DRAFT REPORT OF THE VALIDATION OF THE 21-DAY FISH ENDOCRINE SCREENING ASSAY FOR THE DETECTION OF ENDOCRINE ACTIVE SUBSTANCES PHASE 2-TESTING NEGATIVE SUBSTANCES

This document is the report of the Phase 2 of the validation of the 21-day fish endocrine screening assay. The fish endocrine screening assay was included in the OECD conceptual framework for the testing and assessment of endocrine disrupting chemicals, for the detection of estrogenic, aromatase inhibiting and androgenic substances in fish. A validation study was initiated in 2003 to demonstrate the relevance and reliability of the assay, in accordance with the draft OECD Guidance Document on validation and international acceptance of new or updated test methods for hazard assessment [final document No.34 (OECD, 2005)]. In 2006, the 18th Meeting of the Working Group of the National Coordinators of the Test Guidelines Programme (WNT) approved the reports of Phase 1A and Phase 1B of the validation of the 21-day fish endocrine screening assay. The Phase 1A was considered to be an optimization phase while the Phase 1B consisted in a multi-chemical inter-laboratory study for the evaluation of the reproducibility of the assay.

The Phase 2 of the validation was performed in 2005-2006, using negative substances for the evaluation of the specificity of the assay for the detection of certain endocrine active substances, as indicated above. In the Phase 2 study, two negative substances were tested in the three fish species.

The complete Phase 2 report of the validation was presented at the meeting of the validation management group for ecotoxicity testing (VMG-eco) in January 2007. Comments were made on the statistical tests used for the data analysis, given the replicate number per treatment level for some datasets (i.e. zebrafish and medaka data). A new data analysis was thus performed for those datasets with appropriate statistical test. A decision flow-chart was elaborated by the statistician to guide the choice of the most appropriate statistical test (Annex 1). Additionally, extensive work was performed to evaluate the power properties of the vitellogenin endpoint in the current design; a brief summary is presented in Annex 2 to the report. The report was revised taking into account the comments from the VMG-eco and comments made at the meeting of the Task Force on Endocrine Disrupters Testing and Assessment (27-28 March 2007). The Phase 2 report was endorsed by the Working Group of the National Coordinators of the Test Guidelines Programme at its 19th meeting (28-30 March 2007).

ACTION REQUIRED: ***The Joint Meeting is invited to agree that the report be declassified.***

DRAFT REPORT

PHASE 2 OF THE VALIDATION OF
THE 21-DAY FISH ENDOCRINE SCREENING ASSAY FOR THE DETECTION OF ENDOCRINE ACTIVE SUBSTANCES
-Testing negative substances-

April 2007

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ACKNOWLEDGEMENT

The preparation of this report is based on the data contributions from the participating laboratories in Germany, Japan and the United States representing government as well as industry resources. The discussions of the Validation Management Group for Ecotoxicity Testing in December 2005 and January 2007 have played an important role in determining the key issues to be addressed in the report, mainly dealing with statistics, and the participation of VMG members in this and previous discussions on the validation of this assay is to be acknowledged. Finally special thanks go to John Green, DuPont, who has delivered an important piece of work on the statistical analysis and the power simulations.

SUMMARY

i) This report provides the results from an OECD inter-laboratory study conducted in 2005 to examine the specificity of a standardized OECD protocol on adult fish for the detection of endocrine active substances (i.e. estrogen, aromatase inhibitors, androgen). This work is in a continuum to Phase 1A and then Phase 1B of the validation of the fish screening assay for the detection of endocrine active substances. This Phase 2 was primarily performed to experimentally assess the possibility of false-positive outcomes generated when testing negative substances. Fathead minnow (*Pimephales* promelas), medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) were used.

ii) The protocol followed the same features as in Phase 1B. Reproductively active male and female fish were exposed to test chemical for 21 days and four core endpoints were measured, namely: i) gross morphology (i.e., secondary sex characteristics), ii) spawning status of fish, iii) vitellogenin (VTG) levels, and iv) gonadal histology. Three fish species, i.e., fathead minnow (*Pimephales promelas*), medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) were used as test fish. The negative substances were potassium permanganate (225, 450 and 900 µg/l) and *n*-octanol (0.32, 1.0, 3.2 mg/l), respectively.

iii) Four laboratories in 4 countries participated in this Phase 2 study. One laboratory in the United States participated to the potassium permanganate studies using fathead minnow. One laboratory in Japan participated to the potassium permanganate and n-octanol studies using medaka. One laboratory in Germany participated to the potassium permanganate and n-octanol studies using zebrafish. One laboratory in Germany participated to the potassium permanganate and n-octanol studies using fathead minnow.

iv) Measured concentrations of the potassium permanganate in fathead minnow, medaka and zebrafish studies showed that the nominal concentrations of these chemicals remained consistent throughout the exposure period, although those in one of the fathead minnow study decreased to about 50–80% of the nominal concentrations. Measured concentrations of *n*-octanol in medaka study almost remained within the 80-120% of nominal concentrations during the exposure period. Measured concentrations of n-octanol in the fathead minnow and zebrafish study decreased dramatically, probably caused by the biodegradation of the substance and the lower volume exchange compared to the medaka study.

v) Potassium permanganate exposure had no affect on VTG levels in both sexes of medaka, fathead minnow and zebrafish. The secondary sex characteristics in male medaka and fathead minnow were not affected in potassium permanganate and ocatnol studies, except in male fathead minnow exposed to potassium permanganate where the total score of nuptial tubercles in decreased at the highest concentration. The results of spawning status showed that potassium permanganate caused a reduction in fecundity and fertility of medaka and zebrafish due to the toxicity of the substance, while no clear effect was observed in the fathead minnow study.

vi) *n*-Octanol exposure had no effect on VTG levels in both sexes of medaka, fathead minnow and zebrafish, and no effects on and secondary sex characteristics in meadka and fathead minnow. The results of spawning status showed that the fertility and fecundity decreased in the highest concentration in the zebrafish study, although no clear effect was observed in medaka and fathead minnow studies.

vii) Gonad histology of Phase 2 studies sometimes showed some slight chemical exposure-related alterations associated with the general toxicity of the test substances.

viii) Overall, it is concluded from Phase 2 studies that the 21-day Fish Screening Assay, including the vitellogenin and secondary sexual characteristics endpoints, is relatively specific for endocrine active substances. However, care should be taken when evaluating the reduction in male secondary sex characteristics and spawning status, in light of other information available (e.g. other signs of toxicity, response on other endpoints, etc.). Vitellogenin measurement is a relevant, reliable and relatively specific endpoint for the detection of endocrine activity of chemical substances. Secondary sex characteristics are also relavant, reliable and relatively specific, but may need to be restricted to induction in female fish, and not reduction in male fish, to avoid false positive outcomes.

BACKGROUND

1. At the fourth meeting of the Validation Management Group for ecotoxicity testing, held in December 2004 in Paris, participants discussed the validation status of the 21-day fish screening assay. Members of the VMG, upon a proposal from BIAC, agreed that the testing of at least one negative substance would be useful to give an estimation of false positives outcomes generated by this test method. Following the December meeting, a small group was formed to identify candidate negative substances. With great difficulty to come to an agreement, it was finally proposed that both potassium permanganate and *n*-octanol would be tested, the VMG was informed accordingly on 12th July 2006, and no alternative proposal received. The Secretariat canvassed interest – and availability of funds - of laboratories to do the experimental work. One laboratory in Japan tested both substances with medaka, one laboratory in Germany tested both substances with zebrafish, one laboratory in the United States tested one substance (potassium permanganate) on the fathead minnow, and one laboratory, funded by CEFIC, did both test substances and methoxyethanol on fathead minnow .

Methodology for the identification of candidate substances

2. A small group of experts started with a review of the existing databases and scientific literature. In view of the scarcity of data from *in vivo* fish studies, criteria were used to select putative negative substances. One reason why it took six months to agree on a substance (and eventually two) was due to diverging views on the criterion to be used; even in some cases the usefulness of negative testing was questioned in relation to the intended screening use of the test method. Again, because the specific purpose of the assay remains unclear, it was difficult to select proper negatives.

3. On the one side, it is expected that the assay should be used to detect endocrine and reproductive toxicants (4 endpoints); on the other side, it is thought that the assay should be used for the detection of endocrine modes of action only (2 endpoints). For the former, the candidate negative substances should be known to be toxic on other organs, but not on the gonads; for the latter, the candidate negative substances should be chosen among substances that are likely targets for an endocrine disrupter testing programme, that are likely reproductive toxicants, but not through the endocrine system, hence a recommendation for organic substances, preferably narcotics in their mode of action. Following this, a number of organic substances were proposed: 1- *n*-hexanol (CASRN 111-27-3), 2,4-dinitrophenol (CASRN 51-28-5), 3,4-dichloroaniline (CASRN 95-76-1), and Pentachlorophenol (CASRN 131-52-2).

4. In the absence of sufficient data on the toxicological profile of the 4 substances in fish, Japan accepted to conduct *in vitro* receptor binding assays (ERα, AR) and transcription activation assays (ERα, AR) for each substance. These in vitro studies demonstrated the following (Table 1):

Table 1: Summary of the in vitro studies for selecting negative substances.

Substances	Receptor binding assay		Reporter gene transcriptional assay	
	ER alpha	AR	ER alpha	AR
17beta-Estradiol	100%		100%	
5alpha-Dihydrotestosterone				100%
R1881		100%		
1-Hexanol	Negative	N.D.	Negative	Negative
3,4-Dichloroaniline	N.D.	0.0012	Negative	Negative
2,4-Dinitrophenol	Negative	Negative	Negative	Negative
Pentachlorophenol	Negative	N.D.	Negative	Negative
Potassium permanganate	Negative	Negative	Negative	Negative
n-octanol*	Negative	N.D.	Negative	Negative

Notes: 1)"Negative" in receptor binding assay means that the standard ligand was not eliminated even in the highest concentration.
2) "Negative" in reporter gene transcriptional assay means that no transcriptional activity was found even in the highest concentration.
3)"N.D." in receptor binding assay means that IC 50 was not calculated, although the elimination of standard ligand was found.
4)"N.D." in reporter gene transcriptional assay means that EC 50 was not calculated, although transcriptional activity was found.
* *In vitro* assays with *n*-octanol were also conducted at a later date.

5. A teleconference of the Fish Drafting Group was held on 29th June 2005 to review these outcomes. Potassium permanganate and 2,4-dinitrophenol seemed at the best places for negative testing. However, a member of the Fish Drafting Group brought to its attention a publication (1) demonstrating that 2,4-dinitrophenol is active o the endocrine system of fish. Finally, it was agreed that to test *n*-octanol (CAS RN.111-87-5) which is also considered to be a narcotic and for which there is no presumption of endocrine activity.

Test concentrations

6. The following information served as a basis for deciding on the test concentrations.

n-octanol

7. The 96-hr acute LC50 value in juvenile fathead minnows is 13.5 mg/L (2). Pickering et al. (3) report a 7-day survival NOEC using larval FHM of 6.0 mg/L. The growth-based LOEC and NOEC in the same report were 3.0 and 1.5 mg/L respectively. The 48-hr acute LC50 value for medaka is 16.5 mg/L (4). Thus the concentration range was recommended to be 0.32, 1.0, 3.2 mg/L.

potassium permanganate

8. The 96-hr LC50 with mature fathead minnow under conditions of the reproduction assay was determined to be 2.5 mg/L. Thus the concentration range was set as follows: 225, 450, and 900 ug/L.

Test design used for the negative substances testing

The laboratories testing medaka and zebrafish used the same test design as recommended in Phase 1B, i.e. 3 concentrations and 2 replicates per concentration, with 5 males and 5 females in each replicate. The laboratory testing fathead minnow used a slightly different test design, already well-documented by Ankley et al. (5) where each experimental unit is composed of 4 femalesand 2 males (instead of 5♀:5♂) and 4 experimental units (instead of 2) are allocated for each treatment group. This amendment was recommended in the Phase 1B report to reduce aggressiveness of territorial males of males; 5 males in the same aquarium was simply too many and disturbed spawning behavior.

STATISTICAL ANALYSIS: PROCEDURE FOLLOWED

9. Statistical analysis was performed on secondary sex characteristics of fathead minnow and medaka, and VTG of three fish species. Spawning status and gonad histology were excluded because these data are qualitative and does not lend itself to a straightforward statistical analysis. The statistical analysis of optional data (e.g., egg numbers or steroid hormone levels) has not been made. An ANOVA using a two-side test was performed because the initial hypothesis considers both outcomes (increase and decrease of VTG and of Secondary sex characteristics) as damageable outcomes.

10. For the fathead minnow studies using four replicates per concentration, 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. 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.

11. For the medaka and zebrafish studies using 2 replicates per concentration, the concentration means are examined for dose-response monotonicity. This could be done visually from a plot, numerically from computed means, or formally. Formal tests for monotonicity are described in *Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application*, OECD Series on Testing and Assessment, No. 54 (OECD, 2006). A lack of monotonicity was evident in most cases, which means the Williams and Jonckheere tests were not applicable. The Mann-Whitney test (either the exact or standard version) has zero power to detect effects regardless of magnitude. This leaves only the Dunnett and Dunn tests. The Dunnett test is shown to be much more powerful, but it assumes normality and variance homogeneity. Dunn's test is applicable when the normality or variance homogeneity assumptions are violated, but as indicated, has lower power. If the dose-response is not consistent with monotonicity, then a pairwise analysis is appropriate. The general approach followed is described below.

12. Which pairwise test depends on whether the data are normally distributed or a normalizing transform can be found. If so, the test further depends on whether the variances are homogeneous, either as reported

or after a normalizing, variance stabilizing transform.

13. The individual fish were analyzed using a nested variance structure, with fish nested in vessel and vessel nested in concentration. There are then two variance components taken into account in the mixed model ANOVA. This approach still treats the vessel as the unit of analysis because of the way the nested variance structure is treated. Again, the data were examined for normality and variance homogeneity using the same general procedures indicated above, except the residuals are from a nested ANOVA with concentration as the only fixed effect and Vessel(Concentration) and fish(Vessel(Concentration)) are random effects. Dunnett or Tamhane-Dunnett tests were applied. These tests were modified to use the correct nested variance structure. If the data were not normally distributed, there is no non-parametric procedure analogous to Dunn's test or the Mann-Whitney test that could be applied.

14. In these four experiments, when the dose-response was not monotone and pairwise methods must be used, the approach described above was the soundest approach whenever the data were normally distributed (possibly after a transformation). **Annex 1** describes the decision logic followed.

RESULTS

Analytical chemistry

Analytical chemistry of potassium permanganate studies

15. Measured concentrations of the potassium permanganate in medaka and zebrafish studies showed that the nominal concentrations of these chemicals remained consistent throughout the exposure period, although those in fathead minnow study decreased to about 50–80% of the nominal concentrations.

Table 2: The means of measured potassium permanganate in the test solutions.

		Means measured concentrations (% nominal)			
Nominal values		**112.5µg/L**	**225 µg/L**	**450 µg/L**	**900 µg/L**
LAB 1	Fathead minnow	-	150 (76)	260 (58)	683 (76)
LAB 4	Fathead minnow	95 (85)	190 (84)	435 (96)	-
LAB 2	Medaka	-	200 (89)	380(84)	778(86)
LAB 3	Zebrafish	-	249(110)	471(105)	961(107)

Analytical chemistry of n-octanol studies

16. Although the mean measured concentration of *n*-octanol were 79% and more of the nominal concentrations in LAB 2, those of this substance in the test solutions decreased to about 10–30% of the nominal concentrations in LAB 3.

Table 3: The means of measured *n*-octanol in the test solutions.

		Mean measured concentrations (% nominal)		
Nominal values		**0.320 mg/L**	**1.00 mg/L**	**3.20 mg/L**
LAB 2	Medaka	0.252 (79)	0.814(81)	2.83(88)
LAB 3	Zebrafish	0.0781(24)	0.149(15)	0.385(12)
LAB 4	Fathead minnow	0.23 (71)	0.68 (68)	1.86 (58)

Mortality, abnormal behavior and appearance

Potassium permanganate studies

17. The validity criterion is to have less than 10% mortality in the control. In the medaka study, fish at the highest concentration displayed reduced activity, including feeding, light body color, partial loss of equilibrium and lethargy; fish started to die after 6 days of exposure and none remained alive at the end in the highest exposure group. In fathead minnow study from LAB 1, 12.5% of the fish died in the highest concentration group. In the fathead minnow study from LAB 4, high mortalities were observed, and the fish survived at 450µg/L showed various signs of intoxication, i.e. strong ventilation, swimming mainly near the water surface, reduced feeding, apathy, lying on the side or back on the bottom. These mortalities at high concentrations raise the question of defining more systematically the maximum tolerated dose, based on the LC_{50}, on which to calculate the dose range to apply.

Table 4: Mortality in the potassium permanganate studies at the end of exposure.

Laboratories		**Mortalities (%)**				
		Cont.	**112.5µg/L**	**225 µg/L**	**450 µg/L**	**900 µg/L**
LAB 1	Fathead minnow	0	-	4.2	0	12.5
LAB 4	Fathead minnow	0	4.2	20.8	63	-
LAB 2	Medaka	0	-	0	5	100
LAB 3	Zebrafish	0	-	0	10	0

n-octanol studies

18. Mortalities in the *n*-octanol studies were ≤ 5% in all studies during the exposure period, except in the fathead minnow study in LAB 4 where the validity criterion was not met. In that study, one fish per replicate died in the control and in the lowest concentration, but no sign of intoxication was observed otherwise; at the highest concentration, seven fish died and some of the surviving fish showed signs of intoxication at that concentration. Overall, mortality was not significantly higher in the treated fish compared to the control in LAB 4.

Table 5: Mortality in the *n*-octanol studies at the end of exposure.

Laboratories		Mortalities (%)			
		Cont.	**0.32 mg/L**	**1.00 mg/L**	**3.20 mg/L**
LAB 2	Medaka	0	0	0	5
LAB 3	Zebrafish	0	0	0	0
LAB 4	Fathead minnow	16	16	20	29

Spawning status

Spawning status of potassium permanganate studies

19. In fathead minnow study from LAB 1, the potassium permanganate did not inhibit the fertility and fecundity during the exposure period (Fig. 1, 2). In the fathead minnow study from LAB 4, despite the fact that spawning in the control was sub-optimal, a trend towards decreased fecundity could be observed in treated fish. An explanation for the low fecundity in the control vessels might be that young fish were used at the start of the test and the difference between males and females was not obvious, leading to an inverted sex ratio: 4 males and 2 females in some vessels. Statistically, the outcome of the Mann-Whitney U-test showed a statistically significant decrease in spawning at 225 and 450µg/L; this outcome should be interpreted with care given the high toxicity of the substance and other conditions described above. In the medaka study in LAB 2, no eggs were produced after 4 days of exposure in the 900 µg/L treatment group (toxic effect). In the 450- and 225-µg/L treatment groups, egg production was observed every day. However, the cumulative number of eggs was reduced compared to the controls (Fig. 3). The mean fertility was also reduced dose-dependently (Fig. 4). In the zebrafish study in LAB 3, a dose-dependent reduction in fertility and in fecundity was observed, similar to the case of medaka (Fig. 5, 6).

Table 6: Spawning status in the potassium permanganate studies during the exposure period.

No. of days with spawning "yes" (average of tank 1 and 2) / observed days						
Nominal values		**Cont.**	**112.5µg/L**	**225 µg/L**	**450 µg/L**	**900 µg/L**
LAB 1	Fathead minnow	No data	-	No data	No data	No data
LAB 4	Fathead minnow	4/21	2.5/21	0/21	0.25/21	-
LAB 2	Medaka	21/21	-	21/21	21/21	2.5/20
LAB 3	Zebrafish	14/15	-	14/15	12/15	7/15

Table 7: Daily recording of the spawning status in the potassium permanganate studies.

DAY	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
LAB 2 (Medaka)																						
Control		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
225 µg/L		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
450 µg/L		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
900 µg/L		1	1	1/2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
LAB 3 (Zebrafish)																						
Control		1	1	1			1	1	1	1	1			1	1	1/2	1	1/2			1	1
225 µg/L		1	1	1			1	1/2	1	1	1			1/2	1	1	1	1			1	1
450 µg/L		1	0	1/2			1	1	1	1	1			1	1	1/2	1/2	1			1/2	1
900 µg/L		1/2	1/2	0			0	1/2	0	1/2	1/2			1/2	1	1	1/2	1/2			1/2	1/2

Notes: 1: spawning observed in both replicate tanks; 1/2: spawning observed in one of replicate tanks; 0: no spawning observed in any of the two replicate tanks.

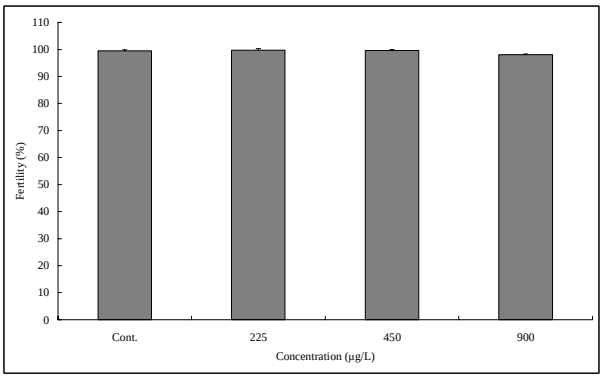

Fig. 1: The means of fertility in fathead minnow exposed to potassium permanganate.
(Optional data of LAB 1)

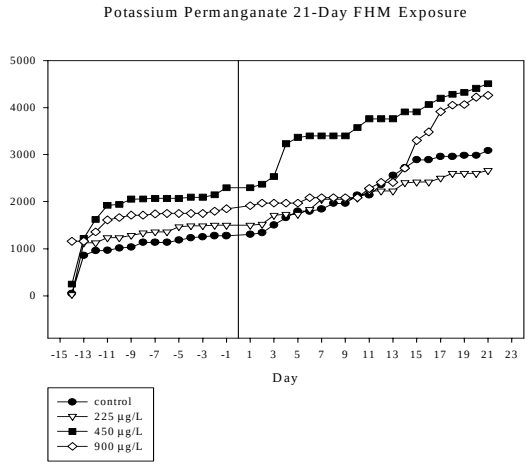

Fig. 2: The cumulative number of eggs in fathead minnow exposed to potassium permanganate.
(Optional data of LAB 1)

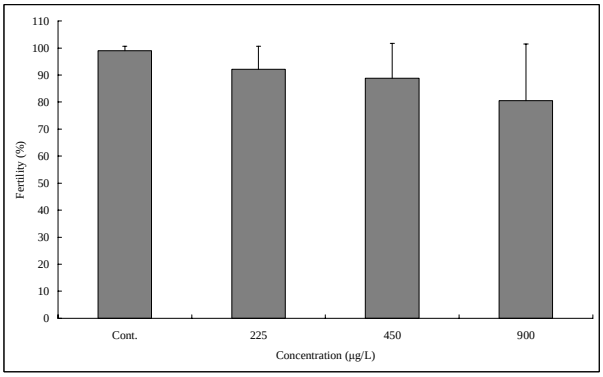

Fig. 3: The means of fertility in medaka exposed to potassium permanganate.
(Optional data of LAB 2)

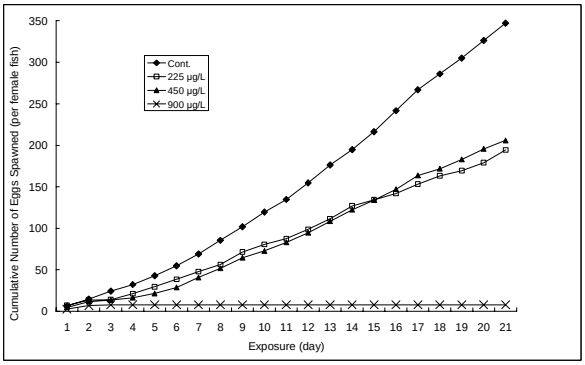

Fig. 4: The cumulative number of eggs spawned in medaka exposed to potassium permanganate.
(Optional data of LAB 2)

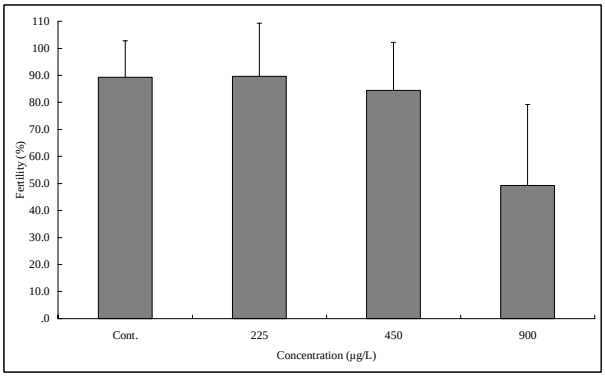

Fig. 5: The means of fertility in zebrafish exposed to potassium permanganate.
(Optional data of LAB 3)

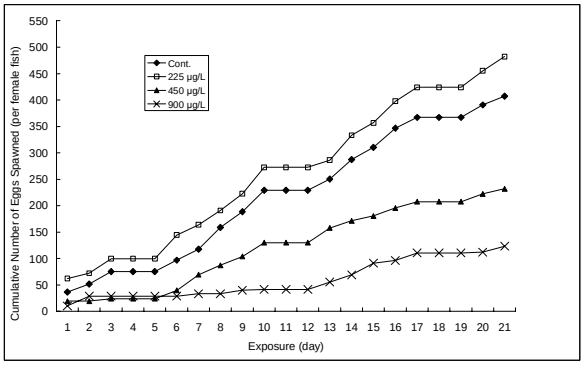

Fig. 6: The cumulative number of eggs spawned in zebrafish exposed to potassium permanganate.
(Optional data of LAB 3)

Spawning status of n-octanol studies

20. In the fathead minnow study from LAB 4, fecundity was sub-optimal in the control vessels, therefore no clear trend in fecundity could be drawn, therefore the transitory significant increase of number of eggs/ female survived at 1mg/L (Mann-Whitney U-test) should be interpreted with caution. Although not clearly stated in the laboratory report, the young age of the fish at the start of the test might be an explanation for the low fecundity in the control vessels, similar to the potassium permanganate study; the sex ratio of the fish in the aquaria was not followed throughout controls and treatments. In the medaka study in LAB 2, there was no difference in fecundity and fertility between treatments and control, while a slight reduction in fertility in the highest concentration group could be observed (Fig. 7). In the zebrafish study in LAB 3, fecundity and fertility decreased at the highest concentration (Fig. 9, 10).

Table 8: Spawning status in the *n*-octanol studies during the exposure period.

No. of days with spawning "yes" (average of tank 1 and 2) / observed days					
Nominal values		**Cont.**	**0.32 mg/L**	**1.00 mg/L**	**3.20 mg/L**
LAB 4	Fathead minnow	2.75/21	5.25/21	7/21	5.25/21
LAB 2	Medaka	20.5/21	21/21	20.5/21	20.5/21
LAB 3	Zebrafish	12/15	14/15	14/15	8/15

Table 9: Daily recording of the spawning status in the *n*-octanol studies.

DAY	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
LAB 2 (Medaka)																						
C		1/2	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		1/2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
H		1/2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
LAB 3 (Zebrafish)																						
C				1	1	1/2	1	1			1	1	1/2	1/2	1/2			1/2	1	1	1	1/2
L				1	1	1	1	1			1	1/2	1/2	1	1			1	1	1	1	1
M				1/2	1	1	1	1			1	1	1	1	1			1	1	1	1	1/2
H				1/2	1/2	1/2	0	1/2			1/2	1	1/2	1/2	1/2			1/2	1	1/2	1	0

Notes: 1: spawning observed in both replicate tanks; 1/2: spawning observed in one of replicate tanks; 0: no spawning observed in any of the two replicate tanks; C, L, M and H mean control, lowest concentration (225 µg/L), medium concentration (450 µg/L), and highest concentration (900 µg/L), respectively.

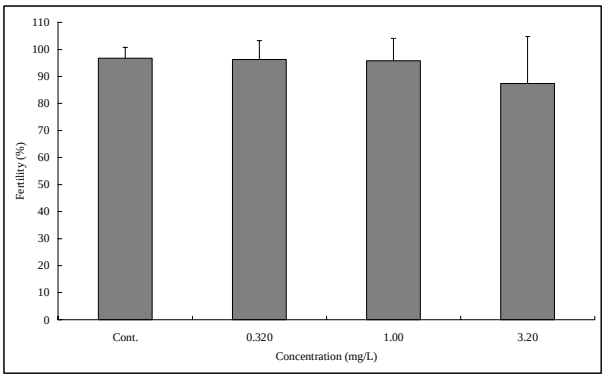

Fig. 7: The means of fertility in medaka exposed to *n*-octanol.
(Optional data of LAB 2)

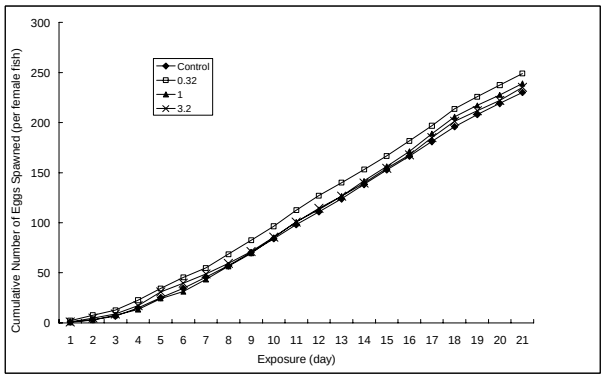

Fig. 8: The cumulative number of eggs spawned in medaka exposed to *n* -octanol.
(Optional data of LAB 2)

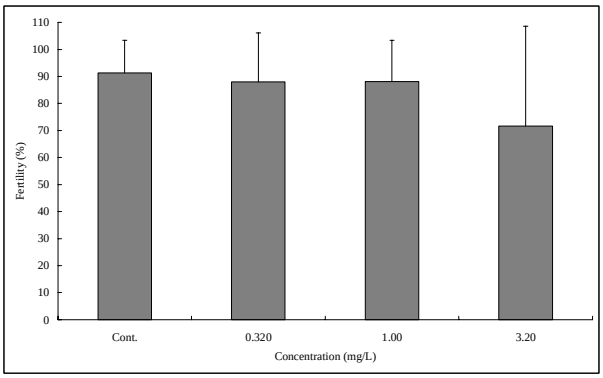

Fig. 9: The means of fertility in zebrafish exposed to *n* -octanol.
(Optional data of LAB 3)

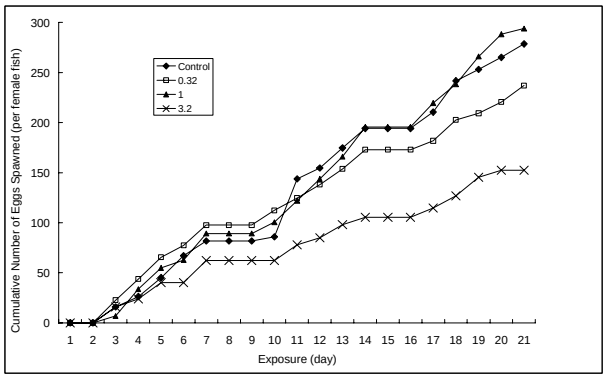

Fig. 10: The cumulative number of eggs spawned in zebrafish exposed to *n*-octanol.
(Optional data of LAB 3)

Vitellogenin measurements (VTG)

VTG measurements in studies using potassium permanganate

LAB	Sex		Potassium permanganate nominal concentration (µg/l)			
			Cont.	**225**	**450**	**900**
LAB.1	**Male**	mean	508	375	1,984	666
		S.D.	265	0	2,064	503
Fathead minnow		n	4	4	4	4
	Female	mean	176E+06	73E+06	6,4E+06*****	27E+06
		S.D.	252E+06	35E+06	6,1E+06	55E+06
		n	4	4	4	4
LAB.4	**Male**	mean	147.4	103	123	4.25
		S.D.	184	126	119	8.5
Fathead minnow		n	4	4	4	4
	Female	mean	1,9E+06	1,6E+06	1,3E+06	1.6E+-5
		S.D.	1,8E+06	1,1E+06	1,1E+06	2.2E+05
		n	4	4	4	4
			Cont.	225	450	900
LAB.2	**Male**	mean	5.0E-01	5.0E-01	5.0E-01	0.0E+00
		S.D.	±0.0E+00	±0.0E+00	±0.0E+00	±0.0E+00
Medaka		n	10	10	9	0
	Female	mean	1.2E+03	1.4E+03	1.3E+03	0.0E+00
		S.D.	±3.8E+02	±3.4E+02	±3.1E+02	±0.0E+00
		n	10	10	10	0
LAB.3	**Male**	mean	2.6E+02	1.4E+02	9.5E+02	2.1E+02
		S.D.	±3.7E+02	±6.6E+01	±1.8E+03	±1.4E+02
		n	9	10	8	11
Zebrafish	**Female**	mean	6.4E+06	3.4E+06	3.6E+06	4.3E+06
		S.D.	±3.6E+06	±1.7E+06	±3.2E+06	±4.8E+06
		n	10	10	10	8

Table 10: Vitellogenin levels in fish exposed to potassium permanganate.
Note: For LAB 4, the concentrations range was 112.5, 225, 450µg/l.
*: statistical significance detected.

21. In the fathead minnow studies from LAB 1 and LAB 4, the Mann-Whitney U-test was used to determine statistical significance. This test possesses acceptable power when at least 4 replicates per treatment level are used. In LAB 1, there was a statistically significant decrease in females at the intermediate test concentration. No statistical difference between treatments and control could be detected in LAB 4.

22. In the medaka and zebrafish studies in LAB 2 and LAB 3 respectively, Mann-Whitney's test and Dunn's test should not be used based on 2 replicates/treatment level, because they have no to low power to detect a significant response. For the sake of comparison and completeness, these tests were applied though. The suitability to apply other tests having good statistical power was investigated thoroughly. Annex 2 reports on the power properties of the various statistical tests described below.

23. In the medaka study from LAB 2, the male data did not vary across control and treatments and there

was thus no point to analyze the data. The female dataset from the medaka study from LAB 2 and the male and female datasets from the zebrafish study from LAB 3 were found to be normally distributed, but of unequal variance across treatments, and did not follow a monotone dose-response. For these reasons, the step-down Jonckheere-Terpstra test or William's test are not indicated. Instead an ANOVA was done with vessel nested in concentration, and fish nested within vessel. This analysis found the data to be normally distributed with homogeneous variances, so a standard Dunnett test could be performed; the female zebrafish dataset needed to be log-transformed to be normalized. All analyses reached the conclusion that there was no statistically significant response on VTG in the low and intermediate concentration (all fish died at the highest concentration).

24. Interestingly, for the female dataset of the study using potassium permanganate, an analysis was performed where replicate mean was ignored and individual fish was treated as the unit of analysis. Dunn's test found a statistically significantly lower median VTG value in the low concentration compared to the control- *the median VTG levels in the two higher test concentrations were not significantly different from the control. It is observed that the mean VTG level in the treatment groups ranges form 53% to 61% of the control mean.* A 47% change from the control mean can happen when there is no treatment related effect expected indicates a potentially serious issue for the use of VTG as a tool for evaluating potentially endocrine disrupting chemicals. The chance that all three treatment groups show such large decreased mean response compared to the control when there is no real treatment effect is thought to be small. An increase in the number of replicates from 2 to 4 should reduce the chance of such false positives. A power study will explore the likelihood of false positives as well as that of false negatives. This study will be reported separately when complete.

25. If replicate vessels are ignored and the individual fish is treated as the unit of analysis, then the NOEC is below the lowest tested concentration. The issue of the appropriate unit of analysis thus is important.

VTG measurements in studies using n-octanol studies

LAB	**Sex**		**Octanol nominal concentration (µg/l)**			
			Cont.	**0.32**	**1.00**	**3.20**
LAB.4	**Male**	mean	150	152	47	681
		S.D.	281	213	76	1,321
Fathead minnow		n	4	4	4	4
	Female	mean	1,9E+06	9E+05	1,4E+06	8.4E+05
		S.D.	2,1E+06	6.7E+05	1,4E+06	6.9E+05
		n	4	4	4	4
LAB.2	**Male**	mean	76	50	50	50
		S.D.	±82	±0	±0	±0
		n	2	2	2	2
Medaka	**Female**	mean	990	1000	1100	820
		S.D.	±170	±200	±230	±110
		n	2	2	2	2
LAB.3	**Male**	mean	150	130	730	7800
		S.D.	±170	±220	±1100	±12000
		n	2	2	2	2
Zebrafish	**Female**	mean	3.2E+06	7.3E+06	4.4E+06	5.8E+06
		S.D.	±3.0E+06	±4.9E+06	±3.7E+06	±6.5E+06
		n	2	2	2	2

Table 11: Vitellogenin levels in fish exposed to octanol.

26. In the fathead minnow study from LAB 4, the Mann-Whitney U-test was used to determine statistical significance. No difference between treatments and control could be found.

27. In the medaka and zebrafish studies from LAB 2 and LAB 3 respectively, the same approach as for the potassium permanganate studies was followed.

28. In the medaka study from LAB 2, there was only one male medaka with measured VTG level different from 0.5 and this was in the control. No statistical analysis of the male data is needed (and no meaningful analysis is possible) to conclude that no statistically significant treatment related effect in VTG was observed in the males.

29. In the medaka study from LAB 2, the female replicate mean VTG levels were analyzed. These data were found to be normally distributed but of unequal variances across treatments (i.e., variance heterogeneity was observed). No transformation was found that maintained normality and stabilized the variances. There was also minor evidence of a non-monotone dose-response. While such minor deviation from montonicity is not a problem for the Jonckheere-Terpstra test, it seemed appropriate that an alternative pairwise analysis should also be done. The Tamhane-Dunnett robust version of Dunnett's test was the preferred method for these normally distributed, heterogeneous data. However, the power of this test for the case of two replicates is low. For comparative purposes, the Dunn non-parametric analysis was also performed, but with only two replicate vessels per test concentration, Dunn's test has low power.

30. An alternative analysis was performed using the nested variance structure of the experiment. That is, an ANOVA was done with vessel nested in concentration, and fish nested within vessel. This analysis found the data to be normally distributed with homogeneous variances, so a standard Dunnett test could be performed. All analyses (Jonckheere-Terpstra, Dunn, and Tamhane-Dunnett on replicate means, and Dunnett on the nested variance structure) reached the same conclusion, that there was no statistically significant effect on VTG levels at any tested concentration.

31. In the zebrafish study from LAB 3, the mean male responses in the control and three positive concentrations were 151, 117, 775, and 6715, respectively, so that except for a 23% decrease from control to low concentration, there is a monotone increasing dose-response. A step-down Jonckheere-Terpstra test is appropriate. Nonetheless, a pair-wise analysis was also performed. The data were found not to be normally distributed. A log-transform normalized the data but the variances were heterogeneous, so a Tamhane-Dunnett pairwise comparison was done as well as a step-down Jonckheere-Terpstra test. For comparison, Dunn's non-parametric analysis was also done. Both the Tamhane-Dunnett and Dunn tests have low power when there are only two reps per test concentration.

32. Another analysis was performed using the nested variance structure of the experiment. That is, an ANOVA was done with vessel nested in concentration, and fish nested within vessel. The data were found not to be normally distributed but a log-transform normalized the data and also yielded homogeneous variances for the full dataset (i.e., individual fish with the nested variance structure). Under the log-transform, the ratio of the between-rep to within-rep variance was 2.1. A standard Dunnett test based on the nested variance structure was thus appropriate. Under the step-down Jonckheere-Terpstra, Dunn, and Tamhane-Dunnett tests on the replicate means and the Dunnett test on the individual fish data using a nested variance structure, there was no statistically significant effect at any test concentration.

33. In the zebrafish study from LAB 3, the female data were found to be normally distributed with heterogeneous variances, the latter attributable primarily to the difference in replicate means in the high concentration. There was also some indication of non-monotonicity in the dose-response, as the observed response means in the control and three positive concentrations were 4109, 7289, 4356, and 5827, respectively. (Each mean should be multiplied by 1000.) However, a step-down Jonckheere-Terpstra test

was done. Given the variance heterogeneity, Williams' test is not appropriate. A Tamhane-Dunnett test is the primary pair-wise comparison method to be employed in this situation. A non-parametric Dunn test was also used for comparison. Both of these pairwise tests have low power when there are only two reps per test concentration.

34. Another analysis was performed using the nested variance structure of the experiment. The data were found to be normally distributed with homogeneous variances. Given that the between-vessel variance was zero, there was little difference between this analysis and an analysis ignoring vessel. The data were found to be normally distributed with homogeneous variances, so a standard Dunnett test could be performed. By all methods considered, there was no statistically significant effect on VTG at any test concentration.

Secondary sex characteristics

Secondary sex characteristics in studies using potassium permanganate

35. Quantifiable secondary sex characteristics are responsive to androgen stimulation in fathead minnow and medaka, not in zebrafish. In the fathead minnow study in LAB 1, the number of nuptial tubercles in male fathead minnow decreased dose-dependently, resulting in significant difference in the 900-µg/L treatment group. In the fathead minnow study in LAB 4 (not represented on Fig.11), there was no significant decrease in the number of tubercles between treated male and control male fish. The mean number of tubercles of male fish decreased from 16 in the control to 10 in the highest test concentration. The mean score of tubercles for male fish of one concentration, which is based on the rating of the tubercle size (1=present, 2=enlarged, 3=pronounced) decreased from 33 (100%) in the control to 12 (36%) in the highest test concentration of nominal 0.45 mg test item/L. However, this data based on the males survived until the end of the test and in the highest test concentration only three male fish survived. Therefore, this data should be interpreted with caution. The vertical bands strongly decreased in male fish of the highest test concentration of nominal 0.45 mg test item/L. Furthermore the male fish in this concentration showed no more territorial aggressiveness after the first week until the end of the test. These results are a consequence of the toxicity of potassium permanganate, because the fish survived showed also many signs of intoxication.

36. In the medaka study in LAB 2, number of the joint plate with papillary processes in male fish was not affected when exposed to potassium permanganate. In female fish no induction of nuptial tubercle or papillary process was observed during the exposure period.

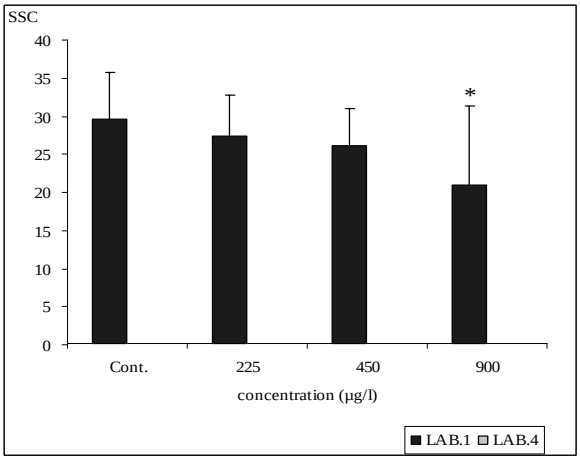

Fig. 11: Total score of nuptial tubercles in male fathead minnow exposed to potassium permanganate. *, significant difference from the control (p<0.05).

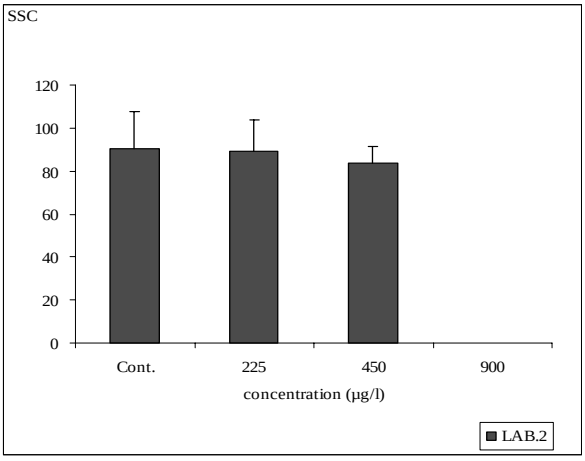

Fig. 12: Number of the joint plate with papillary processes in male medaka exposed to potassium permanganate. All fish died at 900µg/L.

Secondary sex characteristics in studies using n-octanol

37. In the fathead minnow study in LAB 4, there was no significant change in the number of tubercles between control male fish and male fish of all test concentrations (data not reported in Fig.13). The mean number of tubercles of male fish was 16.9, 20.4, 20.6 and 23.3 in the control and in the test concentrations of nominal 0.32, 1.0 and 3.2 mg test item/L, respectively. The mean score of tubercles for male fish, which is based on the rating of the tubercle size (1=present, 2=enlarged, 3=pronounced) did not differ significantly between the control and all test concentrations (see Table 4). The mean score of the fish was 33.3, 38, 38.8 and 38.6 in the control and in the test concentrations of nominal 0.32, 1.0 and 3.2 mg test item/L, respectively. The presence of vertical bands of the surviving fish did not differ between all test concentrations and the control. Furthermore, the surviving male fish showed normal territorial aggressiveness in all test concentrations and in the control.

38. In the medaka study in LAB 2, no significant difference was found in male fish (Fig.13). In female fish no induction of papillary process was observed during the exposure period.

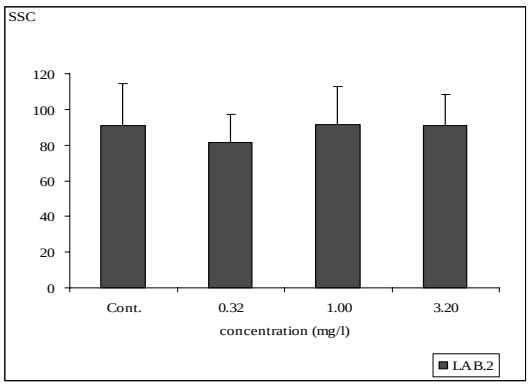

Fig. 13: Number of the joint plate with papillary processes in male medaka exposed to *n*-octanol.

Gonad histology

Gonad histology of potassium permanganate studies

39. The results presented in this section are short descriptive summaries of the findings. In some cases more details are available in the laboratory report, in other cases only the Excel spreadsheet is available with relevant findings coded.

40. No details are available on the findings from the fathead minnow study from LAB 1.

41. In the fathead minnow study from LAB 4, a part from a few conspicuous alterations described below, most effects after exposure to potassium permanganate were only slight and can be interpreted as normal variability within a fish population. The major changes in fish exposed to potassium permanganate comprise three main effects: (1) general increase of fibrotic tissue in both sexes, (2) increased incidence of testicular degeneration and intraluminal histolytic cells in male testes; (3) decreased maturity in females

after exposure to potassium permanganate. Changes after exposure to potassium permanganate indicate a more or less dose-dependent general toxicity, which is more obvious in male fish. Nevertheless, from the results, there is no indication for any endocrine action of potassium permanganate in fathead minnow after 21 days of exposure.

42. In the medaka study performed in LAB 2, all male fish in control and treatment vessels appeared to be in a development stage of 2, also called mid-spermatogenic stage in males, where spermatocytes, spermatids, and spermatozoa are present in roughly equal proportions. Some intraluminal histiocytic cells were found in controls and treated fish. All females fish in control and treatment vessels appeared to be in a development stage of 2, also called mid-development in females, where at least half of observed follicles are early and mid-vitellogenic. Four cases of slight oocyte atresia (immature) were observed in treated fish.

43. In the zebrafish study from LAB 3, male fish in control and treatment vessels had testis at various stages of development, between 1 and 3, and there was no trend towards early or late development following chemical exposure. Male fish in the low and medium concentration level had increased Sertoli cells. Female fish in control had ovaries mainly at development stage 2, whereas there was a trend towards equal distribution of ovaries' development between stages 0, 2, 3 and 4 with increasing concentrations of potassium permanganate.

Gonad histology of n-octanol studies

44. In the study on medaka performed in LAB 2, all male fish in control and treatment vessels appeared to be in a development stage of 2, also called mid-spermatogenic stage in males, where spermatocytes, spermatids, and spermatozoa are present in roughly equal proportions. Some intraluminal histiocytic cells were found in controls and treated fish. One cae of testicular degeneration was reported from a control fish. All females fish in control and treatment vessels appeared to be in a development stage of 2, also called mid-development in females, where at least half of observed follicles are early and mid-vitellogenic. Three cases of slight oocyte atresia (immature) were observed in treated fish. Two cases of increased macrophage aggregates were observed in treated fish.

45. In the study on fathead minnow performed in LAB 4, apart from a few conspicuous alterations, most effects after exposure to Octanol were only slight and can be interpreted as normal variability within a fish population. The overall changes in fish exposed Octanol reveal three main effects. (1) dose-dependent hyperplasia of the ovarian wall in females exposed to Octanol; (2) phenomenons related to a reduced egg quality like increased occurrence of egg debris in the gonoduct and atresis of mature and immature oocytes; (3) decreased maturity in males of the highest concentration paired with an increased number of spermatids in mid-vitellogenic individuals that are probably linked general toxic stress after exposure to Octanol. Changes after exposure to Octanol indicate a more or less dose-dependent toxic action, which is more obvious in female fish. Nevertheless, from the results there is no indication for endocrine action of octanol in fathead minnow after 21 days of exposure.

46. In the study on zebrafish performed in LAB 3, male fish in control and treatment vessels had testis mainly at stages of development 2 and 3, and there was an increasing number of fish at stage 3 with increasing octanol concentrations. Male fish with testis ova were reported at the medium concentration. Female fish in control and treatment levels had ovaries at various development stages and there was no particular noticeable trend. Other findings like oocyte atresia and interstitial fibrosis were found at all treatments, including in the control.

DISCUSSION

Overview of the Phase 2 studies

47. The use of substances not expected to stimulate a response on the endpoints measured (negative substances) in a test is essential to estimate the rate of false positive generated by the test. This is important for examining the specificity of the test, i.e. whether positive findings are truly active on the endocrine system. This is also important for minimizing the number of higher-tier tests, including the animals used, that would be conducted based on false positive results in a lower-tier test.

48. As far as Phase 2 VTG results are concerned, potassium permanganate and *n*-octanol did not affect VTG levels in either male or female fish of the three fish species, except for one false positive finding (decreased VTG in females) in the fathead minnow study from LAB 1 on potassium permanganate, at the intermediate concentration level, which was not confirmed at the highest concentration. From the zebrafish study on potassium permanganate, there was an interesting observation: when taking the individual fish as the unit of analysis rather than the replicate mean, a statistically significant result was found at the low concentration (decreased VTG in females) which highlights the importance of properly choosing the unit of analysis. Generally in Phase 2 studies mortality was high at the high concentration levels in the potassium permanganate experiments that care should be exercised in the interpretation of results.

49. Potassium permanganate and *n*-octanol did not affect secondary sex characteristics in males and females medaka and fathead minnow, except one false positive finding (decrease number of nuptial tubercles in males) in the fathead minnow study from LAB 1 on potassium permanganate, resulting in a significant difference at the highest concentration. The fecundity and fertility of medaka and zebrafish decreased dose-dependently in the potassium permanganate studies. The reduction in fecundity and fertility was also observed in zebrafish exposed to *n*-octanol. In the fathead minnow studies, spawning was rather irregular at any treatment level, including in the control, despite efforts undertaken to place fathead minnow in optimal spawning conditions (4 females and 2 males per test vessel); thus no trend could be drawn from the fathead minnow spawning data. The sub-optimal spawning conditions in LAB 4 were explained by the young age of fish at the start of the study, thus fish could not be properly sexed and the sex ratio was affected in some test aquaria.

50. As a conclusion from this Phase 2 study using negative substances, the 21-day fish screening assay appears to be rather robust and specific enough to discriminate between substances with endocrine active properties and other substances. More care should be exercised in selecting the appropriate concentrations for the test avoiding toxic doses that will kill the fish at the highest treatment level, and thereby loosing important information. Vitellogenin is a robust endpoint for the detection of estrogenic and aromatase inhibiting substances, and does not seem to be prone to generate false positive outcomes. Secondary sex characteristics (papillary processes in medaka and nuptial tubercles in fathead minnow) also appear to be rather robust, but care should be exercised in interpreting the data, as false positive outcomes may sometimes be encountered. As experience is gained in the laboratories applying the test, the nuptial tubercles count should become more robust. There may be a need to develop guidance on data interpretation for regulatory purpose, based on experience gained in these validation studies and on scientific expertise available from members of the VMG.

51. Following consultation with the VMG-eco in January 2007, a special effort has been put on the statistical analysis of the data to determine the most appropriate statistical test to use based on the characteristics of the data, and also to determine the most suitable number of replicates per treatment level. As a result, a statistical flowchart has been produced (Annex 1) to guide the laboratories for the data

handling. Power simulations have been run to determine the most appropriate statistical test when the number of replicate varies between 2 and 4, and the number of fish fluctuates between 2 and 8. This is presented in Annex 2 to this report. There is evidence that increasing the number of replicates from 2 to 4 will increase the power of the test to detect a difference when it is statistically significant; this will be documented so that a formal decision can be taken on the replicate number.

Detailed discussion of negative substances in each endpoint

Potassium permanganate

General aspects

52. The test results in all studies are valid because the mortality in controls, dissolved oxygen and water temperature in the test solutions were within the acceptability criteria of the protocol (10%), except in one fathead minnow study from LAB 4 where mortality in the control was as high as 16% in the *n*-octanol study. The results of analytical chemistry showed that the measured concentrations of potassium permanganate in LAB 2, LAB 3 and LAB 4 remained > 80% of the nominal concentrations throughout the exposure period, while those in LAB 1 were 50–80% of the nominal. LAB 4 used a lower concentration range: 112.5-450µg/L. In medaka study, all fish in the 900 µg/L treatment group died during the exposure period. Potassium permanganate is known as a strong oxidizer. In this treatment group, various symptoms were observed before the death. Although the preliminary study in the U.S. showed that the 96-hr LC_{50} with mature fathead minnow under conditions of the reproduction assay was determined to be 2.5 mg/L which was higher than the exposure levels of this study (225–900 µg/L), susceptibility of medaka to potassium permanganate may be higher that those of fathead minnow and zebrafish.

VTG

53. In this validation work, the VTG levels in exposed males and females were not affected in fathead minnow, medaka and zebrafish exposed to potassium permanganate, with one exception at one concentration level for female fathead minnow. This result is in concordance with information available from the *in vitro* receptor binding assays (ERα and AR) and transcription activation (ERα and AR) assays which gave negative results (Table 1). Vitellogenin measurement is a robust and specific endpoint in this assay.

Secondary sex characteristics

54. The secondary sex characteristics in medaka and fathead minnow of both sexes were not affected in potassium permanganate studies, except in one study from LAB 1 at the highest concentration. The total score of nuptial tubercles in male fathead minnow decreased dose-dependently in LAB 1, resulting in a significant difference at the highest concentration. A few reports have been published showing that the number of nuptial tubercles decreased in fathead minnow exposed to weak and strong estrogens (6) (7). It is unlikely that the reduction of secondary sex characteristics of male fathead minnow was caused by endocrine disruption associated with androgen or estrogen hormone receptor. Other possible causes are unclear yet, but this false positive outcome may restrict the use of secondary sex characteristics observations to females only, where nuptial tubercles are unequivocally formed in females exposed to androgenic substances.

Spawning status

55. In the observation of spawning status, potassium permanganate caused reduction in fertility and fecundity of medaka and zebrafish; no clear effect was found in fathead minnow study. Although several reports have been made of reduced fecundity on the exposure to endocrine disrupting chemicals in medaka (10-12) and fathead minnow (5, 13, 14). One of the reasons for reduced fecundity may be the toxic effects of potassium permanganate. Another reason of spawning reduction may be the inhibition of sexual

behavior (15). Spawning status is a good and sensitive indicator of the general health of the fish.

***n*-octanol**

General aspects

56. The test results in all studies are valid because the mortality in controls, dissolved oxygen and water temperature in the test solutions were within the acceptability criteria of the protocol (10%0, except in one study in LAB 4 where mortality was 16% in the control. The results of analytical chemistry showed that the measured concentrations of *n*-octanol in LAB 2 remained about >80% of the nominal concentrations throughout the exposure period, while those in LAB 3 and LAB 4 decreased as time advanced, resulting in 12-58% of the nominal concentrations. Reduced concentrations in LAB 3 were probably caused by biodegradation of the *n*-octanol, while biodegradation had low impact in LAB 2. The difference in actual concentrations of *n*-octanol in these laboratories might be caused by the difference in volume exchange of the test solution; 17 times a day in LAB 2 and 5 times a day in LAB 3.

VTG

57. In this validation work, the VTG levels in exposed males and females were not affected in fathead minnow, medaka and zebrafish exposed to *n*-octanol. This result is in accordance with the information from *in vitro* receptor binding assays (ER and AR) and transcription activation (ER and AR) assays which gave negative results for this substance (Table 1). Vitellogenin measurement is a robust and specific endpoint in this assay.

Secondary sex characteristics

58. The secondary sex characteristics were not affected in males and females of fathead minnow and medaka exposed to n-octanol. This result is in accordance with information from *in vitro* receptor binding assays (ER and AR) and transcription activation (ER and AR) assays for this substance which gave negative results for this substance (Table 1).

Spawning status

59. n-Octanol caused a slight reduction in fertility in medaka study, and the fertility and fecundity decreased in the highest concentration of zebrafish study. The study in fathead minnow showed very irregular spawning, despite efforts made to accommodate the protocol to that species, by holding two males and four females in each test vessel to avoid territorial aggressiveness of male fish. Thus it is difficult to derive conclusion on fathead minnow spawning. The reason for the inhibition of reproduction in medaka and zebrafish is uncertain, one cause might simply be the general toxicity of *n*-octanol, as the acute toxicity values of this substance to fish (2–4 mg/L) is quite close to the nominal concentrations in this study. Another possibility might be the growth of bacteria through biodegradation of the test substance, which then caused stresses the test fish, resulting in reduced sexual behavior.

Recommendations and Next steps

60. It was agreed at the EDTA Task Force in April 2006 that the 21-day fish endocrine screening assay should include vitellogenin secondary sex characteristics measurements, which appear to be the most relevant and reliable endpoints for the detection of estrogenic, aromatase inhibitors and androgenic substances.

61. It was agreed that the validation of the assay is now ready for the peer-review stage, at least for the medaka fish species. The data base of the 21-day fish endocrine screening assays conducted on medaka is becoming quite large as Japan has conducted additional studies on several weakly active estrogens. The report of these studies is available at: http://www.env.go.jp/en/chemi/ed/rt_medaka.pdf). To increase the size of the dataset on the fathead minnow and zebrafish, additional studies are in progress for the following

weakly active substances: 4-tert-octylphenol (fathead minnow and zebrafish), androstenedione and pentachlorophenol (fathead minnow only). The results will be reported separately, under Phase 3.

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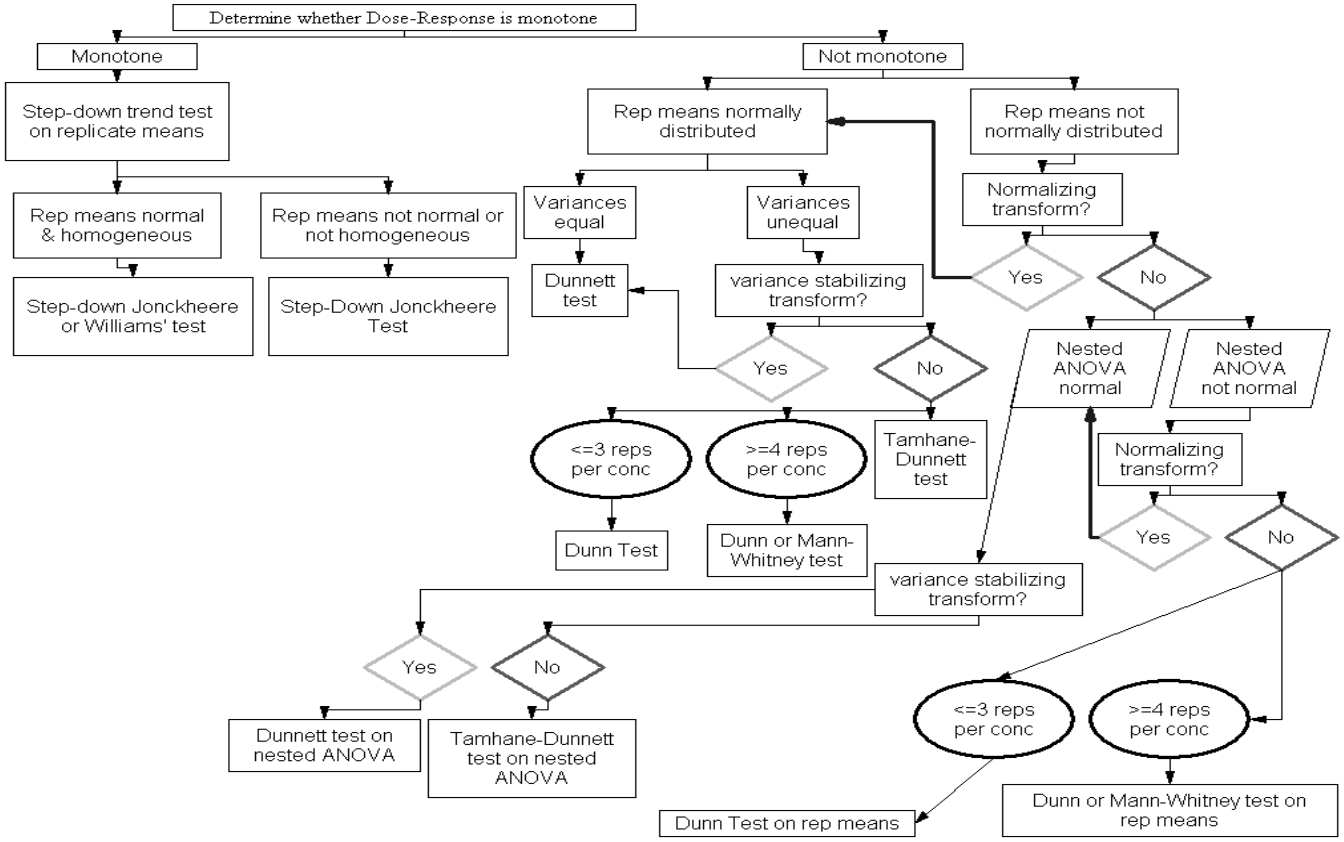
ANNEX 1

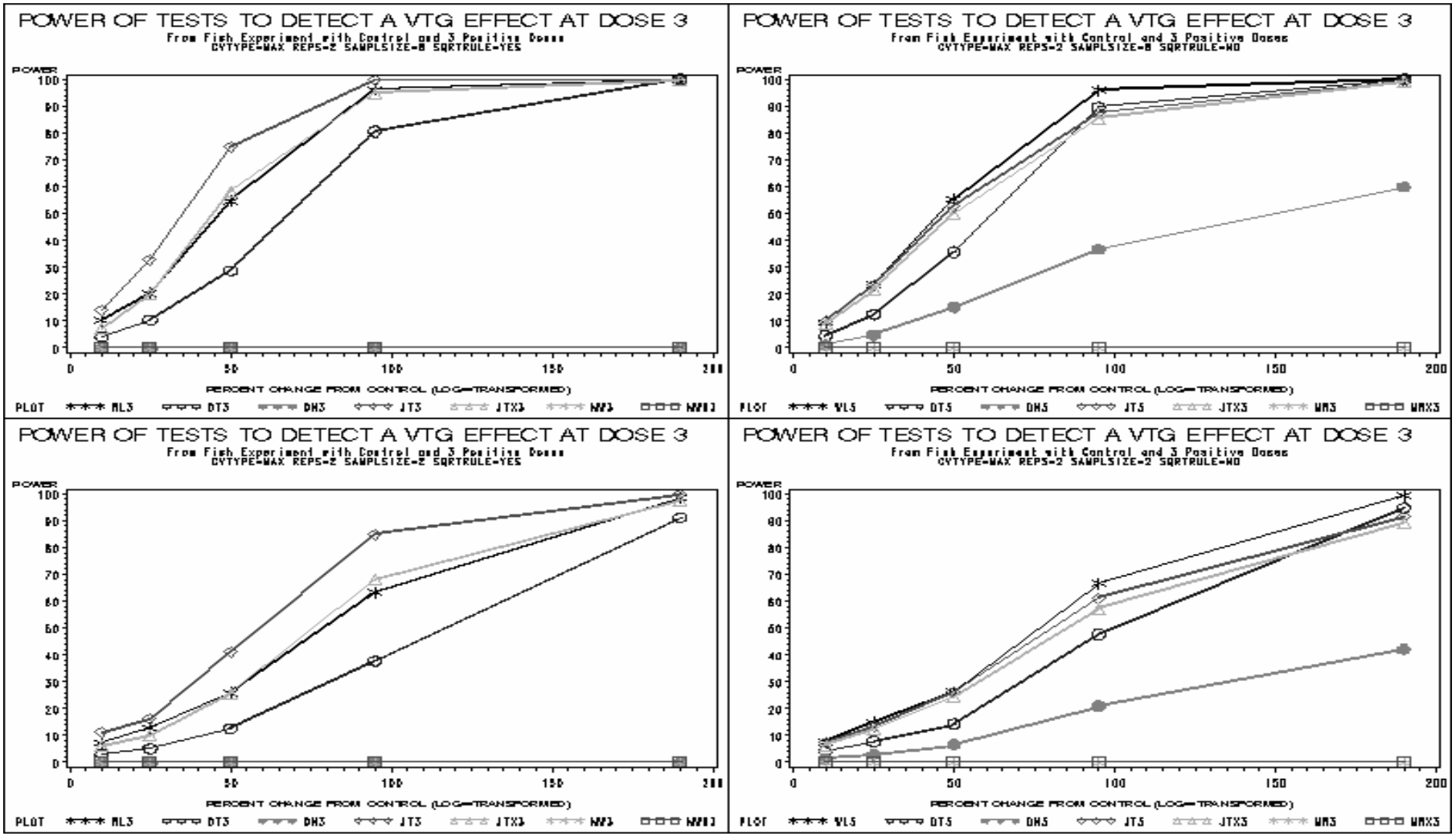

Determine whether Dose-Response is monotone
Monotone
Not monotone
Step-down trend test on replicate means
Rep means normally distributed
Rep means not normally distributed
Rep means normal & homogeneous
Rep means not normal or not homogeneous
Variances equal
Variances unequal
Normalizing transform?
Step-down Jonckheere or Williams' test
Step-Down Jonckheere Test
Dunnett test
variance stabilizing transform?
Yes
No
Yes
No
Nested ANOVA normal
Nested ANOVA not normal
<=3 reps per conc
>=4 reps per conc
Tamhane-Dunnett test
Normalizing transform?
Dunn Test
Dunn or Mann-Whitney test
Yes
No
variance stabilizing transform?
Yes
No
<=3 reps per conc
>=4 reps per conc
Dunnett test on nested ANOVA
Tamhane-Dunnett test on nested ANOVA
Dunn Test on rep means
Dunn or Mann-Whitney test on rep means

ANNEX 2

Power Properties of Tests to Detect Effects on VTG

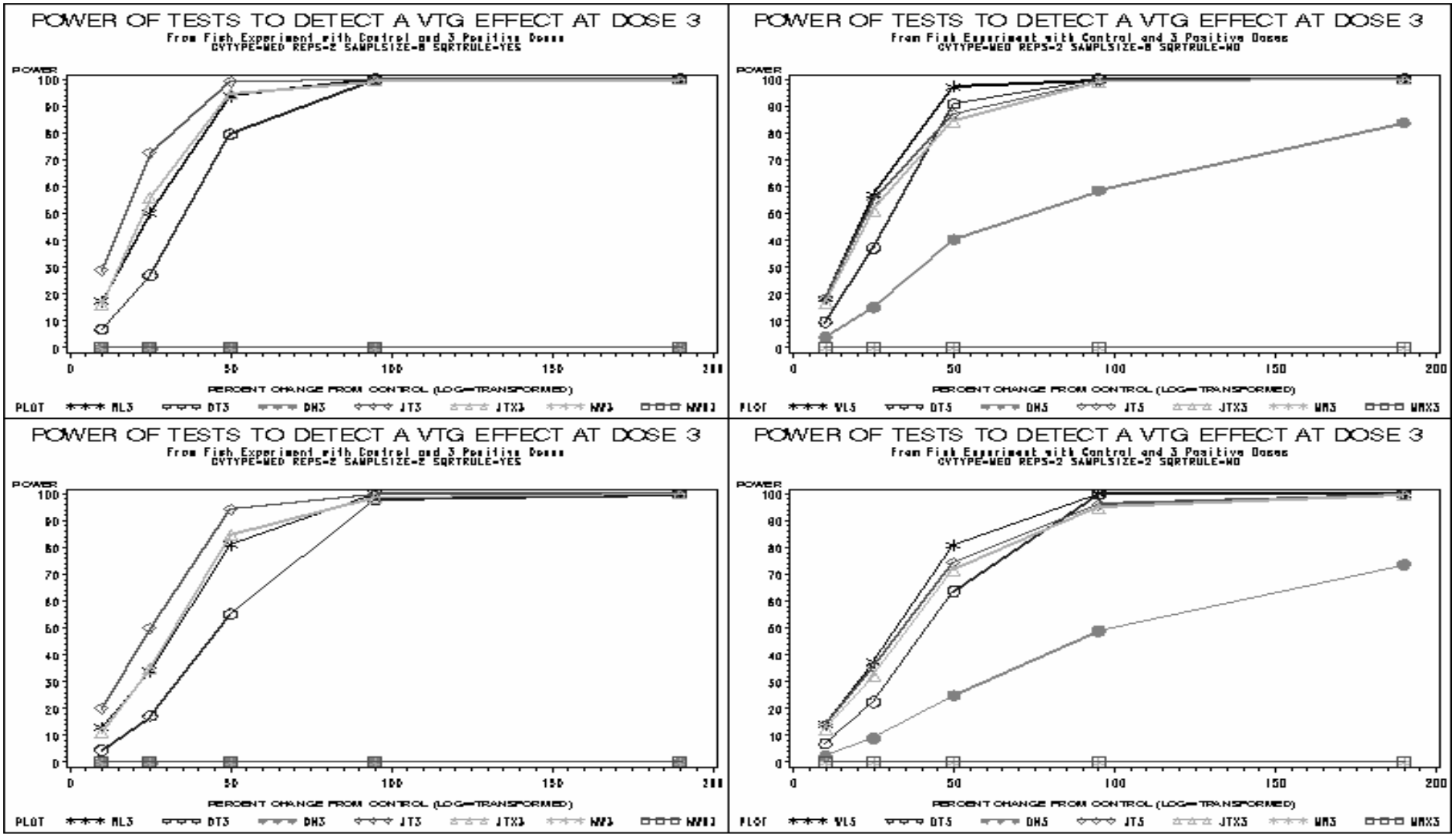
The first three plots below are for two reps per concentration (control or positive test concentration). These three plots show the maximum, median, and minimum variance scenarios, respectively, based on previous data. These plots make clear that the Mann-Whitney test has zero power to detect as statistically significant an effect no matter how large. Dunn's test does have positive power to detect an effect, but the power is markedly less than the power of Dunnett's test or the trend-based Williams and Jonckheere tests. The latter two tests assume a monotone dose-response shape. Both tests are appropriate when the deviation from monotonicity is not severe but are inappropriate for severe non-monotonicity. Dunnett's test assumes normality and variance homogeneity. Dunn's test makes no such assumption.

The last plot shows the power of these tests when there are four replicate vessels per concentration under the maximum variance scenario. The Mann-Whitney and Dunn tests are seen to have very similar power properties only marginally less than Dunnett's test.



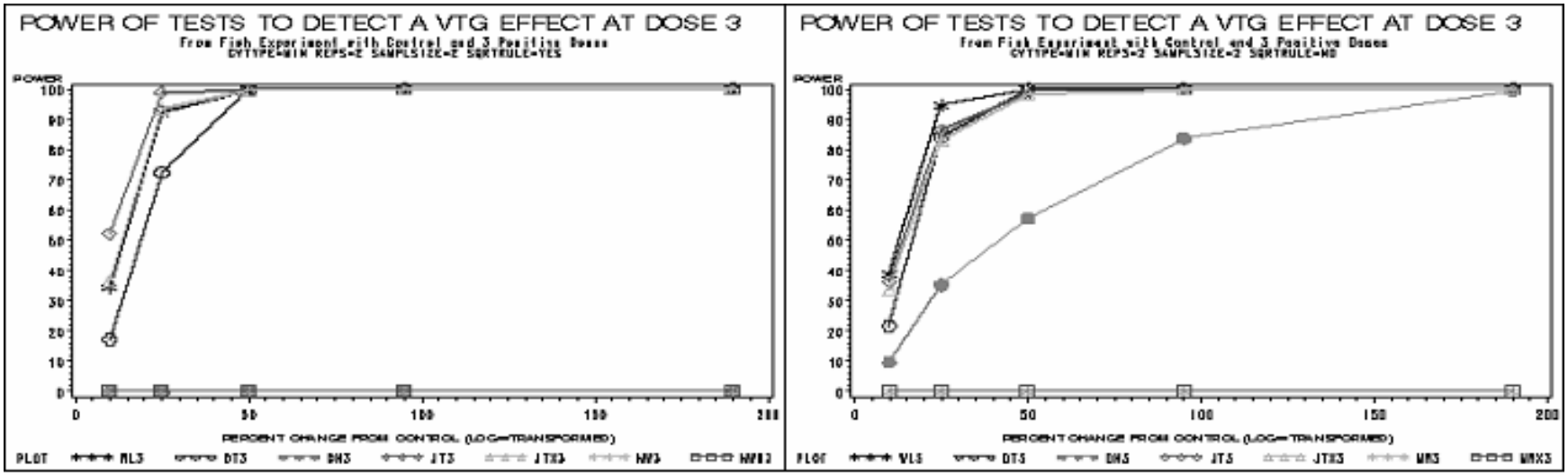
Top row: 2 reps of 8 fish **Bottom row: 2 reps of 2 fish**

Left col: Square-root allocation **Right col: Equal allocation**

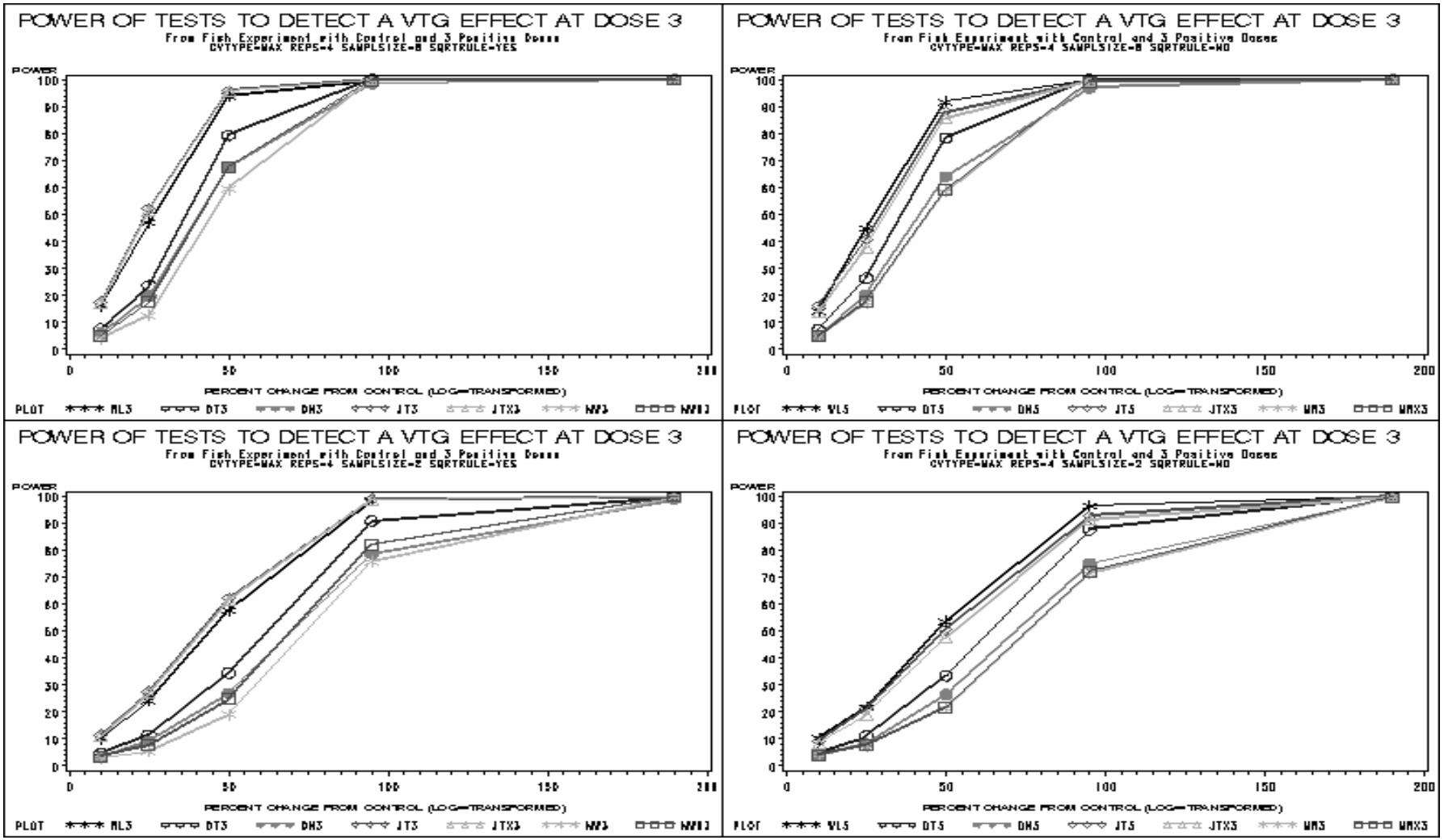


Top row: 2 reps of 8 fish **Bottom row: 2 reps of 2 fish**

Left col: Square-root allocation **Right col: Equal allocation**



Top row: 2 reps of 8 fish Bottom row: 2 reps of 2 fish
Left col: Square-root allocation Right col: Equal allocation



Top row: 4 reps of 8 fish **Bottom row: 4 reps of 2 fish**

Left col: Square-root allocation **Right col: Equal allocation**

ANNEX 3

Details of Statistical Tests Performed on the VTG data from LAB 2 (medaka) and LAB 3 (fathead minnow)

Experiment 1: CERI Medaka, Potassium Permanganate, Females
1a. Nested Analysis

Model VTG = CONC
potassium permanganate
FULL DATA SET
COVPARMS

CovParm	Estimate
VESSEL(conc)	31894
Residual	101083

From this the variance ratio is 31894/101083=0.32.

Model VTG = CONC
potassium permanganate
FULL DATA SET
COVPARMS

Effect	Num DF	Den DF	FValue	ProbF	MSERR	SSQRS	SSERR
conc	3	3	65.85	0.0031	101082.55	19969410.51	303247.65

ANOVA SUMMARY STATISTCS

MODELSS	SSERR	TOTSS	RSQUARE
144512.75	303247.65	447760.40	0.32275

KMNO4 MEDAKA VTG DATA FROM LAB CERI
potassium permanganate
CLASS LEVEL INFORMATION
FULL DATA SET

Class	Levels	Values
conc	4	0 225 450 900
VESSEL	2	1 2

KMNO4 MEDAKA VTG DATA FROM LAB CERI
potassium permanganate
LSMEANS
FULL DATA SET

Effect	conc	Estimate	StdErr	DF	tValue	Probt
conc	0	1175.14	161.42	3	7.28	0.0053
conc	225	1447.27	161.42	3	8.97	0.0029
conc	450	1292.99	161.42	3	8.01	0.0041

KMNO4 MEDAKA VTG DATA FROM LAB CERI
potassium permanganate
T-TEST RESULTS
FULL DATA SET

Effect	conc	_conc	Estimate	StdErr	DF	tValue	Probt	Adjustment	Adjp
conc	225	0	272.14	228.28	3	1.19	0.3189	Dunnett	0.4751
conc	450	0	117.85	228.28	3	0.52	0.6413	Dunnett	0.8357

Thus, the mean VTG level in neither concentration is significantly different from the control mean.

KMNO4 MEDAKA VTG DATA FROM LAB CERI
potassium permanganate
PARAMETER ESTIMATES
FULL DATA SET

Effect	conc	Estimate	StdErr	DF	tValue	Probt
conc	0	1175.14	161.42	3	7.28	0.0053
conc	225	1447.27	161.42	3	8.97	0.0029
conc	450	1292.99	161.42	3	8.01	0.0041

KMNO4 MEDAKA VTG DATA FROM LAB CERI
potassium permanganate
SHAPIRO-WILK TEST OF NORMALITY OF VTG
FULL DATA SET

Obs	Var Name	Test	Test Lab	Stat	pType	p Sign	pValue
1	Resid	Shapiro-Wilk	W	0.952115	Pr < W		0.1926
2	Resid	Kolmogorov-Smirnov	D	0.099985	Pr > D	>	0.1500
3	Resid	Cramer-von Mises	W-Sq	0.060646	Pr > W-Sq	>	0.2500
4	Resid	Anderson-Darling	A-Sq	0.443738	Pr > A-Sq	>	0.2500

Thus, the data are normally distributed.

KMNO4 MEDAKA VTG DATA FROM LAB CERI
potassium permanganate
POSSIBLE OUTLIERS FROM ANOVA ON VTG
FULL DATA SET

Obs	conc	VTG	Pred	Resid	LB	UB
1	0	2033.5	1230.39	803.113	-843.244	779.574

KMNO4 MEDAKA VTG DATA FROM LAB CERI
potassium permanganate
LEVENE TEST FOR VTG
FULL DATA SET

Effect	DF	LEVENE	P_VALUE
conc	2	0.20780	0.82315

NOTE
By Levene's test, the within-group variances are equal. A standard ANOVA will be done.

1b.Analysis of Replicate Means

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 10:14 FRIDAY 09FEB07
MEDAKA MEASUREMENTS FROM DATASET FKMNO4
GROUP STATISTICS FOR VTG BY DOSE w/ Weight=survive, Var-Ratio=0.32

dose	doseval	COUNT	MEAN	MEDIAN	STD_DEV	STD_ERR
1	0	2	1175.14	1175.14	177.044	90.275
2	225	2	1447.27	1447.27	516.943	263.590
3	450	2	1292.99	1292.99	45.422	23.161

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 10:14 FRIDAY 09FEB07
SHAPIRO-WILK TEST OF NORMALITY OF VTG
AQUATIC MEDAKA: FULL DATA

OBS	STD	SKEW	KURT	SW_STAT	P_VALUE	SIGNIF
6	176.823	0	0.75892	0.99164	0.9928	

Thus, the data are normally distributed.

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 10:14 FRIDAY 09FEB07
LEVENE TEST FOR VTG - FULL Model
ANALYSIS OF VARIANCE ON FULL DATA SET

Effect	DF	LEVENE	p_value	SIGNIF
DOSE	2	I	0.0001	**

NOTE: The data was found to be normally distributed but group variances were unequal. A Tamhane-Dunnett analysis is appropriate.

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 10:14 FRIDAY 09FEB07
Tamhane-Dunnett 2-sided test for difference in means in VTG
Using MAXIMUM LIKELIHOOD estimates of variation on MEDAKA values.
FULL DATA USING ALPHA=0.05

dose	MEAN	STDERR	degfree	CONTROL	OBS_DIFF	crit	SIGNIF
2	1447.27	263.590	1.23141	1175.14	272.135	2152.51	
3	1292.99	23.161	1.13108	1175.14	117.854	720.01	

Thus, neither treatment mean VTG value is significantly different from the control mean VTG value.

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 10:14 FRIDAY 09FEB07
MONOTONICITY CHECK OF VTG - FULL DATA
DOSES 0, 225, 450 ug/L
MEDAKA OF AQUATIC MEDAKA

PARM	p_t	SIGNIF
DOSE TREND	0.2945	
DOSE QUAD	0.5899	

A formal test for monotonicity is not significant.

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 10:14 FRIDAY 09FEB07
ANALYSIS OF VTG USING ALPHA=0.05
FULL DATA

```
  ---------- JONCKHEERE-TERPSTRA TEST KEY ----------
  _JT_ IS JONCKHEERE STATISTIC
  Z_JT IS STANDARDIZED JONCKHEERE STATISTIC
  PR_JT IS P-VALUE FOR UPWARD TREND
  PL_JT IS P-VALUE FOR DOWNWARD TREND
  P-VALUES ARE FOR TIE-CORRECTED TEST WITH CONTINUITY CORRECTION FACTOR
  SIGNIF RESULTS ARE FOR Two-sided ALTERNATIVE HYPOTHESIS

Check for Number of Reps in VTG Thru 450 ug/L
Maximum Number of Reps in Any Treatment Group or Control is 2
Total Number of Reps in All Treatment Groups is 6
Exact Permutation Methods Recommended

  Jonckheere Trend Test on Dose 0 + Lowest 2 Doses thru 450 ug/L

  ___JT____        Z_JT   XPL_JT  XPR_JT  SIGNIF  DOSE
       10.5   1.1920791    .      0.1667             3
```

NOTE: The step-down Jonckheere test fins the NOEC to eceed 450 ug/L.

```
 Jonckheere test results are included in the summary table. Group means should be examined to
check for lack-of-fit to a linear trend before trend test results are accepted
```

1b, continued: Dunn Test

```
                    STATISTICAL ANALYSIS LIST FILE
                REPORT CREATED ON 10:23 FRIDAY  09FEB07
                MEDAKA MEASUREMENTS FROM DATASET FKMNO4
   GROUP STATISTICS FOR VTG BY DOSE w/ Weight=survive, Var-Ratio=0.32

  dose    doseval         COUNT      MEAN      MEDIAN    STD_DEV    STD_ERR

    1               0      2      1175.14    1175.14    177.044     90.275
    2             225      2      1447.27    1447.27    516.943    263.590
    3             450      2      1292.99    1292.99     45.422     23.161


      NOTE

      Kruskal-Wallis analysis specifically requested.
      Normality and equality of variance have not been checked.
```

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 10:23 FRIDAY 09FEB07
Kruskal-Wallis Test on VTG
MEDAKA MEASUREMENTS FOR FULL DATA

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable RESPONSE
Classified by Variable dose

dose	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
1	2	4.0	7.0	2.160247	2.00
2	2	8.0	7.0	2.160247	4.00
3	2	9.0	7.0	2.160247	4.50

Kruskal-Wallis Test

Chi-Square	2.0000
DF	2
Pr > Chi-Square	0.3679

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 10:23 FRIDAY 09FEB07
Modified Dunn's Multiple Comparisons (Two-sided) on VTG
MEDAKA MEASUREMENTS FOR FULL DATA USING ALPHA=0.05

dose	doseval	COUNT	N0	MRANK	ABS_DIFF	CRIT_05	CRIT_01	SIGNIF	p_val
1	0	2	2	2.0	0.0	4.19328	5.25148		.
2	225	2	2	4.0	2.0	4.19328	5.25148		0.2850
3	450	2	2	4.5	2.5	4.19328	5.25148		0.1814

Thus, Dunn’s test finds the NOEC to exceed 450 ug/L.

Experiment 2: CERI, Medaka, 1-Octanol, Females

1a. Nested Analysis

Model VTG = CONC
1-OCTANOL
FULL DATA SET
COVPARMS

CovParm	Estimate
VESSEL(conc)	11712
Residual	27758

Thus, the variance ratio is 11712/27758=0.42.

Model VTG = CONC
1-OCTANOL
FULL DATA SET
COVPARMS

Effect	Num DF	Den DF	FValue	ProbF	MSERR	SSQRS	SSERR
conc	4	4	111.11	0.0002	27757.55	12336629.24	111030.19

ANOVA SUMMARY STATISTCS

MODELSS	SSERR	TOTSS	RSQUARE
112621.02	111030.19	223651.21	0.50356

MEDAKA VTG DATA FROM LAB CERI
1-OCTANOL
CLASS LEVEL INFORMATION
FULL DATA SET

Class	Levels	Values
conc	4	0 0.32 1 3.2
VESSEL	2	1 2

MEDAKA VTG DATA FROM LAB CERI
1-OCTANOL
LSMEANS
FULL DATA SET

Effect	conc	Estimate	StdErr	DF	tValue	Probt
conc	0	988.41	92.9085	4	10.64	0.0004
conc	0.32	1034.05	92.9085	4	11.13	0.0004
conc	1	1068.11	92.9085	4	11.50	0.0003
conc	3.2	821.24	94.6866	4	8.67	0.0010

MEDAKA VTG DATA FROM LAB CERI
1-OCTANOL
T-TEST RESULTS
FULL DATA SET

Effect	conc	_conc	Estimate	StdErr	DF	tValue	Probt	Adjustment	Adjp
conc	0.32	0	45.6400	131.39	4	0.35	0.7458	Dunnett	0.9696
conc	1	0	79.7050	131.39	4	0.61	0.5768	Dunnett	0.8752
conc	3.2	0	-167.17	132.66	4	-1.26	0.2761	Dunnett	0.5196

Thus, Dunnett's test finds the NOEC exceeds 3.2 mg/L.

MEDAKA VTG DATA FROM LAB CERI
1-OCTANOL
PARAMETER ESTIMATES
FULL DATA SET

Effect	conc	Estimate	StdErr	DF	tValue	Probt
conc	0	988.41	92.9085	4	10.64	0.0004

conc	0.32	1034.05	92.9085	4	11.13	0.0004
conc	1	1068.11	92.9085	4	11.50	0.0003
conc	3.2	821.24	94.6866	4	8.67	0.0010

MEDAKA VTG DATA FROM LAB CERI
1-OCTANOL
SHAPIRO-WILK TEST OF NORMALITY OF VTG
FULL DATA SET

Obs	Var Name	Test	Test Lab	Stat	pType	p Sign	pValue
1	Resid	Shapiro-Wilk	W	0.977424	Pr < W		0.6103
2	Resid	Kolmogorov-Smirnov	D	0.075484	Pr > D	>	0.1500
3	Resid	Cramer-von Mises	W-Sq	0.027916	Pr > W-Sq	>	0.2500
4	Resid	Anderson-Darling	A-Sq	0.215776	Pr > A-Sq	>	0.2500

Thus, the data are normally distributed.

MEDAKA VTG DATA FROM LAB CERI
1-OCTANOL
POSSIBLE OUTLIERS FROM ANOVA ON VTG
FULL DATA SET

Obs	conc	VTG	Pred	Resid	LB	UB
1	3.2	.	810.280	.	-477.825	476.682

LEVENE TEST FOR VTG

Effect	DF	LEVENE	P_VALUE
conc	3	0.54302	0.67837

NOTE: By Levene's test, the within-group variances are equal. A standard ANOVA will be done.

1b. Analysis if Replicate Means

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 12:52 THURSDAY 08FEB07
MEDAKA MEASUREMENTS FROM DATASET FOCTANOLMNS
GROUP STATISTICS FOR VTG BY DOSE w/ Weight=survive, Var-Ratio=0.43

dose	doseval	COUNT	MEAN	MEDIAN	STD_DEV	STD_ERR
1	0	2	988.41	988.41	99.671	55.940
2	0.32	2	1034.05	1034.05	198.949	111.660
3	1	2	1068.12	1068.12	241.275	135.415
4	3.2	2	821.23	837.39	29.359	16.789

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 12:52 THURSDAY 08FEB07
SHAPIRO-WILK TEST OF NORMALITY OF VTG
AQUATIC MEDAKA: FULL DATA

OBS	STD	SKEW	KURT	SW_STAT	P_VALUE	SIGNIF
8	98.8752	.006271526	-1.30138	0.95761	0.7871	

Thus, the data are normally distributed.

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 12:52 THURSDAY 08FEB07
LEVENE TEST FOR VTG - FULL Model
ANALYSIS OF VARIANCE ON FULL DATA SET

Effect	DF	LEVENE	p_value	SIGNIF
DOSE	3	2.7039E15	0.0001	**

NOTE
The data was found to be normally distributed but group variances were unequal.
A Tamhane-Dunnett analysis is appropriate.

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 12:52 THURSDAY 08FEB07
Tamhane-Dunnett 2-sided test for difference in means in VTG
Using MAXIMUM LIKELIHOOD estimates of variation on MEDAKA values.
FULL DATA USING ALPHA=0.05

dose	MEAN	STDERR	degfree	CONTROL	OBS_DIFF	crit	SIGNIF
2	1034.05	111.660	1.47222	988.41	45.640	997.57	
3	1068.12	135.415	1.33165	988.41	79.705	1170.31	
4	821.23	16.789	1.17223	988.41	-167.180	465.81	

Thus, the NOEC by the Tamhane-Dunnett test exceeds the high concentration, 3.2 mg/L.

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 12:52 THURSDAY 08FEB07
MONOTONICITY CHECK OF VTG - FULL DATA
DOSES 0, 0.32, 1, 3.2 mg/L
MEDAKA OF AQUATIC MEDAKA

PARM	p_t	SIGNIF
DOSE TREND	0.2257	
DOSE QUAD	0.1275	

---------- JONCKHEERE-TERPSTRA TEST KEY ----------

```
  _JT_ IS JONCKHEERE STATISTIC
  Z_JT IS STANDARDIZED JONCKHEERE STATISTIC
  PR_JT IS P-VALUE FOR UPWARD TREND
  PL_JT IS P-VALUE FOR DOWNWARD TREND
  P-VALUES ARE FOR TIE-CORRECTED TEST WITH CONTINUITY CORRECTION FACTOR
  SIGNIF RESULTS ARE FOR Two-sided ALTERNATIVE HYPOTHESIS

Check for Number of Reps in VTG Thru 3.2 mg/L
Maximum Number of Reps in Any Treatment Group or Control is 2
Total Number of Reps in All Treatment Groups is 8
Exact Permutation Methods Recommended

  Jonckheere Trend Test on Dose 0 + Lowest 3 Doses thru 3.2 mg/L

  ___JT____        Z_JT  XPL_JT  XPR_JT  SIGNIF  DOSE
         10  -1.021508  0.1929   .                4
```

NOTE: The Jonckheere test finds the NOEC exceeds the high concentration, 3.2 mg/L.

Jonckheere test results are included in the summary table. Group means should be examined to check for lack-of-fit to a linear trend before trend test results are accepted.

2b, continued: Dunn's test

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 14:18 THURSDAY 08FEB07
MEDAKA MEASUREMENTS FROM DATASET FOCTANOLMNS
GROUP STATISTICS FOR VTG BY DOSE w/ Weight=survive, Var-Ratio=0.42

dose	doseval	COUNT	MEAN	MEDIAN	STD_DEV	STD_ERR
1	0	2	988.41	988.41	100.471	55.940
2	0.32	2	1034.05	1034.05	200.547	111.660
3	1	2	1068.12	1068.12	243.213	135.415
4	3.2	2	821.24	837.39	29.585	16.789

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 14:18 THURSDAY 08FEB07
Kruskal-Wallis Test on VTG
MEDAKA MEASUREMENTS FOR FULL DATA

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable RESPONSE
Classified by Variable dose

dose	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
1	2	10.0	9.0	3.0	5.00
2	2	10.0	9.0	3.0	5.00
3	2	13.0	9.0	3.0	6.50
4	2	3.0	9.0	3.0	1.50

Kruskal-Wallis Test

Chi-Square	4.5000
DF	3
Pr > Chi-Square	0.2123

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 14:18 THURSDAY 08FEB07
Modified Dunn's Multiple Comparisons (Two-sided) on VTG
MEDAKA MEASUREMENTS FOR FULL DATA USING ALPHA=0.05

dose	doseval	COUNT	N0	MRANK	ABS_DIFF	CRIT_05	CRIT_01	SIGNIF	p_val
1	0	2	2	5.0	0.0	5.86403	7.18974		.
2	0.32	2	2	5.0	0.0	5.86403	7.18974		1.0000
3	1	2	2	6.5	1.5	5.86403	7.18974		0.8104
4	3.2	2	2	1.5	3.5	5.86403	7.18974		0.2296

Thus, Dunn’s test finds the NOEC to exceed the highest test concentration, 3.2 mg/L.

Experiment 3: Fraunhofer Lab, Zebrafish, Octanol

3a: Nested ANOVA, Females

Model VTG_1000 = CONC
OCTANOL
FULL DATA SET
COVPARMS

CovParm	Estimate
VESSEL(conc)	0
Residual	21027106

Thus, the variance ratio is 0.

Model VTG_1000 = CONC
OCTANOL
FULL DATA SET
COVPARMS

Effect	Num DF	Den DF	FValue	ProbF	MSERR	SSQRS	SSERR
conc	4	4	10.68	0.0207	21027106.10	898536331.04	84108424.41

ANOVA SUMMARY STATISTCS

MODELSS	SSERR	TOTSS	RSQUARE
47554026.81	84108424.41	131662451.22	0.36118

ZEBRAFISH VTG DATA FROM LAB FRAUNHOFER
OCTANOL
CLASS LEVEL INFORMATION
FULL DATA SET

Class	Levels	Values
conc	4	0 320 1000 3200
VESSEL	2	1 2

ZEBRAFISH VTG DATA FROM LAB FRAUNHOFER
OCTANOL
LSMEANS
FULL DATA SET

Effect	conc	Estimate	StdErr	DF	tValue	Probt
conc	0	4108.92	1733.17	4	2.37	0.0768
conc	320	7288.57	1733.17	4	4.21	0.0136
conc	1000	4356.22	1528.51	4	2.85	0.0464
conc	3200	5827.29	1733.17	4	3.36	0.0282

ZEBRAFISH VTG DATA FROM LAB FRAUNHOFER
OCTANOL
T-TEST RESULTS
FULL DATA SET

Effect	conc	_conc	Estimate	StdErr	DF	tValue	Probt	Adjustment	Adjp
conc	320	0	3179.65	2451.07	4	1.30	0.2643	Dunnett	0.4967
conc	1000	0	247.30	2310.89	4	0.11	0.9199	Dunnett	0.9989
conc	3200	0	1718.37	2451.07	4	0.70	0.5219	Dunnett	0.8243

By Dunnett's test, there is no statistically significant effect at any test concentration.

ZEBRAFISH VTG DATA FROM LAB FRAUNHOFER
OCTANOL
PARAMETER ESTIMATES
FULL DATA SET

Effect	conc	Estimate	StdErr	DF	tValue	Probt
conc	0	4108.92	1733.17	4	2.37	0.0768
conc	320	7288.57	1733.17	4	4.21	0.0136
conc	1000	4356.22	1528.51	4	2.85	0.0464
conc	3200	5827.29	1733.17	4	3.36	0.0282

ZEBRAFISH VTG DATA FROM LAB FRAUNHOFER
OCTANOL
SHAPIRO-WILK TEST OF NORMALITY OF VTG_1000
FULL DATA SET

Obs	Var Name	Test	Test Lab	Stat	pType	p Sign	pValue
1	Resid	Shapiro-Wilk	W	0.936352	Pr < W		0.0726
2	Resid	Kolmogorov-Smirnov	D	0.09415	Pr > D	>	0.1500
3	Resid	Cramer-von Mises	W-Sq	0.064512	Pr > W-Sq	>	0.2500
4	Resid	Anderson-Darling	A-Sq	0.491676	Pr > A-Sq		0.2113

The data are normally distributed.

ZEBRAFISH VTG DATA FROM LAB FRAUNHOFER
OCTANOL
LEVENE TEST FOR VTG_1000
FULL DATA SET

Effect	DF	LEVENE	P_VALUE
conc	3	1.08303	0.45157

The variances are homogeneous.

ZEBRAFISH VTG DATA FROM LAB FRAUNHOFER
OCTANOL
LEVENE TEST FOR VTG_1000
FULL DATA SET

NOTE
By Levene's test, the within-group variances are equal. A standard ANOVA will be done.

3b: Analysis of Replicate Means

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 20:56 THURSDAY 08FEB07
ZEBRAFISH MEASUREMENTS FROM DATASET FFRAUNHOFEROCTMNS
GROUP STATISTICS FOR VTG/1000 BY DOSE w/ Weight=survive, Var-Ratio=0

dose	doseval	COUNT	MEAN	MEDIAN	STD_DEV	STD_ERR
1	0	2	4108.92	3618.11	1499.45	566.74
2	320	2	7288.57	6792.50	1515.52	572.81
3	1000	2	4356.22	4464.80	364.18	121.39
4	3200	2	5827.29	8336.25	7665.01	2897.10

SHAPIRO-WILK TEST OF NORMALITY OF VTG/1000
AQUATIC ZEBRAFISH: FULL DATA

OBS	STD	SKEW	KURT	SW_STAT	P_VALUE	SIGNIF
8	1640.26	-0.75698	2.68639	0.90246	0.3040	

The data are normally distributed.

Outliers & Influential Observations
VTG/1000 FROM FULL DATA w/ Weight=survive, Var-Ratio=0
AQUATIC ZEBRAFISH: Concentrations in ug/L

Obs	ZEBRAFISH	dose	doseval	GROUP	OBSER	Pred
1	1	4	3200	4	8336.25	5827.29
2	2	4	3200	4	2482.00	5827.29

Obs	SE_PRED	L95M	U95M	Resid	LB	UB
1	1505.11	1648.42	10006.15	2508.96	-2220.48	2384.96
2	1505.11	1648.42	10006.15	-3345.29	-2220.48	2384.96

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 20:56 THURSDAY 08FEB07
LEVENE TEST FOR VTG/1000 - FULL Model
ANALYSIS OF VARIANCE ON FULL DATA SET

Effect	DF	LEVENE	p_value	SIGNIF
DOSE	3	I	0.0001	**

NOTE: The variances are heterogeneous. No variance stabilizing transform was found.
The data was found to be normally distributed but group variances were unequal. A Tamhane-Dunnett analysis is appropriate.

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 20:56 THURSDAY 08FEB07
Tamhane-Dunnett 2-sided test for difference in means in VTG/1000
Using MAXIMUM LIKELIHOOD estimates of variation on ZEBRAFISH values.
FULL DATA USING ALPHA=0.05

dose	MEAN	STDERR	degfree	CONTROL	OBS_DIFF	crit	SIGNIF
2	7288.57	572.81	1.99977	4108.92	3179.65	9557.60	
3	4356.22	121.39	1.11757	4108.92	247.30	6917.54	
4	5827.29	2897.10	1.07642	4108.92	1718.37	35014.01	

```
                        STATISTICAL ANALYSIS LIST FILE
                    REPORT CREATED ON 20:56 THURSDAY  08FEB07
                    MONOTONICITY CHECK OF VTG/1000 - FULL DATA
                        DOSES 0, 320, 1000, 3200 ug/L
                        ZEBRAFISH OF AQUATIC ZEBRAFISH

                           PARM          p_t    SIGNIF

                        DOSE TREND    0.7935
                        DOSE QUAD     0.7261

-------------------------------------------------------------------------------

  STATISTICAL ANALYSIS LIST FILE
  REPORT CREATED ON 20:56 THURSDAY  08FEB07
  ANALYSIS OF VTG/1000 USING ALPHA=0.05
  FULL DATA

  ---------- JONCKHEERE-TERPSTRA TEST KEY ----------
  _JT_ IS JONCKHEERE STATISTIC
  Z_JT IS STANDARDIZED JONCKHEERE STATISTIC
  PR_JT IS P-VALUE FOR UPWARD TREND
  PL_JT IS P-VALUE FOR DOWNWARD TREND
  P-VALUES ARE FOR TIE-CORRECTED TEST WITH CONTINUITY CORRECTION FACTOR
  SIGNIF RESULTS ARE FOR Two-sided ALTERNATIVE HYPOTHESIS

Check for Number of Reps in VTG/1000 Thru 3200 ug/L
Maximum Number of Reps in Any Treatment Group or Control is 2
Total Number of Reps in All Treatment Groups is 8
Exact Permutation Methods Recommended

  Jonckheere Trend Test on Dose 0 + Lowest 3 Doses thru 3200 ug/L

  ___JT____        Z_JT  XPL_JT  XPR_JT  SIGNIF  DOSE
         14           0  0.5492   .                4
```

NOTE: The Jonckheere test found no significant effect at any test concentration.

```
Jonckheere test results are included in the summary table.  Group means should be examined to
check for lack-of-fit to a linear trend before trend test results are accepted.
```

3b, continued: Dunn's test

```
                        STATISTICAL ANALYSIS LIST FILE
                  REPORT CREATED ON 21:16 THURSDAY  08FEB07
           ZEBRAFISH MEASUREMENTS FROM DATASET FFRAUNHOFEROCTMNS
      GROUP STATISTICS FOR VTG BY DOSE w/ Weight=survive, Var-Ratio=0

  dose doseval       COUNT    MEAN      MEDIAN     STD_DEV    STD_ERR

    1             0   2   4108919.86 3618109.75 1499449.65  566738.69
    2           320   2   7288571.43 6792500.00 1515523.25  572813.95
    3          1000   2   4356222.22 4464800.00  364180.94  121393.65
    4          3200   2   5827285.71 8336250.00 7665012.50 2897102.41

 NOTE
 Kruskal-Wallis analysis specifically requested.
 Normality and equality of variance have not been checked.
-------------------------------------------------------------------------------
```

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 21:16 THURSDAY 08FEB07
Kruskal-Wallis Test on VTG
ZEBRAFISH MEASUREMENTS FOR FULL DATA

Wilcoxon Scores (Rank Sums) for Variable RESPONSE
Classified by Variable dose

dose	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
1	2	7.0	9.0	3.0	3.50
2	2	13.0	9.0	3.0	6.50
3	2	7.0	9.0	3.0	3.50
4	2	9.0	9.0	3.0	4.50

Kruskal-Wallis Test

Chi-Square	2.0000
DF	3
Pr > Chi-Square	0.5724

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 21:16 THURSDAY 08FEB07
Modified Dunn's Multiple Comparisons (Two-sided) on VTG
ZEBRAFISH MEASUREMENTS FOR FULL DATA USING ALPHA=0.05

dose	doseval	COUNT	N0	MRANK	ABS_DIFF	CRIT_05	CRIT_01	SIGNIF	p_val
1	0	2	2	3.5	0	5.86403	7.18974		.
2	320	2	2	6.5	3	5.86403	7.18974		0.3310
3	1000	2	2	3.5	0	5.86403	7.18974		1.0000
4	3200	2	2	4.5	1	5.86403	7.18974		1.0000

Dunn's test finds no statistically significant effect any test concentration.

3c: Nested ANOVA, Males

Model VTG_1000 = CONC
OCTANOL
FULL DATA SET
COVPARMS

CovParm	Estimate
VESSEL(conc)	19.7493
Residual	16.0240

Thus, the variance ratio is 19.7493 / 16.0240=1.2

SHAPIRO-WILK TEST OF NORMALITY OF VTG_1000
FULL DATA SET

Obs	Var Name	Test	Test Lab	Stat	pType	p Sign	pValue
1	Resid	Shapiro-Wilk	W	0.537236	Pr < W	<	0.0001
2	Resid	Kolmogorov-Smirnov	D	0.339823	Pr > D	<	0.0100
3	Resid	Cramer-von Mises	W-Sq	1.735862	Pr > W-Sq	<	0.0050
4	Resid	Anderson-Darling	A-Sq	8.326933	Pr > A-Sq	<	0.0050

The data are not normally distributed. A log-transform normalizes the data and stabilizes the variances, as shown below.

Model LOGVTG = CONC
OCTANOL
FULL DATA SET
COVPARMS

CovParm	Estimate
VESSEL(conc)	0.4754
Residual	0.2247

The variance ratio under a log-transform is 0.4754 / 0.2247=2.1

Model LOGVTG = CONC
OCTANOL
FULL DATA SET
COVPARMS

Effect	Num DF	Den DF	FValue	ProbF	MSERR	SSQRS	SSERR
conc	4	4	20.64	0.0062	0.22468	18.5538	0.89872

ANOVA SUMMARY STATISTCS

MODELSS	SSERR	TOTSS	RSQUARE
0.64370	0.89872	1.54242	0.41733

MALE ZEBRAFISH LOGVTG DATA FROM LAB FRAUNHOFER
OCTANOL
CLASS LEVEL INFORMATION
FULL DATA SET

Class	Levels	Values
conc	4	0 320 1000 3200
VESSEL	2	1 2

MALE ZEBRAFISH LOGVTG DATA FROM LAB FRAUNHOFER
OCTANOL
LSMEANS
FULL DATA SET

Effect	conc	Estimate	StdErr	DF	tValue	Probt
conc	0	1.9308	0.5128	4	3.77	0.0197
conc	320	1.8353	0.5069	4	3.62	0.0223
conc	1000	2.4204	0.5082	4	4.76	0.0089
conc	3200	2.9172	0.5109	4	5.71	0.0047

MALE ZEBRAFISH LOGVTG DATA FROM LAB FRAUNHOFER
OCTANOL
T-TEST RESULTS
FULL DATA SET

Effect	conc	_conc_	Estimate	StdErr	DF	tValue	Probt	Adjustment	Adjp
conc	320	0	-0.09552	0.7210	4	-0.13	0.9010	Dunnett	0.9981
conc	1000	0	0.4896	0.7219	4	0.68	0.5349	Dunnett	0.8387
conc	3200	0	0.9863	0.7238	4	1.36	0.2447	Dunnett	0.4683

Dunnett's test finds no statistically significant effect at any test concentration.

MALE ZEBRAFISH LOGVTG DATA FROM LAB FRAUNHOFER
OCTANOL
PARAMETER ESTIMATES
FULL DATA SET

Effect	conc	Estimate	StdErr	DF	tValue	Probt
conc	0	1.9308	0.5128	4	3.77	0.0197
conc	320	1.8353	0.5069	4	3.62	0.0223
conc	1000	2.4204	0.5082	4	4.76	0.0089
conc	3200	2.9172	0.5109	4	5.71	0.0047

MALE ZEBRAFISH LOGVTG DATA FROM LAB FRAUNHOFER
OCTANOL
SHAPIRO-WILK TEST OF NORMALITY OF LOGVTG
FULL DATA SET

Obs	Var Name	Test	Test Lab	Stat	pType	p Sign	pValue
1	Resid	Shapiro-Wilk	W	0.965297	Pr < W		0.2277
2	Resid	Kolmogorov-Smirnov	D	0.117811	Pr > D		0.1468
3	Resid	Cramer-von Mises	W-Sq	0.106857	Pr > W-Sq		0.0906
4	Resid	Anderson-Darling	A-Sq	0.60542	Pr > A-Sq		0.1100

The data are normally distributed under a log-transform.

MALE ZEBRAFISH LOGVTG DATA FROM LAB FRAUNHOFER
OCTANOL
POSSIBLE OUTLIERS FROM ANOVA ON LOGVTG
FULL DATA SET

Obs	conc	LOGVTG	Pred	Resid	LB	UB
4	320	2.92247	1.87627	1.04619	-1.06529	1.04069

MALE ZEBRAFISH LOGVTG DATA FROM LAB FRAUNHOFER
OCTANOL
LEVENE TEST FOR LOGVTG
FULL DATA SET

Effect	DF	LEVENE	P_VALUE
conc	3	1.03865	0.46592

By Levene's test, the variances under a log-transform are homogeneous.
NOTE: By Levene's test, the within-group variances are equal. A standard ANOVA will be done.

3d: Analysis of Replicate Means, Males

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 22:56 THURSDAY 08FEB07
ZEBRAFISH MEASUREMENTS FROM DATASET MFRAUNHOFEROCTMNS
GROUP STATISTICS FOR VTG BY DOSE w/ Weight=survive, Var-Ratio=1

dose	doseval	COUNT	MEAN	MEDIAN	STD_DEV	STD_ERR
1	0	2	150.62	100.700	65.12	50.95
2	320	2	117.46	167.486	67.00	51.26
3	1000	2	773.81	150.583	821.80	632.07
4	3200	2	6677.47	119.600	8738.24	6788.04

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 22:56 THURSDAY 08FEB07
SHAPIRO-WILK TEST OF NORMALITY OF VTG
AQUATIC ZEBRAFISH: FULL DATA

OBS	STD	SKEW	KURT	SW_STAT	P_VALUE	SIGNIF
8	3647.98	0.18795	3.37316	0.80192	0.0300	**

The data are not normally distributed. A normalizing transform is found below.

Outliers & Influential Observations
VTG FROM FULL DATA w/ Weight=survive, Var-Ratio=1
AQUATIC ZEBRAFISH: Concentrations in ug/L

Obs	ZEBRAFISH	dose	doseval	GROUP	OBSER	Pred
1	1	4	3200	4	119.60	6677.47
2	2	4	3200	4	13703.75	6677.47

Obs	SE_PRED	L95M	U95M	Resid	LB	UB
1	3409.19	-2787.96	16142.89	-6557.87	-1364.46	1373.10
2	3409.19	-2787.96	16142.89	7026.28	-1364.46	1373.10

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 22:56 THURSDAY 08FEB07
Kruskal-Wallis Test on VTG
ZEBRAFISH MEASUREMENTS FOR FULL DATA

Wilcoxon Scores (Rank Sums) for Variable RESPONSE
Classified by Variable dose

dose	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
1	2	8.0	9.0	3.0	4.00
2	2	6.0	9.0	3.0	3.00
3	2	11.0	9.0	3.0	5.50
4	2	11.0	9.0	3.0	5.50

Kruskal-Wallis Test

Chi-Square	1.5000
DF	3
Pr > Chi-Square	0.6823

Modified Dunn's Multiple Comparisons (Two-sided) on VTG
ZEBRAFISH MEASUREMENTS FOR FULL DATA USING ALPHA=0.05

dose	doseval	COUNT	N0	MRANK	ABS_DIFF	CRIT_05	CRIT_01	SIGNIF	p_val
1	0	2	2	4.0	0.0	5.86403	7.18974		.
2	320	2	2	3.0	1.0	5.86403	7.18974		1.0000
3	1000	2	2	5.5	1.5	5.86403	7.18974		0.8104
4	3200	2	2	5.5	1.5	5.86403	7.18974		0.8104

Thus, Dunn's test finds no statistically significant effect at any test concentration.

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 22:56 THURSDAY 08FEB07
MONOTONICITY CHECK OF VTG - FULL DATA
DOSES 0, 320, 1000, 3200 ug/L
ZEBRAFISH OF AQUATIC ZEBRAFISH

PARM	p_t	SIGNIF
DOSE TREND	0.4339	
DOSE QUAD	0.7437	

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 22:56 THURSDAY 08FEB07
ANALYSIS OF VTG USING ALPHA=0.05
FULL DATA

---------- JONCKHEERE-TERPSTRA TEST KEY ----------
_JT_ IS JONCKHEERE STATISTIC
Z_JT IS STANDARDIZED JONCKHEERE STATISTIC
PR_JT IS P-VALUE FOR UPWARD TREND
PL_JT IS P-VALUE FOR DOWNWARD TREND
P-VALUES ARE FOR TIE-CORRECTED TEST WITH CONTINUITY CORRECTION FACTOR
SIGNIF RESULTS ARE FOR Two-sided ALTERNATIVE HYPOTHESIS

Check for Number of Reps in VTG Thru 3200 ug/L
Maximum Number of Reps in Any Treatment Group or Control is 2
Total Number of Reps in All Treatment Groups is 8
Exact Permutation Methods Recommended

Jonckheere Trend Test on Dose 0 + Lowest 3 Doses thru 3200 ug/L

___JT____	Z_JT	XPL_JT	XPR_JT	SIGNIF	DOSE
17	0.7661309	.	0.2683		4

NOTE: The Jonckheere test finds no statistically significant effect at any test concentration

Jonckheere test results are included in the summary table.
Group means should be examined to check for lack-of-fit
to a linear trend before trend test results are accepted.

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 22:56 THURSDAY 08FEB07
SHAPIRO-WILK TEST OF NORMALITY OF LOG(VTG)
AQUATIC ZEBRAFISH: FULL DATA

OBS	STD	SKEW	KURT	SW_STAT	P_VALUE	SIGNIF
8	1.43688	0.11921	0.46332	0.99010	0.9952	

A log-transform normalizes the data.

NOTE
Untransformed response does not meet normality. LOG transform makes response satisfy normality.

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 22:56 THURSDAY 08FEB07
LEVENE TEST FOR LOG(VTG) - FULL Model
ANALYSIS OF VARIANCE ON FULL DATA SET

Effect	DF	LEVENE	p_value	SIGNIF
DOSE	3	I	0.0001	**

The log-transformed data have heterogeneous variances.
The data was found to be normally distributed but group variances were unequal. A Tamhane-Dunnett analysis is appropriate.

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 17:10 FRIDAY 09FEB07
Tamhane-Dunnett 2-sided test for difference in means in LOG10(VTG)
Using MAXIMUM LIKELIHOOD estimates of variation on ZEBRAFISH values.
FULL DATA USING ALPHA=0.05

dose	MEAN	STDERR	degfree	CONTROL	OBS_DIFF	crit	SIGNIF
2	2.05253	0.20286	1.70802	2.13799	-0.08546	3.7474	
3	2.62002	0.48445	1.15775	2.13799	0.48203	7.4835	
4	2.90137	1.00875	1.04026	2.13799	0.76338	14.4440	

The Tamhane-Dunnett test applied to the log-transformed data finds no significant effect at any test concentration.

Experiment 4: Fraunhofer, Zebrafish, Potassium Permanganate, Females

4a. Nested ANOVA

Model LOGVTG = CONC
Potassium Permanganate
FULL DATA SET
COVPARMS

CovParm	Estimate
VESSEL(conc)	0
Residual	0.1584

Thus, the variance ratio is 0.

Model LOGVTG = CONC
Potassium Permanganate
FULL DATA SET
COVPARMS

Effect	Num DF	Den DF	FValue	ProbF	MSERR	SSQRS	SSERR
conc	4	4	2296.49	<.0001	0.15836	1454.69	0.63344

ANOVA SUMMARY STATISTCS

MODELSS	SSERR	TOTSS	RSQUARE
1.00972	0.63344	1.64317	0.61450

ZEBRAFISH VTG DATA FROM LAB FRAUNHOFER
Potassium Permanganate
CLASS LEVEL INFORMATION
FULL DATA SET

Class	Levels	Values
conc	4	0 225 450 900
VESSEL	2	1 2

ZEBRAFISH VTG DATA FROM LAB FRAUNHOFER
Potassium Permanganate
LSMEANS
FULL DATA SET

Effect	conc	Estimate	StdErr	DF	tValue	Probt
conc	0	6.8059	0.1326	4	51.31	<.0001
conc	225	6.5369	0.1326	4	49.28	<.0001
conc	450	6.4162	0.1407	4	45.60	<.0001
conc	900	6.3629	0.1407	4	45.23	<.0001

ZEBRAFISH VTG DATA FROM LAB FRAUNHOFER
Potassium Permanganate
T-TEST RESULTS
FULL DATA SET

Effect	conc	_conc	Estimate	StdErr	DF	tValue	Probt	Adjustment	Adjp
conc	225	0	-0.2690	0.1876	4	-1.43	0.2248	Dunnett	0.4395
conc	450	0	-0.3897	0.1934	4	-2.02	0.1141	Dunnett	0.2393
conc	900	0	-0.4429	0.1934	4	-2.29	0.0838	Dunnett	0.1794

Thus, Dunnett's test finds the NOEC to exceed the high test concentration, 900 ug/L.

ZEBRAFISH VTG DATA FROM LAB FRAUNHOFER
Potassium Permanganate
PARAMETER ESTIMATES
FULL DATA SET

Effect	conc	Estimate	StdErr	DF	tValue	Probt
conc	0	6.8059	0.1326	4	51.31	<.0001
conc	225	6.5369	0.1326	4	49.28	<.0001
conc	450	6.4162	0.1407	4	45.60	<.0001
conc	900	6.3629	0.1407	4	45.23	<.0001

ZEBRAFISH VTG DATA FROM LAB FRAUNHOFER
Potassium Permanganate
SHAPIRO-WILK TEST OF NORMALITY OF LOGVTG
FULL DATA SET

Obs	Var Name	Test	Test Lab	Stat	pType	p Sign	pValue
1	Resid	Shapiro-Wilk	W	0.962288	Pr < W		0.2832
2	Resid	Kolmogorov-Smirnov	D	0.101899	Pr > D	>	0.1500
3	Resid	Cramer-von Mises	W-Sq	0.062643	Pr > W-Sq	>	0.2500
4	Resid	Anderson-Darling	A-Sq	0.433437	Pr > A-Sq	>	0.2500

Thus, the data are normally distributed under the log transform.

ZEBRAFISH VTG DATA FROM LAB FRAUNHOFER
Potassium Permanganate
POSSIBLE OUTLIERS FROM ANOVA ON LOGVTG
FULL DATA SET

Obs	conc	LOGVTG	Pred	Resid	LB	UB
1	450	5.34044	6.41619	-1.07575	-0.81383	0.85877
2	900	5.49969	6.36293	-0.86325	-0.81383	0.85877

ZEBRAFISH VTG DATA FROM LAB FRAUNHOFER
Potassium Permanganate
LEVENE TEST FOR LOGVTG
FULL DATA SET

Effect	DF	LEVENE	P_VALUE
conc	3	2.00039	0.25633

NOTE: By Levene's test, the within-group variances are equal. A standard ANOVA will be done.

4b. Analysis of Replicate means

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 0:28 THURSDAY 08FEB07
ZEBRAFISH MEASUREMENTS FROM DATASET FFRAUNHOFPOTMNS
GROUP STATISTICS FOR VTG/1000 BY DOSE w/ Weight=survive, Var-Ratio=0

dose	doseval	COUNT	MEAN	MEDIAN	STD_DEV	STD_ERR
1	0	2	6992.22	6276.00	2402.28	800.761
2	225	2	3684.44	3346.00	1135.18	378.392
3	450	2	4188.88	4188.88	144.60	51.125
4	900	2	4273.50	4273.50	748.12	264.500

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 0:28 THURSDAY 08FEB07
SHAPIRO-WILK TEST OF NORMALITY OF VTG/1000
FEMALE ZEBRAFISH: FULL DATA

OBS	STD	SKEW	KURT	SW_STAT	P_VALUE	SIGNIF
8	499.210	0.32820	0.18383	0.99025	0.9954	

Thus, the data are normally distributed.

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 0:28 THURSDAY 08FEB07
LEVENE TEST FOR VTG/1000 - FULL Model
ANALYSIS OF VARIANCE ON FULL DATA SET

Effect	DF	LEVENE	p_value	SIGNIF
DOSE	3	1.428E17	0.0001	**

NOTE
The data was found to be normally distributed but group variances were unequal. A Tamhane-Dunnett analysis is appropriate.

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 0:28 THURSDAY 08FEB07
Tamhane-Dunnett 2-sided test for difference in means in VTG/1000
Using MAXIMUM LIKELIHOOD estimates of variation on ZEBRAFISH values.
FULL DATA USING ALPHA=0.05

dose	MEAN	STDERR	degfree	CONTROL	OBS_DIFF	crit	SIGNIF
2	3684.44	378.392	1.42538	6992.22	-3307.78	11911.43	
3	4188.88	51.125	1.00725	6992.22	-2803.35	10789.06	
4	4273.50	264.500	1.19216	6992.22	-2718.72	11279.72	

Thus, the Tamhane-Dunnett test finds the NOEC to exceed the high test concentration, 900 ug/L

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 0:28 THURSDAY 08FEB07
MONOTONICITY CHECK OF VTG/1000 - FULL DATA
DOSES 0, 225, 450, 900 ug/L
ZEBRAFISH OF FEMALE ZEBRAFISH

PARM	p_t	SIGNIF
DOSE TREND	0.2135	
DOSE QUAD	0.0926	

This formal test finds no problem with monotonicity. A visual inspection of the means raises some doubts about the formal test result.

ANALYSIS OF VTG/1000 USING ALPHA=0.05
FULL DATA

```
  ---------- JONCKHEERE-TERPSTRA TEST KEY ----------
  _JT_ IS JONCKHEERE STATISTIC
  Z_JT IS STANDARDIZED JONCKHEERE STATISTIC
  PR_JT IS P-VALUE FOR UPWARD TREND
  PL_JT IS P-VALUE FOR DOWNWARD TREND
  P-VALUES ARE FOR TIE-CORRECTED TEST WITH CONTINUITY CORRECTION FACTOR
  SIGNIF RESULTS ARE FOR Two-sided ALTERNATIVE HYPOTHESIS

Check for Number of Reps in VTG/1000 Thru 900 ug/L
Maximum Number of Reps in Any Treatment Group or Control is 2
Total Number of Reps in All Treatment Groups is 8
Exact Permutation Methods Recommended

  Jonckheere Trend Test on Dose 0 + Lowest 3 Doses thru 900 ug/L

  ___JT____        Z_JT  XPL_JT  XPR_JT  SIGNIF  DOSE
         11  -0.766131  0.2683   .                4
```

NOTE: The Jonckheere test finds the NOEC to exceed the high test concentration, 900 ug/L.

```
Jonckheere test results are included in the summary table. Group means should be examined to check
for lack-of-fit to a linear trend before trend test results are accepted.
```

4b, continued, Dunn's Test

```
                    STATISTICAL ANALYSIS LIST FILE
                REPORT CREATED ON 12:02 FRIDAY  09FEB07
           ZEBRAFISH MEASUREMENTS FROM DATASET FFRAUNHOFPOTMNS
     GROUP STATISTICS FOR VTG BY DOSE w/ Weight=survive, Var-Ratio=0

 dose  doseval       COUNT     MEAN      MEDIAN    STD_DEV    STD_ERR

   1             0    2    6992222.22  6276000  2402282.36  800760.79
   2           225    2    3684444.44  3346000  1135177.18  378392.39
   3           450    2    4188875.00  4188875   144603.34   51125.00
   4           900    2    4273500.00  4273500   748118.97  264500.00

      NOTE
      Kruskal-Wallis analysis specifically requested.
      Normality and equality of variance have not been checked.

                    STATISTICAL ANALYSIS LIST FILE
                REPORT CREATED ON 12:02 FRIDAY  09FEB07
                       Kruskal-Wallis Test on VTG
                  ZEBRAFISH MEASUREMENTS FOR FULL DATA

                          The NPAR1WAY Procedure

           Wilcoxon Scores (Rank Sums) for Variable RESPONSE
                     Classified by Variable dose

                      Sum of      Expected       Std Dev          Mean
  dose       N        Scores      Under H0      Under H0         Score
  ---------------------------------------------------------------------
  1          2          15.0           9.0           3.0          7.50
  2          2           4.0           9.0           3.0          2.00
  3          2           9.0           9.0           3.0          4.50
  4          2           8.0           9.0           3.0          4.00

                          Kruskal-Wallis Test

                       Chi-Square         5.1667
                       DF                      3
                       Pr > Chi-Square    0.1600

------------------------------------------------------------------------

                    STATISTICAL ANALYSIS LIST FILE
                REPORT CREATED ON 12:02 FRIDAY  09FEB07
          Modified Dunn's Multiple Comparisons (Two-sided) on VTG
           ZEBRAFISH MEASUREMENTS FOR FULL DATA USING ALPHA=0.05
```

dose	doseval	COUNT	N0	MRANK	ABS_DIFF	CRIT_05	CRIT_01	SIGNIF	p_val
1	0	2	2	7.5	0.0	5.86403	7.18974		.
2	225	2	2	2.0	5.5	5.86403	7.18974		0.0371
3	450	2	2	4.5	3.0	5.86403	7.18974		0.3310
4	900	2	2	4.0	3.5	5.86403	7.18974		0.2296

Thus, Dunn's test finds the median response at the low concentration to be significantly lower than the median response in the controls. Since the median responses at the two higher concentrations are not significantly different from the control, it is not clear from this table what to conclude about the NOEC. However, it is observed that the mean responses in the two higher concentrations are 39-40% lower than the control mean response.

4c. Analysis Ignoring Reps

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 1:01 THURSDAY 08FEB07
ZEBRAFISH MEASUREMENTS FROM DATASET FFFRAUNHOFPOT
GROUP STATISTICS FOR VTG/1000 BY DOSE, Unweighted

dose	doseval	COUNT	MEAN	MEDIAN	STD_DEV	STD_ERR
1	0	9	6992.22	6070	3221.38	1073.79
2	225	9	3684.44	3510	1413.76	471.25
3	450	8	4188.88	3685	3361.58	1188.50
4	900	8	4273.50	2490	4844.42	1712.76

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 1:01 THURSDAY 08FEB07
SHAPIRO-WILK TEST OF NORMALITY OF VTG/1000
FEMALE ZEBRAFISH: FULL DATA

OBS	STD	SKEW	KURT	SW_STAT	P_VALUE	SIGNIF
34	3221.09	1.22148	1.62162	0.90584	0.0066	**

Thus, the data are found not to be normally distributed. As shown below, a normalizing transform was found.

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 1:01 THURSDAY 08FEB07
SHAPIRO-WILK TEST OF NORMALITY OF SQRT(VTG/1000)
FEMALE ZEBRAFISH: FULL DATA

OBS	STD	SKEW	KURT	SW_STAT	P_VALUE	SIGNIF
34	23.3300	0.51395	0.47717	0.97839	0.7215	

Thus, the square-root transform normalizes the data.

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 1:01 THURSDAY 08FEB07
LEVENE TEST FOR SQRT(VTG/1000) - FULL Model
ANALYSIS OF VARIANCE ON FULL DATA SET

Effect	DF	LEVENE	p_value	SIGNIF
DOSE	3	2.27802	0.0998	

Thus, the square-root transform stabilizes the variances. A standard Dunnett test can be applied.

Outliers & Influential Observations
SQRT(VTG/1000) FROM FULL DATA Unweighted
FEMALE ZEBRAFISH: Concentrations in ug/L

Obs	ZEBRAFISH	dose	doseval	GROUP	OBSER	Pred
1	1	4	900	4	119.583	56.7949

Obs	SE_PRED	L95M	U95M	Resid	LB	UB
1	8.65101	39.1272	74.4626	62.7877	-54.7025	53.0236

NOTE
The data was found to be normally distributed with equal variances. An analysis of variance will be performed.

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 1:01 THURSDAY 08FEB07
ANALYSIS OF ZEBRAFISH - FULL DATA
DOSES 0, 225, 450, 900 ug/L
ZEBRAFISH OF FEMALE ZEBRAFISH

Obs	Class	Levels	Values
1	dose	4	1 2 3 4

OVERALL F-TESTS FOR ANOVA
SQRT(VTG/1000) FROM FULL DATA Unweighted

Obs	Effect	Num DF	Den DF	FValue	ProbF
1	dose	3	30	2.01	0.1335

LSMEANS - DOSE MEANS ADJUSTED FOR THE EFFECTS OF ZEBRAFISH
SQRT(VTG/1000) FROM FULL DATA Unweighted

Obs	LEVEL	LSMEAN	SE	DDF
1	0	81.7655	8.15625	30
2	225	59.6925	8.15625	30
3	450	58.9312	8.65101	30
4	900	56.7949	8.65101	30

ESTIMATED DOSE EFFECTS & DUNNETT FOR Two-sided ALTERNATIVE
USING ALPHA=0.05 FOR COMPARISONS TO CONTROL
SQRT(VTG/1000) FROM FULL DATA Unweighted

Estimate	Significance	Dunnett 2-sided p-value	Test Group Mean	N
DOSE TREND		0.0532	.	.
DOSE QUAD		0.2451	.	.
DOSE 2 - 1		0.1594	59.6925	9
DOSE 3 - 1		0.1574	58.9312	8
DOSE 4 - 1		0.1110	56.7949	8

Dunnett’s test does not a statistically significant effect at any test concentration.

MONOTONICITY CHECK OF VTG/1000 - FULL DATA
DOSES 0, 225, 450, 900 ug/L
ZEBRAFISH OF FEMALE ZEBRAFISH

PARM	p_t	SIGNIF
DOSE TREND	0.0459	*
DOSE QUAD	0.2160	

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 1:01 THURSDAY 08FEB07
ANALYSIS OF VTG/1000 USING ALPHA=0.05
FULL DATA

---------- JONCKHEERE-TERPSTRA TEST KEY ----------
_JT_ IS JONCKHEERE STATISTIC
Z_JT IS STANDARDIZED JONCKHEERE STATISTIC
PR_JT IS P-VALUE FOR UPWARD TREND
PL_JT IS P-VALUE FOR DOWNWARD TREND
P-VALUES ARE FOR TIE-CORRECTED TEST WITH CONTINUITY CORRECTION FACTOR
SIGNIF RESULTS ARE FOR Two-sided ALTERNATIVE HYPOTHESIS

Check for ties in VTG/1000 (Transform does not affect the Jonckheere test)
Percent of all data tied at 3 most frequently observed values
Since 3 < 25%, 6 < 40% and 9 < 65%
Exact methods (StatXact) are not required

COUNT	SUMWTS	NMISS	NOBS	RESPONSE	TIES	TIEPCT
1	34	2	36	219	1	3
1	34	2	36	316	2	6
1	34	2	36	842	3	9

Check for Number of Reps in VTG/1000 Thru 900 ug/L
Maximum Number of Reps in Any Treatment Group or Control is 9
Total Number of Reps in All Treatment Groups is 34
Exact Permutation Methods Not Required on this Basis

Jonckheere Trend Test on Dose 0 + Lowest 3 Doses thru 900 ug/L

___JT____	Z_JT	PL_JT	PR_JT	SIGNIF	DOSE
203	-2.381611	0.0086	.	**	4

Check for Number of Reps in VTG/1000 Thru 450 ug/L
Maximum Number of Reps in Any Treatment Group or Control is 9
Total Number of Reps in All Treatment Groups is 26
Exact Permutation Methods Not Required on this Basis

Jonckheere Trend Test on Dose 0 + Lowest 2 Doses thru 450 ug/L

___JT____	Z_JT	PL_JT	PR_JT	SIGNIF	DOSE
116	-2.186571	0.0144	.	**	3

Check for Number of Reps in VTG/1000 Thru 225 ug/L
Maximum Number of Reps in Any Treatment Group or Control is 9
Total Number of Reps in All Treatment Groups is 18
Exact Permutation Methods Not Required on this Basis

Jonckheere Trend Test on Dose 0 + Lowest 1 Doses thru 225 ug/L

___JT____	Z_JT	PL_JT	PR_JT	SIGNIF	DOSE
49	-2.428309	0.0076	.	**	2

NOTE: The step-down Jonckheere test applied to the data ignoring reps finds the NOEC to be below the lowest test concentration.

Jonckheere test results are included in the summary table. Group means should be examined to check for lack-of-fit to a linear trend before trend test results are accepted.

Males

4d. Nested ANOVA

ZEBRAFISH VTG DATA FROM LAB FRAUNHOFER
Potassium Permanganate
LSMEANS
FULL DATA SET

Effect	conc	Estimate	StdErr	DF	tValue	Probt
conc	0	254.32	100.48	4	2.53	0.0646
conc	225	136.14	96.6201	4	1.41	0.2316
conc	450	311.01	110.54	4	2.81	0.0481
conc	900	210.62	93.5320	4	2.25	0.0875

SHAPIRO-WILK TEST OF NORMALITY OF VTG

Obs	Var Name	Test	Test Lab	Stat	pType	p Sign	pValue
1	Resid	Shapiro-Wilk	W	0.785503	Pr < W	<	0.0001
2	Resid	Kolmogorov-Smirnov	D	0.195208	Pr > D	<	0.0100
3	Resid	Cramer-von Mises	W-Sq	0.389578	Pr > W-Sq	<	0.0050
4	Resid	Anderson-Darling	A-Sq	2.298457	Pr > A-Sq	<	0.0050

The data are not normally distributed. A log-transform is applied.

Model LOGVTG = CONC
Potassium Permanganate
FULL DATA SET
COVPARMS

CovParm	Estimate
VESSEL(conc)	0.008711
Residual	0.1370

The variance ratio is 0.008711/0.137=0.06.

Model LOGVTG = CONC
Potassium Permanganate
FULL DATA SET
COVPARMS

Effect	Num DF	Den DF	FValue	ProbF	MSERR	SSQRS	SSERR
conc	4	4	243.95	<.0001	0.13699	133.676	0.54797

ANOVA SUMMARY STATISTCS

MODELSS	SSERR	TOTSS	RSQUARE
0.086361	0.54797	0.63433	0.13614

ZEBRAFISH VTG DATA FROM LAB FRAUNHOFER
Potassium Permanganate
CLASS LEVEL INFORMATION
FULL DATA SET

Class	Levels	Values
conc	4	0 225 450 900
VESSEL	2	1 2

ZEBRAFISH VTG DATA FROM LAB FRAUNHOFER

Potassium Permanganate
PARAMETER ESTIMATES
FULL DATA SET

Effect	conc	Estimate	StdErr	DF	tValue	Probt
conc	0	2.1669	0.1401	4	15.47	0.0001
conc	225	2.0864	0.1344	4	15.53	0.0001
conc	450	2.1876	0.1549	4	14.12	0.0001
conc	900	2.2323	0.1298	4	17.20	<.0001

LSMEANS

Effect	conc	Estimate	StdErr	DF	tValue	Probt
conc	0	2.1669	0.1401	4	15.47	0.0001
conc	225	2.0864	0.1344	4	15.53	0.0001
conc	450	2.1876	0.1549	4	14.12	0.0001
conc	900	2.2323	0.1298	4	17.20	<.0001

T-TEST RESULTS

Effect	_conc	conc	Estimate	StdErr	DF	tValue	Probt	Adjustment	Adjp
conc	225	0	-0.08052	0.1941	4	-0.41	0.6996	Dunnett	0.9509
conc	450	0	0.02068	0.2088	4	0.10	0.9259	Dunnett	0.9992
conc	900	0	0.06538	0.1909	4	0.34	0.7493	Dunnett	0.9706

Dunnett's test on the log-transformed data finds no significant effects at any test concentration.

ZEBRAFISH VTG DATA FROM LAB FRAUNHOFER
Potassium Permanganate
SHAPIRO-WILK TEST OF NORMALITY OF LOGVTG
FULL DATA SET

Obs	Var Name	Test	Test Lab	Stat	pType	p Sign	pValue
1	Resid	Shapiro-Wilk	W	0.962104	Pr < W		0.2354
2	Resid	Kolmogorov-Smirnov	D	0.094445	Pr > D	>	0.1500
3	Resid	Cramer-von Mises	W-Sq	0.062935	Pr > W-Sq	>	0.2500
4	Resid	Anderson-Darling	A-Sq	0.431919	Pr > A-Sq	>	0.2500

The log-transformed data are normally distributed.

ZEBRAFISH VTG DATA FROM LAB FRAUNHOFER
Potassium Permanganate
POSSIBLE OUTLIERS FROM ANOVA ON LOGVTG
FULL DATA SET

Obs	conc	LOGVTG	Pred	Resid	LB	UB
1	0	3.07990	2.18535	0.89456	-0.96675	0.88757

LEVENE TEST FOR LOGVTG

Effect	DF	LEVENE	P_VALUE
conc	3	0.90967	0.51143

NOTE: By Levene's test, the within-group variances under a log-transform are equal. A standard ANOVA will be done.

4e. Analysis of Replicate Means

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 1:48 THURSDAY 08FEB07
ZEBRAFISH MEASUREMENTS FROM DATASET MFRAUNHOFPOTMNS
GROUP STATISTICS FOR VTG BY DOSE w/ Weight=survive, Var-Ratio=0.06

dose	doseval	COUNT	MEAN	MEDIAN	STD_DEV	STD_ERR
1	0	2	254.963	349.140	273.470	102.835
2	225	2	136.140	136.140	49.590	17.880
3	450	2	312.454	498.365	502.949	209.414
4	900	2	210.703	224.917	43.744	15.223

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 1:48 THURSDAY 08FEB07
SHAPIRO-WILK TEST OF NORMALITY OF VTG
MALE ZEBRAFISH: FULL DATA

OBS	STD	SKEW	KURT	SW_STAT	P_VALUE	SIGNIF
8	126.590	-0.40603	0.87446	0.96202	0.8292	

The data are normally distributed.

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 1:48 THURSDAY 08FEB07
LEVENE TEST FOR VTG - FULL Model
ANALYSIS OF VARIANCE ON FULL DATA SET

Effect	DF	LEVENE	p_value	SIGNIF
DOSE	3	1.6149E15	0.0001	**

NOTE: The variances are heterogeneous. The data was found to be normally distributed but group variances were unequal.A Tamhane-Dunnett analysis is appropriate.

STATISTICAL ANALYSIS LIST FILE
REPORT CREATED ON 1:48 THURSDAY 08FEB07
Tamhane-Dunnett 2-sided test for difference in means in VTG
Using MAXIMUM LIKELIHOOD estimates of variation on ZEBRAFISH values.
FULL DATA USING ALPHA=0.05

dose	MEAN	STDERR	degfree	CONTROL	OBS_DIFF	crit	SIGNIF
2	136.140	17.880	1.06570	254.963	-118.823	1245.97	
3	312.454	209.414	1.54376	254.963	57.491	2566.50	
4	210.703	15.223	1.05114	254.963	-44.260	1241.57	

By the Tamhane-Dunnett test, there is no statistically significant effect at any test concentration.

MONOTONICITY CHECK OF VTG - FULL DATA
DOSES 0, 225, 450, 900 ug/L
ZEBRAFISH OF MALE ZEBRAFISH

PARM	p_t	SIGNIF
DOSE TREND	0.7962	
DOSE QUAD	0.5795	

ANALYSIS OF VTG USING ALPHA=0.05
FULL DATA

---------- JONCKHEERE-TERPSTRA TEST KEY ----------
_JT_ IS JONCKHEERE STATISTIC
Z_JT IS STANDARDIZED JONCKHEERE STATISTIC
PR_JT IS P-VALUE FOR UPWARD TREND
PL_JT IS P-VALUE FOR DOWNWARD TREND
P-VALUES ARE FOR TIE-CORRECTED TEST WITH CONTINUITY CORRECTION FACTOR
SIGNIF RESULTS ARE FOR Two-sided ALTERNATIVE HYPOTHESIS

```
Check for Number of Reps in VTG Thru 900 ug/L
Maximum Number of Reps in Any Treatment Group or Control is 2
Total Number of Reps in All Treatment Groups is 8
Exact Permutation Methods Recommended

  Jonckheere Trend Test on Dose 0 + Lowest 3 Doses thru 900 ug/L

  ___JT____       Z_JT  XPL_JT  XPR_JT  SIGNIF  DOSE
        15   0.255377   .      0.4508             4
```

NOTE: By the Jonckheere test, there is no statistically significant effect at any test concentration.

```
Jonckheere test results are included in the summary table.
Group means should be examined to check for lack-of-fit
to a linear trend before trend test results are accepted.
```

4e, continued: Dunn's test

```
                    STATISTICAL ANALYSIS LIST FILE
                REPORT CREATED ON 1:51 THURSDAY  08FEB07
          ZEBRAFISH MEASUREMENTS FROM DATASET MFRAUNHOFPOTMNS
   GROUP STATISTICS FOR VTG BY DOSE w/ Weight=survive, Var-Ratio=0.06

  dose   doseval        COUNT     MEAN     MEDIAN   STD_DEV   STD_ERR

    1              0     2     254.963   349.140   273.470   102.835
    2            225     2     136.140   136.140    49.590    17.880
    3            450     2     312.454   498.365   502.949   209.414
    4            900     2     210.703   224.917    43.744    15.223

                    STATISTICAL ANALYSIS LIST FILE
                REPORT CREATED ON 1:51 THURSDAY  08FEB07
                      Kruskal-Wallis Test on VTG
                  ZEBRAFISH MEASUREMENTS FOR FULL DATA

           Wilcoxon Scores (Rank Sums) for Variable RESPONSE
                     Classified by Variable dose

                     Sum of      Expected       Std Dev          Mean
  dose       N       Scores      Under H0      Under H0         Score
  ------------------------------------------------------------------------
  1          2         10.0           9.0           3.0          5.00
  2          2          6.0           9.0           3.0          3.00
  3          2          9.0           9.0           3.0          4.50
  4          2         11.0           9.0           3.0          5.50

                        Kruskal-Wallis Test

                      Chi-Square         1.1667
                      DF                      3
                      Pr > Chi-Square    0.7610
------------------------------------------------------------------------

       Modified Dunn's Multiple Comparisons (Two-sided) on VTG
        ZEBRAFISH MEASUREMENTS FOR FULL DATA USING ALPHA=0.05

dose doseval      COUNT N0 MRANK ABS_DIFF CRIT_05 CRIT_01 SIGNIF  p_val

  1            0   2    2  5.0     0.0   5.86403 7.18974         .
  2          225   2    2  3.0     2.0   5.86403 7.18974        0.6213
  3          450   2    2  4.5     0.5   5.86403 7.18974        1.0000
  4          900   2    2  5.5     0.5   5.86403 7.18974        1.0000
```

By Dunn's test, there is no statistically significant effect at any test concentration.