibitory effect was expected to be more pronounced; however there seems to be a lower affinity of flutamide for the androgen receptor in fish than in mammals.

137. There are at present very few studies with known mammalian anti-androgens, and it remains challenging to interpret study results. One study (35) suggested that the mammalian anti-androgen vinclozolin does not act as anti-androgen in fathead minnow because of low capacity to competitively bind to the androgen receptor. In the case of flutamide, its metabolite hydroxy-flutamide appears to have a greater binding affinity to the androgen receptor than the parent chemical (41).

Gonad histology

Table 41: Staging data for males and females fathead minnow exposed to fluamide

Dose	FHM-Male					
	AZ		SPRING		EPA	
	Median of staging	N	Median of staging	N	Median of staging	N
C	2	10	2	10	2	9
L	2	10	2	9	2	10
M	2	10	3	10	2	11
H	2.5	10	2	9	3	10
Dose	FHM-Female					
	AZ		SPRING		EPA	
	Median of staging	N	Median of staging	N	Median of staging	N
C	3	10	3	10	2	10
L	3	10	2	10	3	10
M	2	9	3	10	3	9
H	2	9	2	9	2	10

Table 42a: Histopathological findings in male fathead minnow exposed to flutamide.

Diagnosis	Dose	LAB 7			LAB 4			LAB 10		
		Average of Grade	N	Obs.	Average of Grade	N	Obs.	Average of Grade	N	Obs.
Increased cells - ICS	C				1	1	9			
	L							1	4	9
	M							2	2	10
	H							1	2	9
Increased cells - SPA	C				1	1	9	1	4	10
	L				1	1	10			
	M				1.2	6	11	1	4	10
	H				1.4	10	10	1	3	9
Increased cells - SPZ	M							1	1	10
Decreased cells - SPC	C							2	2	10
	L									
	M							1	2	10
	H				1.3	4	10	1	1	9
Decreased cells - SPZ	L							2	1	9
	H							3	1	9
Testicular degeneration	C				1	2	9			
	L				1	1	10	2	3	9
	M				1	2	11	1.5	2	10
	H							1.3	3	9
Asynchronous development, spermatocyst	C				3	1	9			
Asynchronous development, gonad	C				2	1	9	1	3	10
	L				1	1	10	1	1	9
	M				1	2	11	1.3	3	10
	H							1	4	9
Asynchronous development, right & left gonads	C							2	1	10
Interstitial fibrosis	L							2	1	9
Sertoli cell hypertrophy	H				2	1	10			9
Granulomatous inflammation	C				2	1	9	1.3	3	10
	L				2	1	10			9
	M				2	1	11	1	2	10
	H				1	2	10	1	2	9
Histiocytic cells, intraluminal	C							2	2	10
	L				1.5	2	10			9
	M				1	1	11	1	2	10
	H				2	2	10	1	3	9
Retained peritoneal attachments	C				1	4	9	1	2	10
	L				1.2	5	10	1.5	2	9
	M				1	1	11	1	2	10
	H				1	6	10	1.4	5	9
Mineralisation, collecting duct	C				1.4	8	9	1.5	4	10
	L				1.3	10	10	1.5	2	9
	M				2	5	11	2	2	10
	H				1.7	3	10	1.0	3	9

C= control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.
ICS: interstitial cells; SPA: spermatogonia; SPZ: spermatozoa; SPC: spermatocytes; SPT: spermatids.
Dark cells: primary diagnoses identified

138. Among the primary diagnoses, increased Leydig cells, increased spermatogonia and testicular degeneration were reported in LAB 4 and LAB 10. In LAB 4 and LAB 10, reported findings increased with treatment level. However, occasionally control fish were affected. In LAB 10, control animals were consitently more affected than the low-dose, which might suggest an inversion between the control and the low-dose group. But this could not be verified. In a previous study on fathead minnow (40), spermatocyte degeneration/necrosis was reported after flutamide exposure in adult males. LAB 7 did not find any histopathological change for this study.

Table 42b: Histopathological findings in female fathead minnow exposed to flutamide

Diagnosis	Dose	LAB 7			LAB 4			LAB 10		
		Average of Grade	N	Obs.	Average of Grade	N	Obs.	Average of Grade	N	Obs.
Oocyte atresia, increased, immature	C				1	1	10	1	1	1
	L	2	2	10	1.4	5	10	1.7	3	3
	M				2	9	9	1.7	3	3
	H	3	1	9	1.9	8	10	2	2	2
Oocyte atresia, increased, mature	C									
	L	2	1	10	3.0	2	10			
	M									
	H							1	1	1
Interstitial fibrosis	C	1	1	10						
Post-ovulatory follicles, increased	L							2	1	1
Granulomatous inflammation	C	3	1	10	1	3	10	1.3	4	4
	L	2.5	2	10	1.4	5	10	1	5	5
	M	1.8	4	10	1	1	9	1	6	6
	H	2	3	9	1	3	10	1	5	5
Egg debris, oviduct	H	3.5	2	9				3	1	1
Ovarian cyst	C				1	1	10			
	L							1	1	1
	H							1	1	1
Ovarian mineralisation	C				2	1	10			
	L				1	1	10			
	H				1	1	10			
Oocyte atresia, increased, late atretic	C				1	1	10			
	L				3.0	3	10			
	M				1	2	9			
	H				1.6	3	10	3	1	1

C= control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.
Dark cells: primary diagnoses identified

139. Findings from all studies suggest an increase in mature/immature oocyte atresia. This is consistent with previous studies on adult fathead minnow (40)(43).

5.4.3 Flutamide studies with zebrafish

Mortality

140. Mortalities in the flutamide treatment groups were ≤ 5% during the exposure period in all studies.

Table 43: Mortality (%)in the flutamide studies with zebrafish at the end of exposure.

	Nominal concentrations				
Laboratories	Cont.	SC [1]	100 µg/L	500 µg/L	1,000 µg/L
LAB 12	0	5	0	0	0
LAB 14	5	0	5	0	5
LAB 6	5	-	5	0	5

[1]: SC=solvent control

Spawning status

141. Control fish spawned relatively well and regularly throughout the 21-day studies. No clear concentration-dependent response on spawning could be noted in any of the studies.

Table 44a: Spawning status in the flutamide studies with zebrafish during the exposure period.

No. of days with spawning "yes" (average of tank 1 and 2) / observed days					
	Nominal concentrations				
Laboratories	Cont.	SC [1]	100 µg/L	500 µg/L	1,000 µg/L
LAB 12	17.5/20	-	19/20	16.5/20	14.5/20
LAB 14	15.5/21	17.5/21	18.5/21	14/21	12.5/21
LAB 6	No data				

[1]: SC=solvent control

Table 44b: Daily recording of spawning status in zebrafish exposed to flutamide.

LAB 12																						
C		1	½	1	1	1	1	½	½	1	½	1	1	½	1	1	1	1	1	-	1	1
L		1	1	½	1	1	1	1	1	1	1	1	1	½	1	1	1	1	1	-	1	1
M		1	½	1	½	1	½	½	½	1	1	1	1	1	½	1	1	1	1	-	½	1
H		½	½	½	½	1	1	1	1	1	1	0	1	1	1	0	1	1	0	-	1	½
LAB 14																						
C		½	1	1	1	1	1	1	1	½	½	1	½	1	0	½	1	½	½	1	1	0
L		½	½	1	1	1	1	1	½	½	1	1	1	1	½	½	1	1	1	1	1	0
M		0	1	½	1	½	½	1	1	½	1	1	½	1	0	½	1	½	1	½	1	0
H		0	½	1	½	1	1	½	1	1	½	1	1	1	0	½	0	½	½	0	½	½

1: spawning observed in both replicate tanks
½ : spawning observed in one of the replicate tanks
0: no spawning observed in any of the two replicate tanks.

LAB 14 (semi-quantitative)

C		0	1	3	4	3	1	2	1	0	0	2	0	2	0	0	2	0	0	3	3	0
		1	2	1	3	1	1	2	1	3	2	4	1	2	0	1	1	2	4	3	3	0
L		2	1	3	1	3	1	1	1	0	1	1	3	2	0	1	1	1	3	1	1	0
		0	0	3	3	4	1	4	0	4	3	3	3	2	3	3	3	3	4	4	3	0
M		0	2	0	1	4	2	2	4	1	3	2	3	1	0	0	3	1	3	2	4	0
		0	2	2	3	0	0	2	3	0	2	2	0	2	0	2	2	0	3	0	4	0
H		0	1	1	0	3	2	1	4	2	2	2	3	2	0	2	0	1	1	0	1	1
		0	0	2	4	3	2	0	3	1	0	2	3	1	0	0	0	0	0	0	0	0

Coding: 0: no egg; 1: <50; 2: 50-100; 3: >100; 4: >>100

142. Under the optional data and further to counting days with spawning, LAB 12 quantitatively evaluated the presence of eggs throughout the study (Figure 19b) and did not report meaningful decrease in any of the treated groups. Only the highest treatment group (1000µg/l) had fewr eggs compared to the control group.
Fertility (Figure 19a) was not affected.

143. In a similar study on adult zebrafish (42) with comparable test concentrations of flutamide, reproductive performance was affected at 1000µg/l. through a significant reduction in the total number of eggs spawned (significant reduction in the number of clutches).

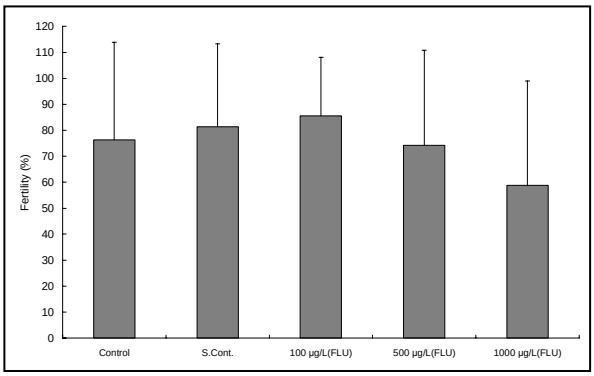


Fig. 19a: The means of fertility in zebrafish exposed to flutamide.
(Optional data of LAB 12)

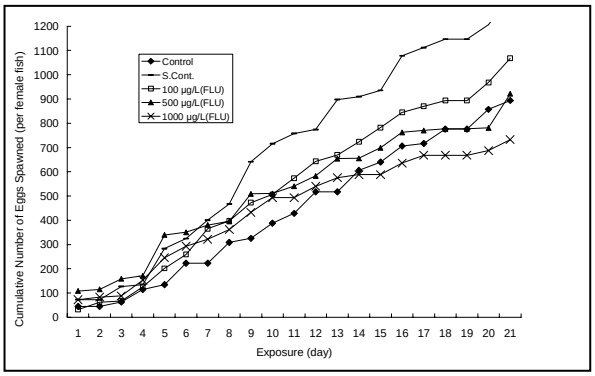


Fig. 19b: The cumulative number of eggs spawned in zebrafish exposed to flutamide.
(Optional data of LAB 12)

Vitellogenin

144. In all studies, neither significant difference nor concentration-dependent response on VTG level could be observed in either male or female of zebrafish exposed to flutamide.

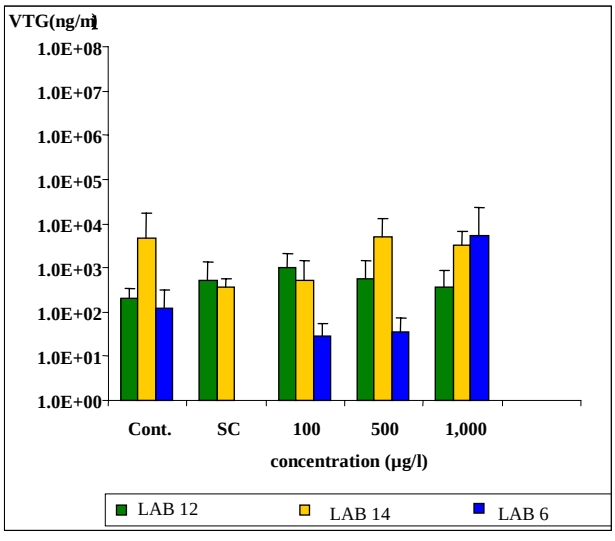


Fig. 20a: VTG in male zebrafish exposed to flutamide.

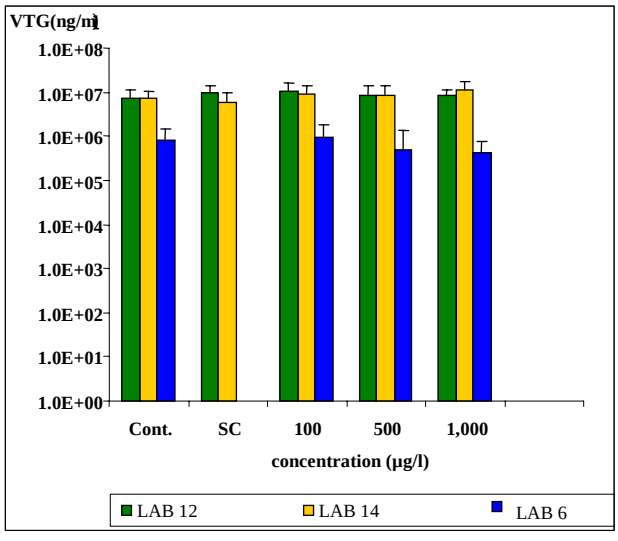


Fig. 20b: VTG in female zebrafish exposed to flutamide.

Gonad histology

Table 45: Staging data for males and females zebrafish exposed to fluamide

Dose	ZBR-Male			
	IME		DHI	
	Median of staging	N	Median of staging	N
C	2	7	2	7
L	2	10	2	8
M	2	10	2	10
H	1	9	2	9
Dose	**ZBR-Female**			
	IME		DHI	
	Median of staging	N	Median of staging	N
C	2	8	3	10
L	3	10	3	9
M	2	8	3	9
H	2	10	2.5	8

Table 46a: Histopathological findings in male zebrafish exposed to flutamide

Diagnosis	**Dose**	**LAB 12**			**LAB 14**		
		Average of Grade	**N**	**Obs.**	**Average of Grade**	**N**	**Obs.**
Increased cells - ICS	C						
	L				1	1	8
	M	1	5	10	1	5	10
	H	1	6	9	1	4	9
Increased cells - SPA	M	2	1	10	1	3	10
	H	1.8	8	9	1	6	9
Increased cells - SPC	M			10	1	1	10
Decreased cells - SPT	H	1	2	9			9
Asynchronous development, spermatocyst	H			9	1	3	9
Interstitial fibrosis	M			10	1	3	10
Sertoli cell hypertrophy	M	1.6	7	10			10
	H	1	9	9	1	2	9

C= control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.
ICS: interstitial cells; SPA: spermatogonia; SPZ: spermatozoa; SPC: spermatocytes; SPT: spermatids.
Dark cells: primary diagnoses identified

145. Both studies suggest a dose-dependent increase of interstitial Leydig cells and an increase of the proportion of spermatogonia. The dose dependent increase relates to the number of animals affected rather than the severity grade of the diagnosis.

146. These findings are consistent with a previous study on adult zebrafish exposed to comparable concentrations of flutamide (42). In that experiment, histological changes in the testis included an increase of interstitial Leydig and Sertoli cells, and an increase of the size of early gonocytes. It was noted in the study that the observed effects of flutamide on Leydig and Sertoli cells and spermatogonia could be explained from disruption of the androgenic stimulation. Authors concluded that flutamide inhibits spermatogenesis in adult zebrafish, although probably confined to early stages. The observed changes were limited, but serious temporal effects of flutamide on spermatogenesis and sperm function could not be excluded.

Table 46b: Histopathological findings in female zebrafish exposed to flutamide

Diagnosis	Dose	LAB 12			LAB 14		
		Average of Grade	N	Obs.	Average of Grade	N	Obs.
Increased cells - PFC	L				1	1	9
Oocyte atresia, increased, mature	C				1	1	10
	L	2.3	3	10	1	2	9
	M	1	1	8			
	H	1.7	3	10			
Proteinaceous fluid, interstitial	C				2.2	5	10
	L				1	1	9
	M				1	4	9
	H				1.3	4	8
Interstitial fibrosis	H				1	1	8
Post-ovulatory follicles, increased	L	3	1	10			
Egg debris, oviduct	C	1	2	8			
	L	2.7	6	10			
	M	2	3	8			
	H	1.6	5	10			

C= control; L= low concentration; M=medium concentration; H= high concentration, P= positive control.
PFC: perifollicular cells;
Dark cells: primary diagnoses identified

147. In female zebrafish exposed to flutamide, there was a dose-dependent increase in oocyte atresia and egg debris in the oviduct in LAB 12. This was not found in LAB 14. In the study carried out by Wester and co-workers on adult zebrafish exposed to flutamide (42), they did not find any histological change in the ovaries.

5.5 Other optional data

148. Measurement of concentrations of persistent organic pollutants (POPs) in food was conducted at LAB 1. The brine shrimp was from Salt Lake in the U.S.A. The results are shown as follows.

Table 47 Measurements of organochlorine pesticides in the brine shrimp.

Substances	Concentrations of organochlorine pesticides in the brine shrimp (ng/g)
Hexachlorobenzene	1.089
β-Hexachlorocychlohexane	0.098
cis-Chlordane	0.025
trans-Nonachlor	0.068
o,p'-DDT	0.026
p,p'-DDT	N.D.*
p,p'-DDE	0.310
p,p'-DDD	0.072

The determination limit of organochlorine pesticides in the brine shrimp: 0.02 ng/g
*: N.D. < 0.02 ng/g

4.6 Compilation of opinions in participating laboratories

149. LAB 1 conducted the survey for Phase 1B of the validation of the Fish Screening Assay to collect various opinions from all participating laboratories. Eight out of 14 laboratories joined the survey, and all opinions are listed in **Annex 6**. Summary of the survey is described below.

Protocol instructions

150. All participating laboratories feel that the present protocol for the Fish Screening Assay is generally sufficient for performing the study.

Selection of test organisms

151. Laboratories for medaka and fathead minnow think that the selection criteria for test fish are sufficient. Several experts point out that it has to take into account that development time generally depends on holding conditions (biomass loading, food, water renewals per day, etc.) and temperature. Participants for zebrafish feel that 15 (±2) weeks seems not to be sufficient for having actively spawning fish, and suggest that fish size should be more important rather than fish age in this species, e.g., Size/Weight: 0,350 g for males and 0,5 g for females.

Endpoints/Spawning status

152. All laboratories recognize the potential necessity for measuring spawning status in Fish Screening Assay. Most of the participating laboratories think that the observation of spawning status in the present protocol is easy and essential. Several experts insist the necessity of quantitative measurement of egg numbers, while another expert for zebrafish suggests that semi-quantitative measurement would be available, because egg counting of zabrafish is time-consuming. However, an expert for medaka points out that spawning status before the exposure should be strictly measured, if the quantitative measurement of the fecundity is adopted in the Fish Screening Assay. An expert suggests the addition of fertility to the Assay. Another expert indicates that senescent or pre-spawning fish should not be selected if this endpoint is used.

Endpoints/Secondary sex characteristics

153. All laboratories for medaka and fathead minnow feel that the SOPs for the measurement of secondary sex characteristics are generally sufficient. Laboratories for medaka point out a minor problem associated with fixation of the fin rays, i.e, crossed fin rays are sometimes observed in the fixed anal fins, which disturbed the measurement of secondary sex characteristics in medaka. In fathead minnow, one expert feels that the SOPs for this species do not cover the early stages of nuptial tubercle formation (i.e., in some instances tubercles were present as white discs, but this stage did not fit into the three category provided). Another expert for fathead minnow suggests that additional drawings of mapping regions and detailed pictures of tubercle size/structures could be helpful, because tubercles could not always be clearly assigned (transition forms). In addition, a minor problem is indicated that quantitatively ranking of tubercles size was not so easy and observations were mostly not completed within 2 minutes. All participating laboratories feel that there is no problem about Excel reporting template, but a minor problem is pointed out by an expert for fathead minnow, i.e., template in SOP dose not identical to that in OECD.

Endpoints/VTG measurement

154. All participants think that the results yielded satisfactory in terms of reproducibility. A few experts feel that the variation of VTG levels can be reduced. An expert for zebrafish points out the difficulty in measurement of the exact blood volume result in variation of this parameter, suggesting that the variation will be minimised if the same person takes the blood samples from all the exposure groups. Another expert for zebrafish think that the more standardized every procedure throughout the experiment is, the lower the observed variations will be.

Other ideas/ observations/ comments

155. Several laboratories indicate the problems associated with preparation for test solution as follow; 1) methods provided need to be suitable for all participants and therefore, avoid the use of specialized equipment, 2) SOP's for the preparation of solvent free dilutions given by LAB 4 were difficult to transform into each laboratories, 3) very few/no information on solubility/stability of the test items and no alternatives on preparation for test solutions were available, resulting in deviations from the protocol, 4) the guidance on how to prepare the test stock solutions was given rather late.

156. Although all participants feel no problems regarding analytical chemistry, a few laboratories suffered from maintenance of the 4-*tert*-penthyphenol concentrations in the test solutions.

157. Regarding the design of and reporting into Excel Spreadsheets, several experts suggest as follows; 1) redesign the Excel Spreadsheets to enter all biological data in each individual, 2) the size of the boxes could have been larger, 3) the information value of flow-through conditions is not quite clear.

General

158. Several participants propose in any future validation exercise as follows; 1) it is needed to distribute the test substances, ELISA kits and VTG standard to each participating laboratory without delay, 2) more communications between lead laboratory and participating laboratories, among lead laboratories may facilitate the overall exercise, 3) technical SOPs should be provided without delay, and 4) it should be easy to integrate the SOPs in existing systems.

6. REPRODUCIBILITY ASPECTS OF TEST RESULTS

159. After a detailed presentation and analysis in Section 4 of the responsiveness of endpoints following chemical exposure, chemical by chemical and species after species, this section is now intended to provide an overview of reproducibility of quantitative endpoints measured in Phase 1B: VTG and secondary sex characteristics.

160. Reproducibility of test results across laboratories using the same fish species, the same protocol and the same chemicals is useful in evaluating the overall reliability of the assay in providing consistent responses when used in different places. A good measure of the reproducibility is the coefficient of variation (CV) of measurements, expressed as [standard deviation / mean] x 100. The CV (%) gives an indication of the level of variability of measurements. In the following description of reproducibility, intra-laboratory CV and inter-laboratory CV have been calculated for VTG and secondary sex characteristics measurements.

161. In this specific case, intra-laboratory CV represents the variability of values representing different individuals ($n \leq 10$) within a group of animals (either control or treated animals). It is important to note that the intra-laboratory CV does not represent variability of the same measurement performed n=10 times on the same biological sample, but the measurement of 10 biological samples subjected to the same treatment. In other words, the intra-laboratory coefficient of variation takes into account the variability of the method used to measure the endpoints, and the inherent variability of individual fish.

162. The inter-laboratory coefficient of variation is measuring the variability of similar results (e.g. feathead minnow male VTG values in control group measured in different laboratories). Coefficients of variations vary according to the treatment level, therefore they are analysed separately. This is because the spread or variability of low values in low parts of the dose response curve (e.g. VTG in control male fish) is much bigger than the spread of high values (e.g. VTG values in estrogen-induced females).

6.1 Reproducibility of results in medaka

6.1.1 Vitellogenin measures in medaka

Intra-laboratory CV

Table 48: Intra-laboratory CV of VTG measurements in medaka

4tPP		Concentration (µg/L)				
Sex	Laboratory	Cont.	100	320	1,000	PC
Male	LAB1	197.5	184.0	58.8	37.0	20.7
	LAB 3	0.0	138.5	60.7	23.3	33.1
	LAB 2	0.0	267.9	132.4	44.4	44.2
	LAB 5	42.0	226.6	217.2	61.7	108.4
Female	LAB 1	65.0	35.0	59.8	95.9	56.9
	LAB 3	31.1	66.5	43.3	44.1	83.9
	LAB 2	77.7	65.8	68.5	45.0	77.1
	LAB 5	110.6	121.8	193.3	139.6	98.5
Prochloraz		Concentration (µg/L)				
Sex	Laboratory	Cont.	20	100	300	PC
Male	LAB 1	0.0	0.0	0.0	0.0	0.0
	LAB 2	52.1	66.1	107.1	61.9	41.6
	LAB 4	207.4	83.0	150.5	108.6	85.8
	LAB 6	268.0	143.5	144.0	158.5	149.6
Female	LAB 1	17.6	45.4	143.9	106.4	107.6

	LAB 2	59.1	69.0	105.7	101.2	128.2
	LAB 4	40.5	65.3	100.0	78.7	75.5
	LAB 6	143.5	138.9	165.1	113.7	316.2
Flutamide		Concentration (µg/L)				
Sex	Laboratory	Cont.	100	500	1,000	
Male	LAB 3	0.0	52.3	0.0	30.3	
	LAB 5	0.0	61.2	61.2	127.3	
	LAB 4	166.6	59.5	148.1	57.6	
	LAB 6	268.0	99.6	86.2	105.8	
Female	LAB 3	41.3	33.9	37.9	34.3	
	LAB 5	118.9	125.1	78.2	140.8	
	LAB 4	63.6	92.6	67.5	91.3	
	LAB 6	143.5	154.8	131.9	101.0	

163. It should be noted that a CV equal to 0 measn that all values in the group were all at the lowest detection limit. The highest CVs (>100%) correspond to low VTG levels, usually found in control males or weakly induced males. As VTG levels increase in e.g. 4tPP exposure, the range of CVs decreases to 30-60%.

164. Interpretation of the CV has to be balanced with the intensity of the effect measured following chemical exposure (VTG increase or decrease). High CV in low parts of the dose-response curve do no mean as such that VTG measurement is not reproducible. As an example, in LAB 1 control males had VTG levels between 0.5 and 12 ng/mg liver (CV=197%). The significance level for 4tPP exposure was reached at the lowest concentration when VTG ranged between 9 and 122 ng/mg liver (CV=184%). At the highest concentration, induced males showed VTG levels between 2815 and 8900 ng/mg (CV=37%). So, the intensity of the response is 1000 time the value in the control group.

165. As said earlier in this section, these coefficients of variation integrate several sources of variability:

- the variability due to the handling of the method: each laboratory used the same method;
- the variability between individual fish: measures were performed once only on each liver sample;

166. Regarding the first point, some laboratories seem to have a better ability to reduce the coefficient of variation as VTG levels increase, other labs maintain high coeffivients of variation even at high VTG levels. For example, LAB 1 and LAB 3 had low coefficients of variation with high VTG values, whereas results from LAB 5 and LAB 6 show a high variability for all VTG values measured.

Inter-laboratory CV

Table 49: Inter-laboratory CV of VTG measurements in medaka

4tPP		Concentration (µg/L)				
Sex		Cont.	100	320	1,000	PC
Male	Lab number	4	4	4	4	4
	CV(%)	74.0	73.3	51.6	52.5	53.5
Female	Lab number	4	4	4	4	4
	CV(%)	75.2	83.8	51.3	45.7	59.0
Prochloraz		Concentration (µg/L)				
Sex		Cont.	20	100	300	PC
Male	Lab number	4	4	4	4	4
	CV(%)	118.5	110.3	95.9	70.0	89.6
Female	Lab number	4	4	4	4	4
	CV(%)	51.5	83.9	72.9	56.1	66.4
Flutamide		Concentration (µg/L)				
Sex		Cont.	100	500	1,000	
Male	Lab number	4	4	4	4	
	CV(%)	183.4	107.9	123.4	112.3	

Female	Lab number	4	4	4	4
	CV(%)	40.0	95.7	110.8	85.6

167. The inter-laboratory coefficient of variation provides an indication of the ability of laboratories to produce similar absolute VTG values with a narrow spread when they are measuring VTG from different fish, exposed to a similar (but not strictly identical) regime and using the same ELISA kit, applied by different technicians. So, it integrates the biological variability of the fish, the variability of the measured concentrations and the variability in the handling of the ELISA kit due to the person using the kit.

168. To remove variability due to the measured test substance concentration, one should only compare control groups among themselves (i.e. where no variability inherent to treatment exists).

6.1.2 Secondary sex characteristics in medaka

Intra-laboratory CV

Table 50: Intra-laboratory CV of secondary sex charactersitics measurements in medaka

4tPP		Concentration (µg/L)				
Sex	Lab	Cont.	100	320	1,000	PC
Male	LAB 1	21.1	22.7	20.1	20.3	18.1
	LAB 3	19.4	21.1	16.1	27.7	19.5
	LAB 2	23.1	27.0	29.5	30.3	17.9
	LAB 5	16.6	35.4	18.0	19.3	11.9
Prochloraz		Concentration (µg/L)				
Sex	Lab	Cont.	20	100	300	PC
	LAB 1	14.1	14.9	23.0	27.9	17.1
	LAB 2	20.7	29.3	10.5	16.2	32.8
	LAB 4	9.5	16.9	11.9	18.1	19.3
Male	LAB 6	11.0	11.1	20.3	26.4	17.2
Flutamide		Concentration (µg/L)				
Sex	Lab	Cont.	100	500	1,000	PC
	LAB 3	18.9	13.6	23.2	16.4	-
	LAB 5	15.8	28.4	19.0	30.0	-
	LAB 4	17.3	11.6	21.5	19.5	-
Male	LAB 6	11.0	12.3	19.9	15.6	-

169. All laboratories used the same standard operating procedures to measure secondary sex charactersitics in medaka. So the CV integrates the biological variability between fish mainly if one compares CV from control groups. The CV in treated groups integrates both the biological variability between fish and the variability of measured concentrations compared to nominal ones.

170. However, in medaka, none of the chemicals induced a significant response of secondary sex characteristics, so it is difficult to comment on the CV with respect with the effect size expected since there was no effect on papillary processes following exposure. In control animals, the group means ranged between 70 and 120 papillary processes (CV=16.5%).

Inter-laboratory CV

Table 51: Inter-laboratory CV of secondary sex characteristics measurements in medaka

4tPP		Concentration (µg/L)				
Sex		Cont.	100	320	1,000	PC
Male	Lab number	4	4	4	4	4
	CV(%)	8.9	12.5	21.8	19.0	7.4
Prochloraz		Concentration (µg/L)				
Sex		Cont.	20	100	300	PC
	Lab number	4	4	4	4	4
Male	CV(%)	15.5	15.1	12.5	8.1	12.0
Flutamide		Concentration (µg/L)				
Sex		Cont.	100	500	1,000	PC
	Lab number	4	4	4	4	-
Male	CV(%)	14.2	16.2	16.9	15.9	-

171. Inter-laboratory CVs were low in the control groups, between 8.9% and 15.5%.

6.2 Reproducibility of results in fathead minnow

6.2.1 Vitellogenin measures in fathead minnow

Intra-laboratory CV

Table 52: Intra-laboratory CV of VTG measurements in fathead minnow

4tPP		Concentration (µg/L)				
Sex	Lab	Cont.	100	320	1,000	PC
Male	LAB 7	74.0	236.8	168.9	37.8	42.0
	LAB 8	0.0	0.0	145.4	43.1	32.0
	LAB 10	-	-	-	-	-
Female	LAB 7	36.9	69.7	39.2	53.4	33.6
	LAB 8	65.5	73.0	108.1	101.7	76.4
	LAB 10	-	-	-	-	-
Prochloraz		Concentration (µg/L)				
Sex	Lab	Cont.	20	100	300	PC
Male	LAB 8	0.0	0.0	0.0	0.0	0.0
	LAB 11	0.0	0.0	0.0	0.0	0.0
	LAB 4	88.1	97.0	278.8	191.6	281.7
Female	LAB 8	85.8	80.4	47.2	144.9	24.9
	LAB 11	58.1	76.5	107.7	216.3	-
	LAB 4	31.5	17.8	52.7	37.0	272.8
Flutamide		Concentration (µg/L)				
Sex	Lab	Cont.	100	500	1,000	PC
Male	LAB 7	0.0	227.2	0.0	107.7	-
	LAB 10	0.0	0.0	0.0	0.0	-
	LAB 4	316.2	246.3	307.2	256.5	-
Female	LAB 7	30.2	47.4	51.2	44.9	-
	LAB 10	95.0	71.6	95.6	74.9	-
	LAB 4	57.6	33.0	38.5	41.0	-

172. Similar to studies in medaka, the coefficient of variation decreases with increasing VTG values: for example in the 4tPP study, for highly induced males and females the CVs vary between 32% and 76% (one CV is outside this range). However, for low and mid-concentration of 4tPP, the coefficient of variation of VTG measurements in males remains elevated, this is because VTG induction was not pronounced at the low and mid-concentrations (especially in LAB 7).

Inter-laboratory CV

Table 53: Inter-laboratory CV of VTG measurements in fathead minnow

4tPP	Concentration (µg/L)					
Sex		Cont.	100	320	1,000	PC
Male	Lab number	2	2	2	2	2
	CV(%)	124.2	115.5	141.2	6.6	19.8
Female	Lab number	2	2	2	2	2
	CV(%)	27.4	20.9	44.8	38.2	107.2
Prochloraz	Concentration (µg/L)					
Sex		Cont.	20	100	300	PC
Male	Lab number	3	3	3	3	3
	CV(%)	83.1	101.5	106.6	111.2	94.2
Female	Lab number	3	3	3	3	3
	CV(%)	73.1	45.3	47.5	99.8	116.9
Flutamide	Concentration (µg/L)					
Sex		Cont.	100	500	1,000	PC
Male	Lab number	3	3	3	3	-
	CV(%)	105.1	149.5	140.4	136.7	-
Female	Lab number	3	3	3	3	-
	CV(%)	89.6	88.0	119.6	97.2	-

173. At high VTG induction levels, the inter-laboratory CV can be as low as 6.6%, such low CV can be achieved when there is a good adequacy between absolute VTG values. But the inter-laboratory CV gets large when there are large differences between absolute VTG values between laboratories.

6.2.2 Secondary sex characteristics in fathead minnow

Intra-laboratory CV

Table 54: Intra-laboratory CV of secondary sex characterstics measurements in fathead minnow

4tPP		Concentration (µg/L)				
Sex	Lab	Cont.	100	320	1,000	PC
Male	LAB 7	22.3	31.0	74.6	0	59.5
	LAB 8	33.7	46.3	59.1	0	70.7
	LAB 9	42.7	-	-	138.2	56.3
Prochloraz		Concentration (µg/L)				
Sex	Lab	Cont.	20	100	300	PC
Male	LAB 8	21.2	28.0	54.4	71.2	28.3
	LAB 9	41.0	42.6	33.4	54.9	37.5
	LAB 11	16.3	29.2	29.5	24.7	17.6
	LAB 4	19.0	33.7	25.4	30.6	19.1
Flutamide		Concentration (µg/L)				
Sex	Lab	Cont.	100	500	1,000	PC
Male	LAB 7	22.8	32.1	29.9	20.7	
	LAB 10	29.4	26.8	19.4	19.0	
	LAB 4	20.3	25.7	34.3	20.0	

174. All laboratories working on the fathead minnow had the same standard operating procedures to count the number of nuptial tubercles. In control groups of male fathead minnow, the intra-laboratory variation ranged between 19% and 42%. These CVs integrate the biological variability between fish and the variability of tubercles counts when performed in different laboratories. In the case of secondary sex characteristics, there is no variability due to the technique used.

175. One observation that can be made when looking at the control groups is that some laboratories seem to constantly have lower CVs (LAB 4 and LAB 7), whereas other have consistently higher CVs (LAB 9).

Inter-laboratory CV

Table 55: Inter-laboratory CV of secondary sex characteristics measurements in fathead minnow

4tPP		Concentration (µg/L)				
Sex		Cont.	100	320	1,000	PC
Male	Lab number	3	2	2	3	2
	CV(%)	21.9	24.5	38.1	173.2	29.9
Prochloraz		Concentration (µg/L)				
Sex		Cont.	20	100	300	PC
Male	Lab number	4	4	4	4	4
	CV(%)	20.1	21.2	15.9	29.5	24.2
Flutamide		Concentration (µg/L)				
Sex		Cont.	100	500	1,000	PC
Male	Lab number	3	3	3	3	-
	CV(%)	10.4	89.2	88.4	95.9	-

176. The inter-laboratory CV in the control groups is quite low (from 10% to 22%). High CVs are often contributed by a real difference in the absolute mean number of tubercles between laboratories rather than by high intra-laboratory CV.

6.3 Reproducibility of results in zebrafish

6.3.1 Vitellogenin measures in zebrafish

Intra-laboratory CV

Table 56: Intra-laboratory CV of VTG measurements in zebrafish

4tPP		Concentration (µg/l)					
Sex	Lab	Cont.	SC	100	320	1000	PC
Male	LAB 12	71.8	134.9	162.7	63.2	47.0	181.0
	LAB 13	92.2	-	108.5	159.0	42.0	47.9
	LAB 14	156.6	-	248.0	299.6	104.5	212.8
Female	LAB 12	63.7	27.6	24.1	42.6	39.0	41.9
	LAB 13	118.7	-	42.5	65.7	95.7	80.1
	LAB 14	96.4	-	73.1	65.3	70.0	65.4
Prochloraz		Concentration (µg/L)					
Sex	Lab	Cont.		20	100	300	PC
Male	LAB 12	248.2		73.8	97.0	40.9	152.2
	LAB 13	186.3		197.2	153.8	163.0	83.9
	LAB 6	138.9		130.8	239.0	181.5	141.6
Female	LAB 12	66.6		35.0	34.7	55.6	26.7
	LAB 13	34.7		43.2	38.4	42.6	247.3
	LAB 6	59.8		137.5	228.1	83.8	96.7
Flutamide		Concentration (µg/l)					
Sex	Lab	Cont.		SC	100	500	1000
Male	LAB 12	70		145	119	162	137
	LAB 14	286		56	194	160	106
	LAB 6	139		-	171	291	333
Female	LAB 12	46		47	55	74	33
	LAB 14	47		71	49	60	64
	LAB 6	60		-	79	120	54

177. Coefficients of variation in zebrafish are generally higher than for the other two species, and there is much disparity between laboratories and between studies. However, low variability can be achieved for elevated VTG levels (CV= 42%), like in other species. It is difficult to identify specific reasons for the high variability observed in the studies, but again, this needs to be balanced with the size of the effect measured. For example, in LAB 12, VTG values ranged between approximately 900 ng/ml plasma (CV=72%) for control males and 21 millions ng/ml plasma (CV=47%) for highly induced males. Another example is LAB 14 where control males had 76000 ng/ml plasma (CV=156%) and induced males had 12 millionsng/ml plasma (CV=104%).

178. In zebrafish studies, it is less clear whether some laboratories work in a more reproducible way than others. Low coefficients of variation are not associated with particular laboratory(ies).

Inter-laboratory CV

Table 57: Inter-laboratory CV of VTG measurements in zebrafish

4tPP		Concentration (µg/L)					
Sex		Cont.	SC	100	320	1,000	Control
Male	Lab number	3	1	3	3	3	3
	CV(%)	164.4	-	49.8	76.1	69.4	158.6
Female	Lab number	3	1	3	3	3	3
	CV(%)	97.0	-	76.0	73.5	42.9	101.2

Prochloraz		Concentration (µg/L)				
Sex		Cont.	20	100	300	PC
Male	Lab number	3	3	3	3	3
	CV(%)	172.4	72.5	113.5	151.9	34.7
Female	Lab number	3	3	3	3	3
	CV(%)	53.7	80.9	38.5	27.8	144.5

Flutamide		Concentration (µg/L)				
Sex		Cont.	SC	100	500	1,000
Male	Lab number	3	3	3	3	3
	CV(%)	148.0	27.6	79.7	126.1	98.7
Female	Lab number	3	2	3	3	3
	CV(%)	70.3	34.3	73.4	74.4	82.0

7. DISCUSSION

7.1 Overview of the validation work of Phase 1B

179. Phase 1B of the validation work demonstrated that the protocol for the Fish 21-day assay using any of the three fish species is able to detect substances acting via the endocrine system such as the weak estrogen 4-*tert*-penthyphenol and the aromatase inhibitor prochloraz, via VTG measurement in males and females respectively. For the anti-androgen mode of action, it appears that the evaluation of gonadal histology is needed to see some changes compared to control.

180. Exposure to 4-*tert*-penthyphenol caused VTG induction in males of the three fish species within the range of concentrations tested. In medaka, a significant induction of VTG was observed at 100 µg/l (*3 labs/4*), in fathead minnow (*2 labs/2*) and zebrafish (*2 labs/3*) at 320 µg/l.

181. Prochloraz exposure inhibited VTG concentrations dose-dependently in females of the three fish species simultaneous with spawning cessation within the range of concentrations tested. A significant reduction of VTG was observed at 300 µg/l in medaka (*4 labs/4*), fathead minnow (*3 labs/3*) and zebrafish (*2 labs/3*). Cessation of spawning was visible in medaka at 100 µg/l (*2 labs/3*); in fathead minnow cessation of spawnin was confounded by a poor spawning in control groups; in zebrafish, spawning decreased but a complete cessation was not clear.

182. Flutamide exposure caused no clear alteration of VTG levels in males or females, no clear alteration of spawning, and no alteration of the secondary sex characteristics. Evaluation of gonadal histology revealed dose-dependent changes in testis and ovaries of fathead minnow and zebrafish. For medaka, histological changes were less clear because control fish were also affected and inter-laboratory consistency was not good.

183. The following table provides a synopsis of responses on endpoints following exposure.

Table 58: Synopsis of Phase 1B responses on core endpoints

Species	Sex	Spawning	VTG	SSC	Histopathology
4-*t*-Pentylphenol (Weak estrogen agonist)					
JMD	M		↑ ** (100 µg/)l	↓	↑ Testicular degeneration
	F	NE	↑ **		↑ Oocyte atresia, immature
FHM	M		↑ **(320 µg/l)	↓ **(1000 µg/l)	↑ Spermatogonia
	F	↓	↑ **		↑ Oocyte atresia, immature
ZBF	M		↑ ** (320 µg/l)		↑ Spermatogonia
	F	NE	↑		NE
Prochloraz (Aromatase inhibitor)					
JMD	M		↓	NE	↑ Spermatozoa, ↑ Interstitial fibrosis
	F	↓	↓ **(300 µg/l)		↑ Oocyte atresia, immature
FHM	M		NE	NE	↑ Spermatozoa
	F	equivocal	↓ **(300 µg/l)		↑ Oocyte atresia, immature
ZBF	M		↓		NE
	F	↓	↓ **(300 µg/l)		↑ Oocyte atresia, immature
Flutamide (Anti-androgen)					
JMD	M		equivocal	NE	NE

	F	equivocal	NE		NE
FHM	M		NE	NE	↑ Spermatogonia
	F	↓	NE		↑ Oocyte atresia, immature
ZBF	M		NE		↑ Interstitial cells, ↑ Spermatogonia
	F	equivocal	NE		NE

SSC: secondary sex characteristics; NE, no effect; shaded cells indicated that the endpoint was not measured in that sex or species
↓ decrease, ↑ increase; ** significant level: 1%

7.2 Application and limitation of the present protocol for the Fish Screening Assay

7.2.1 Weak estrogen

General aspects

184. 4-*tert*-Penthyphenol exposure induced dose-dependent VTG synthesis in males of three fish species, indicating that this protocol can detect estrogenic effect of weak estrogen. Secondary sex characteristics in males and spawning status were inhibited in fathead minnow.

VTG

185. In this validation work, the VTG levels in exposed males were significantly induced in three fish species. There is a report on VTG induction in the fish exposed to weak estrogen. Seki et al. (17) reported that VTG levels clearly increased as low as 51.5 µg/L when medaka were exposed to this alkylphenol from embryo to 101 d posthatch. In addition, several studies have been reported that weak estrogens induce VTG synthesis in adult fish. Ankley et al. (6) exposed adult fathead minnow to weak estrogen, metoxychlor for three weeks, showing that VTG levels increased in male fish. Similar study has been conducted by Kang et al. (12), that demonstrated VTG induction when adult medaka were immersed to weak estrogen, 4-nonylphenol for three weeks. These published reports and this present validation work clearly show that weak estrogen can induce the VTG production of males in adult fish. However, Seki et al. (17) conducted fish full life cycle test with 4-*tert*-penthyphenol using medaka, suggesting that the weak estrogens may exert the estrogenic effects and lethal and sublethal toxicity at similar concentrations. Caution should be exercised in determining test concentrations of weak estrogen, because general toxicity can obscure the estrogenic effects of the chemical.

186. As we have seen, the effect size expected is usually very large. So, even if high variability exists for low VTG levels, it should not impact the detection of significant increase when it is real because the effect is more than 1000 times the value in the control males.

Secondary sex characteristics

187. 4-*tert*-penthyphenol inhibited secondary sex characteristics of male fathead minnow dose-dependently, resulting in significant differences. Although slight reduction of papillary processes in male medaka was observed at highest concentration, it was less responsive than that in fathead minnow, and there was no significant difference except one laboratory. A few reports have been published indicating that the number of nuptial tubercles decreased in fathead minnow exposed to weak and strong estrogens (29) (30), while no significant effect was found in the number of papillary processes in male medaka

exposed to 4-*tert*-penthyphenol (17). This validation study and published reports suggest that the sensitivity of secondary sex characteristics in fathead minnow is higher than that in medaka when exposed to weak estrogen. Secondary sex characteristics in male fish are controlled by endogenous androgens (31) (32), however, it is not clear how estrogenic chemicals inhibit the male secondary sex characteristics. Ankley et al. (6) exposed adult fathead minnow to weak estrogen, metoxychlor for three weeks, indicating the decrease of the concentrations of serum androgens (11-ketotestosterone and testosterone) in males. The author suggested that the inhibition of endogenous androgens might involve the expression of nuptial tubercles in fathead minnow. Therefore, the inhibition of male secondary sex characteristics in this validation work might be caused by the reduction in E_2 levels in the exposed fish.

Spawning status

188. In the observation of spawning status recorded as Yes or No for the presence of eggs, 4-*tert*-penthyphenol caused no clear effect on spawning status of medaka and zebrafish. However, this chemical inhibited the egg production dose-dependently in fathead minnow, in particular, no eggs were produced in the 1,000 µg/L treatment group. The reason for the difference of sensitivity in three fish species is uncertain, but the published reports suggest that medaka may be less sensitive than fathead minnow with regard to fecundity when exposed to estrogens, at the selected range of the present concentrations. Kang et al. (12) reported that exposure of medaka to 463 ng/L E_2 for three weeks decreased fecundity, but that exposure to 227 ng/L had no effect. When fathead minnows were exposed to E_2 for 19 d, the E_2 concentrations expected to cause 50% and 10% inhibition of egg production were 120 and 6.6 ng/L, respectively (33).

189. Therefore, the results of this validation work and the published data suggest that observation of the spawning status may be effective in fathead minnow when exposed to estrogens, but poorly responded in medaka and zebrafish.

190. Although the mechanism of reproductive impairment of fish exposed to estrogen is unclear, previous study by Seki et al. (11) showed that the exposure of paired adult medaka to ethinylestradiol for three weeks induced the development of many previtellogenic oocytes in females, concurrent with decreasing fecundity, suggesting that exposure of estrogens may lead to developmental abnormalities of oocytes, and especially to inhibition of oocyte maturation in the ovary. Another reason of spawning reduction may be the inhibition of sexual behavior (34). However, it may be difficult to standardize this parameter in the Fish 21-day assay.

Gonad histology

191. Exposure to 4tPP cause immature oocyte atresia in medaka and fathead minnow, but not in zebrafish; an increased proportion of spermatogonia and testicular degeneration were noted in fathead minnow, zebrafish and medaka respectively; additionally, male medaka developed testis-ova. There are few studies on adult fish exposed to estrogen and where gonadal histology has been evaluated. Previous studies on medaka in a full life-cycle test (17) identified testis-ova as the main response to estrogen induction in males, no histological change was noted in females.

7.2.2 Aromatase inhibitor

General aspects

192. Prochloraz exposure inhibited VTG concentrations dose-dependently in females of the three fish species simultaneous with spawning cessation. These findings suggest that these responses follow a

cascade triggered by aromatase inhibition. Secondary sex characteristics in both sexes in two fish species was not affected by exposure to prochloraz and the positive control, although slightest sign of masculinization in fathead minnow females was observed in one laboratory.

VTG

193. In this validation work, the VTG levels in exposed females decreased dose-dependently in three fish species, indicating significant reduction, while those in males were not affected because already very low. Although this phenomenon itself seems to be anti-estrogenic, model study with fish exposed to aromatase inhibitor was conducted and the mechanism of the VTG reduction in femals was discussed by Ankley et al. (19). The authors exposed adult fathead minnow to aromatase inhitor, fadrozole, and indicated dose-dependent supression of VTG in females, accompanied by inhibition of plasma 17β-estradiol levels and brain aromatase activity. The authors suggested that the chemical was likely to inhibit the aromatase activity which directly controls endogenous 17β-estradiol, resulting in reduced VTG concentrations in female fish.

194. Therefore, inhibition of VTG in females exposed to prochloraz in this validation work is caused by the reduction of endogenous E_2 which was inhibited by aromatase inhibitor. Ankley et al. (19) reported that VTG and 17β-estradiol concentrations in male fathead minnows were unaffected by exposure to fadrozole. The results of this published report are in agreement with those of the validation work of Phase 1B.

Secondary sex characteristics

195. Prochloraz caused no clear effect on male and female secondary sex characteristics in male medaka and fathead minnow, except one laboratory in fathead minnow. Ankley et al. (19) reported that no significant effects of fadrozole were observed in male and female secondary sex characteristics, although the concentrations of endogenous androgens (11-ketotestosterone and testosterone) were significantly increased in male fish. The reason for the increase of androgen levels in male fish exposed to aromatase inhibitor is uncertain, however, Ankley et al. suggested that inhibition of CYP19 would increase androgen concentrations in males by blocking conversion of testosterine to 17β-estradiol. In this validation work, nuptial tubercles were observed in female fathead minnow exposed to positive control of aromatase inhibitor in one study. Generally, the present validation work is consistent with the report by Ankley et al., and also with other works on fadrozole (43) (18) suggesting the secondary sex characteristics are poorly responsive to aromatase inhibition.

Spawning status

196. Aromatase inhibitor clearly inhibited the spawning of three fish species dose-dependently. In addition, the response of spawning status was rapid, because most of the fish in the highest concentration of prochloraz stopped spawning within 2 or 3 days. Furthermore, this cessation of egg production was associated with inhibition of VTG levels. Ankley et al. (19) reported that the exposure of fathead minnow to fadrozole caused concentration-dependent decrease of fecundity as well as reduction in serum E_2 and VTG in females and marked alterations in ovarian histology. The author suggested that these responses provided the linkage of mechanism-specific information (aromatase inhibition) to a cascade of events through the endocrine system (reduction in 17β-estradiol) and target tissues (liver, ovary), to adverse effects in the whole organism (reduced fecundity). The results of this validation work and the published report by Ankley et al. show that the observation of the spawning status is a useful piece of information as regards the reproductive status of the fish, however it is not diagnostic of an endocrine-mediated response.

197. A question remains on whether egg counts add value to a daily/temporal recording of the spawning status in this type of assay.

Gonad histology

198. Immature oocyte atresia was observed in all three species. Previous studies on fadrozole (19) had also reported that follicles were undergoing atresia rather than proceeding to maturity. In the testis, an increased proportion of spermatozoa was mentioned in medaka and fathead minnow. Ankley et al. found that there was a notable concentration-dependent enlargement of the seminiferous tubules accompanied by an abundant accumulation of sperm in the lumina (19).

7.2.3 Anti-androgen

General aspects

199. Flutamide exposure caused no clear alteration in any of the following endpoints: VTG, secondary sex characteristics, spawning status- except at highest concentration in a fathead minnow study. Interpretation of the Phase 1B results regarding the flutamide study is not obvious and remains challenging. First, it appears that flutamide is a relatively weakly active substance in fish, contrary to expectations from mammalian studies. A couple of studies (18)(40)(41)(42)(43) have documented responses on the core endpoints in fish following flutamide exposure, and not all of them report exactly the same findings. As common features, vitellogenin level in females increased following flutamide exposure (18)(40)(43), without necessarily being significant; fecundity was significantly affected (40)(42)(43); and specific histological changes were noticeable in testis and ovaries (40)(42).

200. It seems that gonadal histopathology evaluation could aid more than the other endpoints in detecting changes following exposure to this representative mammalian anti-androgen.

VTG

201. Flutamide had no effect on VTG levels in males and females in three fish species. Nozaka et al. (39) exposed adult medaka to flutamide (90.4-1,470 µg/L, measured concentrations) for 21 days, indicating that no dose-dependent effect was found in male and female fish. The authors suggested that parameters other than VTG might be appropriate to detect anti-androgen. Panter et al. (18) reported that the exposure of pre-spawning adult fathead minnow to flutamide (95.3, 320.4 and 938.6 µg/L, measured concentrations) for 21 days caused no VTG induction in males, which consistent with the results of this validation work. However, the authors showed that significant VTG induction was found in females exposed to this chemical. Although the reason for this divergence in female VTG responses between this validation work and the published report is uncertain, this may be caused by the differences in sensitivity between spawning and pre-spawning fish. The results of this validation work suggest that the present protocol for Fish Screening Assay may not be able to detect the VTG alteration in the fish exposed to anti-androgen.

Secondary sex characteristics

202. Flutamide inhibited secondary sex characteristics of male fathead minnow at the highest concentraiton, resulting in significant difference in a study conducted at LAB 7, although another study with this species produced no clear effect on this endpoint. Panter et al. (18) reported that the flutamide exposure (938.6 µg/L) for three weeks significantly reduced the nuptial tubercles number in male fathead minnow. The finding of this validation work in LAB 7 concur with the previous study by Panter et al. This reduction of male secondary sex characteristics might be caused by anti-androgenic effect of flutamide, because endogenous androgens control the expression of male sexual characteristics in fish. Although there is no published paper on secondary sex characteristics of medaka exposed to anti-androgen, medaka seems

to be responsive to anti-androgen when exposed from embryo to adult. The fish full-life cycle test with flutamide using medaka showed dose-dependent reduction of papillary processes in male fish (Seki et al, personal communication). This present validation work and the previous studies suggest that flutamide potentially may reduce the secondary sex characteristics in male fish; however, the present protocol of Fish Screening Assay may not be able to detect the effect.

Spawning status

203. Flutamide caused no clear effect on spawning status of medaka and zebrafish; however, this chemical inhibited the egg production of fathead minnow in the 1,000 µg/L treatment group; consistent with other study reports (40)(43). In zebrafish, Wester et al. (42) found that flutamide at 1000 µg/L reduced significantly the number of clutches, thereby reducing the egg number.

Gonad histology

204. In Medaka, gonad histopathology evaluations did not provide consistent information throughout the laboratories. When there appear to be an histological change, it was also reported in control animals, therefore making the interpretation challenging. In fathead minnow and zebrafish, there were noticeable histological changes, consitent across laboratories and with findings reported in the literature (40)(42)(43).

7.3 Outcome of the validation work of Phase 1A and 1B

VTG

205. Results from Phase 1A and 1B demontrate that this protocol can detect estrogenic effects of strong and weak estrogens through VTG induction in males in three fish species. Reproducibility between laboratories was generally good, except when measured concentration of the test substance was outside the expected 80%-120% range. Intra-laboratory coefficients of variation were within ranges reported in other studies (43). VTG also responded to androgenic exposure in Phase 1A: VTG production in females significantly decreased after 17β-trenbolone exposure in the three fish species; however, the mechanism of this reaction is uncertain and not directly mediated via the androgen receptor; other androgens, especially those that undergo aromatization, generally cause other types of response. Aromatase inhibitor also decreased VTG levels in females in three fish species, suggesting that this endpoint may be appropriate to detect the endocrine effects of aromatase inhibitors. VTG poorly responds to anti-androgen; where there was an increase of VTG in females following anti-androgenic exposure, reproducibility across laboratories was not good.

206. In the Phase 1A of the validation work, extreme variability of VTG levels in control males was noted in zebrafish studies. Therefore in preparation for Phase 1B, participating laboratories agreed to use a single, homologous ELISA, method for each species. Standard operating procedures and a unique VTG standard, both available to each participant enable to minimise variations in methodologies applied for e.g. blood sampling and pretreatment. Results from Phase 1B indicated that the variability of VTG levels in control fish still existed. The reasons of this variability could be:

i) determination limit of the VTG levels was not unified in all participating laboratories, because the SOPs for VTG measurement in fathead minnow and zebrafish did not describe the lowest limit of the calibration curve of the VTG standard and the minimal dilution factor of the specimen;

ii) male zebrafish indicated wide range of VTG levels in Phase 1A and 1B, suggesting an intrinsic characteristic of zebrafish;

iii) contamination in food may be of concern in VTG variability, because LAB 1 showed that brine shrimp from Salt Lake contained estrogenic chemicals such as *o,p*'-DDT. The levels of POPs in food seem to be fairly low, however, criteria for food contaminant may be needed.

Secondary sex characteristics

207. This protocol can detect androgenic effect of the chemicals as masculinization of the secondary sex characteristics in females of medaka and fathead minnow. However, we do not have any parameter to identify the androgenic effect of the chemicals in zebrafish, because this species has no clear secondary sex character that can be quantitatively measured. Weak and strong estrogens inhibit the expression of this parameter in male fathead minnow, although the mechanism of this change is uncertain. Weak and strong estrogens cause no alteration of this parameter in male medaka. Anti-androgen may potentially reduce the secondary sex characteristics in male fish, but the present 21-day fish assay does not detect this type of response. Aromatase inhibitor did not cause alteration of this parameter in medaka and fathead minnow.

208. In the validation work of Phase 1A, different response of the secondary sex characteristics was obserbed in a few cases. One of the reason might have come from insufficient experience in measuring this parameter. To minimise the impact of individual laboratory experience in this respect, detailed SOPs for fathead minnow and medaka were prepared by the technical lead laboratories in Phase 1B. As a result, similar outcomes were generally yielded following chemical exposure in Phase 1B studies in both medaka and fathead minnow, suggesting that the level of guidance is now appropriate.

Spawning status

209. This observation was added to the protocol in the in Phase 1B of the validation. This observation allows a verification of the reproductive activity in control group, as a basic principle of the assay. In addition, daily observation in control and treated groups enables to see treatment-related effects on fecundity. For instance, the aromatase inhibitor study indicated that spawning was reduced in three fish species, most probably following a reduction of endogenous estrogen availability. In addition, weak estrogen and anti-androgen exposures inhibited spawning in fathead minnow. Although we did not validate the spawning status in the fish exposed to strong estrogen and androgen in the validation work of phase 1A, some reports on fathead minnow and medaka indicate reproductive impairment when exposed to these chemicals (6) (7) (11) (12) (33).

210. An important point in this parameter is to select reproductively active fish for exposure. If senescent or pre-spawning fish are selected, the spawning status is not valid to detect the reduction of spawning. Several participating laboratories felt that (semi-)quantitative measurement of eggs would add strength to a mere qualitative observation of spawning; however there may be resource implications in terms of time involved. A rapid survey with participating laboratories shows that egg counts demand between one and two hours a day for a study of this type. Reproduction tests developed in fathead minnow (6)(7) and medaka (11)(12)(36) (37) provide the possibility to extend the present protocol in this respect.

Gonad histology

211. Results from Phase 1A demonstrated that this endpoint was able to detect endocrine disputing effect in the exposure of strong estrogen and androgen. However, the experience from Phase 1A had shown that an insufficient level of standardization of procedures and guidance for evaluation were available to participants to read gonad samples in a comparable way and with common criteria and diagnoses. Further work was warranted to develop a consensus guidance document for use in Phase 1B. A group of experienced fish pathologists met after Phase 1A in October 2003, to identify areas where they could contribute further advice regarding the histological procedures and the pathological evaluation.

Considerable efforts were made to draft a comprehensive document, with all necessary standard operating procedures, illustrated with many annotated photos (e.g., dissection procedures, histological slides), providing the diagnostic terminology and a consensus severity scoring system to be applied in Phase 1B. A meeting with pathologists was held after Phase 1B to review slides and findings. The outcome of this meeting was an agreement on four exposure-related diagnoses in males and four exposure-related diagnoses in females. This will considerably reduce time involved in gonad evaluation because pathologists will know what diagnoses are meaningful following exposure to an endocrine active substance. The guidance document will be supplemented with androgenic-related examples and will then be available as an OECD monograph.

Other items

212. For Phase 1B of the validation work, the test design of the 21-day fish assay was improved to increase its biological relevance and optimize the use of animals. First, sampling of fish was fixed to 10 males and 10 females on day-21 of the experiment from each treatment level in Phase 1B, while that was divided in day-14 and day-21 in Phase 1A. This refinement not only simplified the test design, but also increased the number of fish sampled on day-21, resulting in improvement of the statistical power. Furthermore, a 21-day exposure is more appropriate for weak compounds. Second, the age of the test fish was strictly described in the protocol in Phase 1B, i.e., 20 (+/- 2) weeks in fathead minnow and 16 (+/- 2) weeks in medaka and 15 (+/- 2) weeks in zebrafish to exclude the variability associated with fish maturation. This generally contributed to reduce the variability of the results in Phase 1B, but several studies deviated from the protocol because of the delay in receiving test chemicals or ELISA kits for VTG measurement. In addition, a few experts in zebrafish commented that 15 (+/- 2) weeks in zebrafish may be too young. Particularly it is difficult to distinguish males and females in zebrafish from the external characters. Actually most of the laboratories for zebrafish could not exactly select males and females at the beginning of the exposure. Sexual maturity depends on not only fish age but also environmental conditions in each laboratory. Therefore, it may be needed to revise the age criteria for zebrafish. Third, the gonadosomatic index (GSI) was dropped in Phase 1B because GSI was not responsive to estrogen or androgen exposure in Phase 1A. As a result, this change facilitated the dissection procedure in Phase 1B. Forth, male and female fish were exposed together in Phase 1B because separation of males and females with a mesh in Phase 1A caused confounding in gonad histology evaluation, i.e., marked atresia in control ovaries.

8. RECOMMENDATIONS

213. With the view to ascertain the endocrine specificity of responses on core endpoints, it was recommended by the Validation Management Group for Ecotoxicity tests (VMG-eco) to select a substance that will not elicit a response on any of the three core endpoints of the 21-day fish assay.

214. It was also suggested that a different male to female sex ratio may need to be used in a future Test Guideline, especially for fathead minnow which exhibit a territorial behaviour with the current set-up. Territoriality has an impact on the spawning status and may act as a confounder.

215. It was also agreed at the last meeting of the VMG-eco to collect additional quantitative data on fecundity. At this stage, it is not entirely clear what could be the role of fecundity data in the fish 21-day assay. On the one hand, members of the VMG-eco agreed that interpretation of fecundity alone could not be used to qualify the endocrine-disruption nature of a substance, nor could it be used to aid gonad histopathology because individual female contribution to fecundity of the group can not be known. On the other hand, the EDTA Task Force noted that quantitative data on fecundity could form part of a general Test Guideline on fish reproduction, as an enhanced version of the fish 21-day assay. Further discussion on

the matter is warranted in the near future, when additional data on fecundity is collected by the US EPA in 2005.

9. REFFERENCES

1) Gimeno, S., Komen, H., Venderbosch, P.W.M., Bowmer, T. (1997) Disruption of sexual differentiation in genetic male common carp (*Cyprinus carpio*) exposed to an alkylphenol during different life stages. *Environ. Sci. Technol.* 31, 2884-2890.
2) Jobling S, Sheahan D, Osborne JA, Matthiessen P, Sumpter JP. 1996. Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. *Environ Toxicol Chem* 15:194–202.
3) Gray, M.A., Metcalfe, C.D. (1997) Induction of testis-ova in Japanese medaka (*Oryzias latipes*) exposed to *p*-nonylphenol. *Environ. Toxicol. Chem.* 16, 1082-1086.
4) Panter GH, Thompson RS, Sumpter JP. 1998a. Adverse reproductive effects in male fathead minnows (*Pimephales promelas*) exposed to environmentally relevant concentrations of the natural oestrogens, oestradiol and oestrone. *Aquat Toxicol* 42:243-253.
5) Panter GH, Campbell PM, Hutchinson TH, Kime DE, Länge R, Lye C, McCahon P, Sumpter JP, Tattersfield LJ, Tyler CR. 1998b. Effects of endocrine disruptors in the fathead minnow (*Pimephales promelas*). *Abstracts*, 19th Annual Meeting, Society of Environmental Toxicology and Chemistry, Charlotte, NC, USA.
6) Ankley, G.T., Jensen, K.M., Kahl, M.D., Korte, J.J., Makynen, E.A. (2001) Description and evaluation of a short-term reproduction test with the fathead minnow (*Pimephales promelas*). *Environmental Toxicology & Chemistry*, 20, 1276-1290.
7) Ankley, G.T., K.M. Jensen, E.A. Makynen, M.D. Kahl, J.J. Korte, M.W. Hornung, T.R. Henry, J.S. Denny, R.L. Leino, V.S. Wilson, M.C. Cardon, P.C. Hartig and L.E. Gray. 2003. Effects of the androgenic growth promoter 17-B-trenbolone on fecundity and reproductive endocrinology of the fathead minnow (Pimephales promelas). Environ. Toxicol. Chem. 22, 1350-1360.
8) Gray MA and Metcalfe CD (1999). Toxicity of 4-*tert*-octylphenol to early life stages of Japanese medaka (*Oryzias latipes*) *Aquat Toxicol* 46(2):149-154
9) Groonen S, Denslow N, Manning S, Barnes S, Barnes D and Brouwer M (1999) Serum vitellogenin levels and reproductive impairment of male Japanese medaka (*Oryzias latipes*) exposed to 4-tert-octylphenol. *Environ Hlth Perspect* 10: 385-390
10) Shioda T and Wakabayashi M (2000) Effect of certain chemicals on the reproduction of medaka (*Oryzias latipes*) *Chemosphere* 40(3): 239-243
11) Seki M, Yokota H, Matsubara H, Tsuruda Y, Maeda M, Tadokoro H, Kobayashi K. 2002. Effect of ethinylestradiol on the reproduction and induction of vitellogenin and testis-ova in medaka (*Oryzias latipes*). *Environ Toxicol Chem* 21: 1692-1698.
12) Kang IJ, Yokota H, Oshima Y, Tsuruda Y, Yamaguchi T, Maeda M, Imada N, Tadokoro H, Honjo T. 2002. Effects of 17β-estradiol on the reproduction of Japanese medaka (*Oryzias latipes*). *Chemosphere* 47:71–80.
13) Bresch H, Beck H, Ehlermann D, Schlaszus H, Urbanek M (1990) A long-term test comprising reproduction and growth of zebrafish (*Danio rerio*) with 4-chloroaniline. *Arch Environ Contam Toxicol* 19: 419-427
14) Andersen, L., Bengtsson, B.E., Björk, M., Gessbo, Å., Holbech, H., Hylland, K., Norrgren, L., Pedersen, K.L., Lundgren, A., Petersen, G.I., Steinholz, Å. and Örn, S. (2000) Zebrafish for testing endocrine disrupting chemicals. TemaNord 2000:555. ISBN:92-893-0480-4.
15) Andersen, L., Gessbo, Å., Holbech, H. Norrgren, L., Petersen, GI. (2001). Effects of exposure during early development to 17α-ethinylestradiol on the sexual differentiation and induction of vitellogenin in zebrafish (*Danio rerio*). *Aquatic Toxicology*, submitted.
16) Van den Belt K, Verheyen R and Witters H (2001) Reproductiv effects of ethynylestrdadiol and 4t-octylphenol on the Zebrafish (Danio rerio) *Arch Environ Contam Toxicol* 19: 419-427
17) Seki M, Yokota H, Matsubara H, Maeda M, Tadokoro H, Kobayashi K. 2003. Fish full life-cycle

testing for the weak estrogen 4-*tert*-pentylphenol on medaka (*Oryzias latipes*). *Environ Toxicol Chem* 22:1487-1496.
18) Panter GH, Hutchinson TH, Hurd KS, Sherren A, Stanley RD, Tyler CR. 2004. Successful detection of (anti-)androgen adult fathead minnows (*Pimephales promelas*) using easily measured endpoints of sexual development. *Aquat Toxicol* 18: 11-21.
19) Ankley GT, Kahl MD, Jensen KM, Hornung MW, Korte JJ, Makynen EA, Leino RL. 2002. Evaluation of the aromatase inhibitor fadrozole in a shor-term reproduction assay with the fathead minnow (*Pimephales promelas*). *Toxicological sciences* 67:121-130.
20) Panter GH, Hutchinson TH, Lange R, Lye CM, Sumpter JP, Zerulla M, Tyler CR. Utility of a juvenile fathead minnow screening assay for detecting (anti-)estrogenic substances. 2002. Environ Toxicol Chem.; 21(2):319-26.
21) Gimeno S., Komen H., Gerritsen A.G.M., Bowmer T. Feminisation of young males of the common carp, *Cyprinus carpio*, exposed to 4-*tert*-pentylphenol during sexual differentiation. 1998. Aquat Toxicol.43:77-92.
22) Panter GH, Thompson RS, and Sumpter JP. Intermittent Exposure of Fish to Estradiol. 2000. Environ. Sci. Technol.; 2000; 34: 2756 – 2760.
23) Panter GH, Hutchinson TH, LängeR, Whale G, Sumpter JP, Tyler CR. (in preparation).Detection of (anti-) androgens in an adult fathead minnow (*Pimephales promelas*) non-spawning assay. Draft CEFIC-EMSG research report reference AQ006. CEFIC, Brussels, Belgium.
24) Comparative Evaluation of Vitellogenin Methods, draft final report. USEPA, 2002.
25) Ankley GT, Kahl MD, Jensen KM, Hornung MW, Korte JJ, Makynen EA, Leino RL. Evaluation of the aromatase inhibitor fadrozole in a short-term reproduction assay with the fathead minnow (Pimephales promelas). 2002.*Toxicol Sci*.;67(1):121-30.
26) Korte, J.J., Kahl, M.D., Jensen, K.M., et al., 2000. Fathead minnow vitellogenin: complementary DNA sequence and messenger RNA and protein expression after 17b-estradiol treatment. Environ. Toxicol. Chem. 19, 972-981.
27) Tatarazako N, Koshio M, Hori H, Morita M and Iguchi T. 2004. Validation of an Enzyme-Linked Immunosorbent Assay Method for Vitellogenin in the Medaka. *Journal of Health Science* 50:301-308.
28) Holbech H, Andersen L, Petersen GI, Korsgaard B, Pedersen KL, Bjerregaard P, 2001. Development of an ELISA for vitellogenin in whole body homogenate of zebrafish (Danio rerio). Comp Biochem Physiol C Toxicol Pharmacol.;130(1):119-31.
29) Miles-Richardson SR, Kramer VJ, Fitzgerald SD, Render JA, Yamini B, Barbee SJ, Giesy JP. 1999. Effects of waterborne exposure of 17β-estradiol on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*). *Aquat Toxicol* 47:129–145.
30) Harries JE, Runnalls T, Hill E, Harris C, Maddix S, Sumpter JP, Tyler CR. 2000. Development of a reproductive performance test for endocrine disrupting chemicals using pair-breeding fathead minnows (*Pimephales promelas*). *Environ Sci Technol* 34:3003–3011.
31) Kime DE. 1998. *Endocrine Disruption in Fish*. Kluwer Academic, Norwell, MA, USA.
32) Borg B. 1994. Androgens in teleost fishes. *Comp Biochem Physiol C* 109:219–245.
33) Kramer VJ, Miles-Richardson S, Pierens SL, Giesy JP. 1998. Reproductive impairment and induction of alkaline-labile phosphate, a biomarker of estrogen exposure, in fathead minnows (*Pimephales promelas*) exposed to warterborne 17β-estradiol. *Aquat Toxicol* 40:335–360.
34) Gray MA, Teather KL, Metcalfe CD. 1999. Reproductive success and behavior of Japanese medaka (*Oryzias latipes*) exposed to 4-tert-octylphenol. *Environ Toxicol Chem* 18:2587–2594.
35) Makynen EA, Kahl MD, Jensen KM, Tietge JE, Wells KL, Van Der Kraak G, Ankley GT. 2000. Effects of the mammalian antiandrogen vinclozolin on development and reproduction of the fathead minnow (*Pimephales promelas*). *Aquat Toxicol* 48:461–475.
36) Kang IJ, Yokota H, Oshima Y, Tsuruda Y, Oe To, Imada N, Tadokoro H, Honjo T. 2002. Effects of bisphenol A on the reproduction of Japanese medala (*Orizial latipes*). *Environ Toxicol Chem* 21:2394–2400.
37) Kang IJ, Yokota H, Oshima Y, Tsuruda Y, Hano T, Maeda M, Imada N, Tadokoro H, Honjo T. 2003.

Effects of 4-nonylphenol on reproduction of Japanese medaka, *Orizial latipes*. *Environ Toxicol Chem* 22:2438–2445.

38) Nishi, K., Chikae, M., Hatano, Y., Mizukami, H., Yamashita, M., Sakakibara, R., and Tamiya, E. 2002. Development and application of a monoclonal antibody-based sandwich ELISA for quantification of Japanese medaka (Oryzias latipes) vitellogenin. Comparative Biochemistry and Physiology Part C 132: 161-169.
39) Nozaka T, Abe T, Matsuura T, Sakamoto T, Nakano N, Maeda M, Kobayashi K. 2004. Development of vitellogenin assay for endocrine disruptors using medaka (*Oryzias latipes*). *Environmental Sciences* 11:99-111.
40) Jensen KM, Kahl MD, Makynen EA, Korte JJ, Leino RL, Butterworth BC, Ankley GT, 2004. Characterization of responses to the antiandrogen flutamide in a short-term reproduction assay with the fathead minnow. Aquatic Toxicology 70(2):99-110
41) Ankley GT, Defoe DL, Kahl MD, Makynen EA, 2004. Evaluating the model anti-androgen flutamide for assessing the mechanistic basis of responses to an androgen in the fathead minnow. Environmental Science and Technology (38): 6322-6327.
42) Wester PW, van den Brandhof EJ, Vos JH, Van der Ven LTM, 2003. Identification of endocrine disruptive effects in the aquatic environment. A partial life-cycle assay with zebrafish. RIVM report 640920001/2003.
43) US Environment Protection Agency, 2003.