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English - Or. English

**ENVIRONMENT DIRECTORATE**
**JOINT MEETING OF THE CHEMICALS COMMITTEE AND THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY**

**Cancels & replaces the same document of 28 September 2012**

# ANNEXES TO THE VALIDATION REPORT (PHASE 2) FOR THE ZEBRAFISH EMBRYO TOXICITY TEST

**Series on Testing and Assessment**

**No. 179**

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# Annexes I - IV


**Annex I:** Phase 2 Documents, Method Description and Zebrafish Strains used by Laboratories ..... 3

**Annex II** Selection of Chemicals for Phase 2 ..... 7

**Annex IIIa** Analysis of Three Chemicals in Fish Embryo Test Stock and Exposure Solutions for Phase 2b by P&G ..... 23

**Annex IIIb** Analysis of Two chemicals in Fish Embryo Test Stock and Exposure Solutions for Phase 2b by Ipo-Pszczyna ..... 43

**Annex IV** Overview of Runs ..... 65

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# Annex I – Phase 2 Documents, Method Description, Zebrafish Strains used by the Laboratories

## 1. Documents

For Phase 2a and 2b, the following documents were agreed upon by the Validation Management Group (VMG) and distributed to the laboratories by the coordinators:

Phase 2a – Three runs with 3,4-dichloroaniline
- Standard Operating Procedure; SOP_ZFET_OECD_V02.9[1]
- Trial Plan; 20101008_TP_ZFET_OECD_2a_V01 (Annex VII)
- Reporting Template; RT_ZFET_OECD_2a_V01.0

Phase 2b – Testing of thirteen chemicals
- Standard Operating Procedure; SOP_ZFET_OECD_V02.10 (Annex VI)
- Trial Plan; TP_ZFET_OECD_2b_V01 (Annex VII)
- Trial Plan amendment; TP_ZFET_OECD_2b_V01_1 (Annex VII)
- Reporting Template; RT_ZFET_OECD_2b_V01.0

## 2. Brief description of the Zebrafish Embryo Toxicity Test based on the above SOPs:

Newly fertilised zebrafish eggs (20 per test concentration and control) were exposed for 96h to five test concentrations of one chemical with the following controls: negative internal and negative external controls (dilution water) and positive control (3,4-dichloroaniline 4mg/l).

***Zebrafish:***
- A breeding stock of unexposed and healthy mature zebrafish *Danio rerio* with an age between 4 and 18 months was used by the laboratories for the egg production.

***Dilution water***
- Dilution water is prepared according to OECD TG 203 (OECD, 1992).

***Zebrafish egg production***
- Eggs are produced via spawning groups or mass spawning.

***Method***
- The 24-well plates and glass vessels are pre-saturated with the respective test concentrations of the chemicals and controls for at least 24h before the day of the test.

***Distribution of eggs over 24-well plates***
- Fertilised eggs are individually transferred to the freshly prepared 24-well plates (final volume of 2 ml per well) and distributed as the following:
  - 20 eggs for each test concentration (for each concentration one plate)
  - 20 eggs as positive control on one plate;
  - 4 eggs as negative internal control on each of the above plates;
  - 24 eggs as negative external control on one plate

[1] OECD 2011 Series on Testing and Assessment No. 157 - Validation Report (Phase 1) for the Zebrafish Embryo Toxicity Test; Part 2

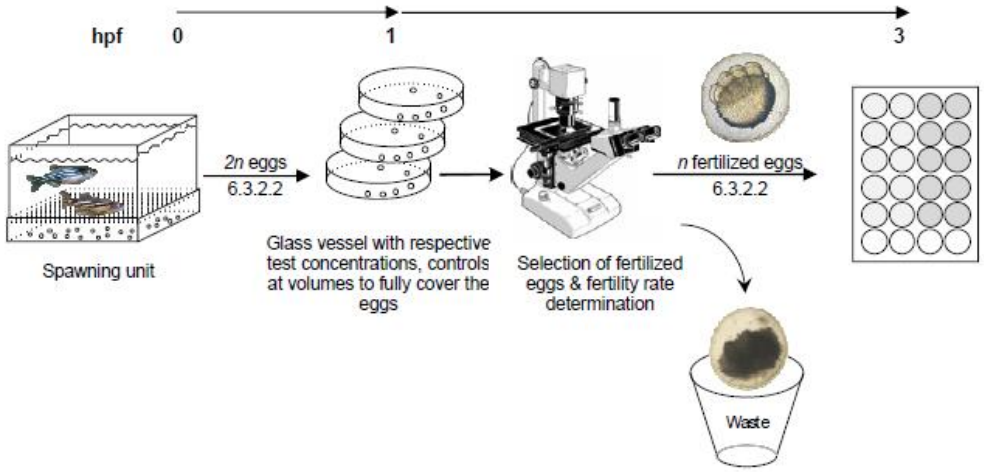


***Fig. 1: Scheme of the ZFET test procedure (from left to right): collection of the eggs, pre-exposure to respective test concentrations/controls in glass vessels immediately after fertilisation, selection of fertilised eggs with an inverted microscope or binocular and distribution of fertilised eggs into prepared 24-well plates, n = number of eggs required for the test run (kindly provided by University of Heidelberg and modified for the study).***

- After the selection step, the fertilised eggs are transferred into 24-well plates covered with self-adhesive foil or lids provided with plates and incubated at 26 ± 1 °C for 96h. Control of the light cycle to 14h light and 10h dark is achieved by keeping the eggs in either an incubator or separate room equipped with an automatic light control.
- Renewal of the test concentrations and the negative control are daily performed with freshly prepared test concentrations from the stock solution.
- Measurements of test conditions such as dissolved oxygen concentration, pH, total hardness, temperature and conductivity are performed for the controls and the highest concentration.

***Recording of toxicity***

Up to four apical endpoints are recorded daily as indicators of acute lethality in fish:

- coagulation of embryo
- lack of somite formation
- non-detachment of tail bud from the yolk sac
- lack of heart-beat

In addition to the four apical endpoints, hatching rate is daily recorded since non-hatching may represent an important toxic effect knowing that zebrafish embryos usually hatch after 72h.

***Acceptance Criteria***

For a run to be considered qualified the following criteria are applied:

- The fertility rate of the parent generation should be ≥70%.
- The dissolved oxygen concentration should be ≥80 % of the air saturation value at the beginning of the test.
- The water temperature should be maintained at 26 ± 1 °C in test chambers at any time during the test.
- Overall survival of embryos in the negative external control should be ≥90% until the end of exposure.
- Exposure to the positive control (e.g., 4.0 mg/L 3,4-dichloroaniline) should result in a minimum mortality of 30 % at the end of the exposure.
- Controls and test solutions must be renewed on a daily basis.

- NOTE: If more than 1 dead embryo is observed in the negative internal control, the plate might be rejected (this criteria was not given in Phase 1).

## 3. Zebrafish Strains used by the Laboratories

The 11 laboratories participating in Phase 1 and Phase 2 of the study reported the following with regard to the zebrafish strains used:

| **Strain** | **Laboratories** |
|---|---|
| unknown | 3 |
| wild type | 1 |
| *Danio rerio* wild type (family 954) | 1 |
| OBI | 1 |
| Westaquarium | 2 |
| AB | 1 |
| Tübinger strain | 1 |
| Own laboratory strain | 1 |

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# Annex II - Selection of Chemicals for Phase 2

## 1. Introduction

The chemical selection is a critical step for a validation study, since it is meant to help to define the applicability domain of the test method. As stated by the OECD GD 34, “a test method’s applicability domain refers for example to chemical classes, mechanisms of action and to the range of responses, for which the test can be used reliably. The applicability domain may also specify known limitations of the test. Furthermore, the test method may be suitable for identifying active substances, but not inactive substances, or vice versa”.

At the 1st meeting of the OECD FET Expert Consultation Meeting (FET I ECM; 9-11 October 2007, Berlin) a Chemical Selection Group (CSG, see Table 1) was established, which presented a first list of 27 candidate chemicals at the 2nd OECD FET ECM (FET II ECM; 14-15 May, 2008, Berlin). From this list, two chemicals (sodium chloride, triclosan) had been selected for Phase 1, and it served as a basis for selecting chemicals for Phase 2.

In the following, the process of and rationale behind the selection will be described.

## 2. The chemicals selection group (CSG)

The initial CSG was re-activated during the course of 2010 and complemented with three additional members.

**Table 1: Composition of the CSG for Phase 2**

| Name | Affiliation/contact |
|---|---|
| François Busquet* | JRC/IHCP/ECVAM |
| Thomas Braunbeck<br>Ruben Strecker* | University of Heidelberg |
| Scott Belanger | Procter & Gamble |
| Jean Bachmann* | UBA |
| Marc Léonard | L’Oreal |

*new members

### 3. Original list of 27 chemicals

As mentioned above, the list of 27 chemicals presented at FET II ECM, served as a basis for selecting the chemicals for Phase 2. This list had considered the recommendations of the OECD FET *ad hoc* expert group:

- definition of the applicability domain for the FET: chemicals should cover a range of chemical classes and areas of use.
- possible barrier function of the chorion: chemicals for which it is known that they might be blocked by the chorion should be proposed (e.g. high molecular weight polymers)
- exposure test concentrations should be measured: chemicals with potential or available quantification analytical method should be preferred.

Further parameters for selection were:

- the mode of action (using the OECD QSAR toolbox - OASIS[2])
- the range of toxicity (fish non-toxic to fish very toxic)
- the log Kow
- the following (non-exhaustive) chemicals classes covering the ones regularly tested by the OECD TG 203 (see Table 2)

**Table 2: Chemical classes to be covered in the OECD ZFET validation study**

| |
|---|
| Polycyclic aromatic hydrocarbons (PAHs) |
| Polychlorobiphenyls (PCBs)* |
| Endocrine disrupters |
| Organotins |
| Pharmaceuticals** |
| Polymers |
| Non-polar narcotics |
| Unspecific reactive substance |
| Surfactant/biocides |

*dropped at FET II ECM since FET data are already available

**OECD TG 203 data may be limited for most compounds, but the development of FET data was still considered useful due to the diversity of modes of action in this chemical group

### 4. Criteria for selection of chemicals for Phase 2

The CSG discussed during several teleconference calls the criteria to be applied for selection of chemicals. The CSG agreed with the VMG to present an extended list of 20 chemicals to the OECD FET *ad hoc* expert group for review and approval. This list should be complementary to the chemicals tested in Phase 1 and would be used to define the final list of 13 chemicals to be tested in Phase 2.

It was agreed to prioritise the list of chemicals taking into account:

a) the area of use (industrial chemicals, pharmaceuticals, pesticides, biocides)
b) the range of fish toxicity (non-toxic, moderately toxic, toxic and very toxic)
c) the availability of a quantification analytical method
d) the availability of fish data and, if possible, FET data

[2] The OASIS concept for predicting the biological activity of chemical compounds. JOURNAL OF MATHEMATICAL CHEMISTRY Volume 4, Number 1, 207-215, 1990

e) the commercial availability of the chemicals

and to some extent considering:

f) the chemical classes
g) the water solubility (use of solvent)
h) the mode of actions (either known or as predicted by other available tools such as the OECD QSAR tool box [OASIS] or USEPA ECOSAR)

## 5. Update of original list of 27 chemicals and its extension to 39 chemicals

It was agreed to revise the original list of 27 chemicals and rearrange the chemicals according to 1) their fish toxicity 2) their main area of use and to 3) complete the list taking into account the concerns of the OECD FET *ad hoc* expert group and the criteria listed above. A list of 39 chemicals was established (see Table 3 and for full details see Appendix 1).

**Table 3: Preliminary list of 39 chemicals**

| **PHASE 2** | INDUSTRIAL CHEMICALS | PHARMACEUTICALS | PESTICIDES & BIOCIDES |
|---|---|---|---|
| FISH NON TOXIC [>100 mg/l] | Diethylene glycol | Sulfamethoxazole* | Morpholine |
| | n-Propylamine | | |
| | Triethylene glycol | | |
| | Acetone | | |
| | N,N-Dimethylformamide | | |
| | Dimethyl sulfoxide | | |
| | 2-Propanol | | |
| | Sodium chloride | | |
| FISH MODERATELY TOXIC [10-100 mg/l] | Diisopropylamine | Carbamazepine* | n-Butylamine |
| | n-Hexylamine | | Cyclohexylamine |
| | 3-Chloroaniline | | |
| | n-Pentylamine | | |
| | 2-Nitroanisole | | |
| | 1-Octanol | | |
| | 2-Nitroaniline | | |
| | 4-Chloroaniline | | |
| FISH TOXIC [1-10 mg/l] | Tridecyl mono-octyl ether | | Prochloraz* |
| | p-tert-Butylphenol | | Malathion* |
| | Merquat 100* | | 2,4,6-Tribromophenol |
| | 4-Nitrophenol | | |
| | Tetradecyl sulfate sodium salt* | | |
| | Luviquat HM 552* | | |
| FISH VERY TOXIC [<1 mg/l] | 2,4-Dinitrophenol | Ivermectin* | 4,6-Dinitro-*o*-cresol |
| | 4-Aminophenol | | Trifloxistrobin* |
| | Methylmercury (II) chloride* | | Copper (II) sulfate pentahydrate* |
| | Triclosan | | Dieldrin* |

Chemicals followed by a (*) are the 12 new chemicals added to the original list of 27 chemicals. The extended list of 20 chemicals submitted for approval to the OECD FET *ad hoc* expert group is shaded in grey. Two chemicals were already tested in Phase 1 (triclosan and sodium chloride).

## 6. Selection of the extended list of 20 chemicals

From the chemicals given in Table 3, an extended list of 20 chemicals (Table 4) was established according to the following reasoning:

- **PAHS** and **Organotins** were not included in Table 3. From an exposure and analytical point of view, PAHs are difficult compounds, because they are photoactivated. The CSG expected that this would increase variability in results, as laboratories utilise different light sources (intensity and wavelengths). Organotins are high on various watch lists (high biohazard level), and in many situations it will be difficult to justify their use (residual material and exposure solutions would be considered hazardous waste in some regulatory jurisdictions).
- Two polymers (**Merquat 100** and **Luviquat HM 552**) and **malathion** were tested in order to assess the barrier function of the chorion. These chemicals are supposed to have a greater post-hatch mortality. Analytical measurement can be available using Gas Phaseous Chromatography for the 2 polymers.
- Three pharmaceuticals were suggested: **sulfamethoxazole**, **ivermectin** and **carbamazepine**. The mode of action of ivermectin is well known in insects and arthropods (paralysis, change of chloride ion channels). Sulfamethoxazole blocks the folic acid synthesis and carbamazepine blocks nerve cell sodium channels. Little is known about their modes of action in fish. Fish embryo data are available for carbamazepine and the information was used to set the test concentrations (Santos *et al.* 2010).
- **Copper (II) sulfate pentahydrate** and **methylmercury (II) chloride** represent the chemical class of inorganic compounds with Cu (II) and Methyl Hg (II) as the active toxicants.
- **1-Octanol** and **2-propanol** were selected as representatives of non-polar narcotics (see Table 2) and due to their different range of toxicity to fish. Their high volatility might result in a higher variability as the preliminary results of Phase 1 showed for 2,3,6-trimethylphenol (50 times more volatile than ethanol).
- **Triethylene glycol** and **diethylene glycol** were selected as representatives of non-polar narcotics and since they are non-toxic to fish.
- **Dimethyl sulfoxide** was selected as a non-toxic chemical. In addition, it is regularly used as a solvent for chemical testing.
- **Tetradecyl sulfate sodium salt** and **dieldrin** were proposed since they are respectively toxic and very toxic to fish, with known fish LC50 data and available quantification analytical methods. As a surfactant and a biocide, they comply with the proposal of the OECD FET *ad hoc* expert group (see Table 2).
- **2,4-Dinitrophenol** is a light-sensitive and, most importantly, an unspecific reactive substance from the CEllSens list (Schirmer *et al.* 2008) suggested by the OECD FET *ad hoc* expert group (see Table 2).
- **n-Butylamine** and **cyclohexylamine** represent the area of use "Pesticides/Biocides". N-butylamine was preferred, since it is less volatile.
- For **4-nitrophenol** and **4-chloroaniline**, FET data were available and could be used for the range-finding testing. Their water solubility is an additional advantage.
- **Prochloraz** is considered as a putative endocrine disrupter (as triclosan tested in Phase 1) and was therefore added to the list to comply with the proposal of the OECD FET *ad hoc* expert group (see Table 2).

## 7. Selection of 13 chemicals from the extended list of 20 chemicals

The extended list of 20 chemicals (Table 4) was presented to the OECD FET *ad hoc* expert group during a teleconference call on 30 June 2010, and 13 chemicals were selected as chemicals to be tested in Phase 2.

**Table 4: Extended list of 20 chemicals and approval of 13 test chemicals (in bold) for Phase 2 by the OECD FET *ad hoc* expert group**

| **PHASE 2** | INDUSTRIALS | PHARMACEUTICALS | PESTICIDES & BIOCIDES |
|---|---|---|---|
| FISH NON TOXIC [>100 mg/l] | Diethylene glycol | **Sulfamethoxazole** | |
| | Triethylene glycol | | |
| | **Dimethyl sulfoxide** | | |
| | 2-Propanol | | |
| FISH MODERATELY TOXIC [10-100 mg/l] | 1-Octanol | **Carbamazepine** | **n-Butylamine** |
| | 4-Chloroaniline | | |
| FISH TOXIC [1-10 mg/l] | **Merquat 100** | | **Prochloraz** |
| | 4-Nitrophenol | | **Malathion** |
| | **Tetradecyl sulfate sodium salt** | | |
| | **Luviquat HM 522** | | |
| FISH VERY TOXIC [<1mg/l] | **2,4-Dinitrophenol** | **Ivermectin** | **Copper (II) sulfate pentahydrate** |
| | Methylmercury (II) chloride | | **Dieldrin** |

## 8. Modifications to the list of 13 chemicals

After discussion within the VMG, sulfamethoxazole was substituted by morpholine, since sulfamethoxazole is light-sensitive and not all laboratories might be equipped to adequately handle the chemical.

During the range-finding tests carried out at two laboratories (University of Heidelberg and P&G), it was not possible to achieve test concentrations inducing mortality in the fish embryos with four chemicals. Taking into account the objective of the study – assess the intra- and interlaboratory reproducibility of the ZFET by comparing the LC50s of the chemicals tested –, the VMG decided to test four other chemicals from the extended list of 20 chemicals which induced mortality. The problems occurred with the four chemicals are described in the following.

- **Ivermectin**: Since it was not possible to dissolve the compound (even not by using solvents as DMSO, acetone and methyl ethyl ketone[3]; 0.1% solvent final concentration) at concentrations inducing lethality (<4 mg/l according to Weil *et al.* 2009), the VMG decided to test **methylmercury (II) chloride** being also highly fish toxic

- **Morpholine**: The highest concentration (2.4 g/l) tested did not trigger any lethality during the range-finding test. Only Brust (1991) reported lethality effects of morpholine to zebrafish

[3] solvent recommended by the supplier in the material safety data sheet.

embryos (48h exposure). Noteworthy, the test solutions had a pH of 9.8 to 10.3. The VMG assumed that this high pH would explain the lethality observed by Brust (Note: This assumption was confirmed by recent tests carried out at the University of Heidelberg). Since the ZFET SOP requests adjustment of the chemical stock solution to pH 7.4, the VMG decided to test **triethylene glycol**, which is also non-toxic to fish.

- **Dieldrin**: At the highest soluble stock solution (1 mg/l in 0.1% acetone final concentration), no lethality was observed in the range-finding test. However, sublethal effects such as hyperactivity were observed and are consistent with the mode of action of dieldrin being a neurotoxicant. The VMG decided to test **4,6-dinitro-*o*-cresol** having the same range of fish toxicity (e.g. very toxic) and being also a pesticide with a specific neurotoxic mode of action.

- **n-Butylamine**: Concentrations up to 1 g/l of n-butylamine did not trigger any lethality in fish embryos, although it had previously been reported as being toxic in the FET (Brust, 2001). As for morpholine, the final pH (8.59-8.93) had not been adjusted to the control (pH 7.4) The VMG substituted n-butylamine with **1-octanol** having an equivalent solubility and fish toxicity.

The final list of chemicals tested in Phase 2 is given in Table 5.

**Table 5: Final list of 13 chemicals for Phase 2**

| **PHASE 2** | INDUSTRIALS | PHARMACEUTICALS | PESTICIDES & BIOCIDES |
|---|---|---|---|
| FISH NON-TOXIC [>100 mg/l] | Triethylene glycol | | |
| | Dimethyl sulfoxide | | |
| FISH MODERATELY TOXIC [10-100 mg/l] | 1-Octanol | Carbamazepine | |
| FISH TOXIC [1-10 mg/l] | Merquat 100 | | Prochloraz |
| | Tetradecyl sulfate sodium salt | | Malathion |
| | Luviquat HM 522 | | |
| FISH VERY TOXIC [<1mg/l] | 2,4-Dinitrophenol | | Copper (II) sulfate pentahydrate |
| | Methylmercury (II) chloride | | 4,6-Dinitro-*o*-cresol |

## 9. References

*Brust, K. (2001). Toxicity of aliphatic amines on the embryos of zebrafish Danio rerio - experimental studies and QSAR. Dissertation zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.) an der Fakultät für Forst-, Geo- und Hydrowissenschaften der Technischen Universität Dresden.*

*Henn, K. and T. Braunbeck (2011). Dechorionation as a tool to improve the fish embryo toxicity test (FET) with the Zebrafish (danio rerio). Comparative Biochemistry and Physiology, Part C 153 91–98.*

*Kamunde, C. and C. M. Wood (2003). The influence of ration size on copper homeostasis during sublethal dietary copper exposure in juvenile rainbow trout, Oncorhynchus mykiss. Aquat Toxicol. 12;62(3):235-54.*

*OECD (1992) Test Guideline 203. OECD Guideline for Testing of Chemicals. Fish, Acute Toxicity Test. Available: [http://www.oecd.org/document/22/0,2340,en_2649_34377_1916054_1_1_1_1,00.html]*

*OECD (2006) Series on Testing and Assessment 34, Guidance document on the validation and international acceptance of new or updated test methods for hazard assessment. [http://www.oecd.org/officialdocuments/displaydocumentpdf/?cote=env/jm/mono(2005)14&doclanguage=en]*

*Orvos, D., D. J. Versteeg, J. Inauen, M. Capdevielle, A. Rothenstein and V. Cunningham. (2002). Aquatic toxicity of triclosan. Environ Toxicol Chem. 21(7):1338-49.*

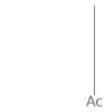
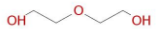
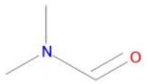
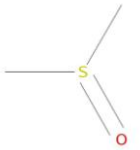
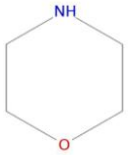
*Santos, L.H.M.L.M., A. N. Araújo, A. Fachini, A. Pena, C. Delerue-Matos,M.C.B.S.M. Montenegro. (2010). Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment. Journal of Hazardous Materials 175: 45–95*

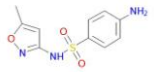
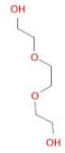
*Schirmer, K., K. Tannenberger, N. I. Kramer, D. Völker, S. Scholz, C. Hafner, L. E. Lee, N. C. Bols, and J. L. Hermens. (2008). Developing a list of reference chemicals for testing alternatives to whole fish toxicity tests. Aquat Toxicol. 11;90(2):128-37.*

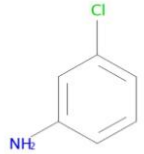
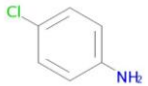
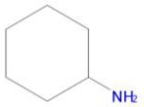
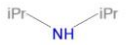
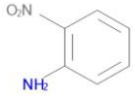
*Weil, M. S. Scholz, M. Zimmer, F. Sacher, and K. Duis. (2009). Gene expression analysis in zebrafish embryos: a potential approach to predict effect concentrations in the fish early life stage test. Environ Toxicol Chem. 28(9):1970-8.*

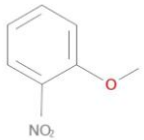
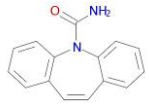
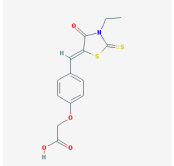
## 10. Appendix 1

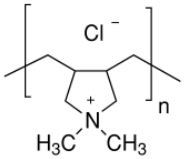
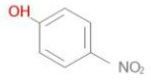
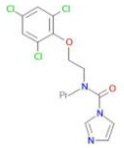
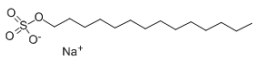
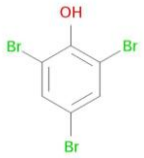
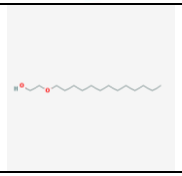
### Properties of the preliminary list of 39 chemicals proposed by the Chemicals Selection Group for Phase 2 of the ZFET validation study

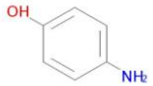
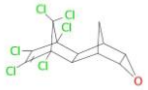
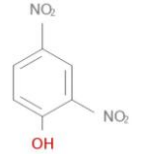
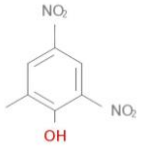
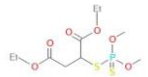
| | Phase 2 | Fish toxicity | Name | Structure | CAS # | Canonical SMILES | MW (g/mol) | Log Kow | HLC (atm $m^3$/ mole) | OECD QSAR MOA (OASIS) | Area of use | REF |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | | - | Acetone |  | 67-64-1 | CC(=O)C | 58.08 | -0.24$^{db}$ | 3.50E-05$^{db}$ | base surface narcotic | industrial | IUCLID 4* |
| **2** | | - | Diethylene glycol |  | 111-46-6 | C(COCCO)O | 106.12 | -1.47$^{est}$ | 2.03 E-09$^{est}$ | base surface narcotic | industrial | IUCLID 4 |
| **3** | | - | N,N-Dimethyl formamide |  | 68-12-2 | CN(C)C=O | 73.1 | -1.01$^{db}$ | 7.38E-08$^{est}$ | unspecific reactivity | industrial | IUCLID 4 |
| **4** | **X** | - | Dimethyl sulfoxide |  | 67-68-5 | CS(=O)C | 78.13 | -1.35$^{db}$ | 4.96 E-08$^{est}$ | unspecific reactivity | industrial | IUCLID 4 |
| **5** | | - | Morpholine |  | 110-91-8 | C1COCCN1 | 87.12 | -0.86$^{db}$ | 1.16 E-06$^{db}$ | narcotic amine | pesticide biocide | IUCLID 4 |

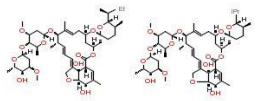
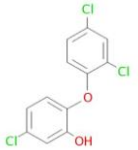
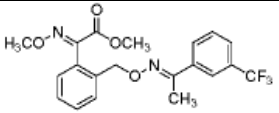
| | Phase 2 | Fish toxicity | Name | Structure | CAS # | Canonical SMILES | MW (g/mol) | Log Kow | HLC (atm $m^3$/ mole) | OECD QSAR MOA (OASIS) | Area of use | REF |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **6** | | - | 2-Propanol |  | 67-63-0 | CC(C)O | 60.1 | 0.05[db] | 8.10 E -06[db] | base surface narcotic | industrial | IUCLID 4 |
| **7** | | - | n-Propyl-amine |  | 107-10-8 | CCCN | 59.11 | 0.48[db] | 1.48 E -05[db] | narcotic amine | industrial | |
| **8** | | - | Sodium chloride |  | 7647-14-5 | [Na+].[Cl-] | 58.44 | -0.46[est] | NA | unspecific reactivity | industrial | IUCLID 4 |
| **9** | | - | Sulfa-methoxa-zole |  | 723-46-6 | CC1=CC(=NO1)NS(=O)(=O)C2=CC=C(C=C2)N | 253.28 | 0.89[db] | 9.56 E-013[est] | unspecific reactivity | pharmaceutical | Santos et al, 2010 |
| **10** | **X** | - | Triethylene glycol |  | 112-27-6 | C(COCCOCCO)O | 150.17 | -1.75[est] | 3.16E-011[est] | base surface narcotic | industrial | IUCLID 4 |
| **11** | | + | n-Butylamine |  | 109-73-9 | CCCCN | 73.14 | 0.97[db] | 1.74E-05[db] | narcotic amine | pesticide biocide | IUCLID 4 |

| | Phase 2 | Fish toxicity | Name | Structure | CAS # | Canonical SMILES | MW (g/mol) | Log Kow | HLC (atm $m^3$/ mole) | OECD QSAR MOA (OASIS) | Area of use | REF |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **12** | | + | 3-Chloro-aniline |  | 108-42-9 | C1=CC(=CC(=C1)Cl)N | 127.57 | 1.88[db] | 1.0E-06[db] | phenol and aniline | industrial | |
| **13** | | + | 4-Chloro-aniline |  | 106-47-8 | C1=CC(=CC=C1N)Cl | 127.57 | 1.83[db] | 1.16E-06[db] | phenol and aniline | industrial | |
| **14** | | + | Cyclohexyl-amine |  | 108-91-8 | C1CCC(CC1)N | 99.18 | 1.49[db] | 1.38E-05[est] | narcotic amine | pesticide biocide | IUCLID 4 |
| **15** | | + | Diisopro-pylamine |  | 108-18-9 | CC(C)NC(C)C | 101.19 | 1.40[db] | 5.17E-05[est] | narcotic amine | industrial | IUCLID 4 |
| **16** | | + | n-Hexylamine |  | 111-26-2 | CCCCCCN | 101.19 | 2.06[db] | 2.68E-05[db] | narcotic amine | industrial | |
| **17** | | + | 2-Nitroaniline |  | 88-74-4 | C1=CC=C(C(=C1)N)[N+](=O)[O-] | 138.13 | 1.85[db] | 5.9E-08[db] | phenol and aniline | industrial | IUCLID 4 |

| | Phase 2 | Fish toxicity | Name | Structure | CAS # | Canonical SMILES | MW (g/mol) | Log Kow | HLC (atm $m^3$/ mole) | OECD QSAR MOA (OASIS) | Area of use | REF |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **18** | | **+** | 2-Nitroanisole |  | 91-23-6 | COC1=CC=CC=C1[N+](=O)[O-] | 153.14 | $1.73^{db}$ | $4.29E-07^{db}$ | base surface narcotic | industrial | IUCLID 4 |
| **19** | **X** | **+** | 1-Octanol |  | 111-87-5 | CCCCCCCCO | 130.23 | $3.00^{db}$ | $2.45E-05^{db}$ | base surface narcotic | industrial | IUCLID 4 |
| **20** | | **+** | n-Pentyl-amine |  | 110-58-7 | CCCCCN | 87.17 | $1.49^{db}$ | $2.43E-05^{db}$ | narcotic amine | industrial | |
| **21** | | **++** | p-tert-butyl-phenol |  | 98-54-4 | CC(C)(C)C1=CC=C(C=C1)O | 150.22 | $3.31^{db}$ | $1.19E-06^{db}$ | phenol and alinine | industrial | IUCLID 4 |
| **22** | **X** | **++** | Carba-mazepine |  | 298-46-4 | C1=CC=C2C(=C1)C=CC3=CC=CC=C3N2C(=O)N | 236.28 | $2.45^{db}$ | $1.08E-10^{est}$ | base surface narcotic | pharmaceutical | IUCLID 4 |
| **23** | **X** | **++** | Luviquat HM 552 |  | 95144-24-4 | CCN1C(=O)C(=CC2=CC=C(C=C2)OCC(=O)O)SC1=S | ~ 400,000 | $1.38^{est}$ | $1.87E-14^{est}$ | NA | industrial | Henn and Braun-beck, 2011 |

| | Phase 2 | Fish toxicity | Name | Structure | CAS # | Canonical SMILES | MW (g/mol) | Log Kow | HLC (atm $m^3$/ mole) | OECD QSAR MOA (OASIS) | Area of use | REF |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **24** | **X** | **++** | Merquat 100 |  | 26062-79-3 | C[N+](C)(CC=C)CC=C.[Cl-] | 200,000-350,000 | -2.49$^{est}$ | 7.2E-12$^{est}$ | | industrial | |
| **25** | | **++** | 4-Nitro-phenol |  | 100-02-7 | C1=CC(=CC=C1[N+](=O)[O-])O | 139.11 | 1.91$^{db}$ | 4.15E-10$^{db}$ | phenol and alinine | industrial | IUCLID 4 |
| **26** | **X** | **++** | Prochloraz |  | 67747-09-5 | CCCN(CCOC1=C(C=C(C=C1Cl)Cl)Cl)C(=O)N2C=CN=C2 | 376.67 | 4.1$^{db}$ | 7.58E-12$^{est}$ | unspecific reactivity | pesticide biocide | IUCLID 4 |
| **27** | **X** | **++** | Tetradecyl suphate sodium salt |  | 1191-50-0 | CCCCCCCCCCCCCCOS(=O)(=O)[O-].[Na+] | 316.43 | 2.67$^{est}$ | 3.25E-07$^{est}$ | | industrial | |
| **28** | | **++** | 2,4,6-Tribromo-phenol |  | 118-79-6 | C1=C(C=C(C(=C1Br)O)Br)Br | 330.8 | 4.13$^{db}$ | 3.55E-8$^{est}$ | phenol and aniline | pesticide biocide | IUCLID 4 |
| **29** | | **++** | Tridecyl mono-octyl ether |  | 24938-91-8 | CCCCCCCCCCCCCOCCO | 244.4 | 4.99$^{est}$ | 1.25E-6$^{est}$ | base surface narcotic | industrial | |

| | Phase 2 | Fish toxicity | Name | Structure | CAS # | Canonical SMILES | MW (g/mol) | Log Kow | HLC (atm $m^3$/ mole) | OECD QSAR MOA (OASIS) | Area of use | REF |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **30** | | **+++** | 4-Amino-phenol |  | 123-30-8 | C1=CC(=CC=C1N)O | 109.13 | 0.04[db] | 3.59E-10[db] | unspecific reactivity | industrial | IUCLID 4 |
| **31** | **X** | **+++** | Copper (II) sulfate penta-hydrate | $CuSO_4 \cdot 5H_2O$ | 7758-99-8 | O.O.O.O.O.[O-]S(=O)(=O)[O-].[Cu+2] | 249.68 | NA | NA | unspecific reactivity | pesticide biocide | Ka-munde and Wood, 2003 |
| **32** | | **+++** | Dieldrin |  | 60-57-1 | C1C2C3C(C1C4C2O4)C5(C(=C(C3(C5(Cl)Cl)Cl)Cl)Cl)Cl | 380.9 | 5.40[db] | 6.81E-08[est] | | pesticide biocide | |
| **33** | **X** | **+++** | 2,4-Dinitro-phenol |  | 51-28-5 | C1=CC(=C(C=C1[N+](=O)[O-])[N+](=O)[O-])O | 184.11 | 1.67[db] | 8.06E-08[db] | unspecific reactivity | industrial | Schir-mer et al., 2008 |
| **34** | **X** | **+++** | 4,6-Dinitro-o-cresol |  | 534-52-1 | CC1=CC(=CC(=C1O)[N+](=O)[O-])[N+](=O)[O-] | 198.14 | 2.13[db] | 1.4E-06[db] | unspecific reactivity | pesticide biocide | |
| **35** | **X** | **+++** | Malathion |  | 121-75-5 | CCOC(=O)CC(C(=O)OCC)SP(=S)(OC)OC | 330.4 | 2.36[db] | 8.39E-10[est] | unspecific reactivity | pesticide biocide | IUCLID 4 |

| | Phase 2 | Fish toxicity | Name | Structure | CAS # | Canonical SMILES | MW (g/mol) | Log Kow | HLC (atm $m^3$/ mole) | OECD QSAR MOA (OASIS) | Area of use | REF |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **36** | | **+++** | Ivermectin |  | 70288-86-7 | CCC(C)C1C(CCC2(O1)CC3CC(O2)CC=C(C(C(C=CC=C4COC5C4(C(C=C(C5O)C)C(=O)O3)O)C)OC6CC(C(C(O6)C)OC7CC(C(C(O7)C)O)OC)OC)C)C.CC1CCC2(CC3CC(O2)CC=C(C(C(C=CC=C4COC5C4(C(C=C(C5O)C)C(=O)O3)O)C)OC6CC(C(C(O6)C)OC7CC(C(C(O7)C)O)OC)OC)C)OC1C(C)C | 1736.2 | NA | NA | unspecific reactivity | pharmaceutical | |
| **37** | **X** | **+++** | Methyl-mercury (II) chloride | $CH_3HgCl$ | 115-09-9 | C[Hg]Cl | 251.08 | 0.41[db] | NA | unspecific reactivity | industrial | IUCLID 4 |
| **38** | | **+++** | Triclosan |  | 3380-34-5 | C1=CC(=C(C=C1Cl)O)OC2=C(C=C(C=C2)Cl)Cl | 289.55 | 4.76[db] | 4.99E-09[est] | phenol and aniline | pesticide biocide | Orvos et al., 2002 |
| **39** | | **+++** | Trifloxi-strobin |  | 141517-21-7 | CC(=NOCC1=CC=CC=C1C(=NOC)C(=O)OC)C2=CC(=CC=C2)C(F)(F)F | 408.37 | 6.62[est] | 1.07E-07[est] | phenol and aniline | pesticide biocide | |

***Fish toxicity: - = non-toxic (LC50>100 mg/L); + = moderately toxic (LC50=10-100 mg/L); ++ = toxic (LC50=1-10 mg/L; +++ = very toxic (LC50<1 mg/L); MW = molecular weight; NA = not available; *IUCLID 4 available on http://ecb.jrc.ec.europa.eu/esis/ HLC: Henry's Law Constant; db = experimental database match; est = estimated***
***Note: log Kow and HLC were estimated using EPISUITE 4.0 (2008) except when measured values were available (cited within EPISUITE)***

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# Annex IIIa - Analysis of Three Chemicals in Fish Embryo Test Stock and Exposure Solutions for Phase 2b by P&G

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Miami Valley Innovation Center
Procter & Gamble
Cincinnati, OH
U.S.A.

12 January 2012

Table of Contents

INTRODUCTION ..... 25
GENERAL METHODS AND APPROACHES ..... 26
Chemicals ..... 26
Preparation of Stock Solutions ..... 26
Preparation of Exposure Solutions ..... 26
Comparison of Aquatic Toxicity Estimates ..... 26
METHODS AND RESULTS: Tetradecyl sulfate sodium salt ..... 26
HPLC/MS/MS Method (TDS Stock and Exposure Concentration Determinations) ..... 26
Preparation of TDS Stock Solutions and Exposure Solutions for Tests at P&G MVIC ..... 27
Analytical Results for TDS ..... 27
METHODS AND RESULTS: 1-Octanol ..... 28
HPLC/MS/MS Method (1-Octanol Stock and Exposure Concentration Determinations) ..... 28
Preparation of 1-Octanol Stock Solutions and Exposure Solutions for Tests at P&G MVIC ..... 28
Analytical Results for 1-Octanol ..... 29
METHODS AND RESULTS: Copper (II) Sulfate pentahydrate ..... 29
ICPMS Method (Copper Stock and Exposure Concentration Determinations) ..... 29
Preparation of Copper Stock Solutions and Exposure Solutions for Tests at P&G MVIC ..... 29
Analytical Results for Copper ..... 29
ADDITIONAL RESULTS ..... 30
OVERALL CONCLUSIONS ..... 31
REFERENCES ..... 32
LIST OF TABLES ..... 33
Table 1. Summary of physical chemical properties of three chemicals entered into Zebrafish Fish Embryo Testing, Phase 2b ..... 34
Table 2. SMILES notation and structures associated with the chemicals ..... 34
Table 3. Dates of studies and nominal exposure concentrations used in each chemical ..... 35
Table 4. Summary of measurements of tetradecyl sulfate concentrations during the conduct of a semi-static, 96-hr Fish Embryo Test ..... 36
Table 5. Summary of stock solution measurements for the 3 chemicals used in this study ..... 36
Table 6. Summary of measurements of 1-Octanol concentrations during the conduct of a semi-static, 96-hr Fish Embryo Test ..... 37
Table 7. Summary of measurements of Copper concentrations during the conduct of a semi-static, 96-hr Fish Embryo Test ..... 37
Table 8. Comparison of 96-hr LC50s calculated based on nominal and measured exposure concentrations ..... 38
LIST OF FIGURES ..... 39
Figure 1. Pattern of measured tetradecyl sulfate sodium salt concentrations in the fish embryo test using a 24-hr renewal, semi-static design ..... 40
Figure2. Pattern of measured 1-octanol concentrations in the fish embryo test using a 24-hr renewal, semi-static design ..... 41
Figure 3. Pattern of measured copper concentrations in the fish embryo test using a 24-hr renewal, semi-static design ..... 42

**INTRODUCTION**

Analytical verification of tetradecyl sulfate sodium salt, 1-octanol and copper (II) sulfate pentahydrate in aqueous stock and exposure solutions utilized during an international validation study of the Fish Embryo Test (FET) was performed by the Trace Analytical Core (Procter & Gamble, Mason Business Center, Cincinnati, Ohio USA) or Pace Analytical Services, Inc. (Columbus, Ohio USA).

Previously, 3,4-DCA (dichloroaniline), which is used as a positive control test chemical in the FET, was assessed under similar circumstances in Phase 1a (Transferability Assessment). In Phase 1b, six chemicals, 6-methyl-5-hepten-2-one, 2,3,6-trimethylphenol, dibutyl maleate, ethanol, sodium chloride and triclosan were assessed. Phase 1a and Phase 1b are summarized in OECD 2011a and OECD 2011b. These studies confirmed that the ZFET was successfully transferred from the lead laboratory to the participating laboratories. The intra- and inter-laboratory reproducibility of the LC50 values was promising. For five chemicals it was very good; however, reproducibility was lower for the volatile chemical 6-methyl-5-hepten-2-one and deemed advisable to establish guidance for testing of volatile chemicals. Analytical measurements confirm exposed test concentrations ≥80% except for 2 chemicals (dibutyl maleate and 6-methyl-5-hepten-2-one). Tests were conducted using a semi-static design. Tests with thirteen chemicals in Phase 2b also followed this experimental design (Annex VII).

The objectives in the present study were to:

- Develop and apply suitable methods to verify stock and exposure solutions using chemical-appropriate methods;
- Verify stock solution concentrations for three chemicals in Phase 2b of the OECD validation program for the FET in one laboratory (P&G); and,
- Determine exposure concentrations for three chemicals in one participating laboratory (P&G).

## GENERAL METHODS AND APPROACHES

*Chemicals*

The three chemicals tested at P&G in Phase 2b are listed in Table 1 along with relevant physical-chemical parameters. Two of the three were organic chemicals. Several of the chemicals could be classified as "challenging chemicals" with low solubility (<10 mg/L). Note that solubility estimates for chemicals are generally made in ion-free water. For example, sodium tetradecyl sulfate is estimated to have a water solubility of ~16 mg/L but in practice was determined experimentally to be 5.13 mg/L (Dyer et al. 1997). Table 2 provides 2-dimensional structures and SMILES notation useful for modeling and chemical characterization.

*Preparation of Stock Solutions*

Stock solution preparation was outlined in the Phase 2b Trial Plan (Annex VI) and is also given in the subsequent sections for each chemical.

*Preparation of Exposure Solutions*

Exposure solutions were prepared from stocks fresh each day of the test (i.e., every 24 hrs). Nominal exposure concentrations are given in Table 3. Exposure solutions were prepared as described in the Phase 2b Trial Plan. Wells of test plates were soaked with the appropriate exposure solution (material and concentrations) at least 24 hr in advance of initiating the definitive test. Sampling of wells for analytical verification was performed before and after renewal.

*Comparison of Aquatic Toxicity Estimates*

Summarization of acute aquatic toxicity of the three chemicals in the FET tests was conducted in the P&G laboratories. In this exercise, studies were summarized as 96-hr LC50s based on the effect (mortality) endpoints described in the SOP – coagulation of the egg, lack of somites, lack of tail detachment, and lack of heartbeat. The influence of measured versus nominal exposure concentrations using 96-hr LC50 determinations was made using the two-parameter logistic function model. This logistic regression model is one of the models recommended by the OECD Series on Testing and Assessment No. 54 for modeling quantal dose-response data (OECD 2006). For the statistical report, see Annex III.

## METHODS AND RESULTS: Tetradecyl sulfate sodium salt

Tetradecyl sulfate sodium salt (TDSS) was analyzed by the P&G Trace Analytical Core at Mason Business Center (M. Karb, K. Wehmeyer). Results are expressed as tetradecyl sulfate (TDS).

*HPLC/MS/MS Method (TDS Stock and Exposure Concentration Determinations)*

Tetradecyl sulfate (TDS), and its stable isotope-labeled internal standard (IS), $2H_{27}$ Tetradecyl sulfate ($d_{27}$ TDS), are determined in reconstituted water (RW) samples by a reversed-phase high performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) method operating under multiple reaction monitoring (MRM) conditions (full details archived at P&G as Method # HCL_13865 v .1_Sodium tetradecyl sulfate ($C_{14}$AS) Revision No.: 0).

TDS stocks and exposure samples were diluted 1:1 (v:v) with methanol (MeOH) at the Environmental Stewardship Organization (ESO) laboratory prior to receipt by the Trace Analytical Core (TAC) with interim storage at ~4°C in amber vials with Teflon-lined caps covered with parafilm. When required, further dilutions of study specimens were performed by TAC with 50:50 MeOH:RW to provide TDS concentrations within the linear range of the standard curve.

TDS working standards (W-STD) and working quality control (W-QC) samples derived from separate chemical stock weighings were prepared in 50:50 MeOH:RW diluents at concentrations ranging from 5 to 250 ng TDS/mL. An aliquot (400 µL) of each W-STD and W-QC was added to each well in a 96-well plate. Internal standard (2500 ng/mL; 20 µL) was added to each W-STD, W-QC sample and exposure solution samples in 96-well plates, followed by immediate covering with a mat cap and mixing. A STD curve was run at the beginning and end of the HPLC/MS/MS run, and exposure solution samples were evenly interspersed with QC samples throughout the run to monitor any bias in results.

Diluted TDS concentrations (ng/mL) were determined by interpolation from a quadratic weighted (1/x) regression of the W-STD concentrations by HPLC/MS/MS instrument response factors (ratio of analyte/internal standard peak areas). Final concentration results in the original RW matrix were then calculated by multiplying by the overall dilution factors applied to the samples.

*Preparation of TDS Stock Solutions and Exposure Solutions for Tests at P&G MVIC*

Approximately 10 mg TDSS was dissolved in 2 L of dilution water for a final stock concentration of 5 mg TDSS/L dilution water or 4.635 mg TDS/L dilution water. Stock solutions were heated at 60°C for a minimum of 1 h in a closed, light proof vessel. Solutions were removed from heat, stirred to ensure the TDSS was completely dissolved, and allowed to stand at room temperature overnight to return to volume and temperature. The stock was stored at room temperature during an individual run. Prior to use of the stock, the solution was stirred at room temperature for 30 min to ensure uniform concentration of the substance. Samples of each stock solution were prepared for concentration confirmation, stored under refrigerated conditions and shipped to the P&G TAC laboratory.

*Analytical Results for TDS*

The average of the stock solution samples from the three independent runs was 112.5% of nominal (4.635 mg/L target, average 5.21 mg/L measured) indicating the stock was accurately prepared (Table 5).

Final exposure concentrations were calculated as the arithmetic mean of the arithmetic means for each time-point. The loss of alkyl sulfates, based on historical knowledge, is known to occur rapidly; therefore, geometric means were not used in this instance to calculate exposure concentrations. Measured exposure concentrations were 0, 0.092, 0.20, 0.33, 0.64 and 2.6 mg TDS/L for the nominal concentrations of 0, 0.145, 0.290, 0.579, 1.16 and 2.32 mg TDS/L, respectively, by HPLC/MS/MS (Table 4). These levels were 55.1-114.2% of nominal. Substantial losses over the 24-hr renewal period were observed across all concentrations (Figure 1).

**METHODS AND RESULTS: 1-Octanol**

1-Octanol was analyzed by the P&G Trace Analytical Core at Mason Business Center (M. Karb, K. Wehmeyer).

*HPLC/MS/MS Method (1-Octanol Stock and Exposure Concentration Determinations)*

Octanol, and its stable isotope-labeled internal standard (IS), $^{2}H_{17}$ Octanol ($d_{17}$ Octanol), were determined in reconstituted water (RW) samples following derivatization with Benzoyl Chloride to form the respective esters, Octyl Benzoate and $^{2}H_{17}$ Octyl Benzoate ($d_{17}$ Octyl Benzoate). The esters are monitored by a reversed-phase high performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) method operating under multiple reaction monitoring (MRM) conditions (full details archived at P&G as Method # HCL_13866_Octanol in Reconstituted Water_ Revision No.: 0).

Octanol stocks and exposure samples were diluted 1:1 (v:v) with acetonitrile (ACN) at the Environmental Stewardship Organization (ESO) laboratory prior to receipt by the Trace Analytical Core (TAC) with interim storage at 4°C in amber vials with Teflon-lined caps covered with parafilm. When required, further dilutions of study specimens were performed by TAC with 50:50 ACN:RW to provide concentrations within the linear range of the standard curve.

Standard (STD) and Quality Control (QC) samples containing known amounts of Octanol were prepared in 50:50 ACN:RW at concentrations ranging from 0.2 to 50 µg Octanol/mL. Internal standard was added to STD samples, QC samples and study specimens, followed by derivatization with Benzoyl Chloride and HPLC/MS/MS analysis. A STD curve was run at the beginning and end of the HPLC/MS/MS run, and exposure solution samples were evenly interspersed with QC samples throughout the run to monitor any bias in results.

Octanol concentrations (µg/mL) were determined by interpolation from a linear (1/x) regression of STD Octanol concentrations (expressed as the original underivatized Octanol concentration) by HPLC/MS/MS instrument response factors for the esters (ratio of analyte/internal standard peak areas). Final Octanol concentration results in the original RW matrix are then calculated by multiplying by the overall dilution factors applied to the samples.

*Preparation of 1-Octanol Stock Solutions and Exposure Solutions for Tests at P&G MVIC*

Approximately 75 mg 1-Octanol was dissolved in 500 mL of dilution water. The substance is a liquid, hence, correction for density at 0.827 g/cm³ was used to disperse the neat material. Ultimately 75 mg corresponds to 90.7 µL of the substance. Stock solutions were stirred in a closed, light proof vessel for 30 minutes at room temperature to ensure that the 1-Octanol was completely dissolved. The stock solution was kept refrigerated in the dark (1-8°C) during a single run. Prior to use of the stock, the solution was stirred at room temperature for 30 min to ensure uniform concentration of the substance. Samples of each stock solution were prepared for concentration confirmation, stored under refrigerated conditions and shipped to the P&G TAC laboratory.

*Analytical Results for 1-Octanol*

The average of the stock solution samples from the three independent runs was 90.0% of nominal (150 mg/L target, average 135.0 mg/L measured) indicating the stock was accurately prepared (Table 5).
Geometric mean measured concentrations throughout the test at 2.5, 5.0, 10, 20, and 40 mg/L were 1.72, 3.92, 8.48, 13.1, and 36.0 mg/L, respectively (Table 6). These levels were 65.4-90.0% of nominal. Substantial losses over the 24-hr renewal period were observed across all concentrations (Figure 2).

**METHODS AND RESULTS: Copper (II) Sulfate pentahydrate**

Copper was analyzed by Pace Analytical Services, Inc. Results are expressed as Copper.

*ICPMS Method (Copper Stock and Exposure Concentration Determinations)*

Concentrations of copper were determined in Reconstituted Water (RW) study exposure samples by inductively coupled plasma – mass spectrometry (ICPMS) USEPA Method 200.8: Determination of Trace Elements in Waters and Wastes.
Copper stocks and exposure samples were added (1 to 5 mLs; samples pooled as necessary) to sample bottles containing Milli-Q diluents at the appropriate volume (45 to 49 mLs) to provide concentrations within the linear range of the standard curve. Samples were stored at room temperature until shipped on ice to Pace Analytical Services, Inc.
Exposure solution samples with nominal test concentrations ranging from 0.0382 to 0.611 mg copper/L of RW were shipped to Pace Analytical Services, Inc.
Final concentration results in the original RW matrix were calculated by multiplying diluted sample copper concentrations by the overall dilution factor applied to the samples.

*Preparation of Copper Stock Solutions and Exposure Solutions for Tests at P&G MVIC*

Approximately 10 mg copper (II) sulfate pentahydrate was dissolved in 2 L of dilution water for a final stock solution concentration of 5 mg copper (II) sulfate pentahydrate/L dilution water or 1.272 mg copper/L dilution water. Stock solutions were stirred in a closed, light proof vessel for a minimum of 1 h at room temperature to ensure that the copper (II) sulfate pentahydrate was completely dissolved. The stock solution was stored in the dark at room temperature. Prior to use of the stock, the solution was stirred at room temperature for 30 min to ensure uniform concentration of the substance. Samples of each stock solution were prepared for concentration confirmation, stored under refrigerated conditions and shipped to Pace Analytical Services, Inc.

*Analytical Results for Copper*

The average of the stock solution samples from the three independent runs was 72.9% of nominal (1.272 mg copper/L target, average 0.93 mg/L measured) (Table 5).
Geometric mean measured concentrations throughout the test at 0.038, 0.076, 0.15, 0.31, and 0.61 mg/L were 0.024, 0.049, 0.099, 0.20, and 0.36 mg copper/L, respectively (Table 7). These

levels were 59.4-64.8% of nominal. Minimal losses over the 24-hr renewal period were observed across all concentrations (Figure 3).

**ADDITIONAL RESULTS**

The studies on the three chemicals described in this report span a wide range of expected acute aquatic toxicities ranging from moderately toxic (10 to 100 mg/L) to highly toxic (< 1 mg/L). Analytical confirmation of exposure has important consequences for LC50 or EC50 determinations. According to OECD technical guidelines (e.g., OECD 1992, 2004) if the measured exposure concentrations depart from nominal by ±20% then measured concentrations should be used in calculating effect concentrations. Initial measured concentrations were within 10% of nominal for 1-Octanol. In the case of TDS, initial measured concentrations in all exposures were >100% of nominal. Evidence from these studies suggests that losses in both the TDS and 1-Octanol studies would clearly result in a need to perform analytical measurements and calculate effect concentrations accordingly (Table 8). Copper concentrations were steady throughout the test; however, initial concentrations were only 64.2-65.8% of the nominal.

As another means to evaluate the importance of quantifying variations from the nominal concentration when using measured concentrations in determining the effect values, we compared 96-hr LC50s using nominal compared to those derived using measured concentrations. Of all the chemicals tested, tetradecyl sulfate had the greatest difference in nominal versus measured 96-hr LC50s declining from 0.573 mg/L to 0.325 mg/L (Table 8), a change of -43.3.5%. This compound is known to be rapidly biodegraded and susceptible to attack by extracellular sulfatases (Konnecker et al. 2011). Biodegradation half lives as short as hours are known from unadapted, low biomass systems. For 1-Octanol and copper, the decline was less substantial but still significant (-26.5 and -36.1%, respectively). While the influences of physical-chemical properties upon changes in the LC50 are not fully obvious, the lower soluble and volatile chemicals do appear problematic (note that degradation here is not discounted but is also a potential contributor).

**OVERALL CONCLUSIONS**

1. Clearly by these studies, determination of exposure concentrations in the Fish Embryo Test can be accomplished by modern analytical methods, even when very low sample volumes and highly toxic substances are involved.
2. Stock solutions for all tested chemicals were consistently prepared but the copper stock solution was only 72.9% of nominal.
3. Analytical confirmation of exposure is likely not essential for every chemical and every study. Some FET tests may provide reliable LC50 determinations under static conditions (versus semi-static) when exposures can be maintained within 20% of nominal concentrations.
4. The most challenging chemicals were characterized by combinations of low solubility, high biodegradability, and being semi-volatile. Results for challenging chemicals appear to be mostly related to the chemistry of the chemical and not a function of the exposure system. In other words, similar types of results would be expected from studies conducted in larger volumes (e.g., volumes relevant to an OECD 203 fish acute toxicity test).
5. Analytical confirmation of exposure for challenging chemicals was necessary and is reflected in lower 96-hr LC50 estimates for all three chemicals.

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OECD 2011b. Validation Report (Phase 1) for the Zebrafish Embryo Toxicity Test Part 2, Series on Testing and Assessment No. 157.

**LIST OF TABLES**

Table 1. Summary of physical chemical properties of chemicals entered into Zebrafish Fish Embryo Testing, Phase 2b
Table 2. SMILES notation and structures associated with the chemicals
Table 3. Dates of studies and nominal exposure concentrations used in each
Table 4. Summary of measurements of tetradecyl sulfate concentrations during the conduct of a semi-static, 96-hr Fish Embryo Test
Table 5. Summary of stock solution measurements for the 3 chemicals used in this study
Table 6. Summary of measurements of 1-octanol concentrations during the conduct of a semi-static, 96-hr Fish Embryo Test
Table 7. Summary of measurements of copper concentrations during the conduct of a semi-static, 96-hr Fish Embryo Test
Table 8. Comparison of 96-hr LC50s calculated based on nominal and measured exposure concentrations

*Table 1. Summary of physical chemical properties of three chemicals entered into Zebrafish Fish Embryo Testing, Phase 2b*

| Chemical | CASNO | MW | Log Kow | HLC (Pas-m3/mole) | Solubility (mg/L) | Fish Expected Toxicity Range | Chemical Purity (%) |
|---|---|---|---|---|---|---|---|
| Tetradecyl sulfate sodium salt | 1191-50-0 | 316.43 | 2.67 | 3.25E-07 | 16.4 | 1 to 10 mg/L | 97.5 |
| 1-Octanol | 111-87-5 | 130.23 | 3.00 | 2.45E-05 | 877 | 10 to 100 mg/L | 99.7 |
| Copper (II) sulfate pentahydrate | 7758-99-8 | 249.68 | N/A | N/A | 230 | <1 mg/L | 99.1 |

*N/A: Not Applicable; HLC: Henry's Law constant; MW: Molecular Weight; note that solubility estimates are made in ion free water and will be lower in environmental waters.*

*Table 2. SMILES notation and structures associated with the chemicals*

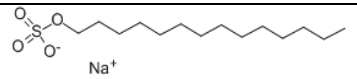
| Chemical | SMILES Notation | Structure |
|---|---|---|
| Tetradecyl sulfate sodium salt | CCCCCCCCCCCCCCOS(=O)(=O)[O-].[Na+] |  |
| 1-Octanol | CCCCCCCCO |  |
| Copper (II) sulfate pentahydrate | Not applicable | $CuSO_4 \cdot 5H_2O$ |

*Table 3. Dates of studies and nominal exposure concentrations used in each chemical*

| Chemical | Nominal Exposure Concentrations (mg/L) | Date Z-FET Study was Initiated | Study for Which Analytical Verification of Exposure was Determined |
|---|---|---|---|
| Tetradecyl sulfate sodium salt<br><br>Tetradecyl sulfate | 0, 0.156, 0.3125, 0.625, 1.25, 2.5<br><br>0, 0.145, 0.290, 0.579, 1.16, 2.32 | 4 Apr 2011<br>2 May 2011<br>20 Jun 2011 | 2 May 2011 |
| 1-Octanol | 0, 2.5, 5, 10, 20, 40 | 2 May 2011<br>16 May 2011<br>13 Jun 2011 | 16 May 2011 |
| Copper (II) sulfate pentahydrate<br><br>Copper | 0, 0.15, 0.3, 0.6, 1.2, 2.4<br><br>0, 0.038, 0.076, 0.15, 0.31, 0.61 | 11 Jul 2011<br>25 Jul 2011<br>8 Aug 2011 | 25 Jul 2011 |

*Table 4. Summary of measurements of tetradecyl sulfate concentrations during the conduct of a semi-static, 96-hr Fish Embryo Test*

| | Arithmetic mean (mg/L) | | | % of nominal | | |
|---|---|---|---|---|---|---|
| Nominal | New | Old | Combined | New | Old | Combined |
| 0.145 | 0.155 | 0.0292 | 0.092 | 107.3 | 20.2 | 63.7 |
| 0.290 | 0.330 | 0.0748 | 0.20 | 113.9 | 25.8 | 69.9 |
| 0.579 | 0.604 | 0.0533 | 0.33 | 104.2 | 9.2 | 56.7 |
| 1.16 | 1.26 | 0.0198 | 0.64 | 108.5 | 1.7 | 55.1 |
| 2.32 | 2.65 | N/A | 2.6 | 114.2 | N/A | 114.2 |

*N/A: Not Applicable*

*Table 5. Summary of stock solution measurements for the 3 chemicals used in this study*

| | n | Nominal Stock Solution (mg/L) | Measured Stock Solution (mg/L) | | % of Nominal | |
|---|---|---|---|---|---|---|
| | | | Average | Stdev | Average | Stdev |
| Tetradecyl sulfate | 3 | 4.635 | 5.21 | 0.21 | 112.5 | 4.6 |
| 1-Octanol | 3 | 150 | 135.0 | 10.7 | 90.0 | 7.2 |
| Copper | 3 | 1.272 | 0.93 | 0.13 | 72.9 | 9.9 |

*Note – Tetradecyl sulfate sodium salt nominal stock solution 5.0 mg/L; Copper (II) sulfate pentahydrate nominal stock solution 5.0 mg/L.*

*Table 6. Summary of measurements of 1-Octanol concentrations during the conduct of a semi-static, 96-hr Fish Embryo Test*

| | Geometric mean (mg/L) | | | % of nominal | | |
|---|---|---|---|---|---|---|
| Nominal | New | Old | Combined | New | Old | Combined |
| 2.5 | 2.34 | 1.26 | 1.72 | 93.6 | 50.5 | 68.7 |
| 5 | 4.85 | 3.17 | 3.92 | 96.9 | 63.4 | 78.4 |
| 10 | 9.69 | 7.41 | 8.48 | 96.9 | 74.1 | 84.8 |
| 20 | 19.3 | 8.85 | 13.1 | 96.7 | 44.2 | 65.4 |
| 40 | 40.3 | 32.2 | 36.0 | 100.7 | 80.5 | 90.0 |

*Table 7. Summary of measurements of Copper concentrations during the conduct of a semi-static, 96-hr Fish Embryo Test*

| | Geometric mean (mg/L) | | | % of nominal | | |
|---|---|---|---|---|---|---|
| Nominal | New | Old | Combined | New | Old | Combined |
| 0.038 | 0.025 | 0.023 | 0.024 | 65.5 | 61.1 | 63.6 |
| 0.076 | 0.050 | 0.048 | 0.049 | 65.8 | 62.6 | 64.1 |
| 0.15 | 0.10 | 0.10 | 0.099 | 65.8 | 63.6 | 64.8 |
| 0.31 | 0.20 | 0.19 | 0.20 | 64.2 | 63.7 | 64.0 |
| 0.61 | 0.40 | 0.33 | 0.36 | 65.2 | 54.1 | 59.4 |

*Table 8. Comparison of 96-hr LC50s calculated based on nominal and measured exposure concentrations*

| | | Nominal LC50 | | | Nominal LC50 | | | Measured LC50 | | | % Change in the Nominal LC50 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Compound | Run | LC50 (mg/L) | LCL (mg/L) | UCL (mg/L) | Mean (mg/L) | STDEV (mg/L) | % CV | LC50 (mg/L) | LCL (mg/L) | UCL (mg/L) | |
| TDS | 1 | 0.393 | 0.336 | 0.464 | 0.435 | 0.123 | 28.2% | | | | |
| | 2 | 0.573 | 0.487 | 0.613 | | | | 0.325 | 0.320 | 0.331 | -43.28% |
| | 3 | 0.339 | 0.293 | 0.400 | | | | | | | |
| 1-Octanol | 1 | 26.2 | 22.3 | 31.2 | 20.7 | 5.8 | 27.9% | | | | |
| | 2 | 14.7 | 12.3 | 17.4 | | | | 10.8 | 9.56 | 12.2 | -26.5% |
| | 3 | 21.2 | 20.3 | 29.0 | | | | | | | |
| Copper | 1 | 0.308 | 0.288 | 0.363 | 0.302 | 0.039 | 12.8% | | | | |
| | 2 | 0.338 | 0.284 | 0.406 | | | | 0.216 | 0.184 | 0.254 | -36.1% |
| | 3 | 0.261 | 0.221 | 0.302 | | | | | | | |

*LCL: lower confidence limit; UCL: upper confidence limit*

**LIST OF FIGURES**

Figure 1. Pattern of measured tetradecyl sulfate sodium salt concentrations in the fish embryo test using a 24-hr renewal, semi-static design
Figure 2. Pattern of measured 1-octanol concentrations in the fish embryo test using a 24-hr renewal, semi-static design
Figure 3. Pattern of measured copper concentrations in the fish embryo test using a 24-hr renewal, semi-static design

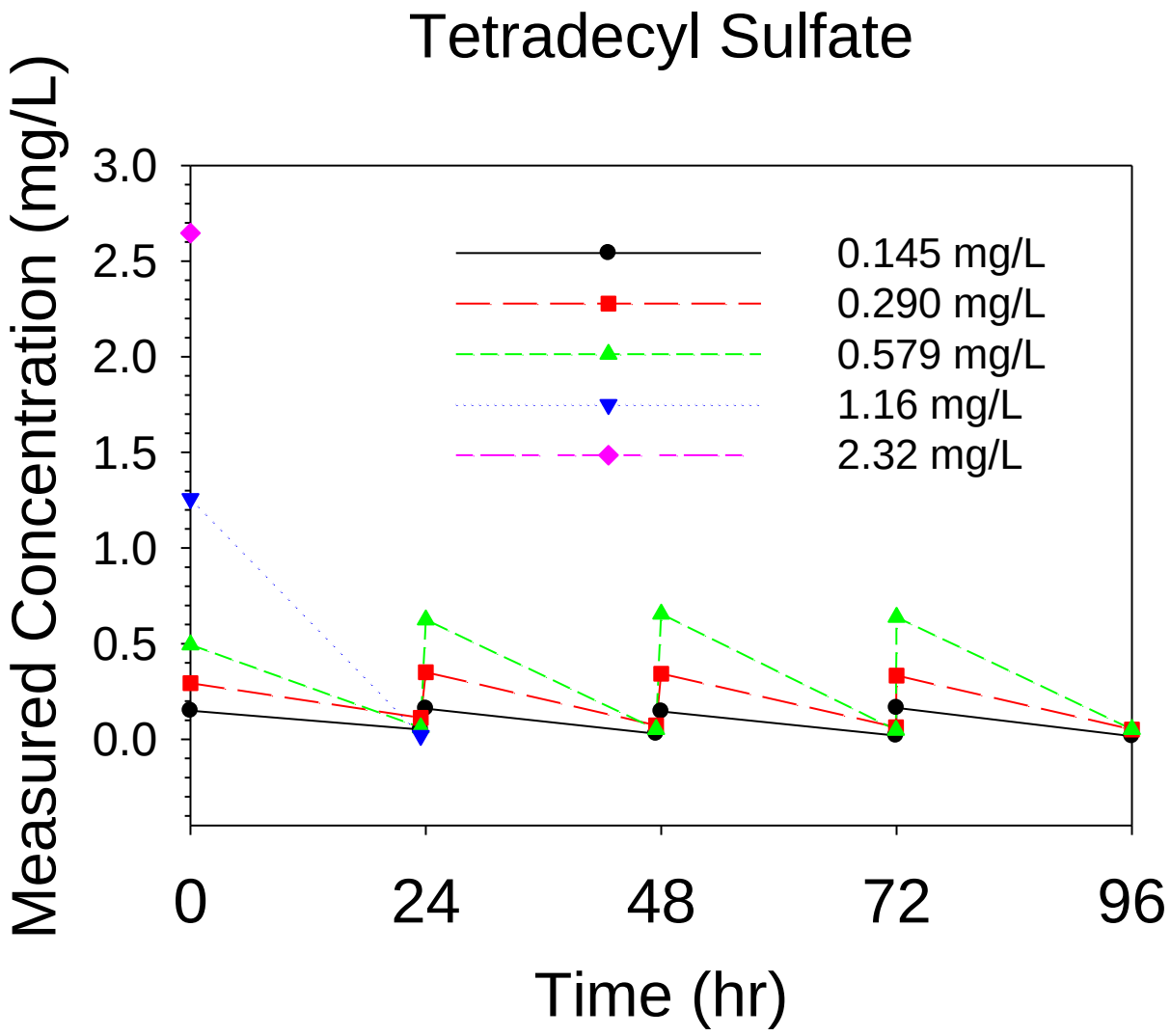



*Figure 1. Pattern of measured tetradecyl sulfate sodium salt concentrations in the fish embryo test using a 24-hr renewal, semi-static design*

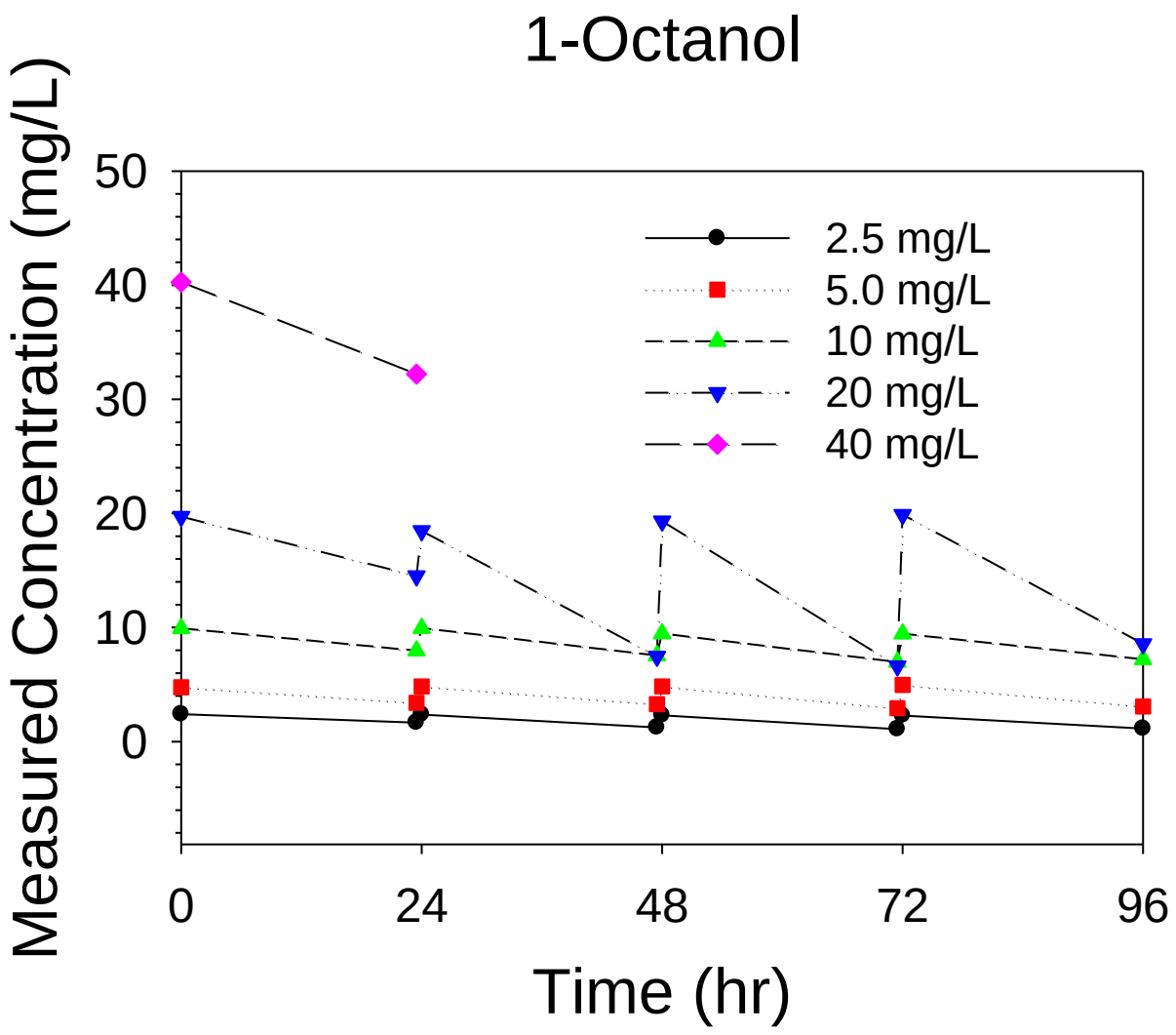



*Figure2. Pattern of measured 1-octanol concentrations in the fish embryo test using a 24-hr renewal, semi-static design*

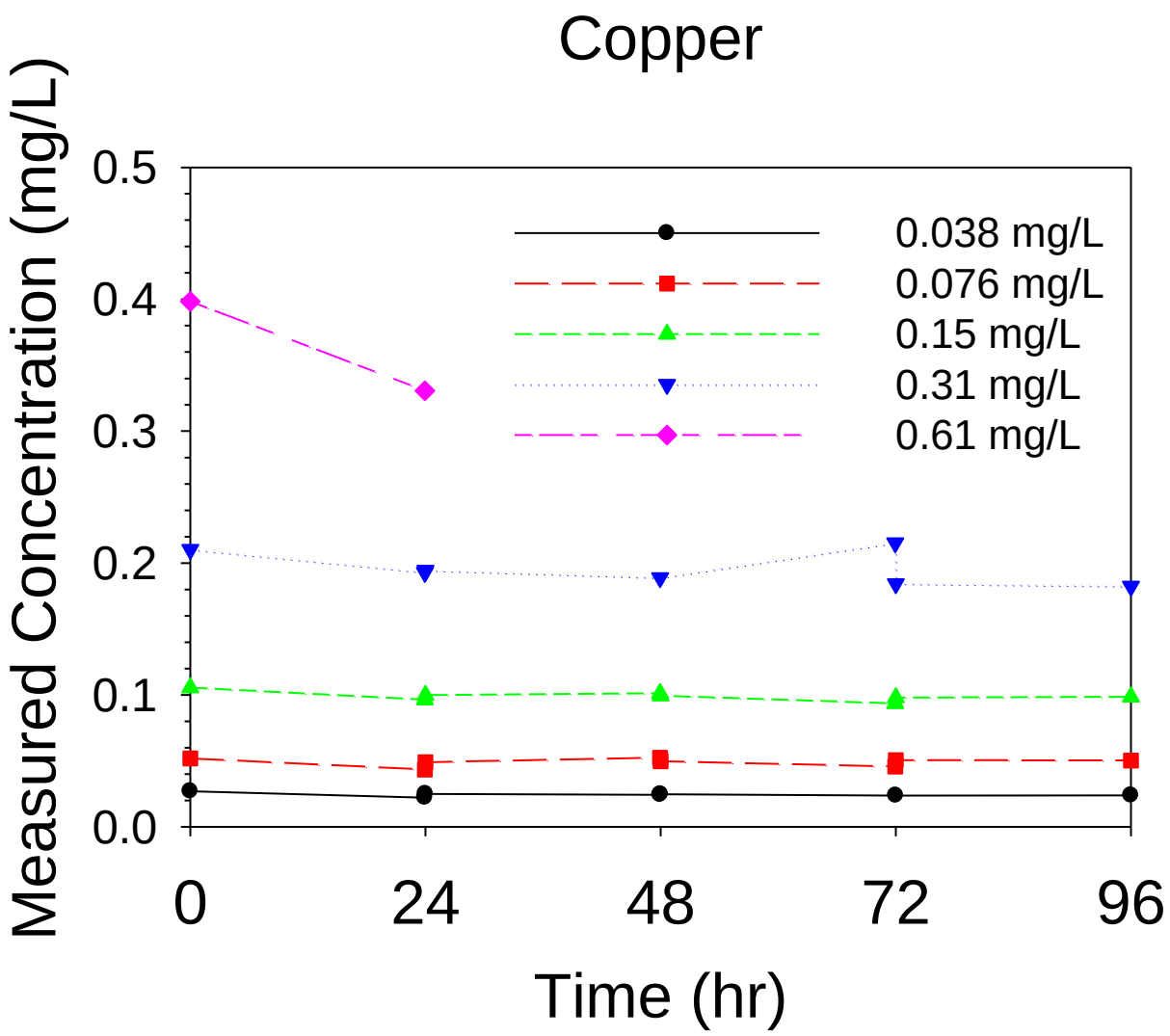



*Figure 3. Pattern of measured copper concentrations in the fish embryo test using a 24-hr renewal, semi-static design*

# Annex IIIb - Analysis of Two chemicals in Fish Embryo Test Stock and Exposure Solutions for Phase 2b by Ipo-Pszczyna

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## ANALYTICAL DETERMINATIONS OF CARBAMAZEPINE AND PROCHLORAZ

Institute of Industrial Organic Chemistry Branch Pszczyna

Department of Ecotoxicology

Doswiadczalna 27, 43-200 Pszczyna, Poland

## 1. DETERMINATION OF CARBAMAZEPINE IN TEST SOLUTIONS

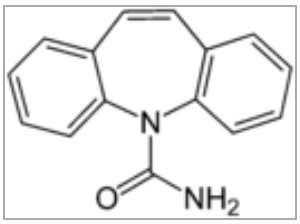
### 1.1. Detected Substance

**Carbamazepine:** 5*H*-dibenzo[*b*,*f*]azepine-5-carboxamide

CAS number: [298-46-4]

Molecular formula: $C_{15}H_{12}N_2O$

Molecular weight: 236.27 g/mol




### 1.2. Analytical Procedure

1.2.1. Reagents and solvents

- acetonitrile for HPLC,
- deionized water,
- universal buffer pH 2.36 (2.47 g of boric acid, 2.7 mL of orthophosphoric acid, 2.3 mL of acetic acid combined into a volumetric flask containing 500 mL of deionized water and filled up to total volume of 1000 mL with deionized water)
- mixture of acetonitrile : universal buffer (70 : 30, v/v),
- carbamazepine used as standard, purity 100% (TLC), Sigma product number C4024, Lot: 119K1317V,

- standard solution of 1 mg/mL carbamazepine in deionised water and stock solutions: 0.01, 0.05, 0.1, 0.5, 1.0, 5.0, 10.0 and 100.0 μg/mL of carbamazepine in deionised water.

1.2.2. Apparatus

- laboratory glassware,
- analytical balance,
- liquid chromatograph Varian Prostar with UV-VIS detector (Varian, USA).

The following liquid chromatography parameters were used:

| | |
|---|---|
| Column | Microsorb-MV 100-5 C18, l = 250 mm, ϕ = 4,6 mm |
| Mobile phase | acetonitrile : universal buffer (70 : 30, v/v) |
| Wavelength | 220 nm |
| Flow rate | 1.0 mL/min. |
| Injected volume | 20 μL |

**1.3. Preparation of Samples for Chromatography Analysis**

From each sample (deionised water, deionised water spiked with carbamazepine, test sample) 20 μL was injected to the chromatographic column. If necessary, the sample was diluted with deionized water (section 1.5).

**1.4. Validation of Analytical Procedure**

Linearity of response, specificity, precision, recovery, limit of quantification and detection for carbamazepine were assessed to validate the analytical procedure.

Calibration curve

Stock solutions containing 0.01, 0.05, 0.1, 0.5, 1.0, 5.0 and 10.0 μg/mL of carbamazepine were injected successively to the chromatographic column and chromatograms were recorded. The standard curve (field of the peak versus quantity of the standard) is linear with a regression coefficient ($r^2$) of 0.999953. The linearity of the analytical procedure ranges from 0.01 μg/mL to 10.0 μg/mL. The standard curve of carbamazepine is presented in Figure 1.

Specificity

The specificity of the analytical procedure was estimated based on analysis of chromatograms generated for control sample (i.e. deionised water) and fortification samples (i.e. samples of deionised water spiked with carbamazepine). Considering the results of the analysis no signal of

carbamazepine was overlapping with matrix signal of control sample (i.e. deionised water) in experimental conditions. Therefore the specificity of the analytical procedure was demonstrated.

Precision

Precision is determined as the repeatability (RSD – relative standard deviation, %). The results of repeatability for carbamazepine determined in fortification samples (i.e. samples of deionised water spiked with carbamazepine) are presented in Table 1.

Extraction recovery level

In order to study the recovery, the stock solution of carbamazepine was added to control sample (i.e. deionised water) and analysed by the analytical procedure. The results are presented in Table 1.

Calibration Curve Report
File: c:\star\data\carbamazepine\carbamazepine.mth
Detector: ProStar 325 UV-Vis Detector, Address: 44, Channel ID: 1

carbamazepine

External Standard Analysis
Curve Type: Linear
Origin: Force
y = +1.144067e+006x

Resp. Fact. RSD: 8.116%
Coeff. Det.($r^2$): 0.999953

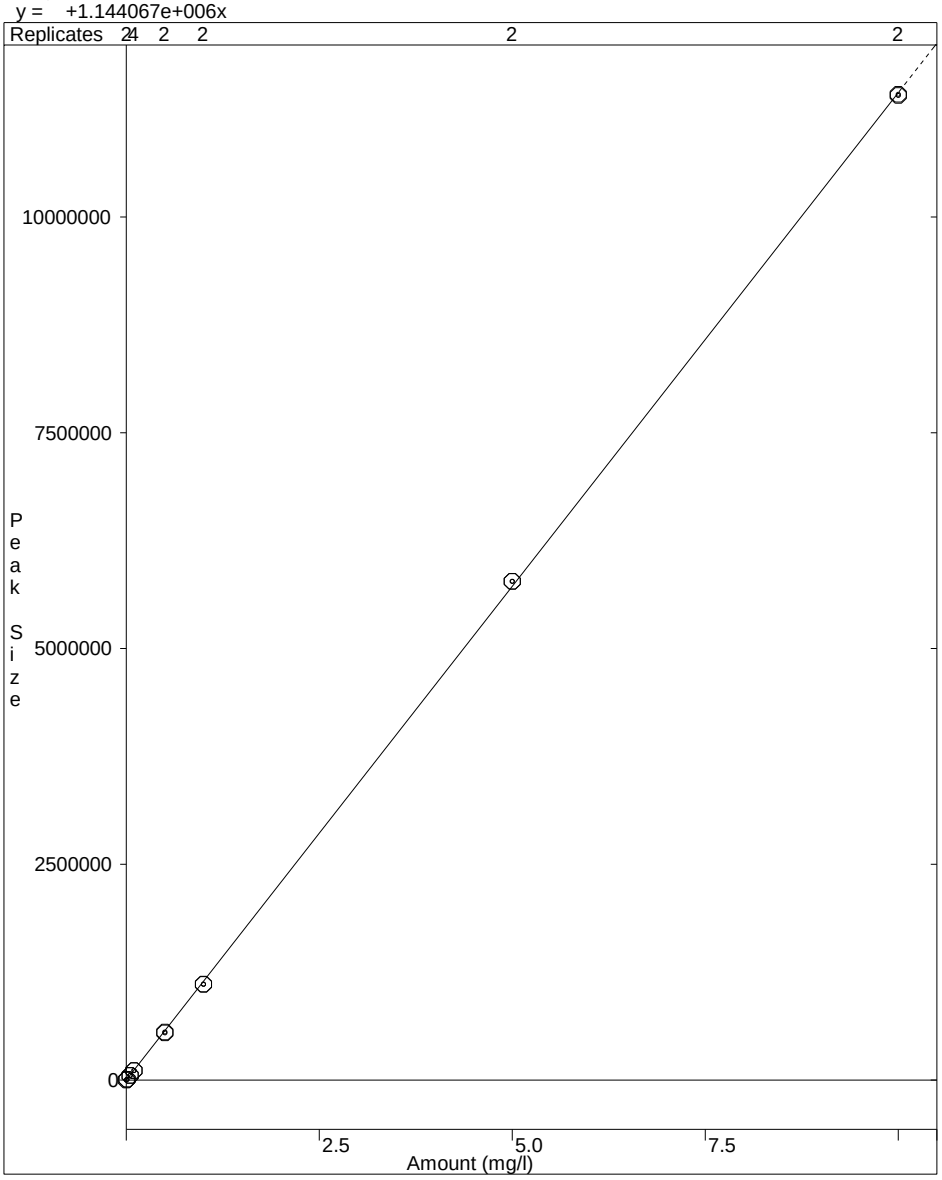



**Figure1. Standard curve for carbamazepine**

**Table 1. The recovery level for carbamazepine**

| Fortification level [mg/L] | Determined concentration of carbamazepine (n=5) [mg/L] | | | | | Mean determined concentration of carbamazepine [mg/L] | SD [mg/L] | RSD [%] | Recovery [%] |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | | | | |
| control | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | - | - |
| 0.01 | 0.011 | 0.011 | 0.010 | 0.011 | 0.011 | 0.011 | 0.000 | 3.1 | 106.2 |
| 10.00 | 9.775 | 9.765 | 9.862 | 9.981 | 9.840 | 9.844 | 0.087 | 0.9 | 98.4 |

SD – standard deviation,
RSD –relative standard deviation

Limit of quantification and detection

*Limit of Quantification* was estimated as the lowest concentration of carbamazepine, at which an acceptable mean recovery is obtained (normally 70 – 100 % with a relative standard deviation of preferably ≤ 20 %). *Limit of Detection* was estimated as the lowest concentration of carbamazepine that the analytical procedure can reliably differentiate from background noise. The results are presented in Table 2.

**Table 2. Limit of Quantification and Detection of carbamazepine in water**

| Detected substance | Limit of Quantification [mg/L] | Limit of Detection [mg/L] |
|---|---|---|
| Carbamazepine | 0.010 | 0.005 |

## 1.5. Analytical procedure during the ZFET test

The stock solution of carbamazepine was freshly prepared for each run and stored according to Trial Plan (TP_ZFET_OECD_2bV01.1 of 17$^{th}$ January 2011). All test vessels were pre-saturated with the respective nominal test concentrations and controls before test initiation. At test initiation and at each renewal fresh solutions were prepared and test samples were collected before division into wells in plates. At each renewal and at test termination test samples of spent solutions were combined from wells in plates.

The determinations were performed in triplicates for each test sample of respective nominal concentration of carbamazepine and control. Test samples were diluted with deionized water (Table 3) and then analysed by the analytical procedure described in section 1.2.

**Table 3. Preparation of samples for analysis**

| Nominal concentration of carbamazepine [mg/L] | Volume of test sample [mL] | Volume of deionised water [mL] | Total volume of diluted sample [mL] |
|---|---|---|---|
| control | 2.0 | 0 | 2.0 |
| 54.7 | 1.0 | 5.0 | 6.0 |
| 76.5 | 1.0 | 9.0 | 10.0 |
| 107.1 | 1.0 | 10.0 | 11.0 |
| 150.0 | 1.0 | 19.0 | 20.0 |
| 210.0 | 1.0 | 29.0 | 30.0 |

### 1.6. Results

Test samples of fresh solutions at test initiation, fresh and spent solutions at each renewal and spent solutions at test termination were analysed.

In samples of fresh solutions in Run 1 (Table 4) the concentration of carbamazepine was determined in the range of 87.4 – 98.4% of nominal concentration. In samples of fresh solutions in Run 2 (Table 5) the concentration of carbamazepine was determined in the range of 89.8 – 98.6% of nominal concentration. In samples of fresh solutions in Run 3 (Table 6) the concentration of carbamazepine was determined in the range of 80.9 – 102.0% of nominal concentration. In samples of fresh solutions in Run 4 (Table 7) the concentration of carbamazepine was determined in the range of 86.0 – 106.6% of nominal concentration. The results generated confirm correct preparation of test solutions.

In samples of spent solutions in Run 1 (Table 4) the concentration of carbamazepine was determined in the range of 93.7 – 113.8% of initial determined concentration. In samples of spent solutions in Run 2 (Table 5) the concentration of carbamazepine was determined in the range of 95.9 – 109.6% of initial determined concentration. In samples of spent solutions in Run 3 (Table 6) the concentration of carbamazepine was determined in the range of 97.6 – 117.3% of initial determined concentration. In samples of spent solutions in Run 4 (Table 7) the concentration of carbamazepine was determined in the range of 96.6 – 110.5% of initial determined concentration. The results generated confirm that the concentration of carbamazepine was stable in periods between renewals.

**Table 4. Results – Run 1 21.03.2011 – 25.03.2011**

| | Sample: Nominal concentration of carbamazepine [mg/L] | Determined concentration of carbamazepine in sample (triple analysis) [mg/L] | | | Mean determined concentration of carbamazepine [mg/L] | SD [mg/L] | RSD [%] | Recovery of nominal concentration [%] | Recovery of initial determined concentration [%] |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | | | | | |
| fresh solutions day 0 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 50.7 | 50.6 | 50.4 | **50.5** | 0.2 | 0.3 | 92.4 | n/a |
| | 76.5 | 69.6 | 69.4 | 69.8 | **69.6** | 0.2 | 0.3 | 90.9 | n/a |
| | 107.1 | 96.7 | 97.5 | 96.8 | **97.0** | 0.5 | 0.5 | 90.6 | n/a |
| | 150.0 | 135.9 | 136.6 | 136.0 | **136.2** | 0.4 | 0.3 | 90.8 | n/a |
| | 210.0 | 187.8 | 191.3 | 191.1 | **190.1** | 2.0 | 1.0 | 90.5 | n/a |
| fresh solutions day 1 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 50.3 | 50.0 | 50.4 | **50.2** | 0.2 | 0.4 | 91.8 | n/a |
| | 76.5 | 68.5 | 67.8 | 67.3 | **67.9** | 0.6 | 0.9 | 88.7 | n/a |
| | 107.1 | 95.9 | 96.0 | 95.9 | **95.9** | 0.1 | 0.1 | 89.6 | n/a |
| | 150.0 | 137.5 | 137.2 | 138.2 | **137.6** | 0.5 | 0.4 | 91.8 | n/a |
| | 210.0 | 191.9 | 192.9 | 191.7 | **192.1** | 0.6 | 0.3 | 91.5 | n/a |
| spent solutions day 1 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 49.5 | 45.5 | 47.2 | **47.4** | 2.0 | 4.2 | 86.6 | 93.7 |
| | 76.5 | 69.1 | 69.9 | 70.7 | **69.9** | 0.8 | 1.2 | 91.4 | 100.5 |
| | 107.1 | 95.3 | 95.8 | 96.5 | **95.9** | 0.6 | 0.6 | 89.5 | 98.8 |
| | 150.0 | 140.6 | 139.4 | 143.2 | **141.0** | 2.0 | 1.4 | 94.0 | 103.6 |
| | 210.0 | 187.1 | 188.8 | 188.0 | **188.0** | 0.9 | 0.5 | 89.5 | 98.9 |
| fresh solutions day 2 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 47.7 | 48.0 | 47.8 | **47.8** | 0.2 | 0.4 | 87.4 | n/a |
| | 76.5 | 69.5 | 69.5 | 69.5 | **69.5** | 0.0 | 0.1 | 90.9 | n/a |
| | 107.1 | 97.3 | 95.2 | 97.5 | **96.7** | 1.3 | 1.3 | 90.3 | n/a |
| | 150.0 | 138.0 | 138.6 | 138.1 | **138.2** | 0.3 | 0.2 | 92.1 | n/a |
| | 210.0 | 196.4 | 196.8 | 196.9 | **196.7** | 0.2 | 0.1 | 93.7 | n/a |
| spent solutions day 2 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 47.7 | 47.7 | 47.0 | **47.5** | 0.4 | 0.8 | 86.8 | 94.5 |
| | 76.5 | 70.5 | 70.4 | 70.7 | **70.5** | 0.1 | 0.2 | 92.2 | 103.9 |
| | 107.1 | 97.0 | 95.9 | 96.9 | **96.6** | 0.6 | 0.6 | 90.2 | 100.7 |
| | 150.0 | 138.1 | 138.0 | 135.2 | **137.1** | 1.7 | 1.2 | 91.4 | 99.6 |
| | 210.0 | 193.6 | 194.2 | 191.9 | **193.2** | 1.2 | 0.6 | 92.0 | 100.6 |
| fresh solutions day 3 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 50.7 | 50.7 | 50.8 | **50.8** | 0.1 | 0.2 | 92.8 | n/a |
| | 76.5 | 72.5 | 72.3 | 72.7 | **72.5** | 0.2 | 0.3 | 94.7 | n/a |
| | 107.1 | 99.7 | 99.7 | 100.1 | **99.9** | 0.2 | 0.2 | 93.2 | n/a |
| | 150.0 | 143.5 | 141.8 | 142.7 | **142.7** | 0.8 | 0.6 | 95.1 | n/a |
| | 210.0 | 206.4 | 206.8 | 206.6 | **206.6** | 0.2 | 0.1 | 98.4 | n/a |
| spent solutions day 3 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 49.1 | 50.7 | 50.3 | **50.0** | 0.9 | 1.7 | 91.5 | 104.7 |
| | 76.5 | 72.9 | 73.2 | 73.2 | **73.1** | 0.1 | 0.2 | 95.6 | 105.2 |
| | 107.1 | 98.5 | 97.2 | 97.7 | **97.8** | 0.6 | 0.7 | 91.3 | 101.2 |
| | 150.0 | 140.6 | 141.7 | 140.9 | **141.0** | 0.6 | 0.4 | 94.0 | 102.0 |
| | 210.0 | 224.9 | 223.1 | 223.5 | **223.8** | 0.9 | 0.4 | 106.6 | 113.8 |
| spent solutions day 4 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 50.7 | 51.1 | 49.1 | **50.3** | 1.0 | 2.1 | 91.9 | 99.1 |
| | 76.5 | 73.1 | 73.2 | 73.0 | **73.1** | 0.1 | 0.1 | 95.5 | 100.8 |
| | 107.1 | 97.1 | 97.0 | 98.8 | **97.6** | 1.0 | 1.0 | 91.2 | 97.8 |
| | 150.0 | 140.5 | 141.2 | 142.0 | **141.2** | 0.8 | 0.5 | 94.1 | 99.0 |
| | 210.0 | 220.2 | 221.1 | 217.9 | **219.7** | 1.6 | 0.7 | 104.6 | 106.4 |

below LoQ – below Limit of Quantification, i.e. below 0.010 mg/L; SD – standard deviation; RSD – relative standard deviation; n/a – not applicable.

**Table 5. Results – Run 2 04.04.2011 – 08.04.2011**

| | Sample: Nominal concentration of carbamazepine [mg/L] | Determined concentration of carbamazepine in sample (triple analysis) [mg/L] | | | Mean determined concentration of carbamazepine [mg/L] | SD [mg/L] | RSD [%] | Recovery of nominal concentration [%] | Recovery of initial determined concentration [%] |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | | | | | |
| fresh solutions day 0 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 53.2 | 52.7 | 53.6 | **53.2** | 0.4 | 0.8 | 97.2 | n/a |
| | 76.5 | 74.4 | 74.4 | 74.4 | **74.4** | 0.0 | 0.1 | 97.2 | n/a |
| | 107.1 | 103.9 | 104.6 | 104.4 | **104.3** | 0.4 | 0.4 | 97.4 | n/a |
| | 150.0 | 146.9 | 146.0 | 145.9 | **146.2** | 0.6 | 0.4 | 97.5 | n/a |
| | 210.0 | 188.1 | 188.6 | 189.2 | **188.7** | 0.5 | 0.3 | 89.8 | n/a |
| fresh solutions day 1 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 51.0 | 50.9 | 51.0 | **51.0** | 0.1 | 0.1 | 93.2 | n/a |
| | 76.5 | 71.5 | 71.9 | 71.8 | **71.7** | 0.2 | 0.2 | 93.8 | n/a |
| | 107.1 | 102.3 | 102.3 | 102.0 | **102.2** | 0.2 | 0.2 | 95.4 | n/a |
| | 150.0 | 140.9 | 141.2 | 141.3 | **141.1** | 0.2 | 0.2 | 94.1 | n/a |
| | 210.0 | 201.2 | 200.5 | 200.2 | **200.6** | 0.5 | 0.3 | 95.5 | n/a |
| spent solutions day 1 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 51.5 | 51.1 | 51.3 | **51.3** | 0.2 | 0.5 | 93.8 | 96.5 |
| | 76.5 | 70.8 | 71.6 | 71.6 | **71.4** | 0.5 | 0.6 | 93.3 | 95.9 |
| | 107.1 | 104.4 | 104.3 | 104.2 | **104.3** | 0.1 | 0.1 | 97.4 | 100.0 |
| | 150.0 | 144.9 | 144.1 | 144.5 | **144.5** | 0.4 | 0.3 | 96.3 | 98.8 |
| | 210.0 | 203.9 | 203.8 | 203.3 | **203.7** | 0.4 | 0.2 | 97.0 | 108.0 |
| fresh solutions day 2 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 53.4 | 53.0 | 53.3 | **53.3** | 0.2 | 0.4 | 97.4 | n/a |
| | 76.5 | 71.7 | 71.5 | 71.4 | **71.5** | 0.2 | 0.2 | 93.5 | n/a |
| | 107.1 | 101.6 | 101.7 | 101.0 | **101.4** | 0.4 | 0.4 | 94.7 | n/a |
| | 150.0 | 143.5 | 144.0 | 144.0 | **143.8** | 0.3 | 0.2 | 95.9 | n/a |
| | 210.0 | 208.5 | 205.8 | 207.2 | **207.2** | 1.3 | 0.7 | 98.6 | n/a |
| spent solutions day 2 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 51.1 | 51.4 | 51.4 | **51.3** | 0.2 | 0.4 | 93.8 | 100.7 |
| | 76.5 | 74.4 | 74.5 | 74.4 | **74.4** | 0.0 | 0.1 | 97.3 | 103.8 |
| | 107.1 | 107.4 | 106.7 | 105.6 | **106.6** | 1.0 | 0.9 | 99.5 | 104.3 |
| | 150.0 | 147.3 | 149.7 | 150.5 | **149.2** | 1.6 | 1.1 | 99.5 | 105.7 |
| | 210.0 | 209.0 | 209.6 | 209.6 | **209.4** | 0.4 | 0.2 | 99.7 | 104.4 |
| fresh solutions day 3 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 51.8 | 52.1 | 52.0 | **52.0** | 0.1 | 0.3 | 95.0 | n/a |
| | 76.5 | 73.4 | 73.2 | 72.1 | **72.9** | 0.7 | 0.9 | 95.3 | n/a |
| | 107.1 | 99.7 | 100.0 | 100.1 | **99.9** | 0.2 | 0.2 | 93.3 | n/a |
| | 150.0 | 143.1 | 143.2 | 143.2 | **143.1** | 0.0 | 0.0 | 95.4 | n/a |
| | 210.0 | 204.9 | 204.4 | 204.5 | **204.6** | 0.3 | 0.1 | 97.4 | n/a |
| spent solutions day 3 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 53.0 | 52.9 | 52.7 | **52.9** | 0.2 | 0.3 | 96.7 | 99.3 |
| | 76.5 | 76.3 | 76.6 | 76.6 | **76.5** | 0.2 | 0.2 | 100.0 | 106.9 |
| | 107.1 | 103.7 | 104.4 | 104.1 | **104.1** | 0.3 | 0.3 | 97.2 | 102.6 |
| | 150.0 | 146.2 | 148.3 | 149.2 | **147.9** | 1.5 | 1.0 | 98.6 | 102.9 |
| | 210.0 | 226.3 | 224.7 | 230.2 | **227.1** | 2.9 | 1.3 | 108.1 | 109.6 |
| spent solutions day 4 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 52.5 | 52.6 | 52.1 | **52.4** | 0.3 | 0.5 | 95.8 | 100.8 |
| | 76.5 | 73.5 | 73.2 | 73.8 | **73.5** | 0.3 | 0.4 | 96.1 | 100.8 |
| | 107.1 | 102.1 | 101.0 | 101.8 | **101.6** | 0.6 | 0.6 | 94.9 | 101.7 |
| | 150.0 | 142.3 | 142.7 | 142.8 | **142.6** | 0.2 | 0.2 | 95.1 | 99.6 |
| | 210.0 | 213.9 | 214.1 | 214.7 | **214.2** | 0.4 | 0.2 | 102.0 | 104.7 |

below LoQ – below Limit of Quantification, i.e. below 0.010 mg/L; SD – standard deviation; RSD – relative standard deviation; n/a – not applicable.

**Table 6. Results – Run 3 18.04.2011 – 22.04.2011**

| | Sample: Nominal concentration of carbamazepine [mg/L] | Determined concentration of carbamazepine in sample (triple analysis) [mg/L] | | | Mean determined concentration of carbamazepine [mg/L] | SD [mg/L] | RSD [%] | Recovery of nominal concentration [%] | Recovery of initial determined concentration [%] |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | | | | | |
| fresh solutions day 0 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 49.9 | 48.7 | 50.8 | **49.8** | 1.0 | 2.1 | 91.0 | n/a |
| | 76.5 | 71.6 | 71.7 | 70.6 | **71.3** | 0.6 | 0.9 | 93.2 | n/a |
| | 107.1 | 97.8 | 97.4 | 97.7 | **97.6** | 0.2 | 0.2 | 91.2 | n/a |
| | 150.0 | 139.8 | 140.5 | 140.1 | **140.2** | 0.3 | 0.2 | 93.4 | n/a |
| | 210.0 | 201.8 | 200.9 | 202.8 | **201.8** | 1.0 | 0.5 | 96.1 | n/a |
| fresh solutions day 1 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 50.0 | 51.4 | 51.2 | **50.9** | 0.8 | 1.5 | 93.0 | n/a |
| | 76.5 | 72.5 | 72.3 | 72.6 | **72.5** | 0.1 | 0.2 | 94.7 | n/a |
| | 107.1 | 101.7 | 101.5 | 101.5 | **101.6** | 0.1 | 0.1 | 94.8 | n/a |
| | 150.0 | 143.2 | 143.0 | 142.8 | **143.0** | 0.2 | 0.2 | 95.3 | n/a |
| | 210.0 | 206.8 | 208.1 | 206.6 | **207.2** | 0.8 | 0.4 | 98.7 | n/a |
| spent solutions day 1 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 49.3 | 49.3 | 49.4 | **49.3** | 0.1 | 0.1 | 90.2 | 99.1 |
| | 76.5 | 78.6 | 78.3 | 78.5 | **78.5** | 0.1 | 0.2 | 102.6 | 110.1 |
| | 107.1 | 98.8 | 98.9 | 98.8 | **98.9** | 0.1 | 0.1 | 92.3 | 101.3 |
| | 150.0 | 140.0 | 139.7 | 139.6 | **139.8** | 0.2 | 0.1 | 93.2 | 99.7 |
| | 210.0 | 205.2 | 205.5 | 204.9 | **205.2** | 0.3 | 0.1 | 97.7 | 101.7 |
| fresh solutions day 2 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 44.2 | 44.3 | 44.2 | **44.2** | 0.0 | 0.1 | 80.9 | n/a |
| | 76.5 | 72.9 | 72.7 | 72.9 | **72.8** | 0.1 | 0.2 | 95.2 | n/a |
| | 107.1 | 101.0 | 101.4 | 100.8 | **101.1** | 0.3 | 0.3 | 94.4 | n/a |
| | 150.0 | 143.7 | 144.3 | 143.7 | **143.9** | 0.3 | 0.2 | 95.9 | n/a |
| | 210.0 | 213.1 | 213.4 | 213.1 | **213.2** | 0.2 | 0.1 | 101.5 | n/a |
| spent solutions day 2 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 52.4 | 52.3 | 52.2 | **52.3** | 0.1 | 0.3 | 95.6 | 102.8 |
| | 76.5 | 73.3 | 73.3 | 73.3 | **73.3** | 0.0 | 0.1 | 95.8 | 101.2 |
| | 107.1 | 101.4 | 101.8 | 101.6 | **101.6** | 0.2 | 0.2 | 94.9 | 100.0 |
| | 150.0 | 142.5 | 145.8 | 145.8 | **144.7** | 1.9 | 1.3 | 96.5 | 101.2 |
| | 210.0 | 213.7 | 212.5 | 215.4 | **213.9** | 1.5 | 0.7 | 101.8 | 103.2 |
| fresh solutions day 3 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 46.4 | 46.6 | 46.2 | **46.4** | 0.2 | 0.4 | 84.8 | n/a |
| | 76.5 | 70.8 | 71.3 | 71.2 | **71.1** | 0.2 | 0.3 | 92.9 | n/a |
| | 107.1 | 99.9 | 99.9 | 100.3 | **100.0** | 0.2 | 0.2 | 93.4 | n/a |
| | 150.0 | 143.4 | 143.4 | 143.5 | **143.4** | 0.0 | 0.0 | 95.6 | n/a |
| | 210.0 | 214.1 | 214.2 | 214.2 | **214.2** | 0.0 | 0.0 | 102.0 | n/a |
| spent solutions day 3 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 51.8 | 51.9 | 51.9 | **51.9** | 0.0 | 0.1 | 94.9 | 117.3 |
| | 76.5 | 74.6 | 74.5 | 74.6 | **74.5** | 0.0 | 0.1 | 97.4 | 102.3 |
| | 107.1 | 102.5 | 101.3 | 102.0 | **101.9** | 0.6 | 0.6 | 95.2 | 100.9 |
| | 150.0 | 147.4 | 147.3 | 146.5 | **147.1** | 0.5 | 0.3 | 98.0 | 102.2 |
| | 210.0 | 207.8 | 207.4 | 209.2 | **208.1** | 1.0 | 0.5 | 99.1 | 97.6 |
| spent solutions day 4 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 51.7 | 51.4 | 51.8 | **51.6** | 0.2 | 0.4 | 94.3 | 111.3 |
| | 76.5 | 72.7 | 72.7 | 72.2 | **72.5** | 0.2 | 0.3 | 94.8 | 102.0 |
| | 107.1 | 99.3 | 98.8 | 98.9 | **99.0** | 0.3 | 0.3 | 92.4 | 99.0 |
| | 150.0 | 142.7 | 143.0 | 143.1 | **142.9** | 0.2 | 0.1 | 95.3 | 99.7 |
| | 210.0 | 218.3 | 218.5 | 219.0 | **218.6** | 0.3 | 0.1 | 104.1 | 102.1 |

below LoQ – below Limit of Quantification, i.e. below 0.010 mg/L; SD – standard deviation; RSD – relative standard deviation; n/a – not applicable.

**Table 7. Results – Run 4 (Repeated Run 3 since the positive control was below the acceptance criteria) 13.06.2011 – 17.06.2011**

| | Sample: Nominal concentration of carbamazepine [mg/L] | Determined concentration of carbamazepine in sample (triple analysis) [mg/L] | | | Mean determined concentration of carbamazepine [mg/L] | SD [mg/L] | RSD [%] | Recovery of nominal concentration [%] | Recovery of initial determined concentration [%] |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | | | | | |
| fresh solutions day 0 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 48.7 | 48.7 | 48.5 | **48.6** | 0.1 | 0.2 | 88.9 | n/a |
| | 76.5 | 74.6 | 74.5 | 74.3 | **74.5** | 0.2 | 0.3 | 97.3 | n/a |
| | 107.1 | 106.0 | 106.4 | 105.5 | **106.0** | 0.5 | 0.5 | 98.9 | n/a |
| | 150.0 | 152.8 | 152.4 | 153.3 | **152.9** | 0.5 | 0.3 | 101.9 | n/a |
| | 210.0 | 209.6 | 220.2 | 219.6 | **216.5** | 6.0 | 2.8 | 103.1 | n/a |
| fresh solutions day 1 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 47.5 | 47.5 | 46.1 | **47.0** | 0.8 | 1.7 | 86.0 | n/a |
| | 76.5 | 75.3 | 75.1 | 75.5 | **75.3** | 0.2 | 0.3 | 98.4 | n/a |
| | 107.1 | 105.9 | 105.6 | 105.4 | **105.7** | 0.3 | 0.2 | 98.6 | n/a |
| | 150.0 | 148.8 | 148.9 | 149.0 | **148.9** | 0.1 | 0.1 | 99.3 | n/a |
| | 210.0 | 218.9 | 221.6 | 221.0 | **220.5** | 1.4 | 0.6 | 105.0 | n/a |
| spent solutions day 1 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 52.9 | 52.8 | 52.8 | **52.8** | 0.1 | 0.1 | 96.6 | 108.6 |
| | 76.5 | 75.3 | 75.1 | 75.4 | **75.3** | 0.2 | 0.2 | 98.4 | 101.1 |
| | 107.1 | 105.3 | 105.2 | 105.0 | **105.2** | 0.1 | 0.1 | 98.2 | 99.2 |
| | 150.0 | 147.1 | 147.4 | 148.3 | **147.6** | 0.6 | 0.4 | 98.4 | 96.6 |
| | 210.0 | 214.3 | 212.8 | 214.1 | **213.7** | 0.8 | 0.4 | 101.8 | 98.7 |
| fresh solutions day 2 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 52.5 | 49.2 | 52.3 | **51.4** | 1.9 | 3.7 | 93.9 | n/a |
| | 76.5 | 75.4 | 76.0 | 75.6 | **75.7** | 0.3 | 0.4 | 98.9 | n/a |
| | 107.1 | 106.5 | 106.5 | 106.0 | **106.3** | 0.3 | 0.2 | 99.3 | n/a |
| | 150.0 | 137.3 | 137.7 | 137.6 | **137.5** | 0.2 | 0.2 | 91.7 | n/a |
| | 210.0 | 222.5 | 222.8 | 224.4 | **223.2** | 1.0 | 0.5 | 106.3 | n/a |
| spent solutions day 2 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 51.5 | 51.2 | 51.6 | **51.4** | 0.2 | 0.3 | 94.0 | 109.4 |
| | 76.5 | 74.4 | 74.5 | 75.1 | **74.7** | 0.4 | 0.5 | 97.6 | 99.2 |
| | 107.1 | 105.2 | 105.3 | 105.5 | **105.4** | 0.1 | 0.1 | 98.4 | 99.7 |
| | 150.0 | 148.8 | 148.5 | 147.4 | **148.2** | 0.7 | 0.5 | 98.8 | 99.5 |
| | 210.0 | 225.9 | 226.1 | 225.8 | **226.0** | 0.2 | 0.1 | 107.6 | 102.5 |
| fresh solutions day 3 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 49.2 | 49.4 | 50.7 | **49.8** | 0.8 | 1.7 | 91.0 | n/a |
| | 76.5 | 75.3 | 75.6 | 75.5 | **75.5** | 0.2 | 0.2 | 98.7 | n/a |
| | 107.1 | 105.2 | 104.0 | 104.8 | **104.7** | 0.6 | 0.6 | 97.7 | n/a |
| | 150.0 | 147.4 | 147.3 | 147.1 | **147.2** | 0.2 | 0.1 | 98.1 | n/a |
| | 210.0 | 225.1 | 223.3 | 223.3 | **223.9** | 1.0 | 0.5 | 106.6 | n/a |
| spent solutions day 3 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 52.2 | 52.1 | 52.4 | **52.2** | 0.1 | 0.2 | 95.5 | 101.7 |
| | 76.5 | 75.4 | 75.5 | 74.1 | **75.0** | 0.8 | 1.0 | 98.1 | 99.2 |
| | 107.1 | 104.4 | 104.6 | 103.9 | **104.3** | 0.4 | 0.4 | 97.4 | 98.1 |
| | 150.0 | 139.1 | 139.2 | 139.3 | **139.2** | 0.1 | 0.1 | 92.8 | 101.2 |
| | 210.0 | 237.7 | 237.9 | 237.4 | **237.7** | 0.3 | 0.1 | 113.2 | 106.5 |
| spent solutions day 4 | control | 0.0 | 0.0 | 0.0 | **below LoQ** | 0.0 | - | - | - |
| | 54.7 | 53.6 | 53.1 | 53.4 | **53.4** | 0.2 | 0.5 | 97.6 | 107.2 |
| | 76.5 | 76.2 | 76.6 | 76.9 | **76.6** | 0.3 | 0.5 | 100.1 | 101.4 |
| | 107.1 | 106.7 | 106.7 | 106.8 | **106.7** | 0.1 | 0.1 | 99.6 | 102.0 |
| | 150.0 | 147.0 | 146.9 | 147.1 | **147.0** | 0.1 | 0.0 | 98.0 | 99.8 |
| | 210.0 | 247.1 | 247.9 | 247.1 | **247.4** | 0.5 | 0.2 | 117.8 | 110.5 |

below LoQ – below Limit of Quantification, i.e. below 0.010 mg/L; SD – standard deviation; RSD – relative standard deviation; n/a – not applicable.

## Compilation of results for carbamazepine

Carbamazepine - Zebrafish Embryo Toxicity Test Run 1 21.03.2011 – 25.03.2011

| | | nominal concentration of carbamazepine [mg/L] | | | | |
|---|---|---|---|---|---|---|
| | sample | 54.7 | 76.5 | 107.1 | 150 | 210 |
| Mean determined concentration of carbamazepine [mg/L] | fresh solution day 0 | 50.5 | 69.6 | 97.0 | 136.2 | 190.1 |
| | fresh solution day 1 | 50.2 | 67.9 | 95.9 | 137.6 | 192.1 |
| | spent solution day 1 | 47.4 | 69.9 | 95.9 | 141.0 | 188.0 |
| | fresh solution day 2 | 47.8 | 69.5 | 96.7 | 138.2 | 196.7 |
| | spent solution day 2 | 47.5 | 70.5 | 96.6 | 137.1 | 193.2 |
| | fresh solution day 3 | 50.8 | 72.5 | 99.9 | 142.7 | 206.6 |
| | spent solution day 3 | 50.0 | 73.1 | 97.8 | 141.0 | 223.8 |
| | spent solution day 4 | 50.3 | 73.1 | 97.6 | 141.2 | 219.7 |
| | average | 49.9 | 71.4 | 98.3 | 140.6 | 202.2 |
| | recovery | 91.2% | 93.3% | 91.8% | 93.7% | 96.3% |
| | TWA | 49.9 | 70.8 | 97.2 | 139.4 | 201.2 |
| | recovery | 91.2% | 92.6% | 90.8% | 92.9% | 95.8% |
| | GA | 49.3 | 70.7 | 97.2 | 139.4 | 200.9 |
| | recovery | 90.1% | 92.4% | 90.8% | 92.9% | 95.7% |

Carbamazepine - Zebrafish Embryo Toxicity Test Run 2 04.04.2011 – 08.04.2011

| | | nominal concentration of carbamazepine [mg/L] | | | | |
|---|---|---|---|---|---|---|
| | sample | 54.7 | 76.5 | 107.1 | 150 | 210 |
| Mean determined concentration of carbamazepine [mg/L] | fresh solution day 0 | 53.2 | 74.4 | 104.3* | 146.2 | 188.7 |
| | fresh solution day 1 | 51.0 | 71.7 | 102.2 | 141.1 | 200.6 |
| | spent solution day 1 | 51.3 | 71.4 | 104.3 | 144.5 | 203.7 |
| | fresh solution day 2 | 53.3 | 71.5 | 101.4 | 143.8 | 207.2 |
| | spent solution day 2 | 51.3 | 74.4 | 106.6 | 149.2 | 209.4 |
| | fresh solution day 3 | 52.0 | 72.9 | 99.9 | 143.1 | 204.6 |
| | spent solution day 3 | 52.9 | 76.5 | 104.1 | 147.9 | 227.1 |
| | spent solution day 4 | 52.4 | 73.5 | 101.6 | 142.6 | 214.2 |
| | average | 52.5 | 73.6 | 103.5 | 145.4 | 207.3 |
| | recovery | 96.0% | 96.2% | 96.6% | 96.9% | 98.7% |
| | TWA | 52.2 | 73.3 | 103.0* | 144.8 | 206.9 |
| | recovery | 95.4% | 95.8% | 96.2% | 96.5% | 98.5% |
| | GA | 52.2 | 73.3 | 103.0 | 144.8 | 206.7 |
| | recovery | 95.4% | 95.8% | 96.2% | 96.5% | 98.4% |

Average: arithmetic average
TWA: time-weighted mean, calculated according to OECD Guideline 211 of October 2008, Annex 6 (TWA is applicable for semi-static tests where the concentration of the test item declines over the period between renewals)
GA: geometric average
Recovery: percent of nominal value
* where the determined concentrations of carbamazepine were the same for fresh and spent solutions mathematical error occurs in calculation of TWA. In order to calculate TWA the value of 0.01 was added to value of Mean determined concentration of carbamazepine for fresh solution

Carbamazepine - Zebrafish Embryo Toxicity Test Run 3 18.04.2011 – 22.04.2011

| | | nominal concentration of carbamazepine [mg/L] | | | | |
|---|---|---|---|---|---|---|
| | sample | 54.7 | 76.5 | 107.1 | 150 | 210 |
| Mean determined concentration of carbamazepine [mg/L] | fresh solution day 0 | 49.8 | 71.3 | 97.6 | 140.2 | 201.8 |
| | fresh solution day 1 | 50.9 | 72.5 | 101.6* | 143.0 | 207.2 |
| | spent solution day 1 | 49.3 | 78.5 | 98.9 | 139.8 | 205.2 |
| | fresh solution day 2 | 44.2 | 72.8 | 101.1 | 143.9 | 213.2 |
| | spent solution day 2 | 52.3 | 73.3 | 101.6 | 144.7 | 213.9 |
| | fresh solution day 3 | 46.4 | 71.1 | 100.0 | 143.4 | 214.2 |
| | spent solution day 3 | 51.9 | 74.5 | 101.9 | 147.1 | 208.1 |
| | spent solution day 4 | 51.6 | 72.5 | 99.0 | 142.9 | 218.6 |
| | average | 50.1 | 73.7 | 101.0 | 143.9 | 210.2 |
| | recovery | 91.6% | 96.3% | 94.3% | 95.9% | 100.1% |
| | TWA | 49.5 | 73.3 | 100.2* | 143.1 | 210.3 |
| | recovery | 90.5% | 95.8% | 93.6% | 95.4% | 100.1% |
| | GA | 49.5 | 73.3 | 100.2 | 143.1 | 210.2 |
| | recovery | 90.5% | 95.8% | 93.6% | 95.4% | 100.1% |

Carbamazepine - Zebrafish Embryo Toxicity Test Run 4 13.06.2011 – 17.06.2011

| | | nominal concentration of carbamazepine [mg/L] | | | | |
|---|---|---|---|---|---|---|
| | sample | 54.7 | 76.5 | 107.1 | 150 | 210 |
| Mean determined concentration of carbamazepine [mg/L] | fresh solution day 0 | 48.6 | 74.5 | 106.0 | 152.9 | 216.5 |
| | fresh solution day 1 | 47.0 | 75.3 | 105.7 | 148.9 | 220.5 |
| | spent solution day 1 | 52.8 | 75.3 | 105.2 | 147.6 | 213.7 |
| | fresh solution day 2 | 51.4 | 75.7 | 106.3 | 137.5 | 223.2 |
| | spent solution day 2 | 51.4 | 74.7 | 105.4 | 148.2 | 226.0 |
| | fresh solution day 3 | 49.8 | 75.5 | 104.7 | 147.2 | 223.9 |
| | spent solution day 3 | 52.2 | 75.0 | 104.3 | 139.2 | 237.7 |
| | spent solution day 4 | 53.4 | 76.6 | 106.7 | 147.0 | 247.4 |
| | average | 51.3 | 75.5 | 105.7 | 146.5 | 224.3 |
| | recovery | 91.2% | 93.3% | 91.8% | 93.7% | 96.3% |
| | TWA | 50.8 | 75.3 | 105.5 | 147.1 | 226.0 |
| | recovery | 92.9% | 98.4% | 98.5% | 98.1% | 107.6% |
| | GA | 50.8 | 75.3 | 105.5 | 146.0 | 225.9 |
| | recovery | 92.9% | 98.4% | 98.5% | 97.3% | 107.6% |

Average: arithmetic average
TWA: time-weighted mean, calculated according to OECD Guideline 211 of October 2008, Annex 6 (TWA is applicable for semi-static tests where the concentration of the test item declines over the period between renewals)
GA: geometric average
Recovery: percent of nominal value
* where the determined concentrations of carbamazepine were the same for fresh and spent solutions mathematical error occurs in calculation of TWA. In order to calculate TWA the value of 0.01 was added to value of Mean determined concentration of carbamazepine for fresh solution

## 2. DETERMINATION OF PROCHLORAZ IN TEST SOLUTIONS

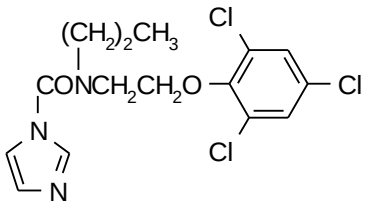
### 2.1. Detected Substance

**Prochloraz:** *N*-propyl-*N*-[2-(2,4,6-trichlorophenoxy)ethyl]imidazole-1-carboxamide

CAS number: [67747-09-5]

Molecular formula: $C_{15}H_{16}Cl_3N_3O_2$

Molecular weight: 376.7 g/mol




### 2.2. Analytical Procedure

2.2.1. Reagents and solvents

- acetonitrile for HPLC,
- deionized water,
- prochloraz standard, Reference material, purity 99.1% (HPLC), Pestanal, Fluka cat. No.45631, Batch SZE6220X,
- standard solution of 1 mg/mL prochloraz in deionised water and stock solutions: 0.1, 0.5, 1.0, 5.0, 10.0 and 100.0 µg/mL of prochloraz in deionised water.

2.2.2. Apparatus

- laboratory glassware,
- analytical balance,
- liquid chromatograph Varian 920-LC with DAD detector (Varian, USA).

The following liquid chromatography parameters were used:

| | |
|---|---|
| Column | Pursuit XRs 3 C18, l = 150 mm, ϕ = 4.6 mm |
| Mobile phase | acetonitrile : water (80 : 20, v/v) |
| Wavelength | 220 nm |
| Flow rate | 0.4 mL/min. |
| Injected volume | 5 µL |

### 2.3. Preparation of Samples for Chromatography Analysis

From each sample (deionised water, deionised water spiked with prochloraz, test sample) 5 µL was injected to the chromatographic column. If necessary, the sample was diluted with deionized water (section 2.5).

## 2.4. Validation of Analytical Procedure

Linearity of response, specificity, precision, recovery, limit of quantification and detection for prochloraz were assessed to validate the analytical procedure.

Calibration curve

Stock solutions containing 0.1, 0.5, 1.0, 5.0 and 10.0 μg/mL of prochloraz were injected successively to the chromatographic column and chromatograms were recorded. The standard curve (field of the peak versus quantity of the standard) is linear with a regression coefficient ($r^2$) of 0.9997. The linearity of the analytical procedure ranges from 0.1 μg/mL to 10.0 μg/mL. The standard curve of prochloraz is presented in Figure 2.

Specificity

The specificity of the analytical procedure was estimated based on analysis of chromatograms generated for control sample (i.e. deionised water) and fortification samples (i.e. deionised water spiked with prochloraz). Considering the results of the analysis no signal of prochloraz was overlapping with matrix signal of control sample (i.e. deionised water) in experimental conditions. Therefore the specificity of the analytical procedure was demonstrated.

Precision

Precision is determined as the repeatability (RSD – relative standard deviation, %). The results of repeatability for prochloraz determined in fortification samples (i.e. samples of deionised water spiked with prochloraz) are presented in Table 8.

Extraction recovery level

In order to study the recovery, the stock solution of prochloraz was added to control sample (i.e. deionised water) and analysed by the analytical procedure. The results are presented in Table 8.

**Table 8. The recovery level for prochloraz**

| Fortification level [mg/L] | Determined concentration of prochloraz (n=5) [mg/L] | | | | | Mean determined concentration of prochloraz [mg/L] | SD [mg/L] | RSD [%] | Recovery [%] |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | | | | |
| control | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | - | - |
| 0.01 | 0.100 | 0.093 | 0.088 | 0.104 | 0.105 | 0.098 | 0.007 | 7.4 | 98.1 |
| 10.00 | 9.860 | 9.579 | 9.956 | 9.587 | 9.609 | 9.718 | 0.177 | 1.8 | 97.2 |

SD – standard deviation
RSD – relative standard deviation

**Calibration Report :**
File : Prochloraz

Component : Prochloraz
Polynom : y = b x +a
a = 0,01360
b = 0,53569
Correlation Coef. : 0,9997
Weighting : None
Force zero : No

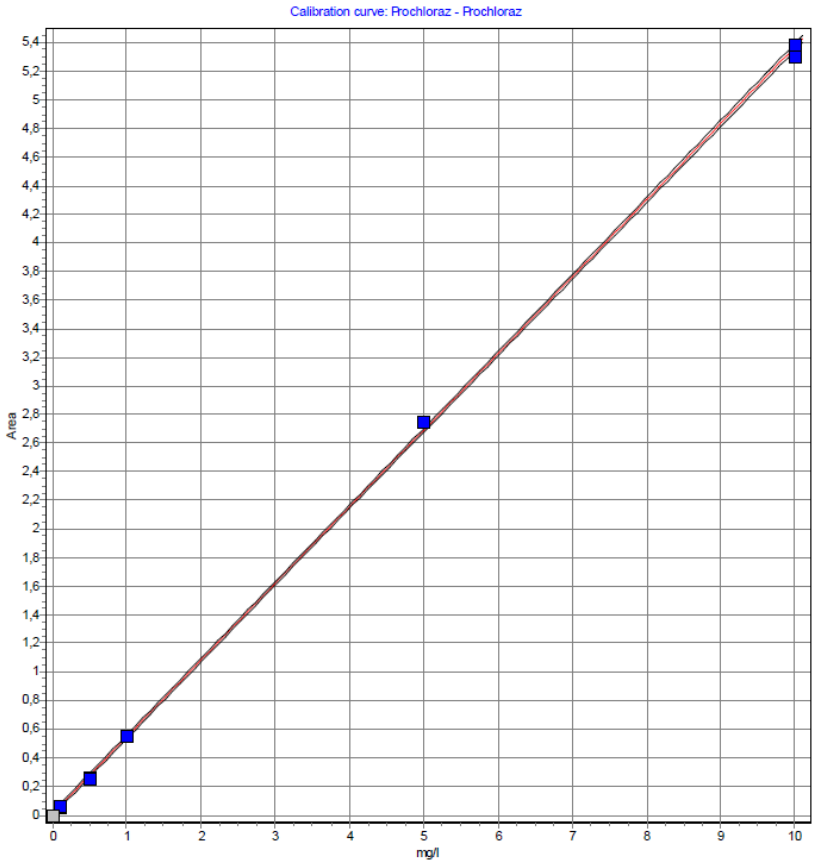



**Figure 2. Standard curve for prochloraz**

Limit of quantification and detection

*Limit of Quantification* was estimated as the lowest concentration of prochloraz, at which an acceptable mean recovery is obtained (normally 70 – 100 % with a relative standard deviation of preferably ≤ 20 %). *Limit of Detection* was estimated as the lowest concentration of prochloraz that the analytical procedure can reliably differentiate from background noise. The results are presented in Table 9.

**Table 9. Limit of Quantification and Detection of prochloraz in water**

| Detected substance | Limit of Quantification [mg/L] | Limit of Detection [mg/L] |
|---|---|---|
| Prochloraz | 0.100 | 0.001 |

## 2.5. Analytical procedure during the ZFET test

The stock solution of prochloraz was freshly prepared for each run and stored according to Trial Plan (TP_ZFET_OECD_2bV01.1 of 17th January 2011). The stock solution was analyzed at test initiation and reanalyzed at each renewal. All test vessels were pre-saturated with the respective nominal concentration and controls before test initiation. At test initiation and at each renewal fresh solutions were prepared and test samples were collected before division into wells in plates. At each renewal and at test termination test samples of spent solutions were combined from wells in plates.

The determinations were performed in triplicates for each test sample of respective nominal concentration of prochloraz and control. Test samples were diluted with deionized water (Table 10) and then analysed by the analytical procedure described in section 2.2.

**Table 10. Preparation of samples for analysis.**

| Nominal concentration of prochloraz [mg/L] | Volume of test sample [mL] | Volume of deionised water [mL] | Total volume of sample [mL] |
|---|---|---|---|
| control | 2.0 | 0.0 | 2.0 |
| 0.5 | 2.0 | 0.0 | 2.0 |
| 1.0 | 2.0 | 0.0 | 2.0 |
| 2.0 | 2.0 | 0.0 | 2.0 |
| 4.0 | 2.0 | 0.0 | 2.0 |
| 8.0 | 2.0 | 0.0 | 2.0 |
| 20.0 | 2.0 | 2.0 | 4.0 |

## 2.6. Results

Test samples of fresh solutions at test initiation, fresh and spent solutions at each renewal and spent solutions at test termination were analysed.

In samples of fresh solutions in Run 1 (Table 11) the concentration of prochloraz was determined in the range of 81.9 – 105.3% of nominal concentration. In samples of fresh solutions in Run 3 (Table 13) the concentration of prochloraz was determined in the range of 78.2 – 97.8% of nominal concentration. The results generated in Run 1 and Run 3 confirm correct preparation of test solutions.

In samples of fresh solutions in Run 2 (Table 12) the concentration of prochloraz was determined in the range of 62.8 – 85.9% of nominal concentration. However, the determined concentration of prochloraz in the stock solution used in Run 2 was between 56.8 and 76.8% of nominal (Table 12).

The Trial Plan (TP_ZFET_OECD_2bV01.1 of 17th January 2011) recommends stirring with heating up to 35°C to ensure the complete dissolution of prochloraz (and no separation of possibly non-dissolved prochloraz). The determined concentration of prochloraz in the stock solutions prepared strictly according to these recommendations was in the range of 56.8 – 85.8% of nominal at test initiation. The results generated for the reanalyzed stock solution show that the determined concentration of prochloraz increased with time of storage between renewals. The stock solution prepared for each run was stored between renewals at temperature of approximately 26°C what could enable further dissolution of prochloraz.

In samples of spent solutions in Run 1 (Table 11) the concentration of prochloraz was determined in the range of 88.6 – 108.6% of initial determined concentration. In samples of spent solutions in Run 2 (Table 12) the concentration of prochloraz was determined in the range of 87.1 – 109.3% of initial determined concentration. In samples of spent solutions in Run 3 (Table 13) the concentration of prochloraz was determined in the range of 79.8 – 115.7% of initial determined concentration. The results generated confirm that the concentration of prochloraz was stable in periods between renewals.

**Table 11. Results – Run 1 21.03.2011 – 25.03.2011**

| | Sample: Nominal concentration of prochloraz [mg/L] | Determined concentration of prochloraz in sample (triple analysis) [mg/L] | | | Mean determined concentration of prochloraz [mg/L] | SD [mg/L] | RSD [%] | Recovery of nominal concentration [%] | Recovery of initial determined concentration [%] |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | | | | | |
| fresh solutions day 0 | control | 0.00 | 0.00 | 0.00 | **below LoQ** | 0.00 | - | - | - |
| | 0.5 | 0.43 | 0.42 | 0.40 | **0.42** | 0.01 | 2.7 | 83.3 | n/a |
| | 1.0 | 0.80 | 0.83 | 0.82 | **0.82** | 0.01 | 1.7 | 81.9 | n/a |
| | 2.0 | 1.78 | 1.76 | 1.77 | **1.77** | 0.01 | 0.5 | 88.4 | n/a |
| | 4.0 | 3.44 | 3.45 | 3.51 | **3.47** | 0.04 | 1.1 | 86.6 | n/a |
| | 8.0 | 7.16 | 7.00 | 6.80 | **6.99** | 0.18 | 2.6 | 87.4 | n/a |
| stock | 20.0 | 17.39 | 17.24 | 16.54 | 17.06 | 0.45 | 2.6 | 85.3 | n/a |
| fresh solutions day 1 | control | 0.00 | 0.00 | 0.00 | **below LoQ** | 0.00 | - | - | - |
| | 0.5 | 0.45 | 0.47 | 0.45 | **0.46** | 0.01 | 3.1 | 91.2 | n/a |
| | 1.0 | 0.91 | 0.86 | 0.91 | **0.89** | 0.03 | 3.2 | 89.4 | n/a |
| | 2.0 | 1.85 | 1.79 | 1.91 | **1.85** | 0.06 | 3.4 | 92.5 | n/a |
| | 4.0 | 3.86 | 3.92 | 3.98 | **3.92** | 0.06 | 1.5 | 98.1 | n/a |
| | 8.0 | 8.20 | 7.98 | 7.79 | **7.99** | 0.21 | 2.6 | 99.9 | n/a |
| stock | 20.0 | 18.68 | 18.92 | 18.29 | 18.63 | 0.32 | 1.7 | 93.1 | n/a |
| spent solutions day 1 | control | 0.00 | 0.00 | 0.00 | **below LoQ** | 0.00 | - | - | - |
| | 0.5 | 0.43 | 0.45 | 0.47 | **0.45** | 0.02 | 5.1 | 90.4 | 108.6 |
| | 1.0 | 0.80 | 0.83 | 0.83 | **0.82** | 0.02 | 2.0 | 82.0 | 100.2 |
| | 2.0 | 1.64 | 1.60 | 1.62 | **1.62** | 0.02 | 1.0 | 81.0 | 91.6 |
| | 4.0 | 3.12 | 3.14 | 3.14 | **3.13** | 0.01 | 0.4 | 78.4 | 90.4 |
| | 8.0 | 6.40 | 6.59 | 6.53 | **6.51** | 0.10 | 1.5 | 81.3 | 93.1 |
| fresh solutions day 2 | control | 0.00 | 0.00 | 0.00 | **below LoQ** | 0.00 | - | - | - |
| | 0.5 | 0.51 | 0.46 | 0.44 | **0.47** | 0.04 | 7.7 | 93.9 | n/a |
| | 1.0 | 0.98 | 0.99 | 0.97 | **0.98** | 0.01 | 1.4 | 98.0 | n/a |
| | 2.0 | 2.02 | 2.11 | 2.02 | **2.05** | 0.05 | 2.4 | 102.5 | n/a |
| | 4.0 | 4.13 | 4.14 | 4.18 | **4.15** | 0.02 | 0.6 | 103.7 | n/a |
| | 8.0 | 8.31 | 8.42 | 8.36 | **8.36** | 0.05 | 0.6 | 104.5 | n/a |
| stock | 20.0 | 18.46 | 18.75 | 18.45 | 18.56 | 0.17 | 0.9 | 92.8 | n/a |
| spent solutions day 2 | control | 0.00 | 0.00 | 0.00 | **below LoQ** | 0.00 | - | - | - |
| | 0.5 | 0.49 | 0.51 | 0.48 | **0.49** | 0.01 | 3.0 | 98.8 | 108.4 |
| | 1.0 | 0.94 | 0.90 | 0.86 | **0.90** | 0.04 | 4.3 | 90.0 | 100.7 |
| | 2.0 | 1.78 | 1.74 | 1.76 | **1.76** | 0.02 | 1.2 | 88.0 | 95.1 |
| | 4.0 | 3.51 | 3.52 | 3.58 | **3.53** | 0.04 | 1.1 | 88.3 | 90.1 |
| | 8.0 | 7.49 | 7.12 | 7.52 | **7.37** | 0.22 | 3.0 | 92.2 | 92.3 |
| fresh solutions day 3 | control | 0.00 | 0.00 | 0.00 | **below LoQ** | 0.00 | - | - | - |
| | 0.5 | 0.47 | 0.44 | 0.46 | **0.46** | 0.02 | 3.6 | 91.9 | n/a |
| | 1.0 | 0.97 | 0.96 | 0.93 | **0.96** | 0.02 | 2.3 | 95.5 | n/a |
| | 2.0 | 2.03 | 2.02 | 1.99 | **2.01** | 0.02 | 1.0 | 100.6 | n/a |
| | 4.0 | 4.07 | 4.09 | 4.10 | **4.09** | 0.02 | 0.5 | 102.2 | n/a |
| | 8.0 | 8.52 | 8.32 | 8.42 | **8.42** | 0.10 | 1.2 | 105.3 | n/a |
| stock | 20.0 | 19.02 | 19.04 | 18.80 | 18.95 | 0.13 | 0.7 | 94.8 | |
| spent solutions day 3 | control | 0.00 | 0.00 | 0.00 | **below LoQ** | 0.00 | - | - | - |
| | 0.5 | 0.45 | 0.47 | 0.47 | **0.47** | 0.01 | 3.0 | 93.3 | 99.3 |
| | 1.0 | 0.89 | 0.92 | 0.98 | **0.93** | 0.04 | 4.8 | 92.7 | 94.6 |
| | 2.0 | 1.86 | 1.88 | 1.86 | **1.87** | 0.01 | 0.7 | 93.3 | 91.1 |
| | 4.0 | 3.68 | 3.67 | 3.68 | **3.68** | 0.01 | 0.2 | 91.9 | 88.6 |
| | 8.0 | 7.72 | 7.39 | 7.53 | **7.55** | 0.16 | 2.1 | 94.3 | 90.2 |
| spent solutions day 4 | control | 0.00 | 0.00 | 0.00 | **below LoQ** | 0.00 | - | - | - |
| | 0.5 | 0.47 | 0.47 | 0.45 | **0.46** | 0.01 | 2.2 | 92.4 | 100.5 |
| | 1.0 | 0.96 | 0.89 | 0.95 | **0.93** | 0.04 | 4.0 | 93.4 | 97.8 |
| | 2.0 | 1.87 | 1.87 | 1.84 | **1.86** | 0.01 | 0.7 | 93.0 | 92.5 |
| | 4.0 | 3.64 | 3.64 | 3.63 | **3.64** | 0.01 | 0.1 | 90.9 | 89.0 |
| | 8.0 | 7.36 | 7.55 | 7.52 | **7.48** | 0.11 | 1.4 | 93.4 | 88.8 |

below LoQ – below Limit of Quantification, i.e. below 0.100 mg/L; SD – standard deviation,; RSD – relative standard deviation; n/a – not applicable.

**Table 12. Results – Run 2 04.04.2011 – 08.04.2011**

| | Sample: Nominal concentration of prochloraz [mg/L] | Determined concentration of prochloraz in sample (triple analysis) [mg/L] | | | Mean determined concentration of prochloraz [mg/L] | SD [mg/L] | RSD [%] | Recovery of nominal concentration [%] | Recovery of initial determined concentration [%] |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | | | | | |
| fresh solutions day 0 | control | 0.00 | 0.00 | 0.00 | **below LoQ** | 0.00 | - | - | - |
| | 0.5 | 0.32 | 0.34 | 0.29 | **0.32** | 0.02 | 7.0 | 63.2 | n/a |
| | 1.0 | 0.66 | 0.64 | 0.63 | **0.64** | 0.01 | 2.1 | 64.2 | n/a |
| | 2.0 | 1.24 | 1.29 | 1.24 | **1.26** | 0.03 | 2.3 | 62.8 | n/a |
| | 4.0 | 2.50 | 2.52 | 2.56 | **2.53** | 0.03 | 1.2 | 63.2 | n/a |
| | 8.0 | 5.34 | 5.13 | 5.32 | **5.26** | 0.12 | 2.3 | 65.8 | n/a |
| stock | 20.0 | 11.16 | 11.11 | 11.82 | 11.36 | 0.39 | 3.5 | 56.8 | n/a |
| fresh solutions day 1 | control | 0.00 | 0.00 | 0.00 | **below LoQ** | 0.00 | - | - | - |
| | 0.5 | 0.37 | 0.36 | 0.36 | **0.36** | 0.01 | 1.7 | 72.5 | n/a |
| | 1.0 | 0.69 | 0.70 | 0.74 | **0.71** | 0.02 | 3.4 | 70.9 | n/a |
| | 2.0 | 1.44 | 1.34 | 1.42 | **1.40** | 0.05 | 3.6 | 70.0 | n/a |
| | 4.0 | 2.89 | 2.96 | 2.91 | **2.92** | 0.04 | 1.2 | 72.9 | n/a |
| | 8.0 | 5.96 | 5.74 | 5.91 | **5.87** | 0.11 | 1.9 | 73.4 | n/a |
| stock | 20.0 | 12.88 | 12.34 | 12.78 | 12.66 | 0.29 | 2.3 | 63.3 | n/a |
| spent solutions day 1 | control | 0.00 | 0.00 | 0.00 | **below LoQ** | 0.00 | - | - | - |
| | 0.5 | 0.30 | 0.28 | 0.25 | **0.28** | 0.02 | 8.9 | 55.2 | 87.3 |
| | 1.0 | 0.60 | 0.63 | 0.58 | **0.61** | 0.02 | 3.9 | 60.6 | 94.5 |
| | 2.0 | 1.19 | 1.20 | 1.25 | **1.21** | 0.03 | 2.8 | 60.7 | 96.6 |
| | 4.0 | 2.46 | 2.29 | 2.28 | **2.35** | 0.10 | 4.4 | 58.6 | 92.7 |
| | 8.0 | 4.91 | 4.88 | 4.67 | **4.82** | 0.13 | 2.8 | 60.2 | 91.6 |
| fresh solutions day 2 | control | 0.00 | 0.00 | 0.00 | **below LoQ** | 0.00 | - | - | - |
| | 0.5 | 0.36 | 0.32 | 0.34 | **0.34** | 0.02 | 5.7 | 67.9 | n/a |
| | 1.0 | 0.74 | 0.71 | 0.67 | **0.71** | 0.03 | 4.6 | 70.7 | n/a |
| | 2.0 | 1.55 | 1.60 | 1.53 | **1.56** | 0.03 | 2.1 | 77.9 | n/a |
| | 4.0 | 3.15 | 3.25 | 3.19 | **3.20** | 0.05 | 1.6 | 79.9 | n/a |
| | 8.0 | 6.37 | 6.39 | 6.44 | **6.40** | 0.04 | 0.6 | 80.0 | n/a |
| stock | 20.0 | 14.90 | 14.67 | 14.71 | 14.76 | 0.12 | 0.8 | 73.8 | n/a |
| spent solutions day 2 | control | 0.00 | 0.00 | 0.00 | **below LoQ** | 0.00 | - | - | - |
| | 0.5 | 0.36 | 0.38 | 0.36 | **0.37** | 0.01 | 2.3 | 73.7 | 101.6 |
| | 1.0 | 0.69 | 0.69 | 0.67 | **0.68** | 0.01 | 1.0 | 68.3 | 96.3 |
| | 2.0 | 1.33 | 1.53 | 1.33 | **1.40** | 0.12 | 8.3 | 69.8 | 99.6 |
| | 4.0 | 2.61 | 2.67 | 2.63 | **2.64** | 0.03 | 1.0 | 65.9 | 90.3 |
| | 8.0 | 5.46 | 5.25 | 5.52 | **5.41** | 0.14 | 2.6 | 67.6 | 92.2 |
| fresh solutions day 3 | control | 0.00 | 0.00 | 0.00 | **below LoQ** | 0.00 | - | - | - |
| | 0.5 | 0.36 | 0.36 | 0.35 | **0.35** | 0.01 | 2.4 | 71.0 | n/a |
| | 1.0 | 0.77 | 0.78 | - | **0.77** | 0.00 | 0.5 | 77.5 | n/a |
| | 2.0 | 1.59 | 1.63 | 1.63 | **1.61** | 0.02 | 1.5 | 80.7 | n/a |
| | 4.0 | 3.46 | 3.38 | 3.46 | **3.43** | 0.04 | 1.3 | 85.9 | n/a |
| | 8.0 | 6.58 | 6.90 | 7.00 | **6.83** | 0.22 | 3.2 | 85.3 | n/a |
| stock | 20.0 | 15.15 | 15.57 | 15.37 | 15.36 | 0.21 | 1.4 | 76.8 | n/a |
| spent solutions day 3 | control | 0.00 | 0.00 | 0.00 | **below LoQ** | 0.00 | - | - | - |
| | 0.5 | 0.36 | 0.39 | 0.37 | **0.37** | 0.01 | 3.6 | 74.2 | 109.3 |
| | 1.0 | 0.72 | 0.71 | 0.69 | **0.70** | 0.01 | 1.8 | 70.4 | 99.6 |
| | 2.0 | 1.41 | 1.38 | 1.44 | **1.41** | 0.03 | 2.3 | 70.5 | 90.4 |
| | 4.0 | 2.77 | 2.82 | 2.76 | **2.78** | 0.03 | 1.2 | 69.6 | 87.1 |
| | 8.0 | 5.71 | 5.48 | 5.59 | **5.59** | 0.12 | 2.1 | 69.9 | 87.4 |
| spent solutions day 4 | control | 0.00 | 0.00 | 0.00 | **below LoQ** | 0.00 | - | - | - |
| | 0.5 | 0.35 | 0.34 | 0.36 | **0.35** | 0.01 | 2.7 | 70.6 | 99.5 |
| | 1.0 | 0.74 | 0.74 | 0.76 | **0.75** | 0.01 | 1.7 | 74.8 | 96.5 |
| | 2.0 | 1.57 | 1.52 | 1.58 | **1.56** | 0.03 | 2.1 | 77.9 | 96.6 |
| | 4.0 | 3.04 | 3.08 | 3.04 | **3.05** | 0.02 | 0.7 | 76.3 | 88.8 |
| | 8.0 | 6.19 | 5.89 | 6.20 | **6.10** | 0.18 | 2.9 | 76.2 | 89.3 |

below LoQ – below Limit of Quantification, i.e. below 0.100 mg/L; SD – standard deviation,; RSD – relative standard deviation,; n/a – not applicable.

**Table 13. Results – Run 3 18.04.2011 – 22.04.2011**

| | Sample: Nominal concentration of prochloraz [mg/L] | Determined concentration of prochloraz in sample (triple analysis) [mg/L] | | | Mean determined concentration of prochloraz [mg/L] | SD [mg/L] | RSD [%] | Recovery of nominal concentration [%] | Recovery of initial determined concentration [%] |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | | | | | |
| fresh solutions day 0 | control | 0.00 | 0.00 | 0.00 | **below LoQ** | 0.00 | - | - | - |
| | 0.5 | 0.40 | 0.36 | 0.41 | **0.39** | 0.03 | 7.0 | 78.2 | n/a |
| | 1.0 | 0.86 | 0.81 | 0.86 | **0.84** | 0.03 | 3.6 | 84.5 | n/a |
| | 2.0 | 1.66 | 1.64 | 1.70 | **1.67** | 0.03 | 1.6 | 83.3 | n/a |
| | 4.0 | 3.25 | 3.14 | 3.11 | **3.17** | 0.07 | 2.2 | 79.2 | n/a |
| | 8.0 | 6.56 | 6.41 | 6.65 | **6.54** | 0.12 | 1.8 | 81.7 | n/a |
| stock | 20.0 | 14.16 | 14.52 | 14.65 | 14.45 | 0.25 | 1.7 | 72.2 | n/a |
| fresh solutions day 1 | control | 0.00 | 0.00 | 0.00 | **below LoQ** | 0.00 | - | - | - |
| | 0.5 | 0.41 | 0.44 | 0.43 | **0.43** | 0.02 | 3.9 | 85.3 | n/a |
| | 1.0 | 0.80 | 0.88 | 0.84 | **0.84** | 0.04 | 4.6 | 84.0 | n/a |
| | 2.0 | 1.88 | 1.84 | 1.77 | **1.83** | 0.05 | 2.8 | 91.4 | n/a |
| | 4.0 | 3.63 | 3.62 | 3.35 | **3.54** | 0.16 | 4.5 | 88.4 | n/a |
| | 8.0 | 6.99 | 7.31 | 7.15 | **7.15** | 0.16 | 2.2 | 89.3 | n/a |
| stock | 20.0 | 16.61 | 17.24 | 16.38 | 16.74 | 0.44 | 2.7 | 83.7 | n/a |
| spent solutions day 1 | control | 0.00 | 0.00 | 0.00 | **below LoQ** | 0.00 | - | - | - |
| | 0.5 | 0.42 | 0.37 | 0.40 | **0.40** | 0.02 | 5.4 | 79.5 | 101.6 |
| | 1.0 | 0.70 | 0.67 | 0.65 | **0.67** | 0.02 | 3.4 | 67.4 | 79.8 |
| | 2.0 | 1.33 | 1.46 | 1.42 | **1.40** | 0.07 | 5.0 | 70.1 | 84.1 |
| | 4.0 | 2.82 | 2.74 | 2.77 | **2.78** | 0.04 | 1.5 | 69.4 | 87.7 |
| | 8.0 | 5.86 | 5.81 | 5.54 | **5.74** | 0.17 | 3.0 | 71.7 | 87.7 |
| fresh solutions day 2 | control | 0.00 | 0.00 | 0.00 | **below LoQ** | 0.00 | - | - | - |
| | 0.5 | 0.44 | 0.42 | 0.45 | **0.44** | 0.02 | 3.7 | 87.5 | n/a |
| | 1.0 | 0.84 | 0.88 | 0.91 | **0.88** | 0.03 | 3.9 | 87.6 | n/a |
| | 2.0 | 1.74 | 1.88 | 1.83 | **1.81** | 0.07 | 4.1 | 90.7 | n/a |
| | 4.0 | 3.77 | 3.54 | 3.74 | **3.68** | 0.13 | 3.4 | 92.1 | n/a |
| | 8.0 | 7.81 | 7.44 | 7.69 | **7.65** | 0.19 | 2.5 | 95.6 | n/a |
| stock | 20.0 | 18.00 | 18.61 | 18.76 | 18.46 | 0.40 | 2.2 | 92.3 | n/a |
| spent solutions day 2 | control | 0.00 | 0.00 | 0.00 | **below LoQ** | 0.00 | - | - | - |
| | 0.5 | 0.51 | 0.49 | 0.49 | **0.49** | 0.01 | 2.1 | 98.7 | 115.7 |
| | 1.0 | 0.79 | 0.84 | 0.83 | **0.82** | 0.03 | 3.7 | 81.8 | 97.4 |
| | 2.0 | 1.64 | 1.57 | 1.61 | **1.61** | 0.03 | 2.1 | 80.3 | 87.8 |
| | 4.0 | 3.17 | 3.06 | 3.19 | **3.14** | 0.07 | 2.3 | 78.5 | 88.8 |
| | 8.0 | 6.78 | 6.77 | 6.79 | **6.78** | 0.01 | 0.2 | 84.8 | 94.9 |
| fresh solutions day 3 | control | 0.00 | 0.00 | 0.00 | **below LoQ** | 0.00 | - | - | - |
| | 0.5 | 0.43 | 0.43 | 0.44 | **0.44** | 0.01 | 1.6 | 87.2 | n/a |
| | 1.0 | 0.88 | 0.92 | 0.85 | **0.89** | 0.04 | 4.2 | 88.6 | n/a |
| | 2.0 | 1.85 | 1.94 | 1.91 | **1.90** | 0.05 | 2.6 | 95.0 | n/a |
| | 4.0 | 3.82 | 3.55 | 3.75 | **3.70** | 0.14 | 3.7 | 92.6 | n/a |
| | 8.0 | 7.66 | 7.89 | 7.93 | **7.83** | 0.14 | 1.8 | 97.8 | n/a |
| stock | 20.0 | 18.94 | 18.12 | 18.65 | 18.57 | 0.42 | 2.2 | 92.8 | n/a |
| spent solutions day 3 | control | 0.00 | 0.00 | 0.00 | **below LoQ** | 0.00 | - | - | - |
| | 0.5 | 0.47 | 0.50 | 0.48 | **0.48** | 0.02 | 3.5 | 97.0 | 110.9 |
| | 1.0 | 0.81 | 0.84 | 0.88 | **0.84** | 0.03 | 3.9 | 84.4 | 96.4 |
| | 2.0 | 1.69 | 1.68 | 1.60 | **1.65** | 0.05 | 3.0 | 82.7 | 91.2 |
| | 4.0 | 3.50 | 3.49 | 3.25 | **3.41** | 0.14 | 4.1 | 85.3 | 92.7 |
| | 8.0 | 6.89 | 7.12 | 7.04 | **7.01** | 0.12 | 1.7 | 87.7 | 91.7 |
| spent solutions day 4 | control | 0.00 | 0.00 | 0.00 | **below LoQ** | 0.00 | - | - | - |
| | 0.5 | 0.50 | 0.48 | 0.49 | **0.49** | 0.01 | 2.5 | 97.9 | 112.3 |
| | 1.0 | 0.89 | 0.87 | 0.84 | **0.87** | 0.02 | 2.9 | 86.7 | 97.9 |
| | 2.0 | 1.73 | 1.76 | 1.70 | **1.73** | 0.03 | 1.6 | 86.5 | 91.0 |
| | 4.0 | 3.48 | 3.50 | 3.40 | **3.46** | 0.05 | 1.5 | 86.5 | 93.4 |
| | 8.0 | 7.07 | 7.14 | 7.01 | **7.07** | 0.06 | 0.9 | 88.4 | 90.4 |

below LoQ – below Limit of Quantification, i.e. below 0.100 mg/L; SD – standard deviation,; RSD – relative standard deviation; n/a – not applicable

## Compilation of results for prochloraz

Prochloraz - Zebrafish Embryo Toxicity Test Run 1 21.03.2011 – 25.03.2011

| | sample | nominal concentration of prochloraz [mg/L] | | | | | stock |
|---|---|---|---|---|---|---|---|
| | | 0.5 | 20 | 2 | 4 | 8 | 20 |
| Mean determined concentration of prochloraz [mg/L] | fresh solution day 0 | 0.42 | 0.82* | 1.77 | 3.47 | 6.99 | 17.08 |
| | fresh solution day 1 | 0.46 | 0.89 | 1.85 | 3.92 | 7.99 | 18.63 |
| | spent solution day 1 | 0.45 | 0.82 | 1.62 | 3.13 | 6.51 | - |
| | fresh solution day 2 | 0.47* | 0.98 | 2.05 | 4.15 | 8.36 | 18.56 |
| | spent solution day 2 | 0.49 | 0.90 | 1.76 | 3.53 | 7.37 | - |
| | fresh solution day 3 | 0.46* | 0.96 | 2.01 | 4.09 | 8.42 | 18.95 |
| | spent solution day 3 | 0.47 | 0.93 | 1.87 | 3.68 | 7.55 | - |
| | spent solution day 4 | 0.46 | 0.93 | 1.86 | 3.64 | 7.48 | - |
| | average | 0.46 | 0.91 | 1.87 | 3.73 | 7.63 | 18.64 |
| | recovery | 92.0% | 91.0% | 93.5% | 93.3% | 95.4% | 93.2% |
| | TWA | 0.46* | 0.90* | 1.85 | 3.70 | 7.58 | n/a |
| | recovery | 92.0% | 90.0% | 92.5% | 92.5% | 94.8% | n/a |
| | GA | 0.46 | 0.90 | 1.84 | 3.69 | 7.56 | 18.29 |
| | recovery | 92.0% | 90.0% | 92.0% | 92.3% | 94.5% | 91.5% |

Prochloraz - Zebrafish Embryo Toxicity Test Run 2 04.04.2011 – 08.04.2011

| | sample | nominal concentration of prochloraz [mg/L] | | | | | stock |
|---|---|---|---|---|---|---|---|
| | | 0.5 | 20 | 2 | 4 | 8 | 20 |
| Mean determined concentration of prochloraz [mg/L] | fresh solution day 0 | 0.32 | 0.64 | 1.26 | 2.53 | 5.26 | 11.36 |
| | fresh solution day 1 | 0.36 | 0.71 | 1.40* | 2.92 | 5.87 | 12.66 |
| | spent solution day 1 | 0.28 | 0.61 | 1.21 | 2.35 | 4.82 | - |
| | fresh solution day 2 | 0.34 | 0.71 | 1.56 | 3.20 | 6.40 | 14.76 |
| | spent solution day 2 | 0.37 | 0.68 | 1.40 | 2.64 | 5.41 | - |
| | fresh solution day 3 | 0.35* | 0.77 | 1.61 | 3.43 | 6.83 | 15.36 |
| | spent solution day 3 | 0.37 | 0.7 | 1.41 | 2.78 | 5.59 | - |
| | spent solution day 4 | 0.35 | 0.75 | 1.56 | 3.05 | 6.10 | - |
| | average | 0.36 | 0.73 | 1.49 | 2.99 | 6.03 | 14.83 |
| | recovery | 68.5% | 73.0% | 74.5% | 74.8% | 75.4% | 74.2% |
| | TWA | 0.34* | 0.69 | 1.42* | 2.86 | 5.78 | n/a |
| | recovery | 68.0% | 69.0% | 71.0% | 71.5% | 72.3% | n/a |
| | GA | 0.34 | 0.69 | 1.42 | 2.84 | 5.75 | 13.44 |
| | recovery | 68.0% | 69.0% | 71.0% | 71.0% | 71.9% | 67.2% |

Average: arithmetic average
TWA: time-weighted mean, calculated according to OECD Guideline 211 of October 2008, Annex 6 (TWA is applicable for semi-static tests where the concentration of the test item declines over the period between renewals)
GA: geometric average
Recovery: percent of nominal value
* where the determined concentrations of prochloraz were the same for fresh and spent solutions mathematical error occurs in calculation of TWA. In order to calculate TWA the value of 0.001 was added to value of Mean determined concentration of prochloraz for fresh solution
n/a: not applicable

Prochloraz - Zebrafish Embryo Toxicity Test Run 3 18.04.2011 – 22.04.2011

| | sample | nominal concentration of prochloraz [mg/L] | | | | | stock |
|---|---|---|---|---|---|---|---|
| | | 0.5 | 20 | 2 | 4 | 8 | 20 |
| Mean determined concentration of prochloraz [mg/L] | fresh solution day 0 | 0.39 | 0.84 | 1.67 | 3.17 | 6.54 | 14.45 |
| | fresh solution day 1 | 0.43 | 0.84 | 1.83 | 3.54 | 7.15 | 16.74 |
| | spent solution day 1 | 0.40 | 0.67 | 1.40 | 2.78 | 5.74 | - |
| | fresh solution day 2 | 0.44 | 0.88 | 1.81 | 3.68 | 7.65 | 18.46 |
| | spent solution day 2 | 0.49 | 0.82 | 1.61 | 3.14 | 6.78 | - |
| | fresh solution day 3 | 0.44 | 0.89 | 1.90 | 3.70 | 7.83 | 18.57 |
| | spent solution day 3 | 0.48 | 0.84 | 1.65 | 3.41 | 7.01 | - |
| | spent solution day 4 | 0.49 | 0.87 | 1.73 | 3.46 | 7.07 | - |
| | average | 0.45 | 0.85 | 1.73 | 3.43 | 7.09 | 17.64 |
| | recovery | 90.0% | 85.0% | 86.5% | 85.8% | 88.6% | 88.2% |
| | TWA | 0.44 | 0.83 | 1.70 | 3.36 | 6.97 | n/a |
| | recovery | 88.0% | 83.0% | 85.0% | 84.0% | 87.1% | n/a |
| | GA | 0.44 | 0.83 | 1.69 | 3.35 | 6.94 | 16.97 |
| | recovery | 88.0% | 83.0% | 84.5% | 83.8% | 86.8% | 84.9% |

Average: arithmetic average
TWA: time-weighted mean, calculated according to OECD Guideline 211 of October 2008, Annex 6 (TWA is applicable for semi-static tests where the concentration of the test item declines over the period between renewals)
GA: geometric average
Recovery: percent of nominal value
n/a: not applicable

# Annex IV - Overview of Runs

***Table - Overview of runs from Phase 1 and Phase 2***

| | **Chemicals** | **A** | **B** | **C** | **D** | **E** | **F** | **G** | **H** | **I** | **J** | **K** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1a – 2a | 3,4-Dichloroaniline | 1 | 1 | OK | OK | OK | OK | OK | OK | OK | 1 | OK |
| 1b | Dibutyl maleate | OK | --- | OK | 1 | --- | OK | OK | --- | --- | --- | --- |
| | Ethanol | OK | --- | 1 | OK | --- | OK | OK | --- | --- | --- | --- |
| | 6-Methyl-5-heptene-2-one | --- | OK | OK | --- | --- | OK | OK | --- | --- | --- | --- |
| | Sodium chloride | --- | OK | 2 | --- | --- | OK | OK | --- | --- | --- | --- |
| | Triclosan | --- | OK | OK | --- | --- | OK | OK | --- | --- | --- | --- |
| | 2,3,6-Trimethylphenol | OK | --- | OK | OK | --- | OK | OK | --- | --- | --- | --- |
| 2b | Carbamazepine | --- | --- | --- | 1 | 1 | OK | --- | --- | --- | --- | OK |
| | Copper (II) sulfate pentahydrate | --- | --- | --- | --- | --- | OK | OK | OK | OK | --- | --- |
| | Dimethyl sulfoxide | --- | --- | --- | --- | --- | OK | --- | OK | --- | OK | OK |
| | 4,6-Dinitro-*o*-cresol | --- | --- | --- | --- | 1 | OK | --- | OK | --- | --- | OK |
| | 2,4-Dinitrophenol | --- | --- | --- | 1 | --- | OK | --- | --- | OK | --- | OK |
| | Luviquat HM 522 | --- | --- | --- | --- | --- | OK | --- | OK | OK | --- | OK |
| | Malathion | --- | 1 | --- | --- | --- | OK | --- | --- | --- | 1 | OK |
| | Merquat 100 | --- | --- | --- | --- | --- | OK | --- | OK | --- | OK | OK |
| | Methylmercury (II) chloride | --- | --- | --- | OK | --- | OK | --- | --- | --- | 1 | --- |
| | 1-Octanol | --- | 1 | --- | --- | --- | OK | OK | OK | --- | --- | --- |
| | Prochloraz | --- | --- | --- | --- | OK | OK | --- | OK | 1 | --- | --- |
| | Tetradecyl sulfate sodium salt | --- | OK | --- | --- | --- | OK | OK | OK | --- | --- | --- |
| | Triethylene Glycol | --- | OK | --- | --- | 1 | OK | --- | --- | OK | --- | --- |

( ): number of disqualified runs; ---: not tested;

**TRAINING (1a + 2a) 3 runs out of 33 to be repeated**

3,4-Dichloroaniline

- Laboratory A: temperature during exposure time was <26ºC; the run was not repeated.
- Laboratory B: lethality in the negative external control was >10%; the run was not repeated.
- Laboratory J: fertility rate was <70%; the run was repeated.

**PHASE 1b (4 runs out of 81 to be repeated)**

Ethanol

- Laboratory C: lethality in the negative external control was >10%; the run was repeated.

Sodium chloride

- Laboratory C: for the 2 runs, the lethality in the negative external control was >10%; the runs were repeated.

Dibutyl maleate

- Laboratory D: 100% lethality in all test concentrations due to a mistake in the test concentration preparation; the run was repeated.

**PHASE 2b (10 runs out of 153 to be repeated)**

Malathion

- Laboratory J: lethality in the negative external control was >10%; the run was repeated.
- Laboratory B: lethality in the negative external control was >10%, the run was repeated.

Methylmercury (II) chloride

- Laboratory J: lethality in the negative external control was >10%; the run was repeated.

4,6-Dinitro-*o*-cresol

- Laboratory E: lethality in the internal control was too high for the plate with the highest concentration and there was also a very high lethality in the plate with the lowest concentration; the run was repeated.

Triethylene glycol

- Laboratory E: lethality in the internal controls was too high for the plate with the highest concentration and there was also a very high lethality in the plate with the lowest concentration; the run was repeated.

Carbamazepine

- Laboratory E: lethality in the positive control was below the acceptance criteria (>30%); the run was repeated.
- Laboratory D: lethality in the negative external control was >10%; the run was repeated.

1-Octanol

- Laboratory B: lethality in the negative external control was >10%; the run was repeated.

2,4-Dinitrophenol

- Laboratory D: lethality in the negative external control was >10%; the run was repeated.

Prochloraz

- Laboratory I: lethality in the negative internal control was too high for 5 plates out of 7; the run was repeated.

In summary, out of a total of 267 runs, 17 runs had to be repeated for the following reasons:

- 10 runs due to increased lethality in the negative external control
- 3 runs due to increased lethality in the negative internal control
- 1 run due to decreased lethality in the positive control
- 1 runs due to a mistake in the preparation of the test concentrations
- 1 run due to a decreased fertility rate of the parent generation
- 1 run due to a decreased incubation temperature

# Annex V:

# Statistical Report Phase 2

# –

# Transferability, intra- and inter-laboratory reproducibility of 13 chemicals

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## 1. Overview

This report refers to the statistical analysis as described in Annex 2 of the trial plan (TP_ZFET_OECD_2b_V01, 11 January 2011).

## 2. Methods

### 2.1. Choose appropriate model for estimating the $LC_{50}$ including confidence intervals

The primary model fit to the experimental results of this phase is the two-parameter logistic function. It has two parameters, $LC_{50}$ and $\beta$, where

$$\Pr(Dead) = \frac{1}{1+\exp\left(b\left(x - LC_{50}\right)\right)}$$

Both x and $LC_{50}$ are on the log-scale of concentration. This logistic regression model is one of the models recommended by the OECD Series on Testing and Assessment No. 54 for modelling quantal dose-response data[1]. Note that this model implies that there is no background mortality, and in fact observed background mortality does not contribute to model parameter estimation. Under this model, the control data role is solely to assess experimental quality.

If this model is an obviously poor fit to a given set of experimental data, the estimated LC50 and accompanying confidence intervals may be biased. In these cases, alternative models may be given consideration. For example, a three-parameter logistic model might fit better:

$$\Pr\left(Dead\right) = C + \frac{1 - C}{1+\exp\left(b\left(x - LC_{50}\right)\right)}.$$

In this model the additional parameter C represents a positive non-zero background rate associated with the control group. This model is identical to the two-parameter logistic when $C$=0. While this equivalence is true and the model has the advantage of using the control data to estimate model parameters, there are drawbacks to using the three-parameter model exclusively. One is that when the background parameter is estimated to be very small or zero, the numerical calculations are sometimes unstable. More importantly, because we use at most 20 replicates per group, when background mortality is observed the only nonzero percentages possible are 1/20 = 5% and 2/20 = 10%, so there is a good chance that the parameter C will overestimate background mortality. Each of these is well above our historical experience, so the two models effectively trade bias in one direction for bias in another.

### 2.2. Quality criteria for fitting the model

Because these models must be fit using iterative numerical calculations, convergence of the numerical model fitting process must be confirmed prior to any other evaluation. Upon that confirmation, the fit of the primary model is checked using graphical summaries. If the model shows an obviously inappropriate fit, the estimated $LC_{50}$ values may be biased, and alternative models will be investigated. It is preferable that all estimates and confidence intervals be based on a common model, so any secondary models will only be used if a strong justification exists.

[1] OECD Series on Testing and Assessment No. 54: Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to application. Chapter 6.2, p 63ff

## 2.3. Confidence interval calculation

Confidence interval calculation is via the profile likelihood method[2]. In cases where the data provide adequate information for model estimation, profile likelihood confidence limits are nearly equivalent to the conventional intervals constructed from estimates and their standard errors. It has been shown that in very large samples they will become equivalent. Comparisons performed on data similar to those obtained in this phase demonstrate that the profile likelihood and conventional intervals are practically equivalent when the data are sufficiently informative, or well behaved, for fitting the model (results not shown). The advantage of profile likelihood intervals lies in cases where the data are not well behaved (described below). Unlike the conventional intervals, profile likelihood intervals will not be unrealistically wide, or narrow.

It is assumed that the tested concentrations reasonably bound the concentrations through which the response traverses the 0 to 100% response range. Because the embryos are tested in groups, a 'well-behaved' experimental result can be defined in terms of the number of informative concentration groups. Under the test design used for this validation, the informative groups will usually be the smallest concentration with partial mortality, the largest with partial mortality, plus all groups between, regardless of their mortality rates. More formally, in order to capture special cases, a group is informative if one of the following three conditions holds:

1. The group experiences partial toxicity, in the sense that at least one survival AND at least one death occurs (i.e., the percentage of deaths is NOT 0 or 100%),
2. The group percentage is 0%, but at least one concentration BELOW the one under consideration is anything greater than 0%,
3. The group percentage is 100%, but at least one concentration ABOVE the one under consideration is anything less than 100%.

By these definitions informative groups must be consecutive in the concentration scale, and when two or more occur, that experimental result would be considered 'well-behaved'.

There are of course obvious cases for which an experimental result would be considered suboptimal. For example, if all of the response rates observed are less than 50%, or all greater than 50%, or all nearly equal to 50%, it should be obvious that these data will not provide good information on the $LC_{50}$. More commonly, the experimental results obtained do largely cover the full response range, but fewer than two informative groups occur. The two specific cases of concern are:

1. A single group experiences something between 0 and 100% mortality, and all groups at concentrations below it experience 0% mortality, and all groups at concentrations above it experience 100% (see 2.3.1 Case 1).
2. All groups experience only 0 or 100% mortality, and all of the 0% groups are in concentrations lower than all of the 100% groups (see 2.3.1 Case 2).

When the observed result is suboptimal, there is essentially no information for bounding the steepness of the concentration-response curve. The slope can be arbitrarily steep and, due to artefacts of the numerical computations, the conventional confidence intervals are either far too

[2] Meeker, W.Q., Escobar, L.A. (1995): Teaching about approximate confidence regions based on maximum likelihood estimation. *The American Statistician*, v49, 48-53.

narrow, or too wide.[3] The profile likelihood method for confidence interval construction is not susceptible to these problems. Profile likelihood intervals are more difficult to calculate (and hence the reason that the method is not more commonly implemented). A specialized program was developed to perform the calculations (available on request).

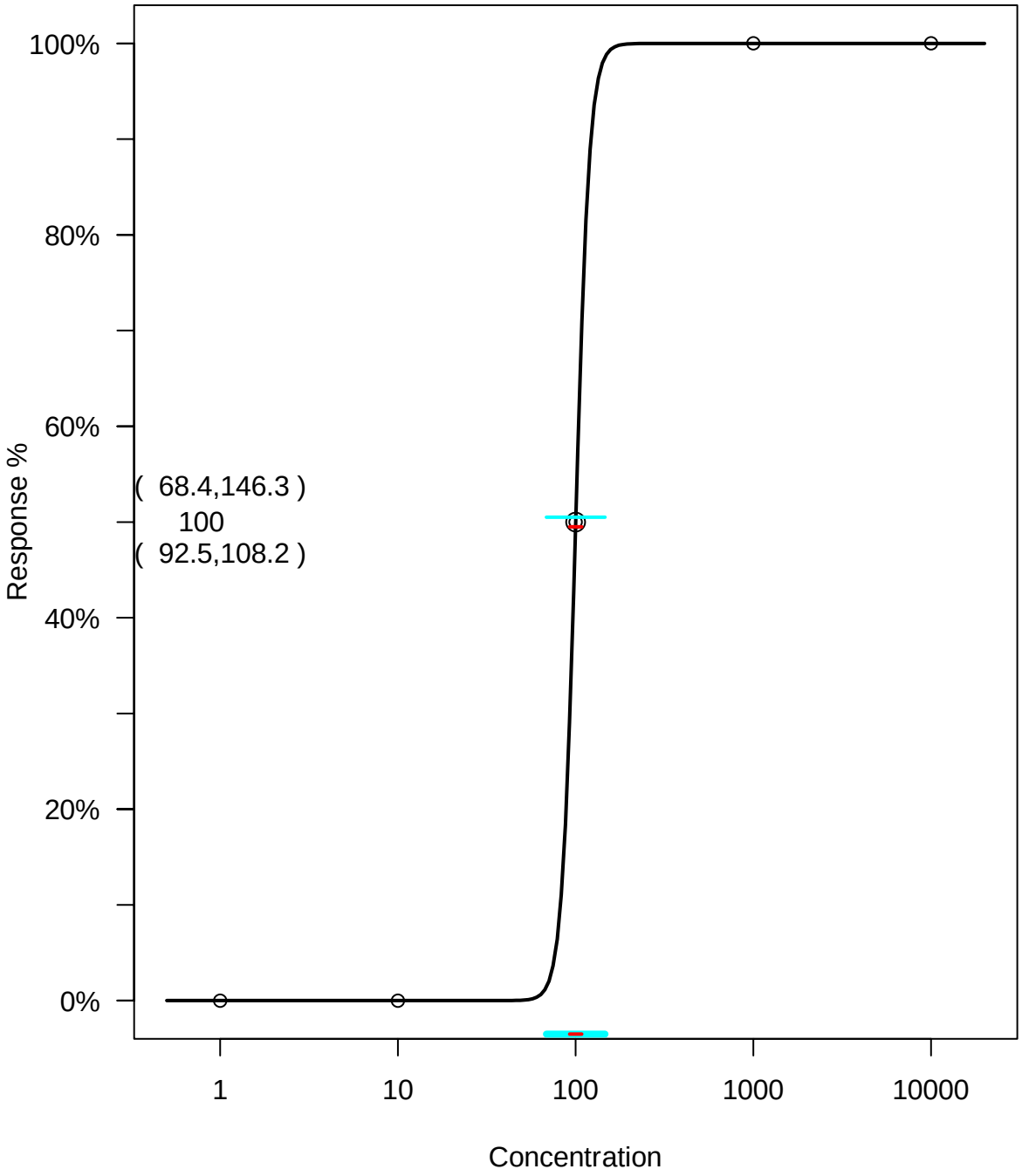
Other approaches were considered in the two suboptimal cases described above, such as the Spearman-Kärber or binomial method. However, this would result in different point estimates for the $LC_{50}$ values for the intra/inter laboratories comparison (reliability) and therefore it was not implemented.

[3] Environment Canada (2005 with amendments from 2007): Guidance document on statistical methods for environmental toxicity tests/Method Development and Application Section. Section 4.

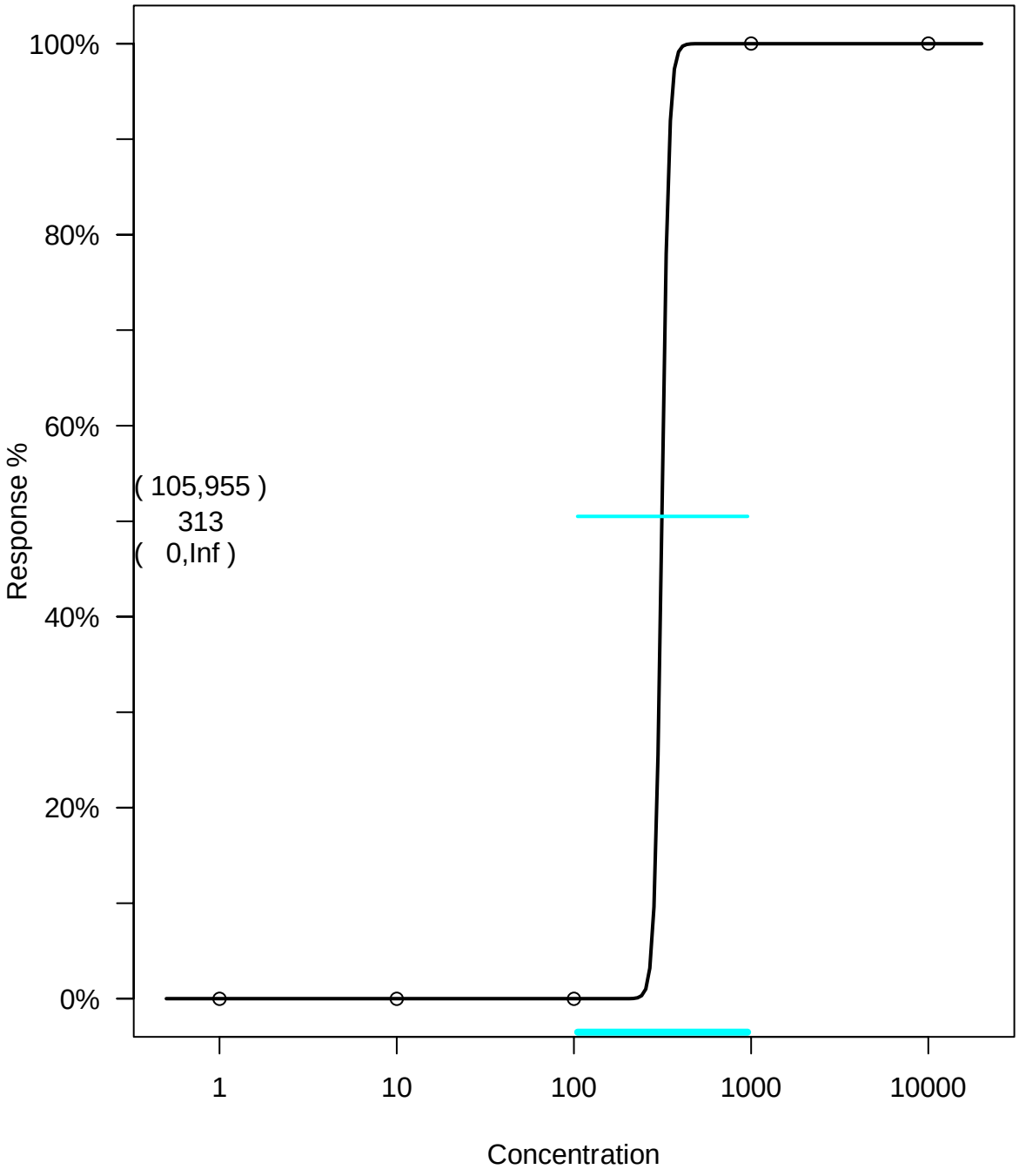
### 2.3.1. Illustrative Examples

Case 1: Single partial response (one informative concentration)

The conventional interval (red, lower set of numbers) is very narrow around the concentration that has 50% response. It cannot be known that the $LC_{50}$ lies in such a narrow window. The profile likelihood interval is much wider (blue, upper set of numbers).



Case 2: No informative concentrations (every group is either 0 or 100%)
In this case, the conventional interval does not even exist, because the estimated standard error of the $LC_{50}$ is zero, so the interval is arbitrarily wide. The profile likelihood interval is intuitively correct: the $LC_{50}$ is somewhere between the two concentrations that bracket the 50% response.



## 2.4. Calculations

All model-based calculations were performed with R version 2.12.0[4], using the standard numerical optimization functions available in the default installation of R. The profile likelihood calculations have been thoroughly tested, and were also found to be in agreement with profile likelihood estimation in SAS/STAT LOGISTIC procedure[5]. Statistical tests on the control data were performed in StatXact v4[6]. In addition to model fit, $LC_{50}$ determinations were deemed acceptable when the $LC_{50}$ was both below the highest exposure concentration and the highest concentration had at least 50% mortality. In this manner, the $LC_{50}$ was not extrapolated beyond the highest exposure concentration (note: by convention, many practitioners seek the highest exposure concentration to have at least 65% mortality in order to "trust" the $LC_{50}$ determination, which again is a desire to not over-interpret the data).

## 2.5. Analysis of 3,4-DCA Qualification Data for New Laboratories

Previously, all participating laboratories were qualified for inclusion in the Trial Plan following demonstration of adherence to the Trial Plan SOP and testing of 3,4-Dichloroaniline (3,4-DCA) (TP_ZFET_OECD_2b_V01_1.doc, 17 January 2011). Analysis of "new" laboratories H, I, J and K consisted of the same models, calculations, and considerations outlined above in Sections 2.1-2.4.

## 2.6. Internal control analysis

The potential effects of a 'halo effect' of the toxicity due to treatment in neighbouring wells will be assessed by appropriate data visualizations, and if warranted, a stratified Cochran-Armitage tests of trend in proportions. The data from internal controls will be summarized at various levels (the strata), where the statistical analysis will attempt to detect that more control deaths occur in plates on which high toxicity is experienced in the neighbouring test article wells, compared to controls tested in plates with low toxicity in the test article wells. Alternatively, a correlation analysis can be conducted in which, rather than the actual exposure concentration, the toxicity of the test article is used as the predictor for control well toxicity.

## 2.7 Summarization of results

This phase of the validation is focused on reproducibility, although it is also possible to evaluate the ability of the ZFET to distinguish these chemicals of widely separated toxicity levels. The estimated $LC_{50}$ values and profile likelihood confidence intervals for the thirteen chemicals (Carbamazepine, Copper(II) sulfate pentahydrate, 4,6-Dinitro-o-cresol (DNOC), 2,4-Dinitrophenol (2,4-DNP), Dimethyl sulfoxide (DMSO), Luviquat HM 552, Malathion, Merquat 100, Methylmercury (II) chloride, 1-Octanol, Prochloraz, Tetradecyl sulfate sodium salt (TSSS), and Triethylene glycol (TEG)) are calculated for each qualified run at 48 and 96h. This information is further summarized graphically and statistically (see Appendix B).

---

[4] R Development Core Team (2011). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.

[5] SAS Institute Inc. 2010. SAS/STAT® 9.22 User's Guide. Cary, NC: SAS Institute Inc.

[6] Cytel Software Corp. 1998. StatXact 4 For Windows: Statistical Software for Exact Nonparametric Inference. Cambridge, MA: CYTEL Software Corp.

In some cases, the results of experiments are ill-suited to the estimation of an $LC_{50}$ and its confidence interval, and these are excluded from the summarizations related to $LC_{50}$ estimation. The simple criterion used is that the prediction model must achieve a response rate of at least 50% at the maximum concentration tested. It should be noted that these experimental results are still included in all other evaluations, such as internal control mortality.

## 3. Results

The designated primary model

$$\Pr(Dead) = \frac{1}{1+\exp\left(b\left(x - LC_{50}\right)\right)}$$

is adequate for all models fit to data from this phase. None of the model fits is sufficiently improved by alternative models to justify this added complexity to the analysis and interpretation.

### 3.1 Transferability for Laboratories H, I, J and K with 3,4-Dichloroaniline

***Table 1: $LC_{50}$ values and confidence intervals of the Zebrafish Embryo Toxicity Test – three runs with 3,4-Dichloroaniline***

| | | 48h (mg/L) | | | | 96h (mg/L) | | | |
|---|---|---|---|---|---|---|---|---|---|
| Lab | Run | $LC_{50}$ | Lower | Upper | Inform | $LC_{50}$ | Lower | Upper | Inform |
| H | 1 | 3.62 | 3.03 | 4.31 | 3 | 2.59 | 2.23 | 3.04 | 2 |
| | 2 | 3.97 | 3.12 | 5.20 | 5 | 2.55 | 2.11 | 3.08 | 3 |
| | 3 | 4.00 | 3.43 | 4.67 | 1 | 2.82 | 2.02 | 3.95 | 0 |
| | Mean | 3.86 | | | | 2.65 | | | |
| I | 1 | 2.82 | 2.02 | 3.95 | 0 | 2.15 | 2.01 | 3.21 | 1 |
| | 2 | 3.15 | 2.60 | 3.81 | 4 | 3.04 | 2.48 | 3.75 | 4 |
| | 3 | 2.46 | 2.03 | 2.99 | 3 | 2.46 | 2.03 | 2.99 | 3 |
| | Mean | 2.81 | | | | 2.55 | | | |
| J | 1 | 4.35 | 3.51 | 5.50 | 4 | 2.94 | 2.37 | 3.68 | 4 |
| | 2 | 2.11 | 2.01 | 3.04 | 1 | 2.11 | 2.01 | 3.04 | 1 |
| | 3 | 3.77 | 3.05 | 4.70 | 4 | 3.05 | 2.46 | 3.79 | 4 |
| | Mean | 3.41 | | | | 2.70 | | | |
| K | 1 | 4.13 | 3.53 | 4.86 | 2 | 4.00 | 3.41 | 4.70 | 2 |
| | 2 | 3.88 | 3.30 | 4.54 | 2 | 2.97 | 2.53 | 3.46 | 2 |
| | 3 | 3.38 | 2.80 | 4.06 | 2 | 2.62 | 2.23 | 3.10 | 2 |
| | Mean | 3.79 | | | | 3.20 | | | |
| | Grand Mean | 3.47 | | | | 2.77 | | | |

Inform = the number of informative concentrations (defined in Section 2.3; see figures in the Appendix A)

3.2 Intra-laboratory reproducibility - coefficients of variation (%) for laboratories H, I, J, and K with 3,4-Dichloroaniline

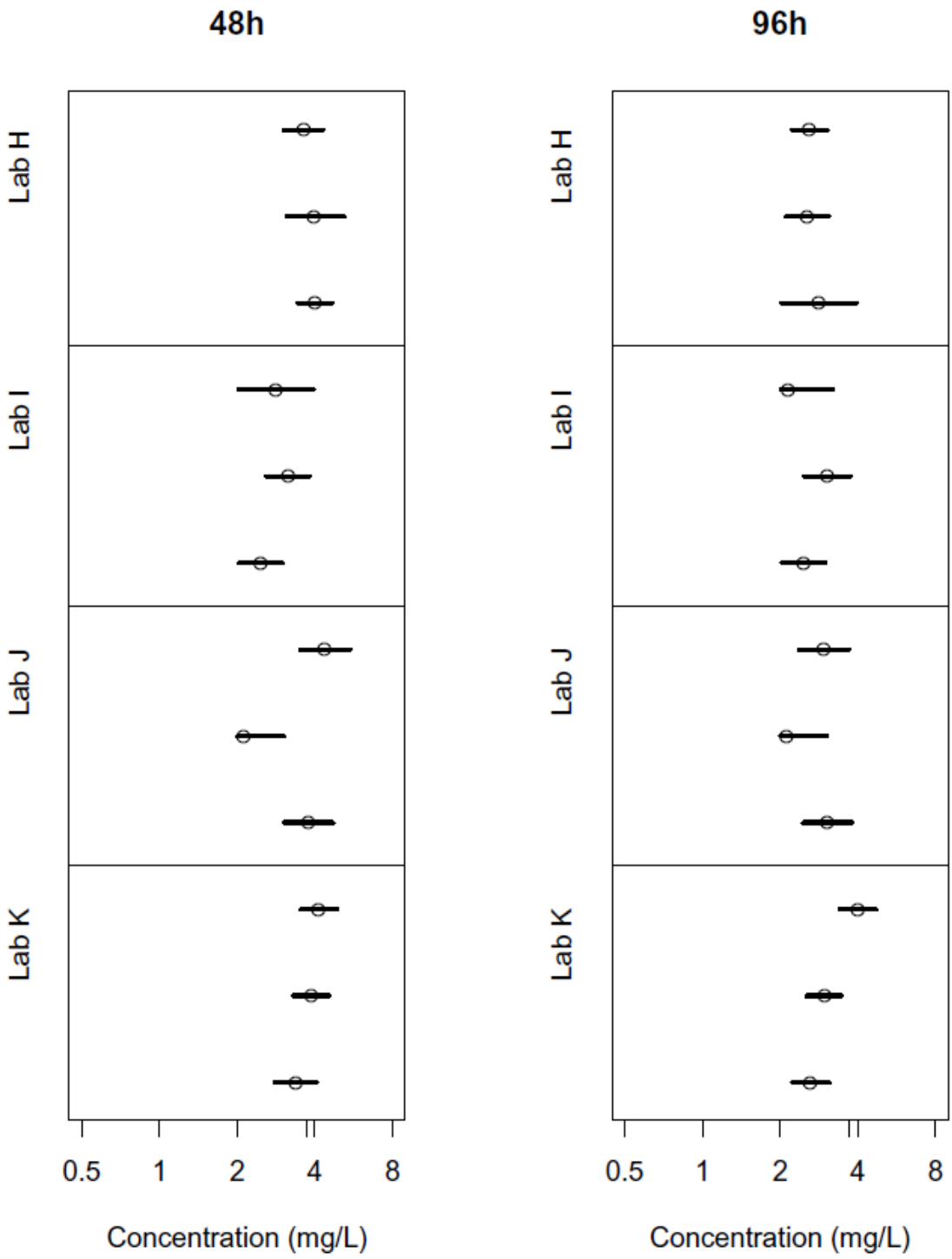
The calculated coefficients of variation per laboratory for 3,4-Dichloroaniline to demonstrate transferability of the SOP and qualification for inclusion in the validation program are given in Table 2.

***Table 2: Intra-laboratory reproducibility - coefficients of variation (%) for 3,4-DCA performed in 4 new laboratories involved in Phase 2 – three runs***

| Laboratory | 48-h Coefficient of Variation (%) | 96-h Coefficient of Variation (%) |
|---|---|---|
| H | 5.47 | 5.50 |
| I | 12.28 | 17.72 |
| J | 34.09 | 19.03 |
| K | 10.08 | 22.42 |

Both $LC_{50}$s and CV statistics indicated laboratories successfully implemented the SOP.

***Figure 1: $LC_{50}$ values and 95% confidence limits for runs of 3,4-DCA for the transferability of Zebrafish Embryo Toxicity Test in new laboratories***



3.2 Run-level summaries

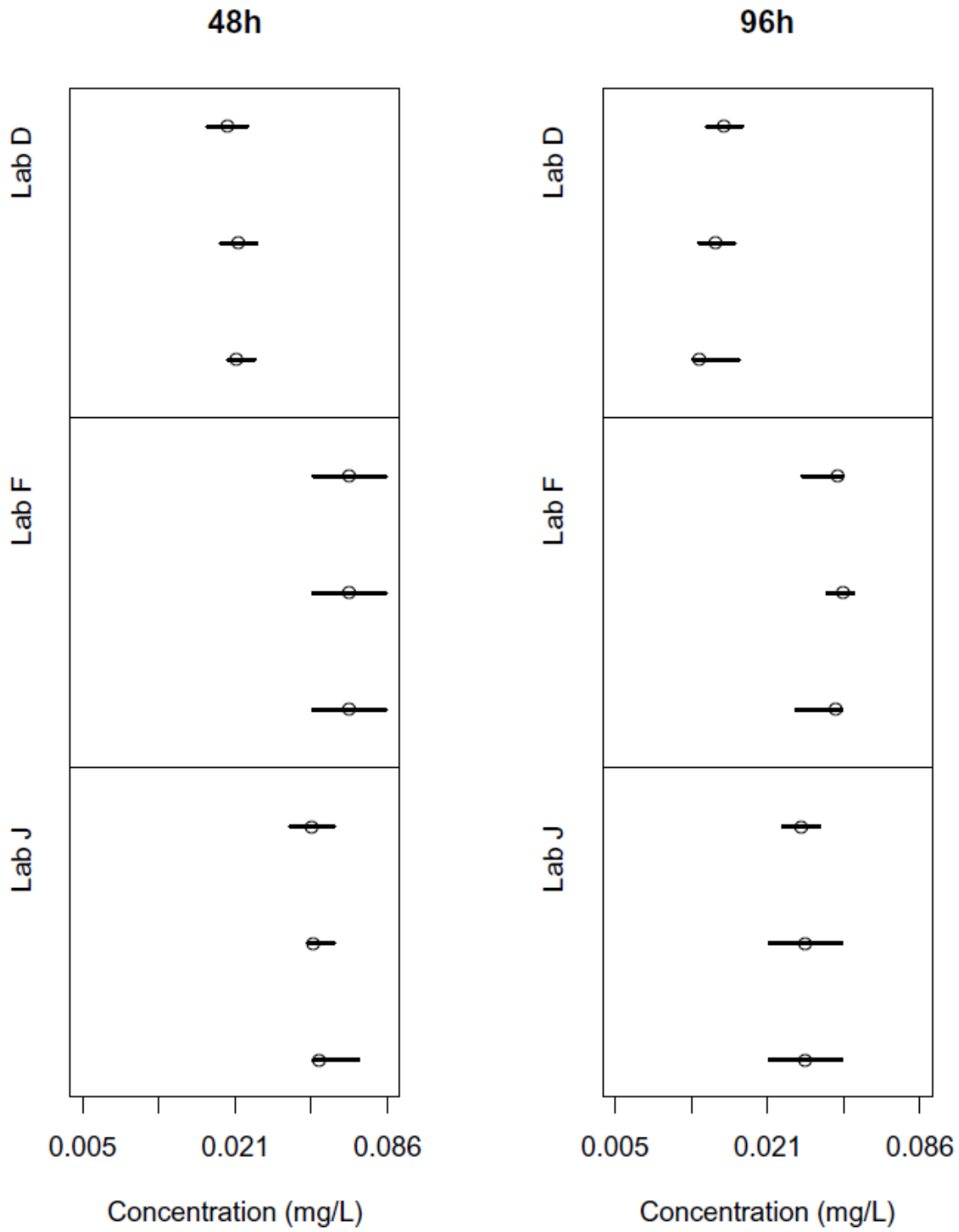
### 3.2.1. Methylmercury (II) Chloride

***Table 3: $LC_{50}$ values and confidence intervals of the Zebrafish Embryo Toxicity Test – three runs with Methylmercury (II) Chloride***

| | | 48h (mg/L) | | | | 96h (mg/L) | | | |
|---|---|---|---|---|---|---|---|---|---|
| Lab | Run | $LC_{50}$ | Lower | Upper | Inform | $LC_{50}$ | Lower | Upper | Inform |
| D | 1 | 0.0201 | 0.0168 | 0.0239 | 3 | 0.0144 | 0.0124 | 0.0170 | 2 |
| | 2 | 0.0222 | 0.0189 | 0.026 | 2 | 0.0134 | 0.0116 | 0.0158 | 2 |
| | 3 | 0.0217 | 0.0203 | 0.0256 | 1 | 0.0115 | 0.0110 | 0.0165 | 1 |
| | Mean | 0.0213 | | | | 0.0131 | | | |
| F | 1 | 0.0606 | 0.0439 | 0.0841 | 0 | 0.0406 | 0.0296 | 0.0423 | 1 |
| | 2 | 0.0606 | 0.0439 | 0.0841 | 0 | 0.0427 | 0.0372 | 0.0468 | 1 |
| | 3 | 0.0606 | 0.0439 | 0.0841 | 0 | 0.0399 | 0.0280 | 0.0420 | 1 |
| | Mean | 0.0606 | | | | 0.0411 | | | |
| J | 1 | 0.0431 | 0.0356 | 0.0526 | 4 | 0.0292 | 0.0249 | 0.0344 | 3 |
| | 2 | 0.0437 | 0.0417 | 0.0527 | 1 | 0.0303 | 0.0219 | 0.0421 | 0 |
| | 3 | 0.0462 | 0.0439 | 0.0658 | 1 | 0.0303 | 0.0219 | 0.0421 | 0 |
| | Mean | 0.0443 | | | | 0.0299 | | | |
| | Grand Mean | 0.0421 | | | | 0.0280 | | | |

Inform = the number of informative concentrations (defined in Section 2.3; see figures in the Appendix B)
Note that the toxicities are expressed in terms of the cation, methyl mercury.

**Figure 2: $LC_{50}$ values and 95% confidence limits for runs of Methylmercury in the Zebrafish Embryo Toxicity Test.**




***Note that nominal test concentrations have been corrected to indicate exposure only to the cation Methylmercury***

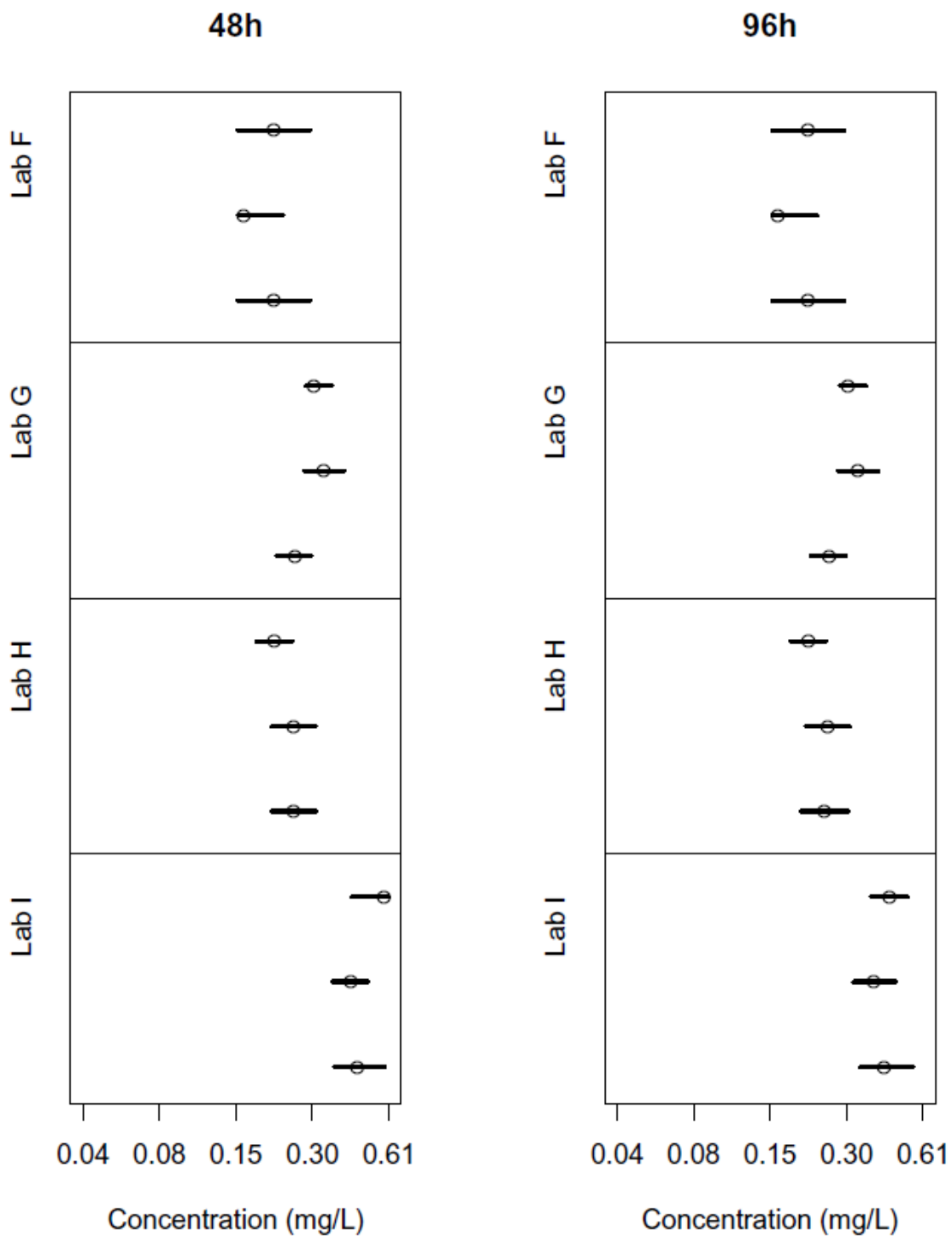
### 3.2.2. Copper (II) Sulphate Pentahydrate

***Table 4: $LC_{50}$ values and confidence intervals of the Zebrafish Embryo Toxicity Test – three runs with Copper (II) Sulphate Pentahydrate***

| | | 48h (mg/L) | | | | 96h (mg/L) | | | |
|---|---|---|---|---|---|---|---|---|---|
| Lab | Run | $LC_{50}$ | Lower | Upper | Inform | $LC_{50}$ | Lower | Upper | Inform |
| F | 1 | 0.215 | 0.156 | 0.298 | 0 | 0.215 | 0.156 | 0.298 | 0 |
| | 2 | 0.164 | 0.156 | 0.234 | 1 | 0.164 | 0.156 | 0.234 | 1 |
| | 3 | 0.215 | 0.156 | 0.298 | 0 | 0.215 | 0.156 | 0.298 | 0 |
| | Mean | 0.198 | | | | 0.198 | | | |
| G | 1 | 0.308 | 0.288 | 0.363 | 1 | 0.308 | 0.288 | 0.363 | 1 |
| | 2 | 0.338 | 0.284 | 0.406 | 3 | 0.338 | 0.284 | 0.406 | 3 |
| | 3 | 0.261 | 0.221 | 0.302 | 2 | 0.261 | 0.221 | 0.302 | 2 |
| | Mean | 0.302 | | | | 0.302 | | | |
| H | 1 | 0.216 | 0.184 | 0.253 | 2 | 0.216 | 0.184 | 0.253 | 2 |
| | 2 | 0.257 | 0.211 | 0.314 | 4 | 0.257 | 0.211 | 0.314 | 4 |
| | 3 | 0.257 | 0.211 | 0.314 | 4 | 0.249 | 0.201 | 0.31 | 4 |
| | Mean | 0.243 | | | | 0.241 | | | |
| I | 1 | 0.582 | 0.437 | 0.603 | 1 | 0.45 | 0.381 | 0.529 | 2 |
| | 2 | 0.431 | 0.367 | 0.506 | 2 | 0.39 | 0.325 | 0.473 | 3 |
| | 3 | 0.458 | 0.373 | 0.588 | 3 | 0.429 | 0.346 | 0.556 | 3 |
| | Mean | 0.491 | | | | 0.423 | | | |
| | Grand Mean | 0.308 | | | | 0.291 | | | |

Inform = the number of informative concentrations (defined in Section 2.3; see figures in the Appendix B)
Note that the toxicities are expressed in terms of the cation, copper.

***Figure 3: $LC_{50}$ values and 95% confidence limits for runs of Copper in the Zebrafish Embryo Toxicity Test.***




**Note that nominal test concentrations have been corrected to indicate exposure only to the cation, copper**

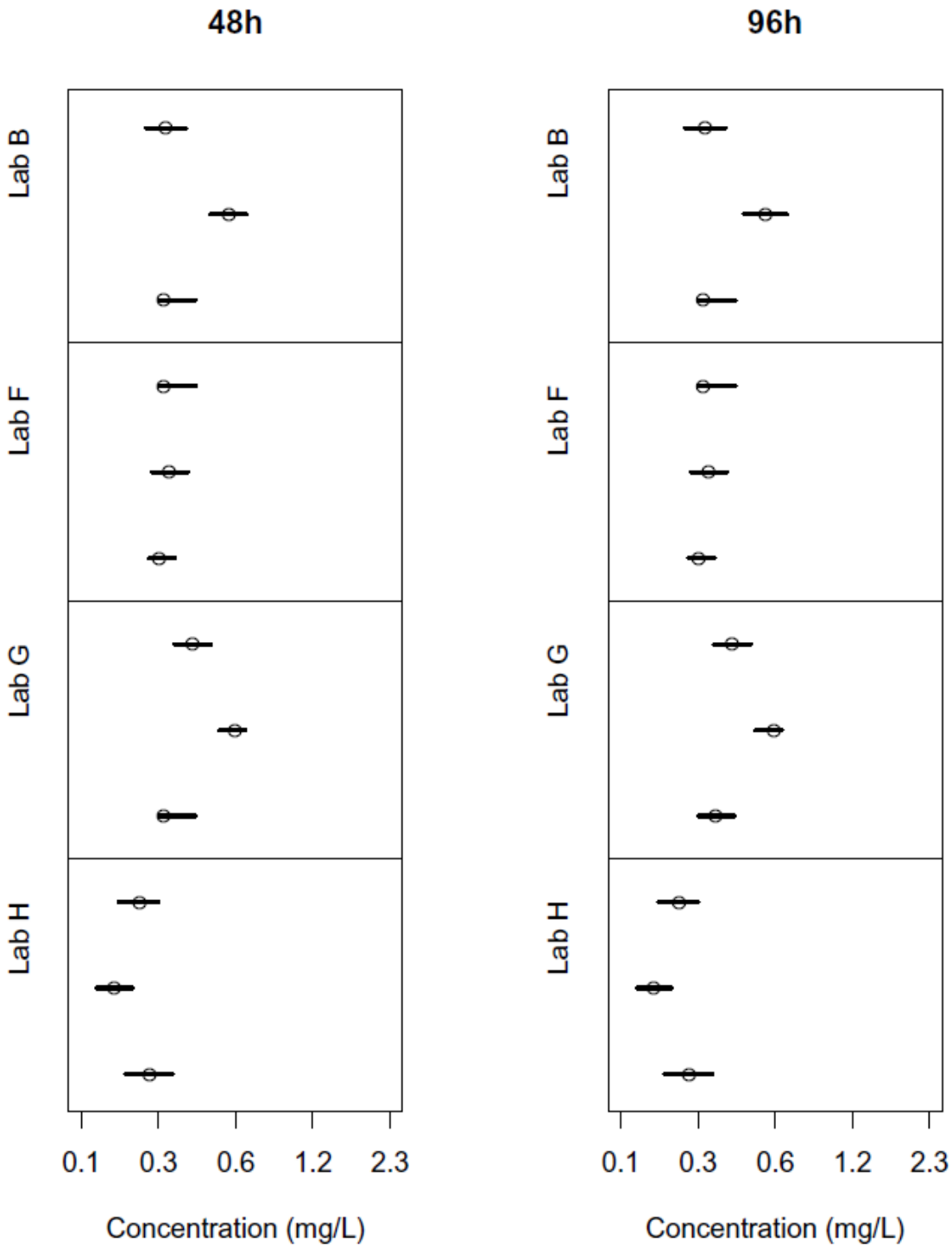
### 3.2.3 Tetradecyl sulphate sodium salt

***Table 5: $LC_{50}$ values and confidence intervals of the Zebrafish Embryo Toxicity Test – three runs with Tetradecyl sulphate sodium salt***

| | | 48h (mg/L) | | | | 96h (mg/L) | | | |
|---|---|---|---|---|---|---|---|---|---|
| Lab | Run | $LC_{50}$ | Lower | Upper | Inform | $LC_{50}$ | Lower | Upper | Inform |
| B | 1 | 0.309 | 0.259 | 0.370 | 2 | 0.309 | 0.259 | 0.370 | 2 |
| | 2 | 0.544 | 0.462 | 0.635 | 2 | 0.530 | 0.438 | 0.642 | 3 |
| | 3 | 0.303 | 0.293 | 0.404 | 1 | 0.303 | 0.293 | 0.404 | 1 |
| | Mean | 0.385 | | | | 0.381 | | | |
| F | 1 | 0.303 | 0.293 | 0.404 | 1 | 0.303 | 0.293 | 0.404 | 1 |
| | 2 | 0.318 | 0.273 | 0.375 | 2 | 0.318 | 0.273 | 0.375 | 2 |
| | 3 | 0.291 | 0.266 | 0.335 | 1 | 0.291 | 0.266 | 0.335 | 1 |
| | Mean | 0.304 | | | | 0.304 | | | |
| G | 1 | 0.393 | 0.336 | 0.464 | 3 | 0.393 | 0.336 | 0.464 | 3 |
| | 2 | 0.576 | 0.502 | 0.631 | 1 | 0.573 | 0.487 | 0.613 | 1 |
| | 3 | 0.303 | 0.293 | 0.404 | 1 | 0.339 | 0.293 | 0.400 | 2 |
| | Mean | 0.424 | | | | 0.435 | | | |
| H | 1 | 0.244 | 0.203 | 0.29 | 2 | 0.244 | 0.203 | 0.29 | 2 |
| | 2 | 0.195 | 0.167 | 0.229 | 2 | 0.195 | 0.167 | 0.229 | 2 |
| | 3 | 0.268 | 0.215 | 0.328 | 2 | 0.268 | 0.215 | 0.328 | 2 |
| | Mean | 0.236 | | | | 0.236 | | | |
| | Grand Mean | 0.337 | | | | 0.339 | | | |

Inform = the number of informative concentrations (defined in Section 2.3; see figures in the Appendix B)
Note that the toxicities are expressed in terms of the anion, tetradecyl sulfate.

***Figure 4: $LC_{50}$ values and 95% confidence limits for runs of Tetradecyl Sulphate in the Zebrafish Embryo Toxicity Test.***




**Note that nominal test concentrations have been corrected to indicate exposure only to the anion, Tetradecyl sulfate**

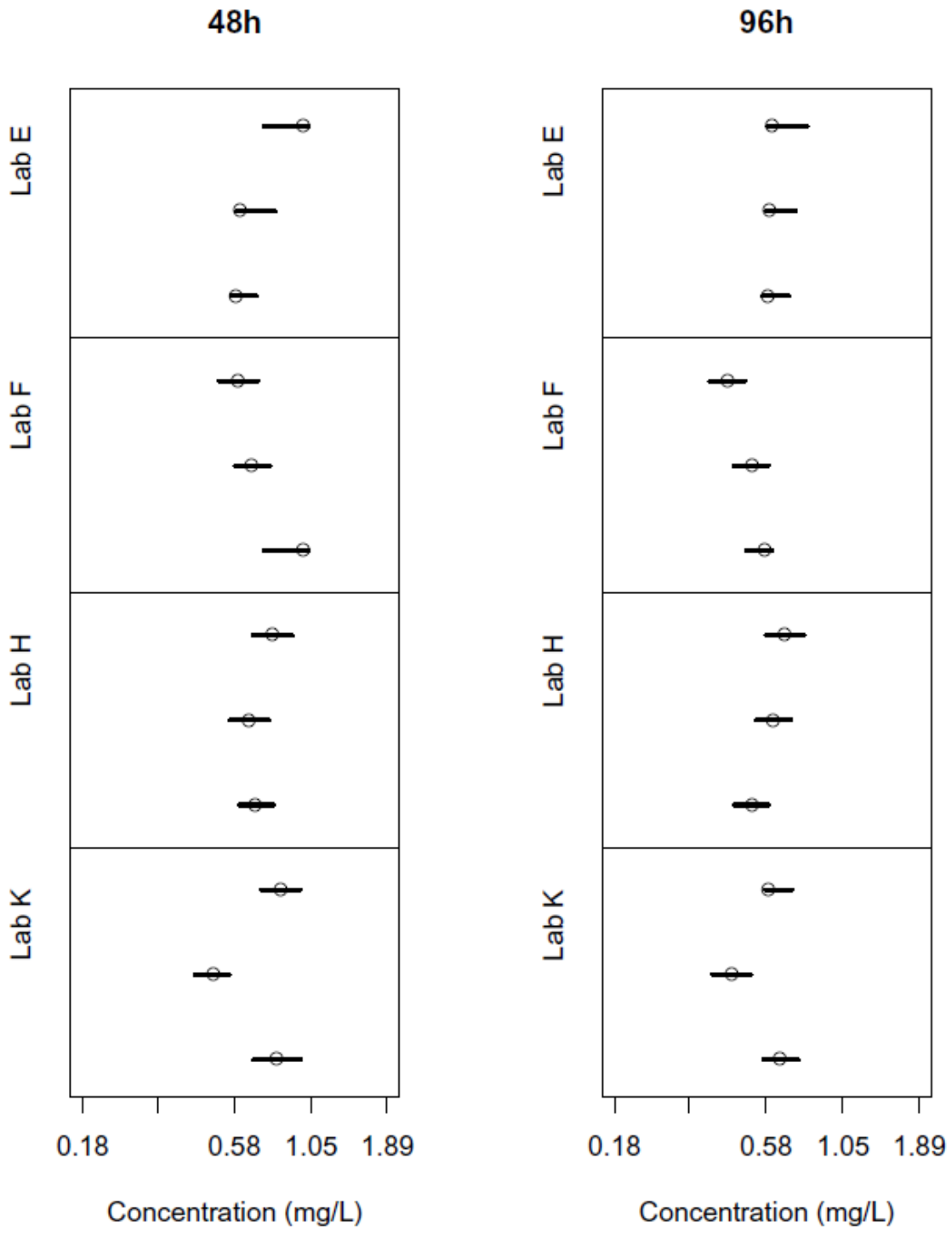
### 3.2.4. 4,6-Dinitro-o-cresol

***Table 6: $LC_{50}$ values and confidence intervals of the Zebrafish Embryo Toxicity Test – three runs with 4,6-Dinitro-o-cresol***

| | | 48h (mg/L) | | | | 96h (mg/L) | | | |
|---|---|---|---|---|---|---|---|---|---|
| Lab | Run | $LC_{50}$ | Lower | Upper | Inform | $LC_{50}$ | Lower | Upper | Inform |
| E | 1 | 0.987 | 0.729 | 1.030 | 1 | 0.608 | 0.588 | 0.798 | 1 |
| | 2 | 0.608 | 0.588 | 0.798 | 1 | 0.594 | 0.581 | 0.728 | 1 |
| | 3 | 0.588 | 0.566 | 0.691 | 1 | 0.588 | 0.566 | 0.691 | 1 |
| | Mean | 0.728 | | | | 0.597 | | | |
| F | 1 | 0.597 | 0.514 | 0.694 | 3 | 0.431 | 0.376 | 0.494 | 3 |
| | 2 | 0.663 | 0.585 | 0.764 | 2 | 0.521 | 0.452 | 0.592 | 2 |
| | 3 | 0.987 | 0.729 | 1.03 | 1 | 0.575 | 0.499 | 0.609 | 1 |
| | Mean | 0.749 | | | | 0.509 | | | |
| H | 1 | 0.780 | 0.671 | 0.909 | 3 | 0.669 | 0.580 | 0.778 | 2 |
| | 2 | 0.650 | 0.562 | 0.756 | 3 | 0.612 | 0.536 | 0.703 | 2 |
| | 3 | 0.682 | 0.602 | 0.785 | 2 | 0.521 | 0.452 | 0.592 | 2 |
| | Mean | 0.704 | | | | 0.601 | | | |
| K | 1 | 0.831 | 0.714 | 0.964 | 2 | 0.591 | 0.577 | 0.709 | 1 |
| | 2 | 0.493 | 0.428 | 0.558 | 2 | 0.445 | 0.384 | 0.515 | 2 |
| | 3 | 0.806 | 0.673 | 0.968 | 4 | 0.646 | 0.568 | 0.743 | 2 |
| | Mean | 0.710 | | | | 0.561 | | | |
| | Grand Mean | 0.723 | | | | 0.567 | | | |

Inform = the number of informative concentrations (defined in Section 2.3; see figures in the Appendix B)

***Figure 5: LC50 values and 95% confidence limits for runs of 4,6-Dinitro-o-cresol in the Zebrafish Embryo Toxicity Test***



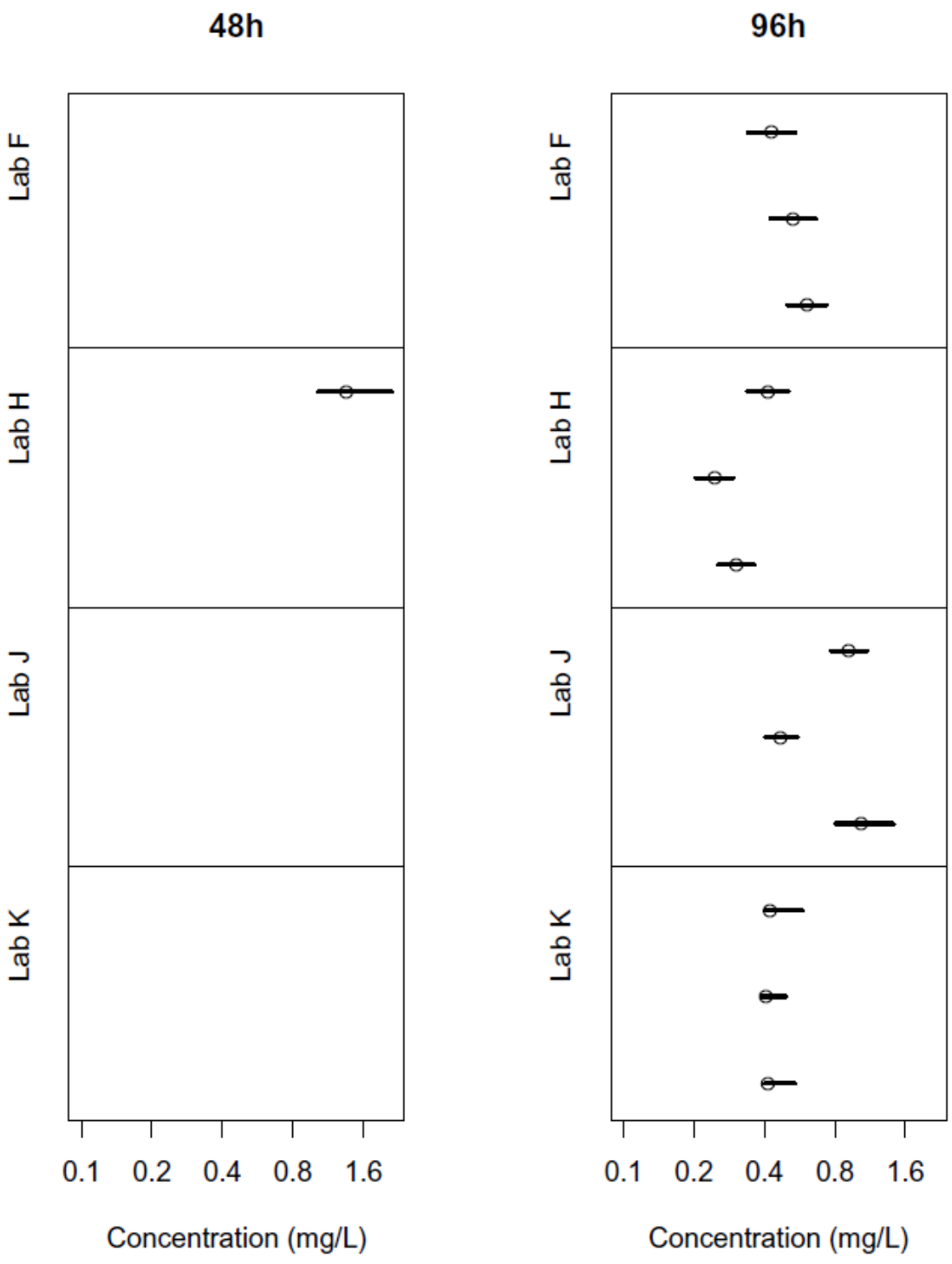
### 3.2.5. Merquat 100

***Table 7: $LC_{50}$ values and confidence intervals of the Zebrafish Embryo Toxicity Test – three runs with Merquat 100***

| | | 48h (mg/L) | | | | 96h (mg/L) | | | |
|---|---|---|---|---|---|---|---|---|---|
| Lab | Run | $LC_{50}$ | Lower | Upper | Inform | $LC_{50}$ | Lower | Upper | Inform |
| F | 1 | CNC | | | | 0.429 | 0.34 | 0.543 | 4 |
| | 2 | CNC | | | | 0.530 | 0.425 | 0.664 | 2 |
| | 3 | CNC | | | | 0.608 | 0.504 | 0.732 | 2 |
| | Mean | | | | | 0.522 | | | |
| H | 1 | 1.36 | 1.03 | 2.12 | 5 | 0.414 | 0.339 | 0.506 | 4 |
| | 2 | CNC | | | | 0.245 | 0.205 | 0.295 | 3 |
| | 3 | CNC | | | | 0.304 | 0.255 | 0.362 | 3 |
| | Mean | 1.36 | | | | 0.321 | | | |
| J | 1 | CNC | | | | 0.917 | 0.772 | 1.100 | 3 |
| | 2 | CNC | | | | 0.468 | 0.404 | 0.552 | 2 |
| | 3 | CNC | | | | 1.040 | 0.81 | 1.420 | 5 |
| | Mean | CNC | | | | 0.808 | | | |
| K | 1 | CNC | | | | 0.423 | 0.406 | 0.580 | 1 |
| | 2 | CNC | | | | 0.407 | 0.388 | 0.491 | 1 |
| | 3 | CNC | | | | 0.414 | 0.403 | 0.539 | 1 |
| | Mean | CNC | | | | 0.415 | | | |
| | Grand Mean | CNC | | | | 0.516 | | | |

Inform = the number of informative concentrations (defined in Section 2.3; see figures in the Appendix B)
CNC – cannot calculate due to insufficient mortality

***Figure 6: $LC_{50}$ values and 95% confidence limits for runs of Merquat 100 in the Zebrafish Embryo Toxicity Test***



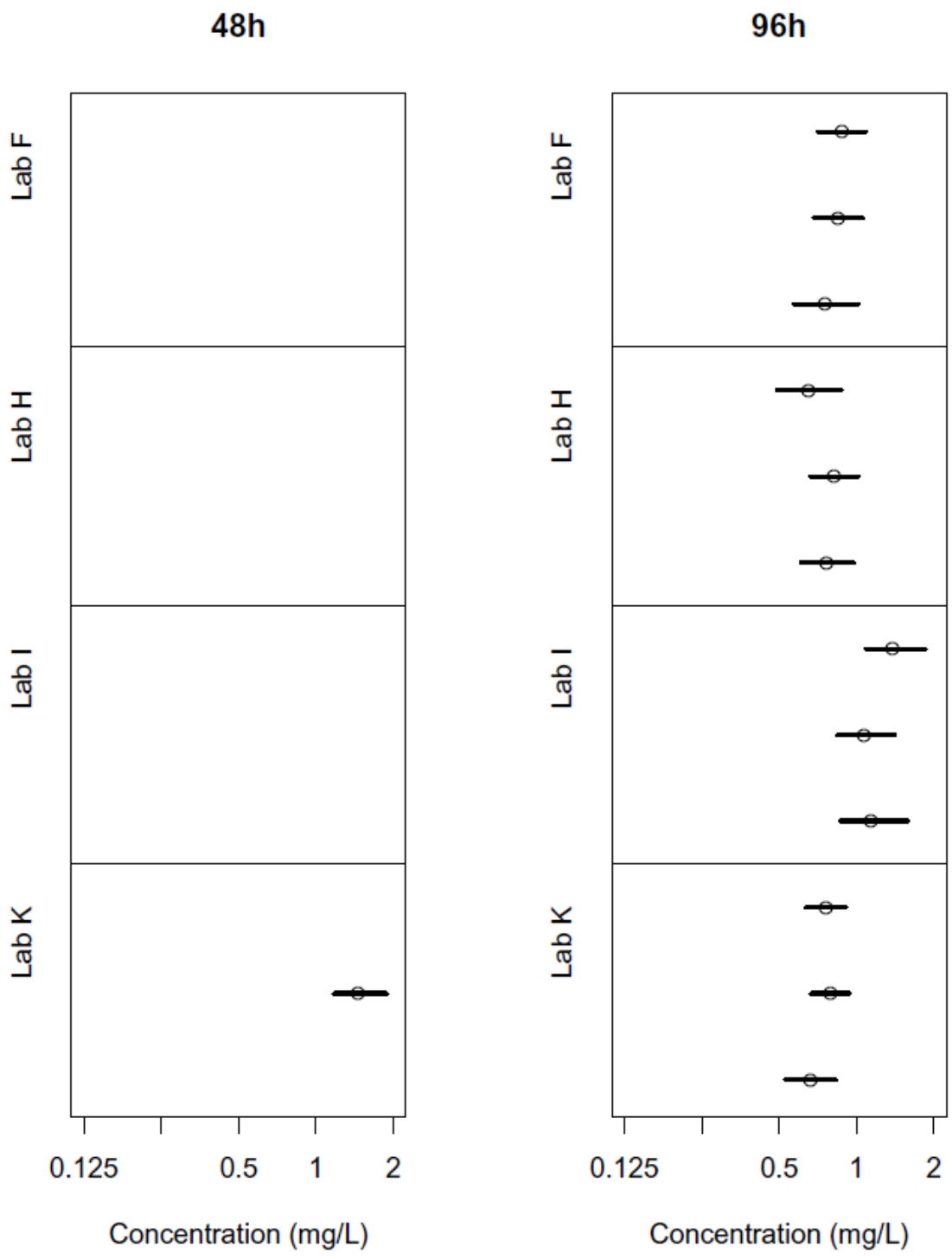
### 3.2.6. Luviquat HM 552

***Table 8: $LC_{50}$ and confidence intervals of the Zebrafish Embryo Toxicity Test – three runs with Luviquat HM 552***

| | | 48h (mg/L) | | | | 96h (mg/L) | | | |
|---|---|---|---|---|---|---|---|---|---|
| Lab | Run | $LC_{50}$ | Lower | Upper | Inform | $LC_{50}$ | Lower | Upper | Inform |
| F | 1 | CNC | | | | 0.876 | 0.711 | 1.090 | 3 |
| | 2 | CNC | | | | 0.847 | 0.683 | 1.060 | 3 |
| | 3 | CNC | | | | 0.754 | 0.572 | 1.020 | 5 |
| | Mean | CNC | | | | 0.826 | | | |
| H | 1 | CNC | | | | 0.649 | 0.491 | 0.872 | 5 |
| | 2 | CNC | | | | 0.817 | 0.661 | 1.020 | 3 |
| | 3 | CNC | | | | 0.765 | 0.607 | 0.973 | 4 |
| | Mean | CNC | | | | 0.744 | | | |
| I | 1 | CNC | | | | 1.380 | 1.090 | 1.850 | 5 |
| | 2 | CNC | | | | 1.070 | 0.844 | 1.410 | 3 |
| | 3 | CNC | | | | 1.140 | 0.871 | 1.590 | 5 |
| | Mean | CNC | | | | 1.200 | | | |
| K | 1 | CNC | | | | 0.761 | 0.637 | 0.905 | 2 |
| | 2 | 1.450 | 1.180 | 1.880 | 2 | 0.791 | 0.666 | 0.931 | 2 |
| | 3 | CNC | | | | 0.662 | 0.531 | 0.830 | 4 |
| | Mean | 1.450 | | | | 0.738 | | | |
| Grand Mean | | | | | | 0.876 | | | |

Inform = the number of informative concentrations (defined in Section 2.3; see figures in the Appendix B)
CNC – cannot calculate due to insufficient mortality

***Figure 7: $LC_{50}$ values and 95% confidence limits for runs of Luviquat HM 552 in the Zebrafish Embryo Toxicity Test***



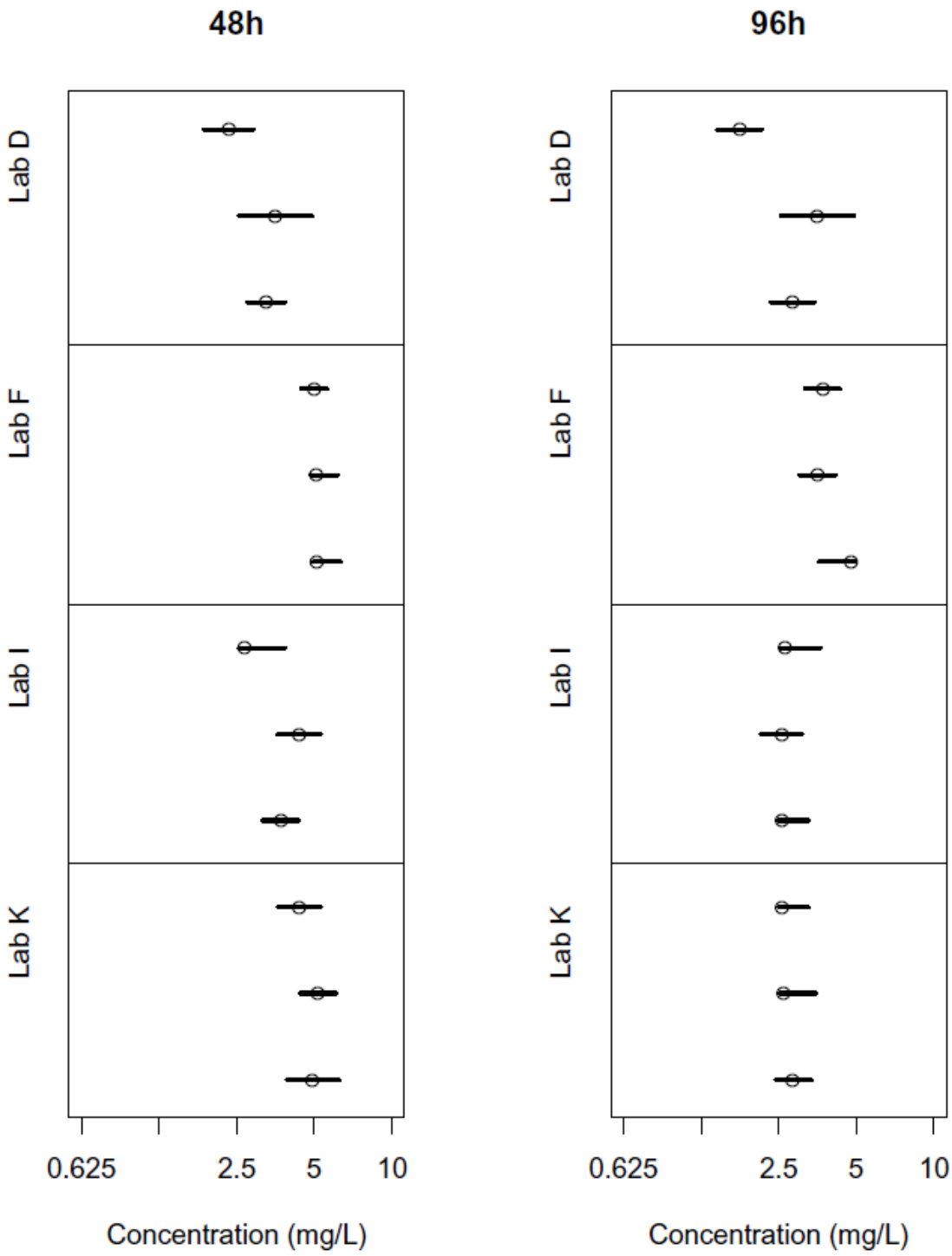
### 3.2.7 2,4-Dinitrophenol

***Table 9: $LC_{50}$ and confidence intervals of the Zebrafish Embryo Toxicity Test – three runs with 2,4-Dinitrophenol***

| | | 48h (mg/L) | | | | 96h (mg/L) | | | |
|---|---|---|---|---|---|---|---|---|---|
| Lab | Run | $LC_{50}$ | Lower | Upper | Inform | $LC_{50}$ | Lower | Upper | Inform |
| D | 1 | 2.33 | 1.87 | 2.9 | 3 | 1.77 | 1.45 | 2.15 | 2 |
| | 2 | 3.52 | 2.55 | 4.9 | 0 | 3.52 | 2.55 | 4.9 | 0 |
| | 3 | 3.25 | 2.76 | 3.86 | 3 | 2.83 | 2.34 | 3.42 | 3 |
| | Mean | 3.04 | | | | 2.71 | | | |
| F | 1 | 5.00 | 4.46 | 5.61 | 1 | 3.72 | 3.16 | 4.32 | 2 |
| | 2 | 5.08 | 4.85 | 6.14 | 1 | 3.54 | 3.01 | 4.15 | 2 |
| | 3 | 5.11 | 4.97 | 6.33 | 1 | 4.78 | 3.59 | 4.95 | 1 |
| | Mean | 5.07 | | | | 4.01 | | | |
| I | 1 | 2.69 | 2.56 | 3.83 | 1 | 2.64 | 2.54 | 3.63 | 1 |
| | 2 | 4.37 | 3.61 | 5.27 | 4 | 2.57 | 2.14 | 3.08 | 3 |
| | 3 | 3.72 | 3.16 | 4.32 | 2 | 2.57 | 2.51 | 3.26 | 1 |
| | Mean | 3.59 | | | | 2.59 | | | |
| K | 1 | 4.37 | 3.62 | 5.28 | 2 | 2.57 | 2.51 | 3.26 | 1 |
| | 2 | 5.16 | 4.41 | 6.08 | 2 | 2.61 | 2.53 | 3.49 | 1 |
| | 3 | 4.90 | 3.92 | 6.23 | 4 | 2.83 | 2.44 | 3.34 | 2 |
| | Mean | 4.81 | | | | 2.67 | | | |
| | Grand Mean | 4.13 | | | | 3.00 | | | |

Inform = the number of informative concentrations (defined in Section 2.3; see figures in the Appendix B)

***Figure 8: $LC_{50}$ values and 95% confidence limits for runs of 2,4-Dinitrophenol in the Zebrafish Embryo Toxicity Test***



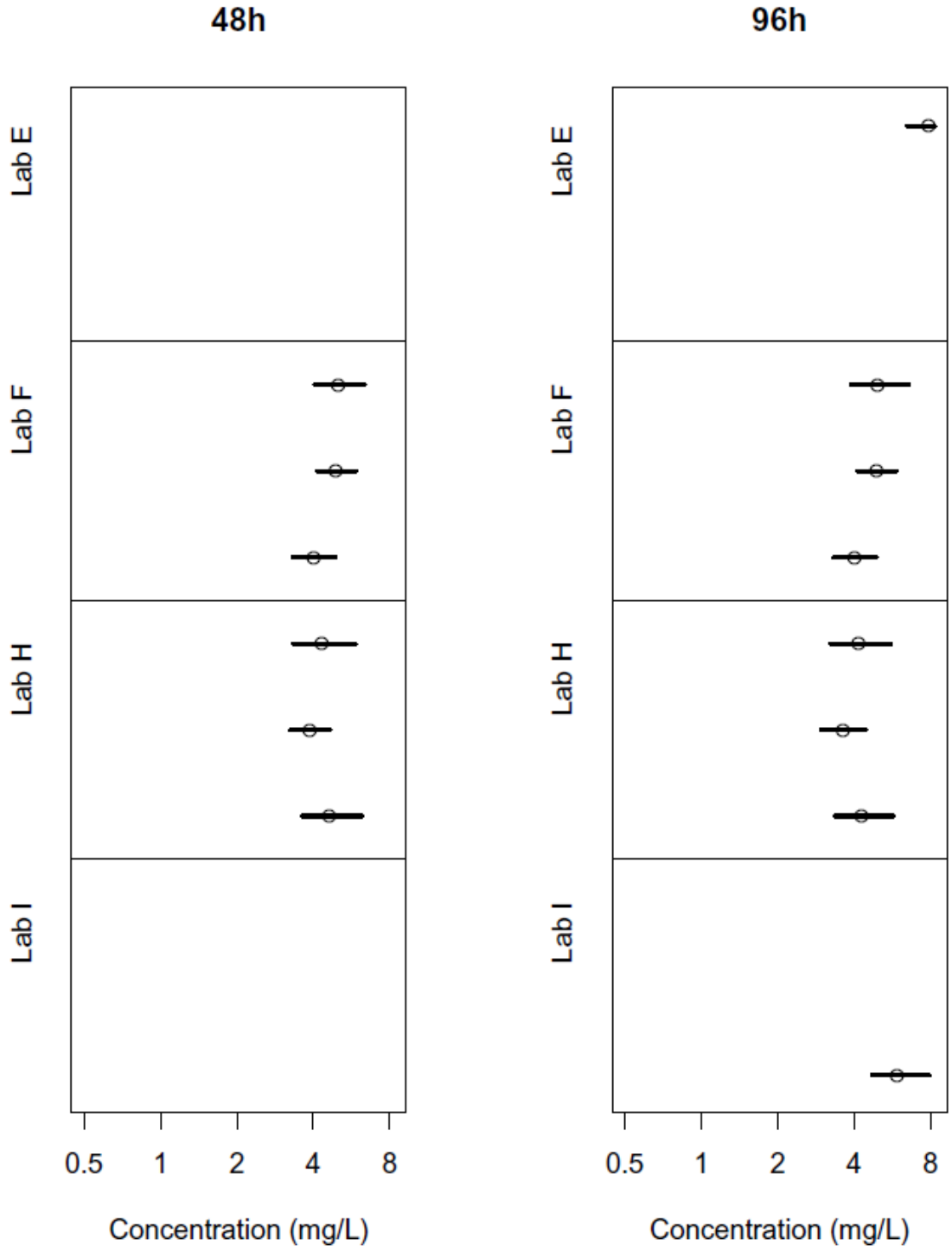
### 3.2.8 Prochloraz

***Table 10: $LC_{50}$ and confidence intervals of the Zebrafish Embryo Toxicity Test – three runs with Prochloraz***

| | | 48h (mg/L) | | | | 96h (mg/L) | | | |
|---|---|---|---|---|---|---|---|---|---|
| Lab | Run | $LC_{50}$ | Lower | Upper | Inform | $LC_{50}$ | Lower | Upper | Inform |
| E | 1 | CNC | | | 1 | 7.87 | 6.49 | 8.29 | 1 |
| | 2 | CNC | | | 4 | | | | 4 |
| | 3 | CNC | | | 1 | | | | 1 |
| | Mean | CNC | | | | 7.87 | | | |
| F | 1 | 5.02 | 4.06 | 6.37 | 3 | 4.94 | 3.90 | 6.56 | 4 |
| | 2 | 4.90 | 4.15 | 5.88 | 2 | 4.90 | 4.15 | 5.88 | 2 |
| | 3 | 4.02 | 3.32 | 4.90 | 4 | 4.02 | 3.32 | 4.90 | 4 |
| | Mean | 4.65 | | | | 4.62 | | | |
| H | 1 | 4.33 | 3.35 | 5.85 | 5 | 4.16 | 3.23 | 5.58 | 5 |
| | 2 | 3.87 | 3.24 | 4.64 | 3 | 3.62 | 2.97 | 4.45 | 3 |
| | 3 | 4.63 | 3.62 | 6.22 | 4 | 4.29 | 3.35 | 5.69 | 4 |
| | Mean | 4.28 | | | | 4.02 | | | |
| I | 1 | CNC | | | 5 | | | | 5 |
| | 2 | CNC | | | 2 | | | | 2 |
| | 3 | CNC | | | 5 | 5.9 | 4.71 | 7.91 | 5 |
| | Mean | CNC | | | | 5.9 | | | |
| | Grand Mean | CNC | | | | 5.6 | | | |

Inform = the number of informative concentrations (defined in Section 2.3; see figures in the Appendix B)
CNC – cannot calculate due to insufficient mortality

***Figure 9: $LC_{50}$ values and 95% confidence limits for runs of Prochloraz in the Zebrafish Embryo Toxicity Test***



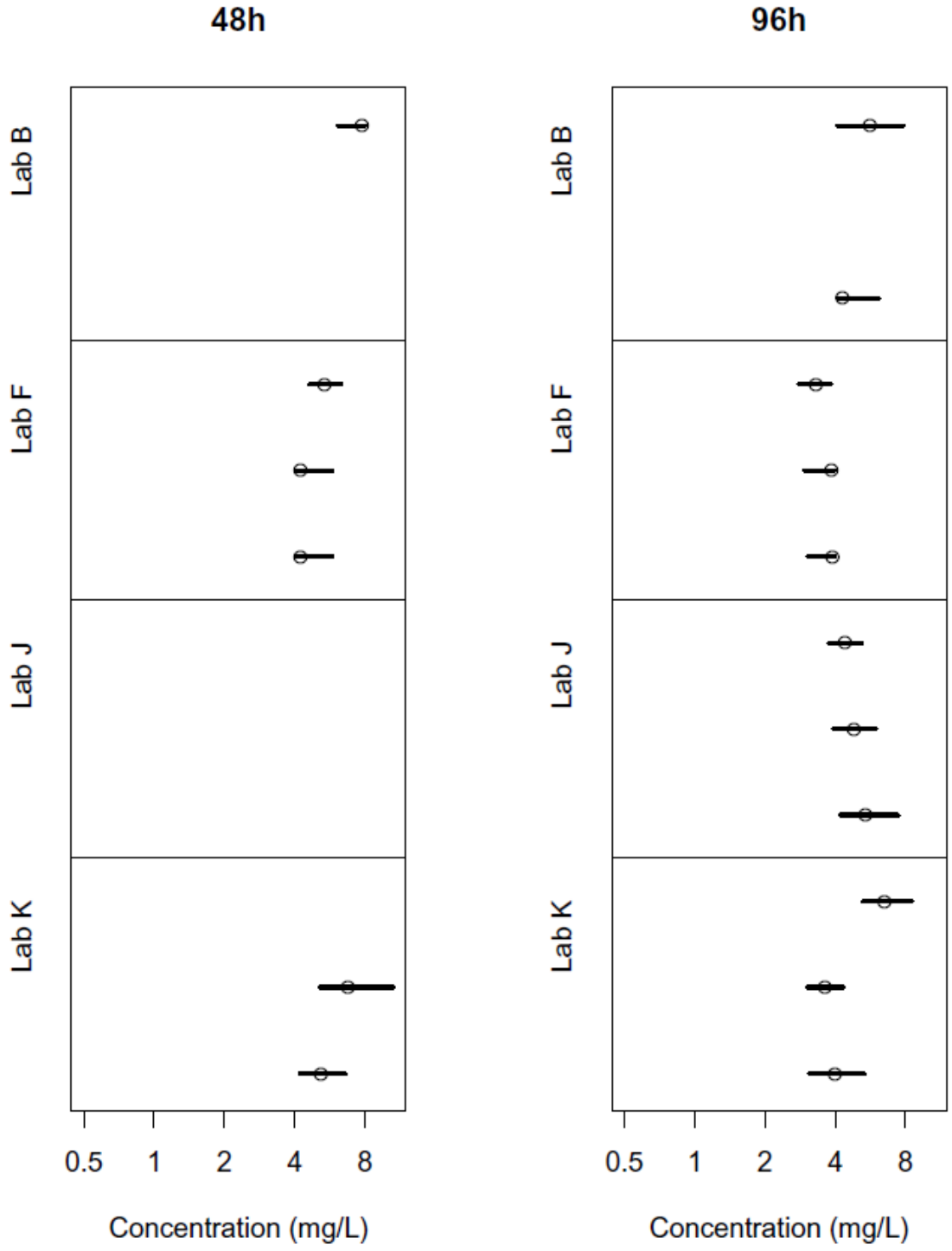
### 3.2.9 Malathion

***Table 11: $LC_{50}$ and confidence intervals of the Zebrafish Embryo Toxicity Test – three runs with Malathion***

| | | 48h (mg/L) | | | | 96h (mg/L) | | | |
|---|---|---|---|---|---|---|---|---|---|
| Lab | Run | $LC_{50}$ | Lower | Upper | Inform | $LC_{50}$ | Lower | Upper | Inform |
| B | 1 | 7.77 | 6.12 | 7.98 | 1 | 5.64 | 4.09 | 7.83 | 0 |
| | 2 | CNC | | | 4 | CNC | | | 4 |
| | 3 | CNC | | | 1 | 4.30 | 4.09 | 6.13 | 1 |
| | Mean | 7.77 | | | | 4.97 | | | |
| F | 1 | 5.38 | 4.63 | 6.33 | 2 | 3.31 | 2.81 | 3.83 | 2 |
| | 2 | 4.23 | 4.07 | 5.80 | 1 | 3.86 | 2.97 | 3.97 | 1 |
| | 3 | 4.23 | 4.07 | 5.80 | 1 | 3.89 | 3.07 | 3.99 | 1 |
| | Mean | 4.61 | | | | 3.69 | | | |
| J | 1 | CNC | | | 3 | 4.40 | 3.78 | 5.20 | 2 |
| | 2 | CNC | | | 5 | 4.81 | 3.94 | 5.98 | 4 |
| | 3 | CNC | | | 3 | 5.39 | 4.21 | 7.43 | 4 |
| | Mean | CNC | | | | 4.87 | | | |
| K | 1 | CNC | | | 3 | 6.50 | 5.28 | 8.52 | 3 |
| | 2 | 6.78 | 5.15 | 10.6 | 3 | 3.62 | 3.03 | 4.31 | 3 |
| | 3 | 5.20 | 4.22 | 6.59 | 4 | 4.00 | 3.11 | 5.32 | 4 |
| | Mean | 5.99 | | | | 4.71 | | | |
| | Grand Mean | 6.88 | | | | 4.56 | | | |

Inform = the number of informative concentrations (defined in Section 2.3; see figures in the Appendix B)
CNC – cannot calculate due to insufficient mortality

***Figure 10: $LC_{50}$ values and 95% confidence limits for runs of Malathion in the Zebrafish Embryo Toxicity Test***



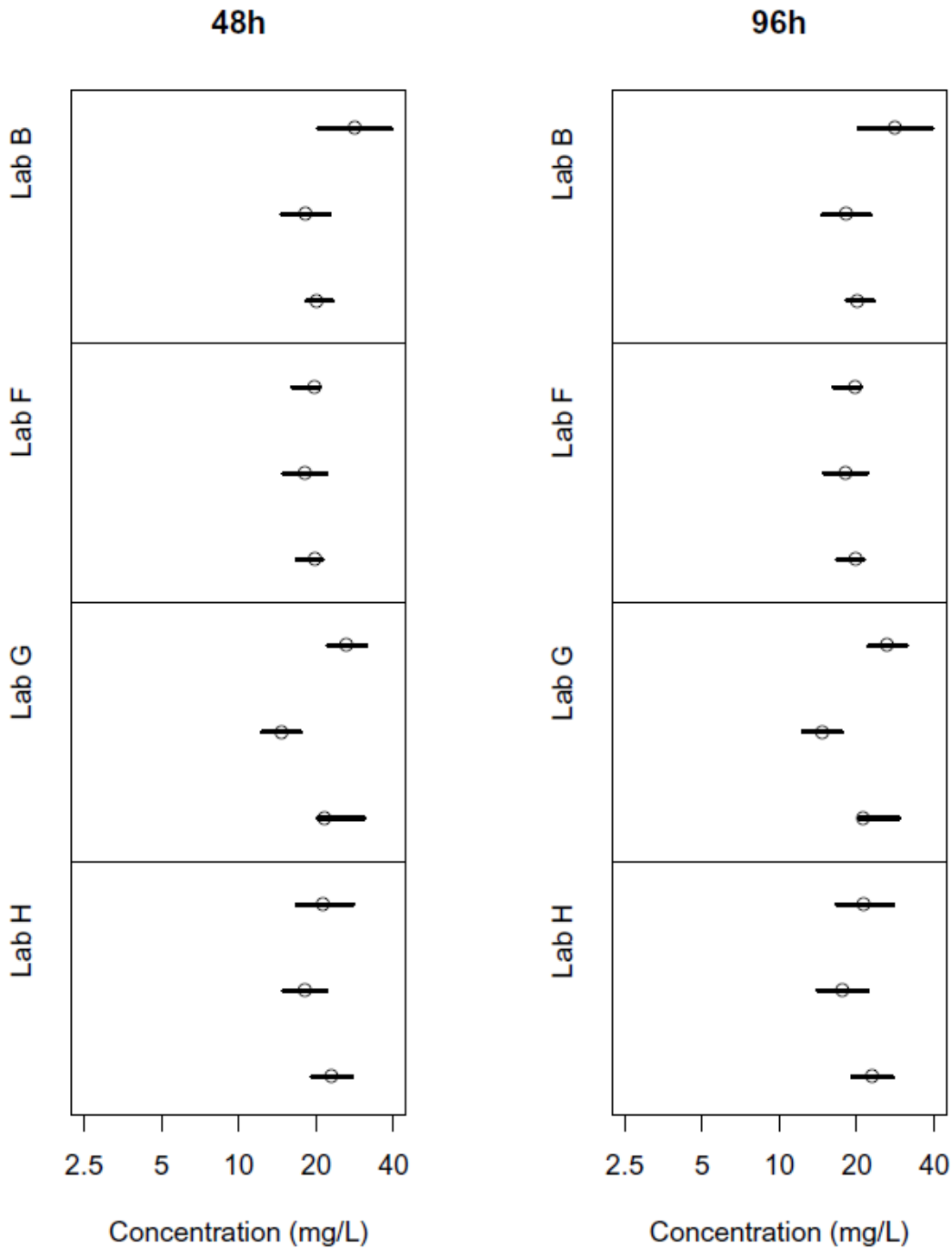
### 3.2.10 1-Octanol

***Table 12: $LC_{50}$ and confidence intervals of the Zebrafish Embryo Toxicity Test – three runs with 1-Octanol***

| | | 48h (mg/L) | | | | 96h (mg/L) | | | |
|---|---|---|---|---|---|---|---|---|---|
| Lab | Run | $LC_{50}$ | Lower | Upper | Inform | $LC_{50}$ | Lower | Upper | Inform |
| B | 1 | 28.2 | 20.4 | 39.2 | 0 | 28.2 | 20.4 | 39.2 | 0 |
| | 2 | 18.2 | 14.7 | 22.6 | 3 | 18.2 | 14.7 | 22.6 | 3 |
| | 3 | 20.1 | 18.4 | 23.1 | 1 | 20.1 | 18.4 | 23.1 | 1 |
| | Mean | 22.2 | | | | 22.2 | | | |
| F | 1 | 19.7 | 16.3 | 20.6 | 1 | 19.7 | 16.3 | 20.6 | 1 |
| | 2 | 18.1 | 15.0 | 21.9 | 4 | 18.1 | 15.0 | 21.9 | 4 |
| | 3 | 19.8 | 16.8 | 21.2 | 1 | 19.8 | 16.8 | 21.2 | 1 |
| | Mean | 19.2 | | | | 19.2 | | | |
| G | 1 | 26.2 | 22.3 | 31.2 | 3 | 26.2 | 22.3 | 31.2 | 3 |
| | 2 | 14.7 | 12.3 | 17.4 | 3 | 14.7 | 12.3 | 17.4 | 3 |
| | 3 | 21.5 | 20.5 | 30.7 | 1 | 21.2 | 20.3 | 29.0 | 1 |
| | Mean | 20.8 | | | | 20.7 | | | |
| H | 1 | 21.3 | 16.8 | 27.7 | 4 | 21.3 | 16.8 | 27.7 | 4 |
| | 2 | 18.1 | 15.0 | 21.9 | 3 | 17.6 | 14.1 | 22 | 4 |
| | 3 | 22.9 | 19.3 | 27.6 | 3 | 22.9 | 19.3 | 27.6 | 3 |
| | Mean | 20.8 | | | | 20.6 | | | |
| | Grand Mean | 20.7 | | | | 20.7 | | | |

Inform = the number of informative concentrations (defined in Section 2.3; see figures in the Appendix B)

***Figure 11: $LC_{50}$ values and 95% confidence limits for runs of 1-Octanol in the Zebrafish Embryo Toxicity Test***



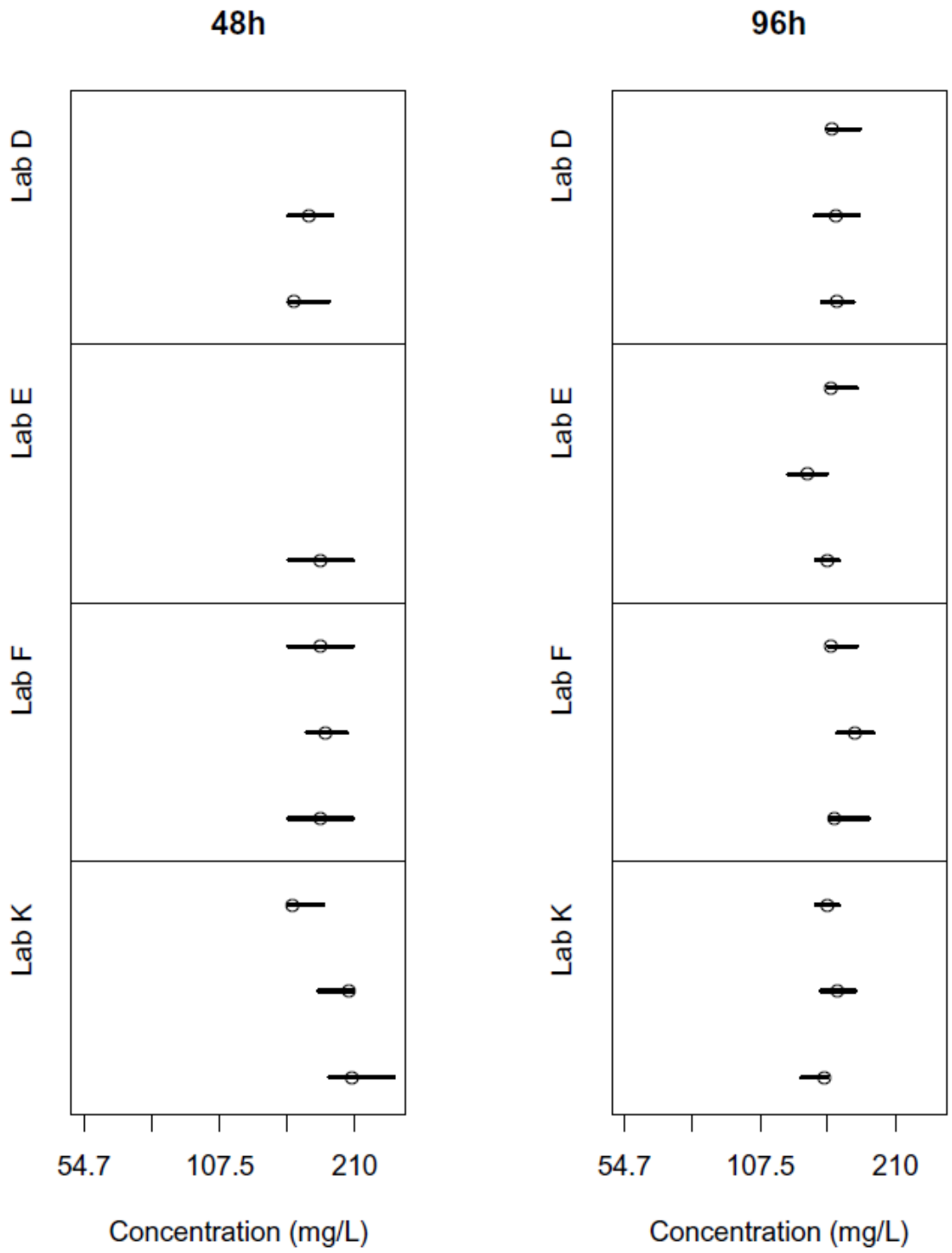
### 3.2.11 Carbamazepine

***Table 13: $LC_{50}$ and confidence intervals of the Zebrafish Embryo Toxicity Test – three runs with Carbamazepine***

| | | 48h (mg/L) | | | | 96h (mg/L) | | | |
|---|---|---|---|---|---|---|---|---|---|
| Lab | Run | $LC_{50}$ | Lower | Upper | Inform | $LC_{50}$ | Lower | Upper | Inform |
| D | 1 | CNC | | | 1 | 153 | 151 | 176 | 1 |
| | 2 | 167 | 151 | 188 | 3 | 156 | 141 | 175 | 3 |
| | 3 | 155 | 152 | 185 | 1 | 157 | 146 | 170 | 2 |
| | Mean | 161 | | | | 156 | | | |
| E | 1 | CNC | | | 1 | 153 | 150 | 173 | 1 |
| | 2 | CNC | | | 4 | 136 | 124 | 149 | 3 |
| | 3 | 177 | 152 | 208 | 0 | 150 | 142 | 159 | 1 |
| | Mean | 177 | | | | 146 | | | |
| F | 1 | 177 | 152 | 208 | 0 | 153 | 150 | 173 | 1 |
| | 2 | 182 | 166 | 202 | 5 | 172 | 158 | 188 | 4 |
| | 3 | 177 | 152 | 208 | 0 | 155 | 152 | 185 | 1 |
| | Mean | 179 | | | | 160 | | | |
| K | 1 | 154 | 151 | 180 | 1 | 150 | 142 | 159 | 1 |
| | 2 | 204 | 175 | 208 | 1 | 158 | 145 | 173 | 3 |
| | 3 | 207 | 185 | 255 | 2 | 148 | 132 | 150 | 1 |
| | Mean | 189 | | | | 152 | | | |
| | Grand Mean | 177 | | | | 153 | | | |

Inform = the number of informative concentrations (defined in Section 2.3; see figures in the Appendix B)
CNC – cannot calculate due to insufficient mortality

***Figure 12: $LC_{50}$ values and 95% confidence limits for runs of Carbamazepine in the Zebrafish Embryo Toxicity Test***



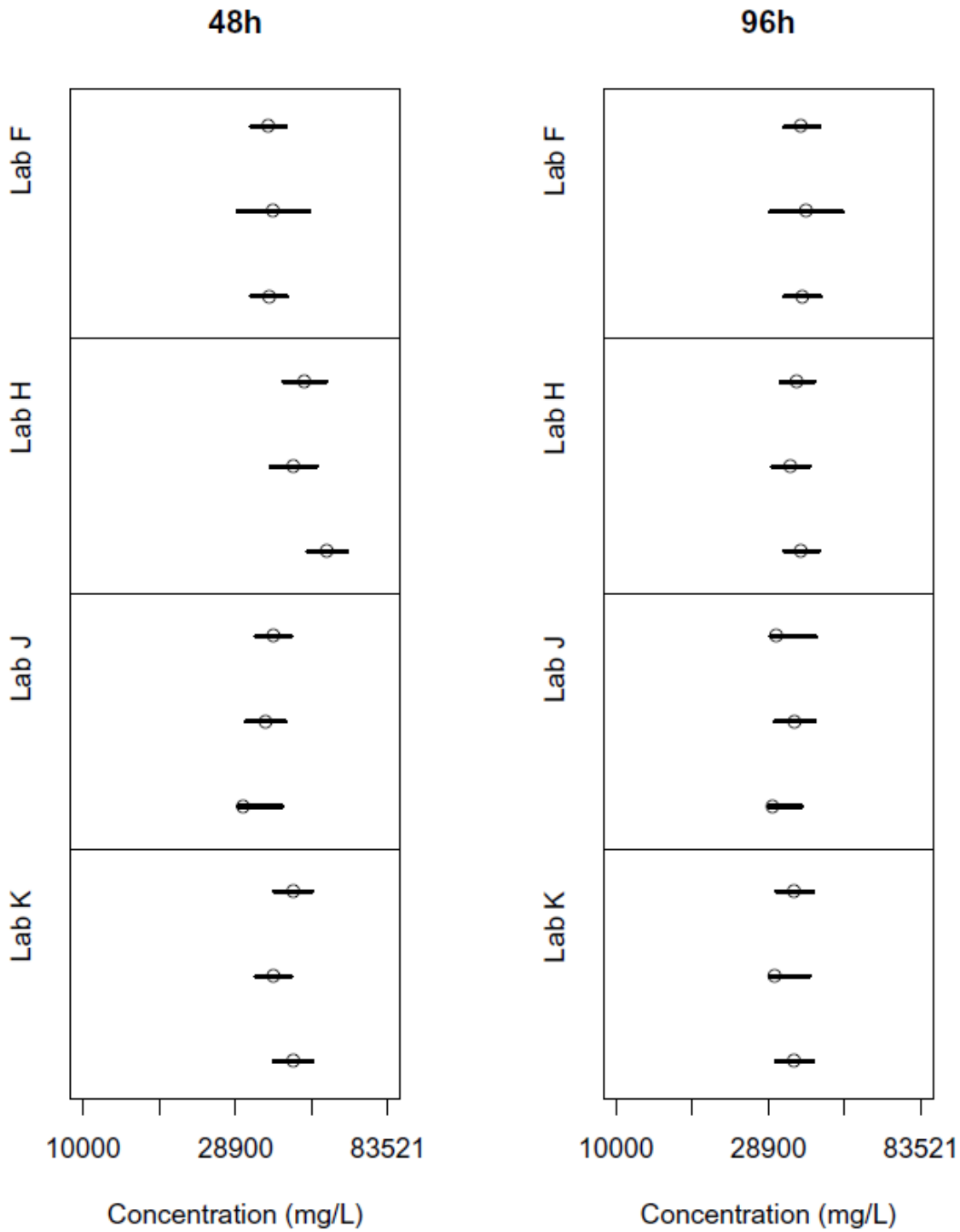
### 3.2.12 Dimethyl Sulfoxide

***Table 14: $LC_{50}$ and confidence intervals of the Zebrafish Embryo Toxicity Test – three runs with Dimethyl Sulfoxide***

| | | 48h (mg/L) | | | | 96h (mg/L) | | | |
|---|---|---|---|---|---|---|---|---|---|
| Lab | Run | $LC_{50}$ | Lower | Upper | Inform | $LC_{50}$ | Lower | Upper | Inform |
| F | 1 | 36300 | 32300 | 41000 | 2 | 36300 | 32300 | 41000 | 2 |
| | 2 | 37600 | 29400 | 48300 | 0 | 37600 | 29400 | 48300 | 0 |
| | 3 | 36500 | 32300 | 41400 | 3 | 36500 | 32300 | 41400 | 3 |
| | Mean | 36800 | | | | 36800 | | | |
| H | 1 | 46700 | 40400 | 54200 | 3 | 46700 | 40400 | 54200 | 3 |
| | 2 | 43200 | 36900 | 50800 | 3 | 43200 | 36900 | 50800 | 3 |
| | 3 | 54500 | 47800 | 62800 | 3 | 54500 | 47800 | 62800 | 3 |
| | Mean | 48200 | | | | 48200 | | | |
| J | 1 | 37700 | 33300 | 42600 | 2 | 37700 | 33300 | 42600 | 2 |
| | 2 | 35600 | 31200 | 40800 | 3 | 35600 | 31200 | 40800 | 3 |
| | 3 | 30500 | 29400 | 40100 | 1 | 30500 | 29400 | 40100 | 1 |
| | Mean | 34600 | | | | 34600 | | | |
| K | 1 | 43300 | 37800 | 49200 | 2 | 43300 | 37800 | 49200 | 2 |
| | 2 | 37700 | 33400 | 42500 | 2 | 37700 | 33400 | 42500 | 2 |
| | 3 | 43300 | 37800 | 49200 | 2 | 43300 | 37800 | 49200 | 2 |
| | Mean | 41400 | | | | 41400 | | | |
| | Grand Mean | 40200 | | | | 40200 | | | |

Inform = the number of informative concentrations (defined in Section 2.3; see figures in the Appendix B)

***Figure 13: $LC_{50}$ values and 95% confidence limits for runs of Dimethyl Sulfoxide in the Zebrafish Embryo Toxicity Test***



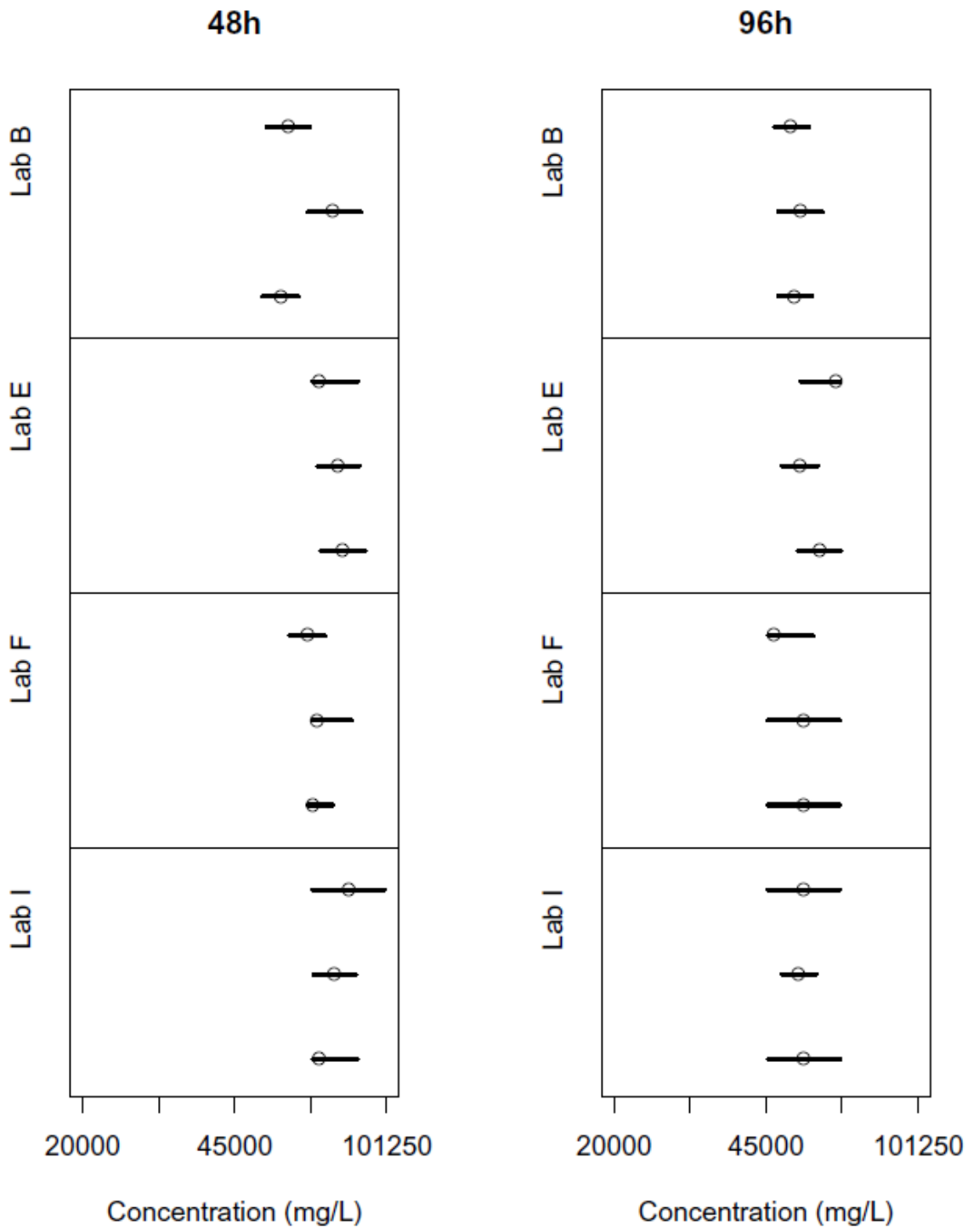
### 3.2.13 Triethylene glycol

***Table 15: $LC_{50}$ and confidence intervals of the Zebrafish Embryo Toxicity Test – three runs with Triethylene glycol***

| | | 48h (mg/L) | | | | 96h (mg/L) | | | |
|---|---|---|---|---|---|---|---|---|---|
| Lab | Run | $LC_{50}$ | Lower | Upper | Inform | $LC_{50}$ | Lower | Upper | Inform |
| B | 1 | 59900 | 53500 | 67100 | 3 | 51200 | 47100 | 56400 | 2 |
| | 2 | 75900 | 66600 | 88400 | 4 | 54000 | 48100 | 60900 | 4 |
| | 3 | 57700 | 52200 | 63300 | 3 | 52300 | 48000 | 57500 | 2 |
| | Mean | 64500 | | | | 52500 | | | |
| E | 1 | 70500 | 68400 | 86600 | 1 | 65300 | 54300 | 66900 | 1 |
| | 2 | 77900 | 70100 | 87300 | 4 | 53800 | 49000 | 59300 | 3 |
| | 3 | 79800 | 71400 | 90300 | 3 | 59900 | 53500 | 67100 | 3 |
| | Mean | 76100 | | | | 59700 | | | |
| F | 1 | 66300 | 60300 | 72700 | 2 | 46900 | 45600 | 57800 | 1 |
| | 2 | 69800 | 68100 | 83900 | 1 | 55000 | 45600 | 66700 | 0 |
| | 3 | 68100 | 66300 | 76100 | 1 | 55000 | 45600 | 66700 | 0 |
| | Mean | 68100 | | | | 52300 | | | |
| I | 1 | 82600 | 68400 | 100000 | 0 | 55000 | 45600 | 66700 | 0 |
| | 2 | 76400 | 68600 | 85800 | 3 | 53500 | 49000 | 58800 | 2 |
| | 3 | 70500 | 68400 | 86600 | 1 | 55000 | 45600 | 66700 | 0 |
| | Mean | 76500 | | | | 54500 | | | |
| | Grand Mean | 71300 | | | | 54800 | | | |

Inform = the number of informative concentrations (defined in Section 2.3; see figures in the Appendix B)

***Figure 14 : $LC_{50}$ values and 95% confidence limits for runs of Triethylene glycol in the Zebrafish Embryo Toxicity Test***



### 3.2. Global Comparisons

The overall spread in the toxicity of chemicals assessed in the validation is given in Table 16. This perspective is in addition to summarizing Coefficients of Variation given below. The ranges quoted in Table 16 are the minimum and maximum mean LC50s for a chemical across the laboratories which tested that chemical. Several observations can be provided. The largest maximum:minimum ratios are approximately 3.5 and this occurs for the most toxic compounds. Many runs for these highly toxic chemicals have very steep exposure-response curves which amplify differencfes between runs and laboratories. Merquat 100 has the single largest ratio (4.24) which is attributable to variation in toxicity caused by lower penetration of the chemical across the chorion and small differences in hatching times (when most of the toxicity actually occurs). Maximum:minimum ratios are also relatively similar at 48 and 96 h.

An overall graphical summary of results appears in Figures 15 and 16. Chemicals are ordered by the average toxicity value estimated in this validation study. The gray shading within each chemical identifies the range of concentrations tested, where the vertical lines within each shaded region, along with the outer vertical edges, are the five concentration levels tested. The horizontal dashed lines within the gray-shaded regions separate the sets of three runs contributed by each participating laboratory. Letter codes for the laboratories are placed in the margins.

Of special note are 48h results for the polymers Merquat 100 and Luviquat HM 552. As expected, toxicity was not observed at this time point. Following hatch, toxicity was observed at 96h. Prochloraz, malathion, and carbamazepine were challenging compounds with testing occurring near the limits of solubility and in some runs were not toxic at or near the highest concentration. For all the compounds the span of $LC_{50}$s was remarkably small.

Estimates and confidence intervals shown in the light red color are calculated results for which the model predicted value at the maximum concentration tested is below 50%. These results are not used in the further summarization of results.

**Table 16: $LC_{50}$ ranges and range ratios**

| Chemical | Time (h) | Min $LC_{50}$ | Max $LC_{50}$ | Ratio |
|---|---|---|---|---|
| Methylmercury (II) chloride | 48 | 0.0201 | 0.0606 | 3.0 |
| | 96 | 0.0115 | 0.0427 | 3.7 |
| Copper (II) sulphate pentahydrate | 48 | 0.164 | 0.582 | 3.55 |
| | 96 | 0.164 | 0.450 | 2.74 |
| Tetradecyl sulfate sodium salt | 48 | 0.195 | 0.576 | 2.95 |
| | 96 | 0.195 | 0.573 | 2.94 |
| 4,6-Dinitro-o-cresol | 48 | 0.493 | 0.987 | 2.00 |
| | 96 | 0.431 | 0.669 | 1.55 |
| Merquat 100 | 48 | A | A | A |
| | 96 | 0.245 | 1.040 | 4.24 |
| Luviquat HM 552 | 48 | A | A | A |
| | 96 | 0.649 | 1.380 | 2.13 |
| 2,4-Dinitrophenol | 48 | 2.33 | 5.16 | 2.21 |
| | 96 | 1.77 | 4.78 | 2.70 |
| Prochloraz | 48 | 3.87 (B) | 5.02 (B) | 1.30 (B) |
| | 96 | 3.62 (C) | 4.94 (C) | 1.36 (C) |
| Malathion | 48 | 4.23 (D) | 7.77 (D) | 1.84 (D) |
| | 96 | 3.31 (E) | 6.50 (E) | 1.96 (E) |
| 1-Octanol | 48 | 14.7 | 28.2 | 1.92 |
| | 96 | 14.7 | 28.2 | 1.92 |
| Carbamazepine | 48 | 154 (F) | 207 (F) | 1.34 (F) |
| | 96 | 136 | 172 | 1.26 |
| Dimethyl Sulfoxide | 48 | 30500 | 54500 | 1.79 |
| | 96 | 29700 | 37600 | 1.27 |
| Triethylene glycol | 48 | 57700 | 82600 | 1.43 |
| | 96 | 46900 | 65300 | 1.39 |

A: Only one 48h $LC_{50}$ was determined in 12 runs
B: $LC_{50}$s were not generated by 6 runs across all labs
C: $LC_{50}$s were not generated by 4 runs across all labs
D: $LC_{50}$s were not generated by 6 runs across all labs
E: $LC_{50}$ was not generated in 1 run across all labs
F: $LC_{50}$s were not generated by 3 runs across all labs

**Figure 15: Global Summary of 48h test results**

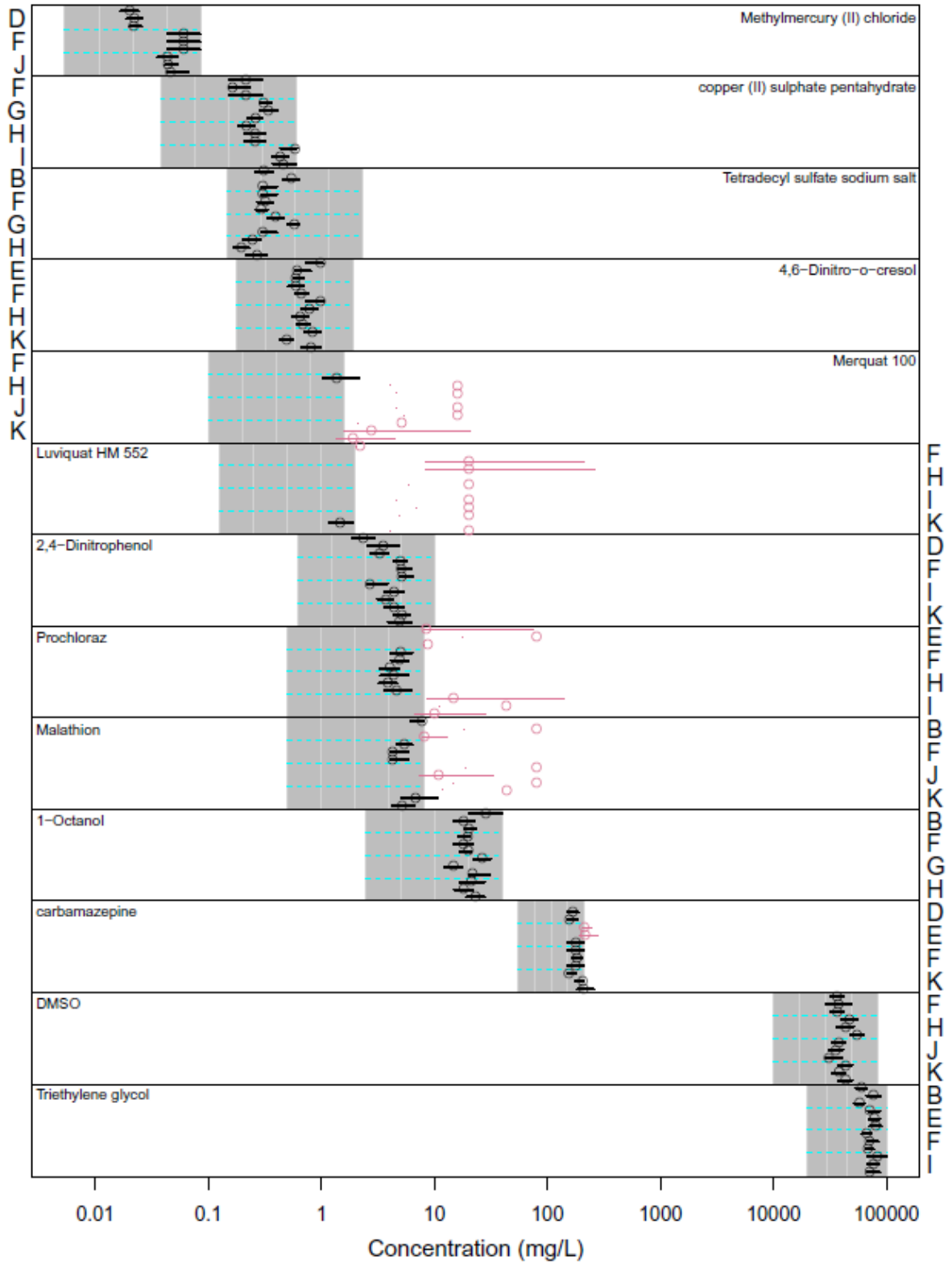
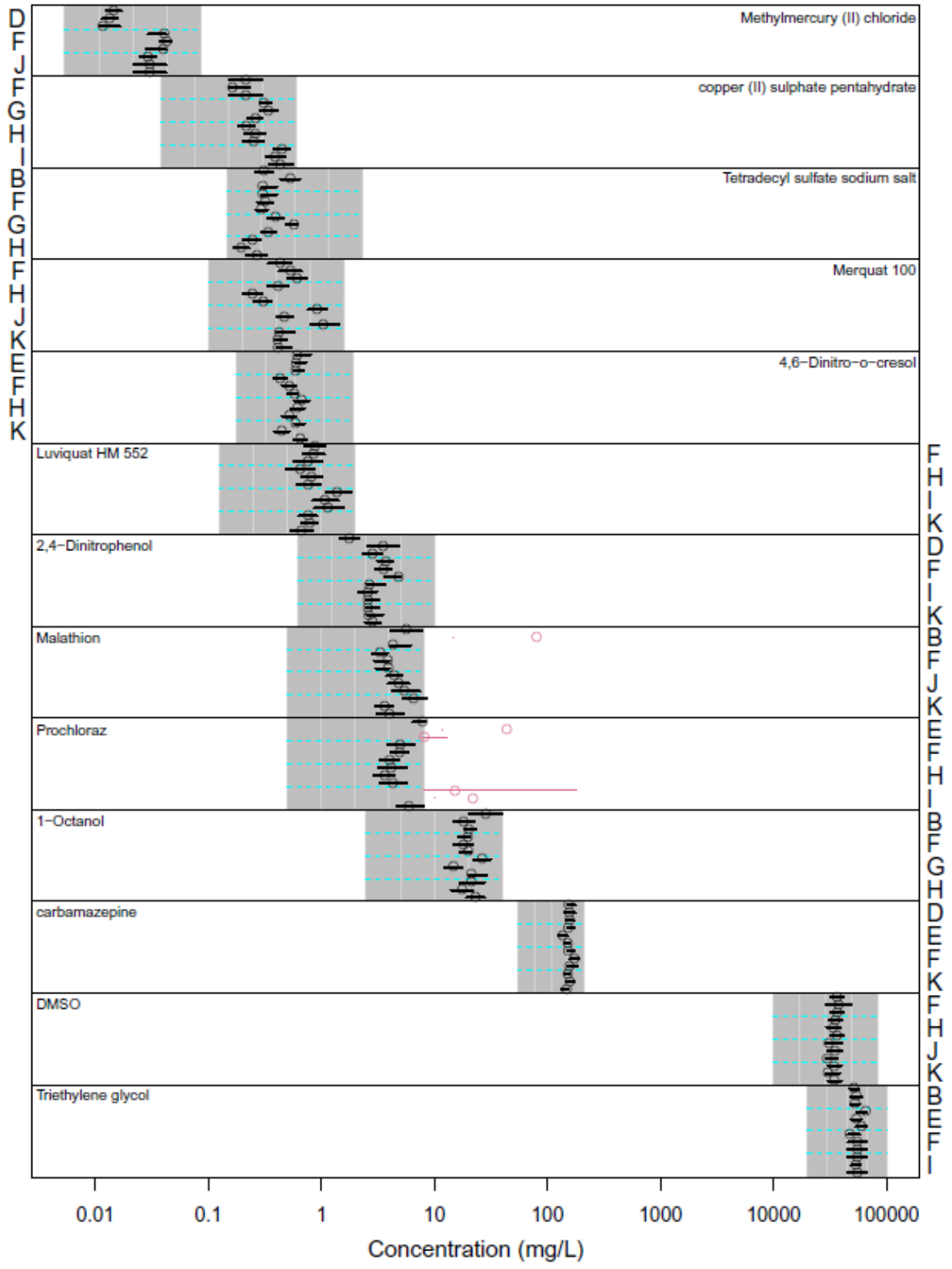


**Figure 16: Global Summary of 96h test results**



### 3.3. Intra-laboratory reproducibility

The calculated coefficients of variation per laboratory and chemical are given in Table 17.

***Table 17: Intra-laboratory reproducibility - coefficients of variation (%) for 13 chemicals at 48h – three runs***

| Chemical | 48h Results | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | B | D | E | F | G | H | I | J | K |
| Methylmercury (II) chloride | - - | 5.1 | - - | 0 | - - | - - | - - | 3.7 | - - |
| Copper (II) sulphate pentahydrate | - - | - - | - - | 14.9 | 12.9 | 9.9 | 16.5 | - - | - - |
| Tetradecyl sulfate sodium salt | 35.7 | - - | - - | 4.5 | 32.8 | 15.9 | - - | - - | - - |
| 4,6-Dinitro-o-cresol | - - | - - | 30.9 | 27.8 | - - | 9.7 | - - | - - | 26.5 |
| Merquat 100 | - - | - - | - - | NA | - - | NA | - - | NA | NA |
| Luviquat HM 552 | - - | - - | - - | NA | - - | NA | NA | - - | NA |
| 2,4-Dinitrophenol | - - | 20.6 | - - | 1.2 | - - | - - | 23.6 | - - | 8.4 |
| Prochloraz | - - | - - | NA | 11.8 | - - | 8.9 | NA | - - | - - |
| Malathion | NA | - - | - - | 14.4 | - - | - - | - - | NA | 18.7 |
| 1-Octanol | 24.1 | - - | - - | 4.9 | 27.9 | 11.8 | - - | - - | - - |
| Carbamazepine | - - | 5.3 | NA | 1.5 | - - | - - | - - | - - | 15.9 |
| DMSO | - - | - - | - - | 1.9 | - - | 12.1 | - - | 10.6 | 7.8 |
| Triethylene glycol | 15.4 | - - | 6.5 | 2.6 | - - | - - | 7.9 | - - | - - |
| | 96h Results | | | | | | | | |
| Methylmercury (II) chloride | - - | 11.2 | - - | 3.5 | - - | - - | - - | 2.1 | - - |
| Copper (II) sulphate pentahydrate | - - | - - | - - | 14.9 | 12.9 | 9.2 | 7.2 | - - | - - |
| Tetradecyl sulfate sodium salt | 34.0 | - - | - - | 4.5 | 28.2 | 15.9 | - - | - - | - - |
| 4,6-Dinitro-o-cresol | - - | - - | 1.7 | 14.3 | - - | 12.4 | - - | - - | 18.5 |
| Merquat 100 | - - | - - | - - | 17.1 | - - | 26.7 | - - | 37.2 | 1.9 |
| Luviquat HM 552 | - - | - - | - - | 7.8 | - - | 11.6 | 13.5 | - - | 9.1 |
| 2,4-Dinitrophenol | - - | 32.7 | - - | 16.8 | - - | NA | 1.6 | - - | 5.3 |
| Prochloraz | - - | - - | NA | 11.3 | - - | 8.7 | NA | - - | - - |
| Malathion | 19.0 | - - | - - | 8.8 | - - | - - | - - | 10.3 | 33.2 |
| 1-Octanol | 24.1 | - - | - - | 4.9 | 28.0 | 13.3 | - - | - - | - - |
| Carbamazepine | - - | 1.3 | 6.1 | 6.5 | - - | - - | - - | - - | 3.4 |
| DMSO | - - | - - | - - | 1.9 | - - | 3.7 | - - | 8.5 | 7.8 |
| Triethylene glycol | 2.7 | - - | 9.7 | 8.9 | - - | - - | 1.6 | - - | - - |

- -: chemical not tested
NA: CV could not be calculated

### 3.3. Inter-laboratory reproducibility

The inter-laboratory coefficients of variation were calculated based on the combined $LC_{50}$ calculations and are given in Table 18. In addition, the combined CV for all laboratories that tested 3,4-Dichloroaniline in Phase 1a and Phase 2a is presented in Table 19.

***Table 18: Inter-laboratory reproducibility - coefficients of variation for 13 chemicals based on means of three runs per laboratory***

| Time (h) | Chemical | CV (%) | Number of labs | Number of labs contributing to CV calculations | Total number of runs contributing to CV calculations |
|---|---|---|---|---|---|
| 48 | Methylmercury (II) chloride | 46.9 | 3 | 3 | 9 |
| | Copper (II) sulphate pentahydrate | 41.7 | 4 | 4 | 12 |
| | Tetradecyl sulfate sodium salt | 25.0 | 4 | 4 | 12 |
| | 4,6-Dinitro-o-cresol | 2.8 | 4 | 4 | 12 |
| | Merquat 100 | NA | 4 | 1 | 1 |
| | Luviquat HM 552 | NA | 4 | 1 | 1 |
| | 2,4-Dinitrophenol | 23.5 | 4 | 4 | 12 |
| | Prochloraz | 5.8 | 4 | 2 | 6 |
| | Malathion | 25.8 | 4 | 3 | 6 |
| | 1-Octanol | 5.9 | 4 | 4 | 12 |
| | Carbamazepine | 6.4 | 4 | 4 | 9 |
| | DMSO | 14.9 | 4 | 4 | 12 |
| | Triethylene glycol | 8.4 | 4 | 4 | 12 |
| 96 | Methylmercury (II) chloride | 50.2 | 3 | 3 | 9 |
| | Copper (II) sulphate pentahydrate | 33.6 | 4 | 4 | 12 |
| | Tetradecyl sulfate sodium salt | 25.8 | 4 | 4 | 12 |
| | 4,6-Dinitro-o-cresol | 7.5 | 4 | 4 | 12 |
| | Merquat 100 | 40.8 | 4 | 4 | 12 |
| | Luviquat HM | 24.8 | 4 | 4 | 12 |
| | 2,4-Dinitrophenol | 22.7 | 4 | 4 | 12 |
| | Prochloraz | 30.4 | 4 | 4 | 8 |
| | Malathion | 13.0 | 4 | 4 | 12 |
| | 1-Octanol | 5.9 | 4 | 4 | 12 |
| | Carbamazepine | 3.8 | 4 | 4 | 12 |
| | DMSO | 6.6 | 4 | 4 | 12 |
| | Triethylene glycol | 6.3 | 4 | 4 | 12 |

***Table 19: Inter-laboratory reproducibility - coefficients of variation for 3,4-DCA based on means of three runs per laboratory for all laboratories participating in Phase 1a and Phase 2a***

| Time (h) | Laboratory | Phase | Mean $LC_{50}$ (mg/L) | CV (%) |
|---|---|---|---|---|
| 48 | A | 1a | 3.7* | |
| | B | 1a | 1.2* | |
| | C | 1a | 3.1 | |
| | D | 1a | 2.8 | |
| | E | 1a | 4.5 | |
| | F | 1a | 3.0 | |
| | G | 1a | 4.3 | |
| | H | 2a | 3.9 | |
| | I | 2a | 2.8 | |
| | J | 2a | 3.4 | |
| | K | 2a | 3.8 | 27.4 |
| 96 | A | 1a | 3.5* | |
| | B | 1a | 1.2* | |
| | C | 1a | 2.4 | |
| | D | 1a | 2.6 | |
| | E | 1a | 4.1 | |
| | F | 1a | 2.6 | |
| | G | 1a | 3.4 | |
| | H | 2a | 2.7 | |
| | I | 2a | 2.6 | |
| | J | 2a | 2.7 | |
| | K | 2a | 3.2 | 26.4 |

*based on two runs
Note that Phase 1a results were previously given in OECD (2011)[7]

## 4. Effect on internal controls due to chemical toxicity in neighbouring wells

Figures 17, 18, and 19 evaluate the correlation of treatment toxicity with internal control mortality. Because internal controls are paired with exactly one chemical, concentration, and time, there is a possibility that internal controls could respond to toxic effects due to treatments applied to neighbouring wells. The premise is that, particularly for volatile substances or highly toxic exposures, there may be a toxic effect on neighbouring control wells, even though no toxic treatment is directly applied.

In these figures, internal control mortality is the percentage calculated by pooling all data across laboratories and runs for a given chemical, concentration, and time, compared to a similar pooling of chemical data on the same plates tested with the identical chemical, concentration, and time. In total there are 74 data points: for each time point there are thirteen chemicals and

[7] OECD 2011 - Series on Testing and Assessment No. 157; Validation Report (Phase 1) for the Zebrafish Embryo Toxicity Test Part 2.

for each chemical there are six treatments (5 chemical concentrations, 1 positive control) which accounts for 13x6 = 78 data points per time, or 156 in total.

As shown in Figure 17, at 48h only two (Luviquat HM 552 and triethylene glycol) of the thirteen chemicals show a positive correlation, and nine with a negative correlation, though none are statistically significant. At 96h, Figure 18 shows five of the thirteen correlations are positive (triethylene glycol, carbamazepine, copper, 2,4-dinitrophenol, and Luviquat HM 552), and again none are significant. The 48h and 96h data are highly correlated in time (i.e., are nearly the same).

All of the data in Figures 17 and 18 are combined into Figure 19, where the colours and symbol styles of Figures 17 and 18 are carried over. A linear regression model is superimposed showing little or no effect of chemical toxicity on matched internal control toxicity. In fact, the slope ( $\hat{b}_{LR} = -0.0001$) is slightly negative, and nowhere close to statistically significant ($p$=0.9).

These data are further broken down by chemical and time in Figures 17 and 18. Nothing noteworthy appears in these displays.

**Figure 17: 48h Correlation of internal control mortality as function of chemical toxicity, for each chemical separately**

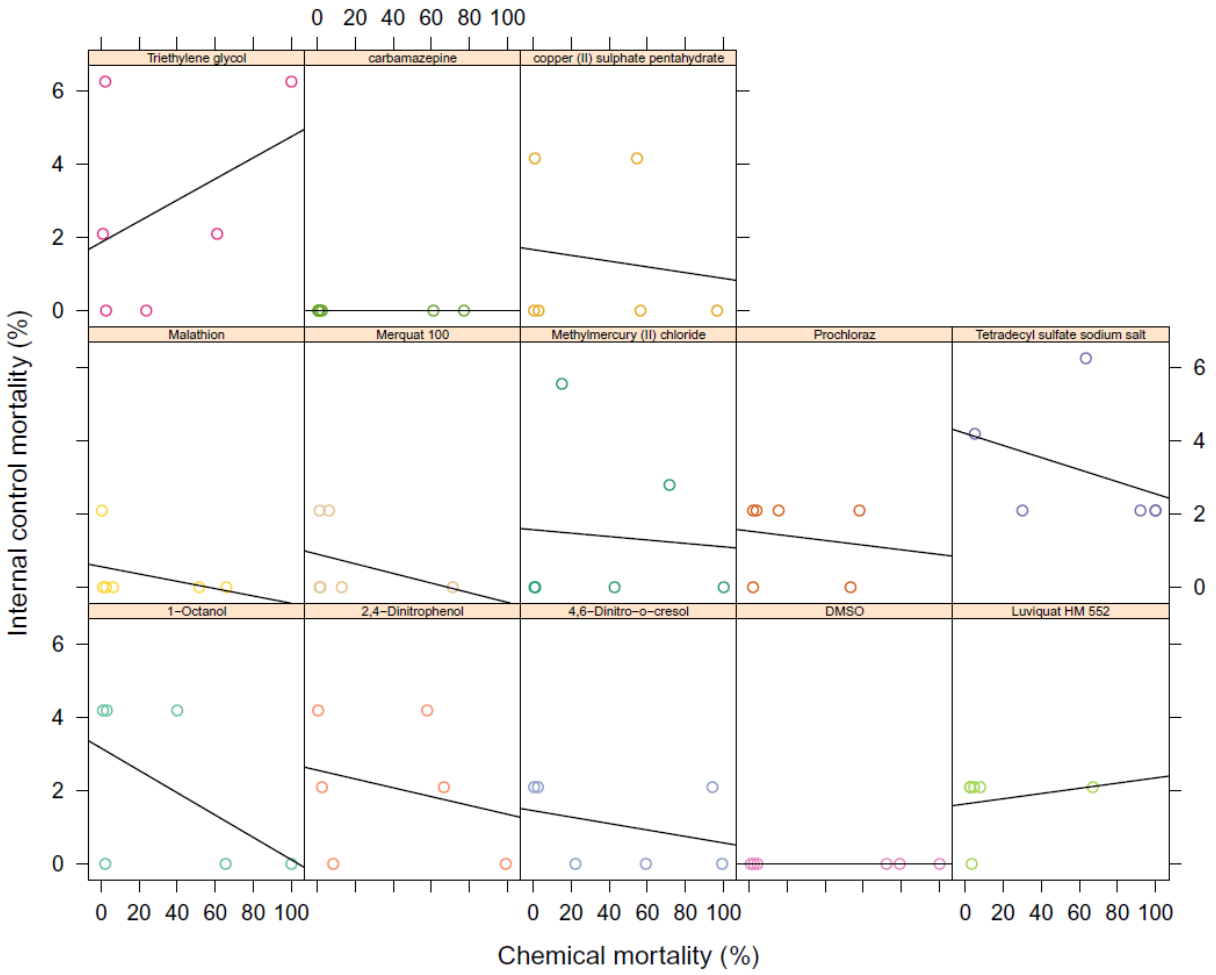


**Figure 18: 96h Correlation of internal control mortality as function of chemical toxicity, for each chemical separately**

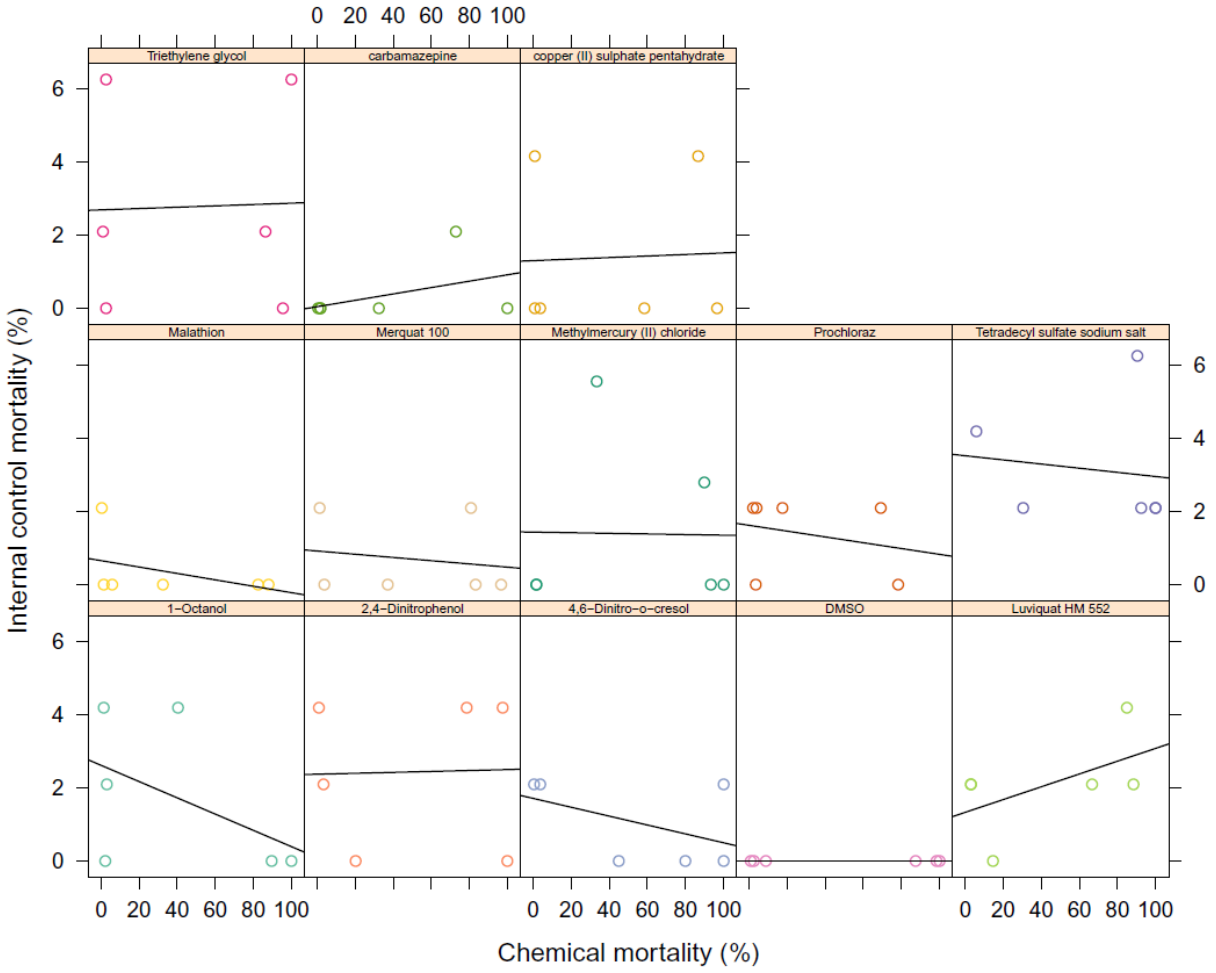
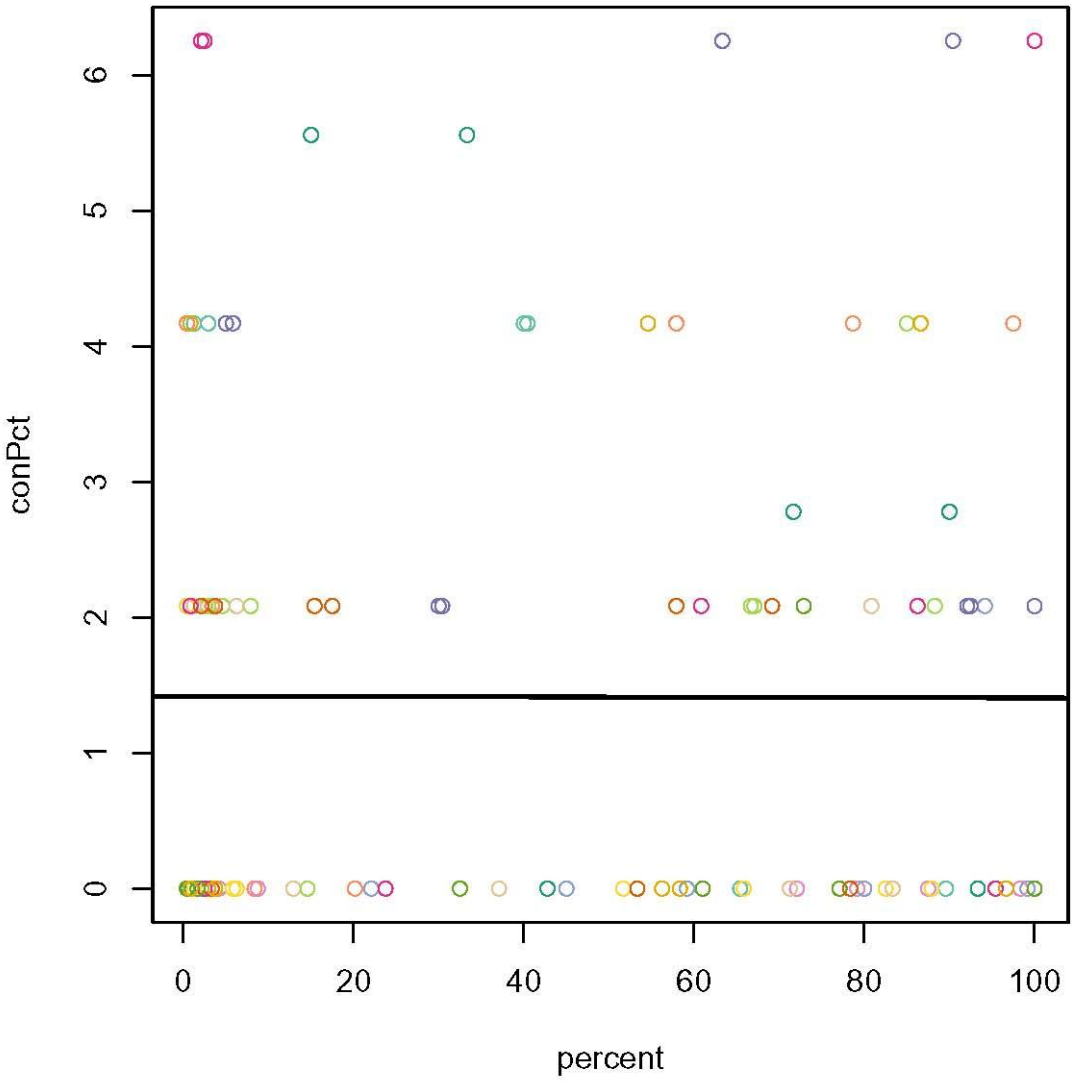


**Figure 19: Correlation of internal control mortality as function of chemical toxicity**



## 5. Overview internal, external and positive controls per laboratory

The external control mortality was somewhat variable across laboratories but overall was slightly lower than observed in Phase 1b (Tables 20.1, 20.2). Laboratory B in particular has the highest mortality for both internal and external control observations, but this is less than the highest from Phase 1b. Laboratories G and J had lower rates of mortality at 96h vs. 48h, most likely as a result of more definitive observations of non-coagulation endpoints at the latter time point. External and internal control mortality rates were entirely consistent with overall observations from Phase 1b (OECD 2011, Report No. 157).

The 3.4-DCA positive control data reported in Table 21 were highly similar to observations in Phase 1b.

***Table 20.1: Summarised overview: external controls***

| Time (h) | Lab | Dead | Total | Percent | Runs |
|---|---|---|---|---|---|
| 48 | B | 8 | 288 | 2.78 | 12 |
| | D | 2 | 216 | 0.93 | 9 |
| | E | 1 | 288 | 0.35 | 12 |
| | F | 15 | 936 | 1.60 | 39 |
| | G | 6 | 216 | 2.78 | 9 |
| | H | 10 | 576 | 1.74 | 24 |
| | I | 2 | 360 | 0.56 | 15 |
| | J | 2 | 288 | 0.69 | 12 |
| | K | 0 | 504 | 0.00 | 21 |
| | Total | 46 | 3672 | 1.25 | 153 |
| 96 | B | 8 | 288 | 2.78 | 12 |
| | D | 2 | 214* | 0.93 | 9 |
| | E | 1 | 288 | 0.35 | 12 |
| | F | 17 | 936 | 1.82 | 39 |
| | G | 7 | 216 | 3.24 | 9 |
| | H | 10 | 576 | 1.74 | 24 |
| | I | 2 | 360 | 0.56 | 15 |
| | J | 2 | 288 | 0.69 | 12 |
| | K | 0 | 504 | 0.00 | 21 |
| | Total | 49 | 3670 | 1.34 | 153 |

* 2 embryos were lost during the daily renewal of the medium

***Table 20.2: Summarised overview: internal controls***

| Time (h) | Lab | Dead | Total | Percent | Runs |
|---|---|---|---|---|---|
| 48 | B | 12 | 288 | 4.17 | 12 |
| | D | 6 | 216 | 2.78 | 9 |
| | E | 1 | 288 | 0.35 | 12 |
| | F | 11 | 936 | 1.18 | 39 |
| | G | 5 | 216 | 2.31 | 9 |
| | H | 12 | 576 | 2.08 | 24 |
| | I | 4 | 360 | 1.11 | 15 |
| | J | 0 | 288 | 0.00 | 12 |
| | K | 0 | 504 | 0.00 | 21 |
| | Total | 51 | 3672 | 1.39 | 153 |
| 96 | B | 11 | 288 | 3.82 | 12 |
| | D | 7 | 216 | 3.24 | 9 |
| | E | 1 | 288 | 0.35 | 12 |
| | F | 11 | 936 | 1.18 | 39 |
| | G | 5 | 216 | 2.31 | 9 |
| | H | 12 | 576 | 2.08 | 24 |
| | I | 5 | 360 | 1.39 | 15 |
| | J | 0 | 288 | 0.00 | 12 |
| | K | 1 | 504 | 0.20 | 21 |
| | Total | 53 | 3672 | 1.44 | 153 |

***Table 21: Summarised overview positive controls***

| Time (h) | Lab | Dead | Total | Percent | Runs |
|---|---|---|---|---|---|
| 48 | B | 235 | 240 | 97.9 | 12 |
| | D | 150 | 176 | 85.2 | 9 |
| | E | 47 | 240 | 19.6 | 12 |
| | F | 437 | 780 | 56.0 | 39 |
| | G | 34 | 180 | 18.9 | 9 |
| | H | 399 | 480 | 83.1 | 24 |
| | I | 155 | 300 | 51.7 | 15 |
| | J | 177 | 240 | 73.8 | 12 |
| | K | 263 | 420 | 62.6 | 21 |
| | Total | 1897 | 3056 | 62.1 | 153 |
| 96 | B | 239 | 240 | 99.6 | 12 |
| | D | 173 | 176 | 98.2 | 9 |
| | E | 147 | 240 | 61.3 | 12 |
| | F | 616 | 780 | 79.0 | 39 |
| | G | 151 | 180 | 83.9 | 9 |
| | H | 470 | 480 | 97.9 | 24 |
| | I | 245 | 298* | 82.2 | 15 |
| | J | 227 | 240 | 94.6 | 12 |
| | K | 292 | 420 | 69.5 | 21 |
| | Total | 2560 | 3054 | 83.8 | 153 |

* 2 embryos were lost during the daily renewal of the medium

## 6. Discussion

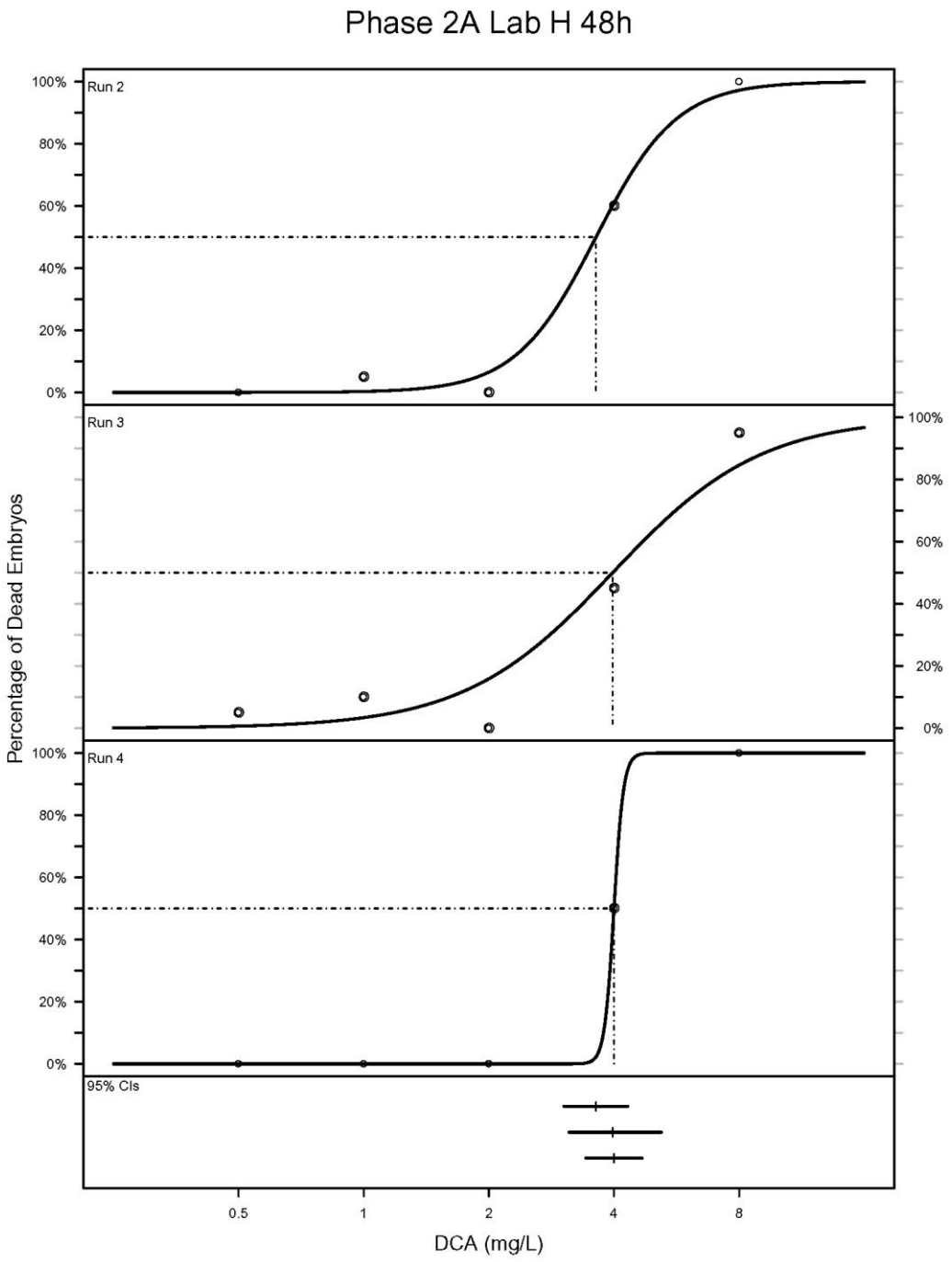
The level of replication (number of laboratories assessing a given chemical) in phase 2 is relatively small but typical of many validation efforts. This limits the statistical summarization to some degree. It is difficult to separate the potential sources of variability in these data (run-to-run variability within lab and chemical, and lab-to-lab within chemical), particularly if they vary as a function of lab, chemical tested, etc.

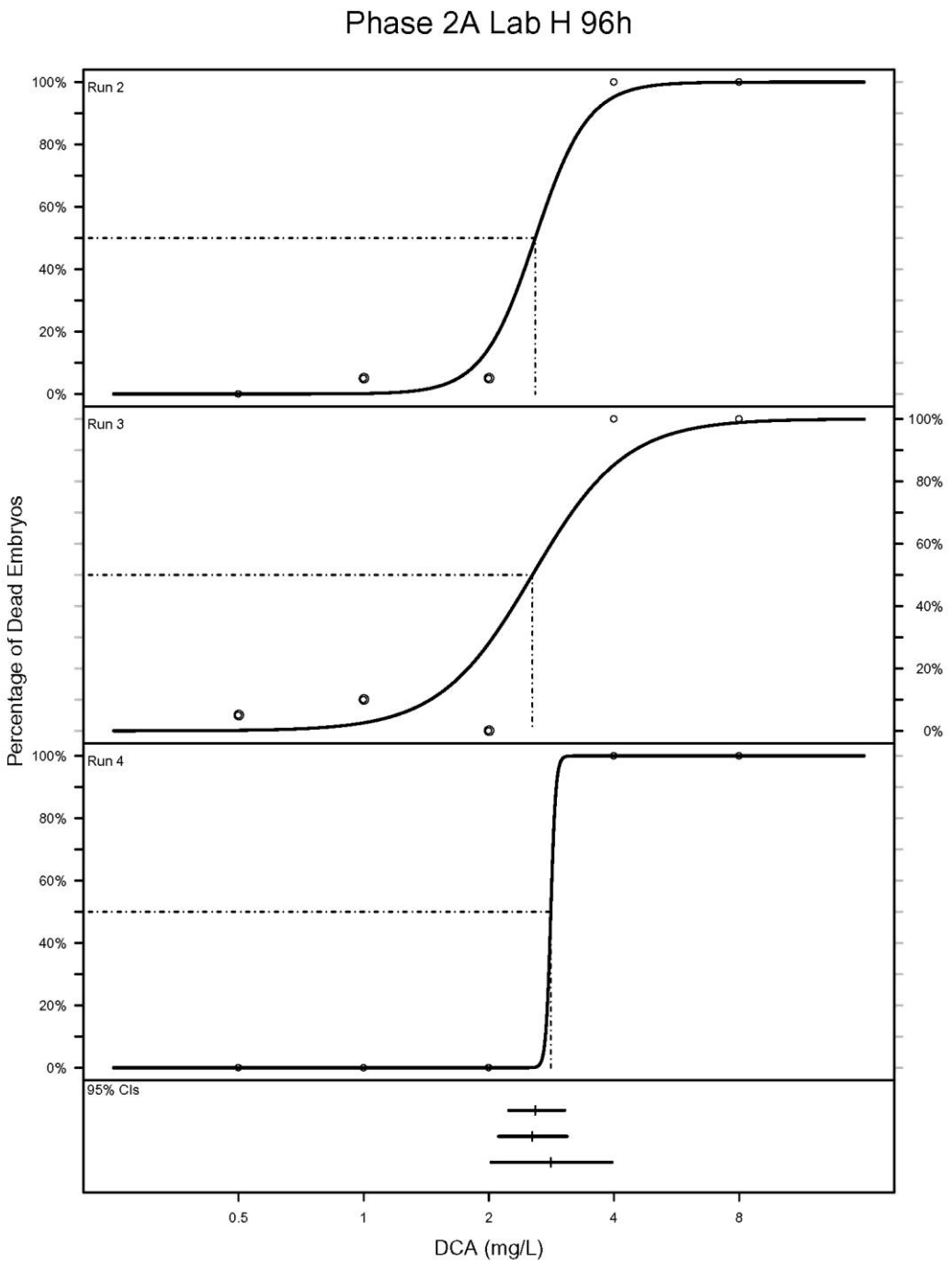
The in-practice application of the ZFET test method, like the one it aims as an alternative, is typically a single laboratory producing a single experimental result for a given chemical exposure. The experiment provides a single estimate of the $LC_{50}$, and a confidence interval. For any of these methods, a single test result provides no estimate of the total variability in the estimate of the $LC_{50}$ (e.g., experiment-to-experiment, lab-to-lab, and other factors such as water quality, health of embryos and so forth). Overall trends, however, are interpretable as seen in Figures 15 and 16. The chemicals included in Phase 2 span a wide range of toxicities, hydrophobicity, solubility and volatility and include "difficult substances" as defined in OECD Guidance Document 23[8]. The large degree of overlap in confidence intervals for a given chemical, the span of low to high $LC_{50}$s, and reasonable Coefficients of Variation based on a small number of laboratories suggest the method is robust.

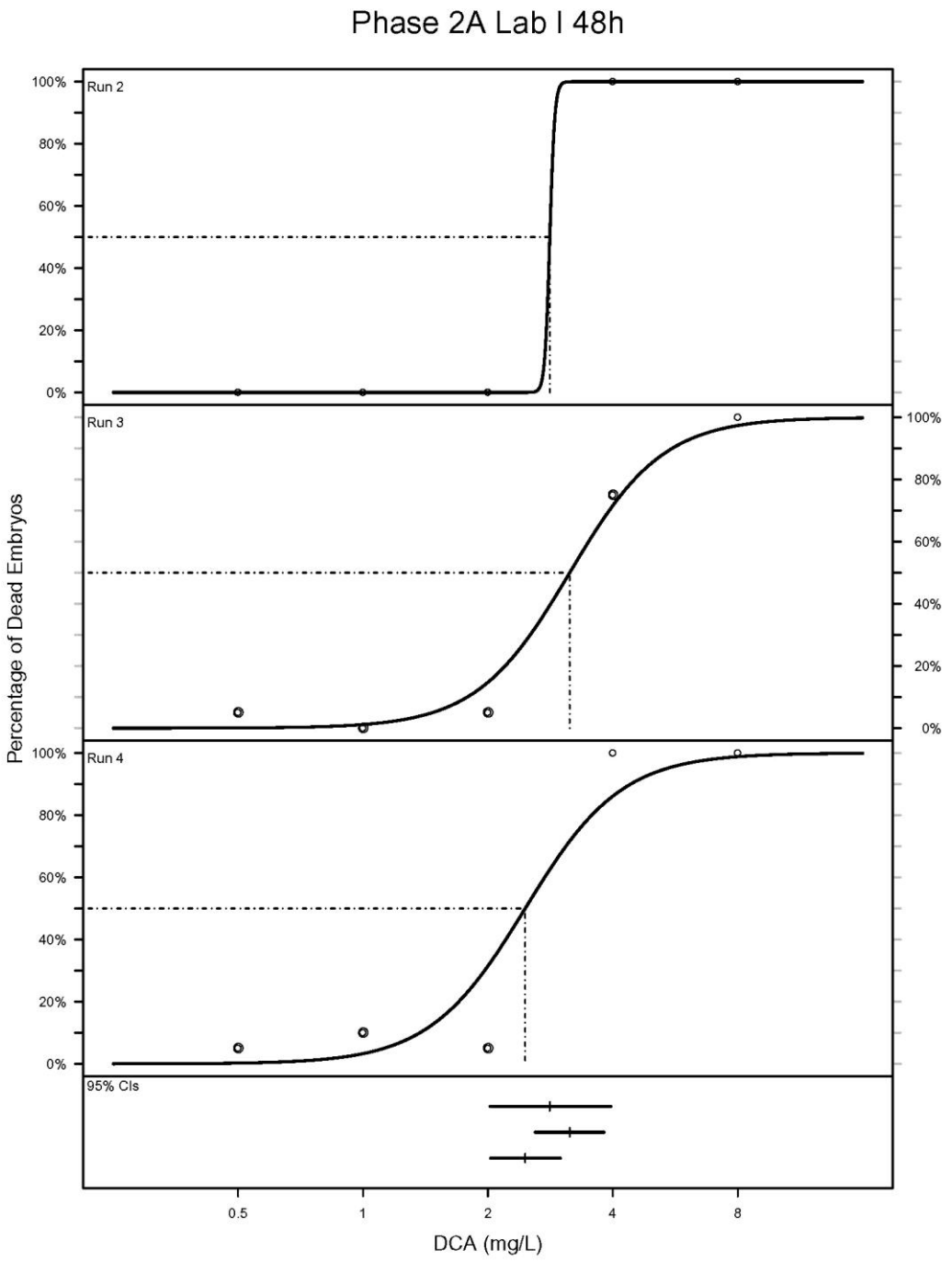
[8] OECD (2000). Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures. OECD Series on Testing and Assessment, Number 23, Paris, France. 53p.

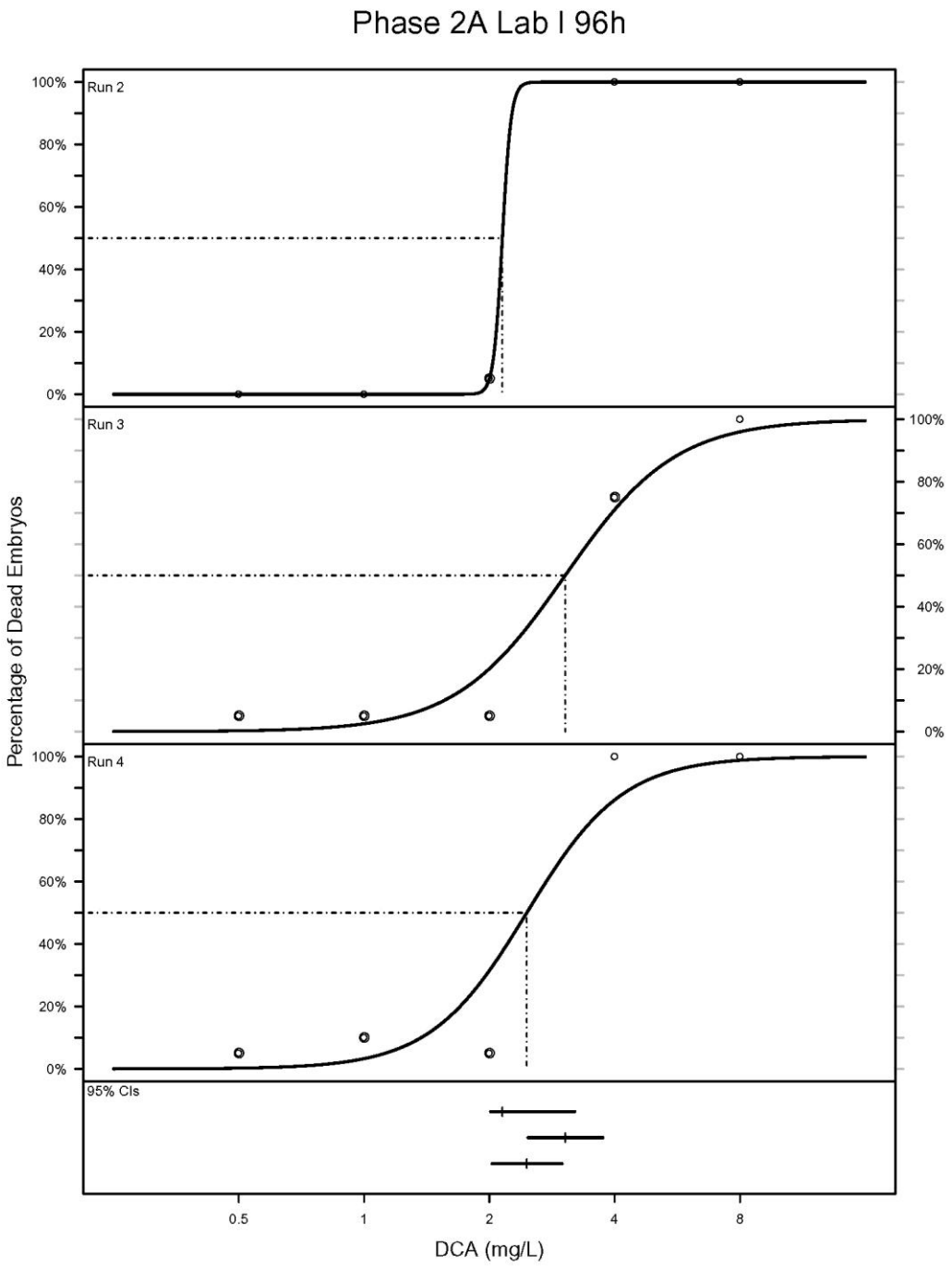
## Appendix A: Phase 2 – Three Runs with 3,4-DCA to Qualify Laboratories H, I, J, and K

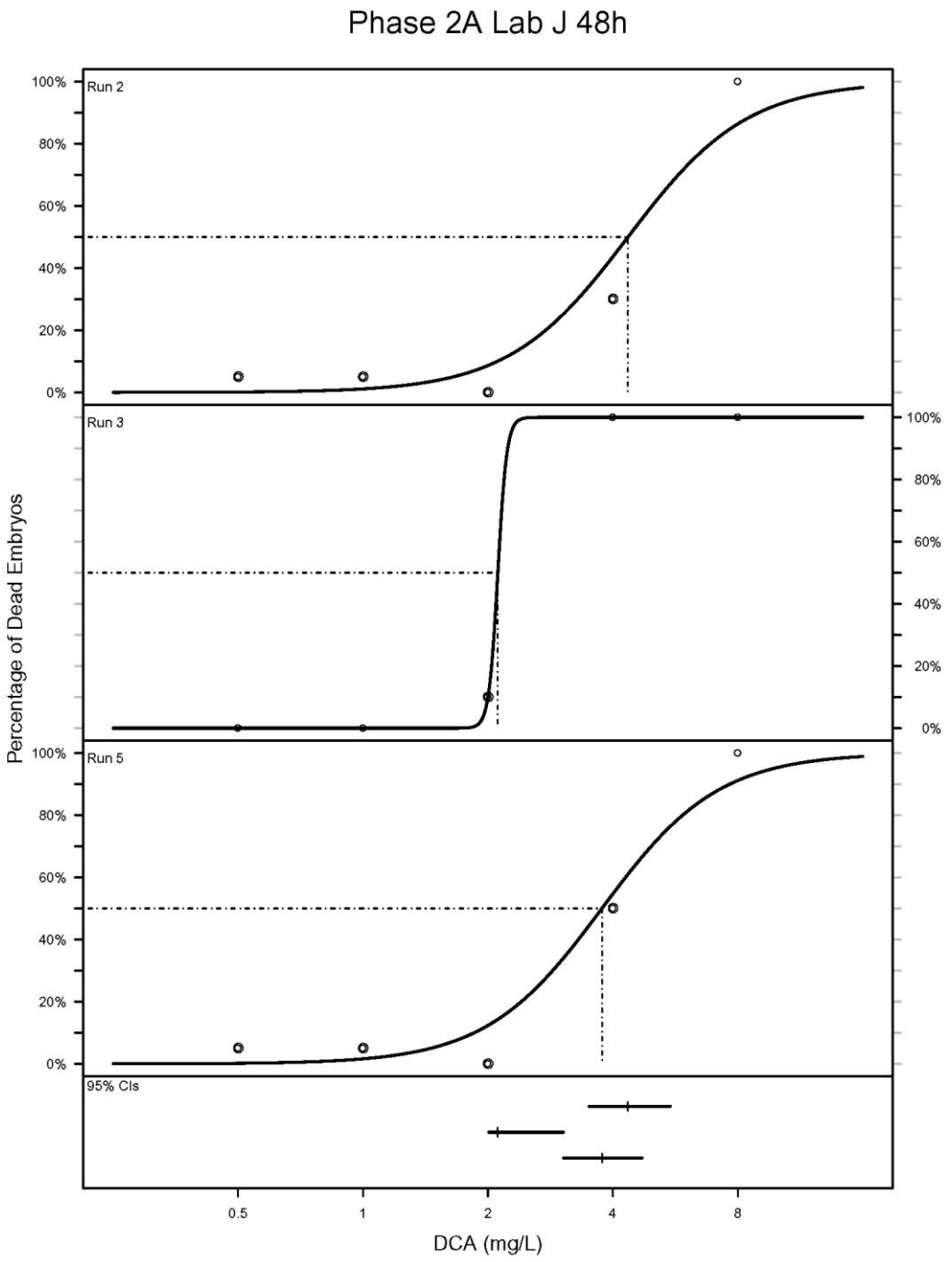
In each figure, the observed percentages of dead embryos are the points and the prediction model is the solid curve. Dashed lines represent the estimated $LC_{50}$, and associated confidence intervals are given in the lowest panel of each display. The larger points are the informative concentrations for fitting the curve. They are particularly important for estimating the slope of the prediction model. Ideally there will be two or more of these informative concentrations. When only one occurs, the slope will be arbitrarily steep very close to that concentration, and when none occur, the slope will be arbitrarily steep midway between the two concentrations that separate all 0% responses from all 100% responses.

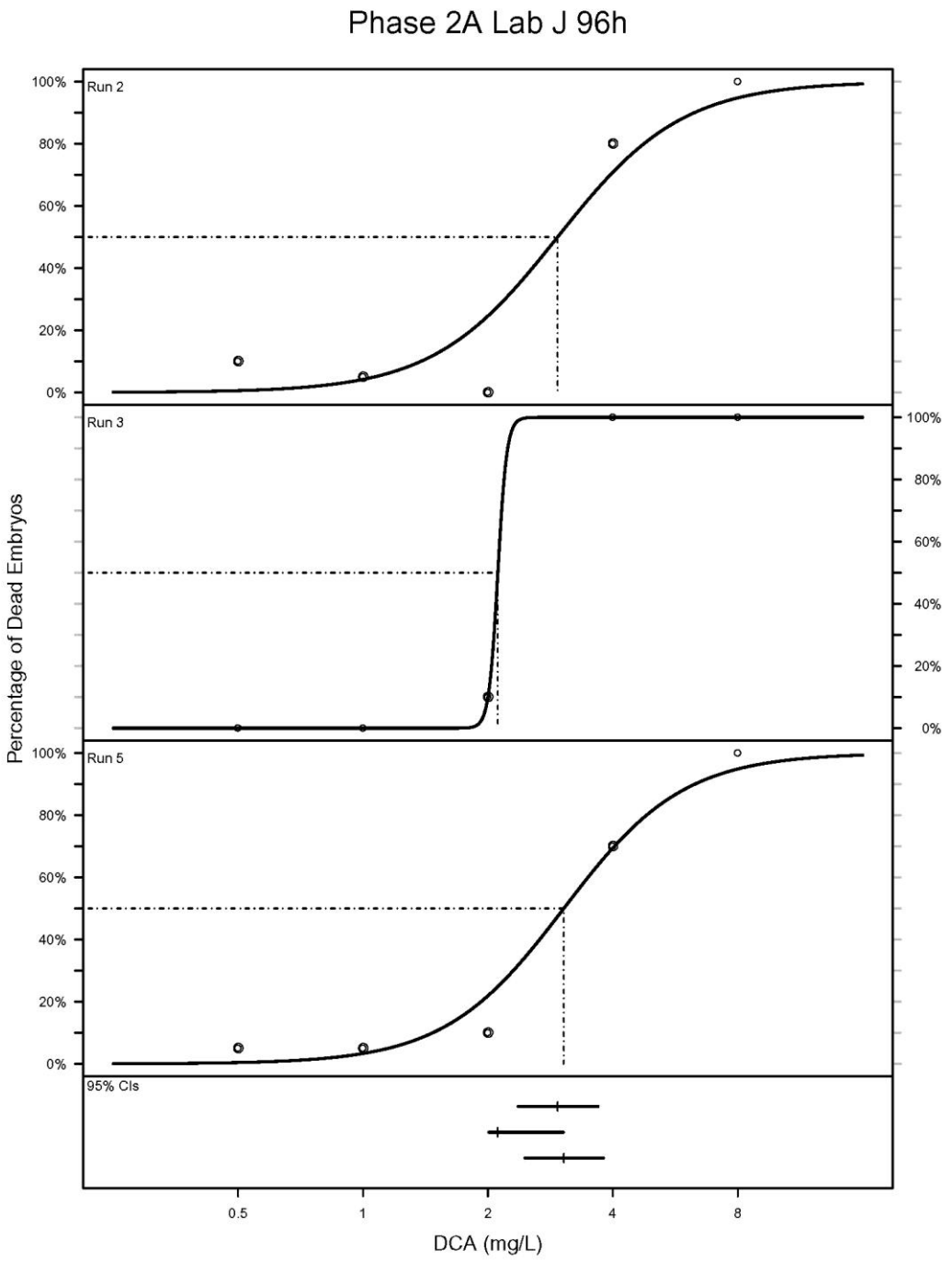

Phase 2A Lab H 48h
Run 2
Run 3
Run 4
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.5
1
2
4
8
DCA (mg/L)

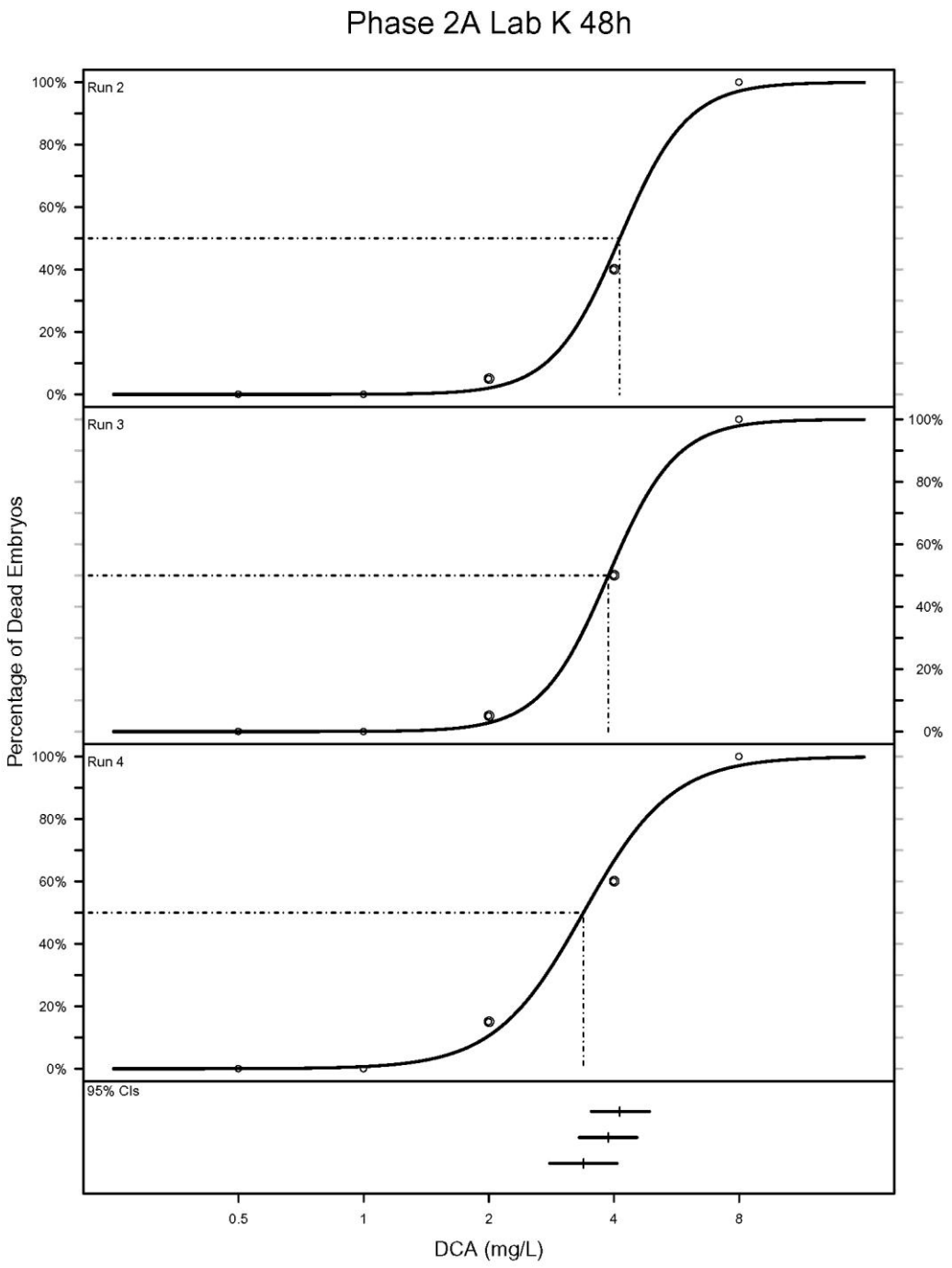

Phase 2A Lab H 96h
Run 2
Run 3
Run 4
95% CIs
Percentage of Dead Embryos
100%
80%
60%
40%
20%
0%
0.5
1
2
4
8
DCA (mg/L)

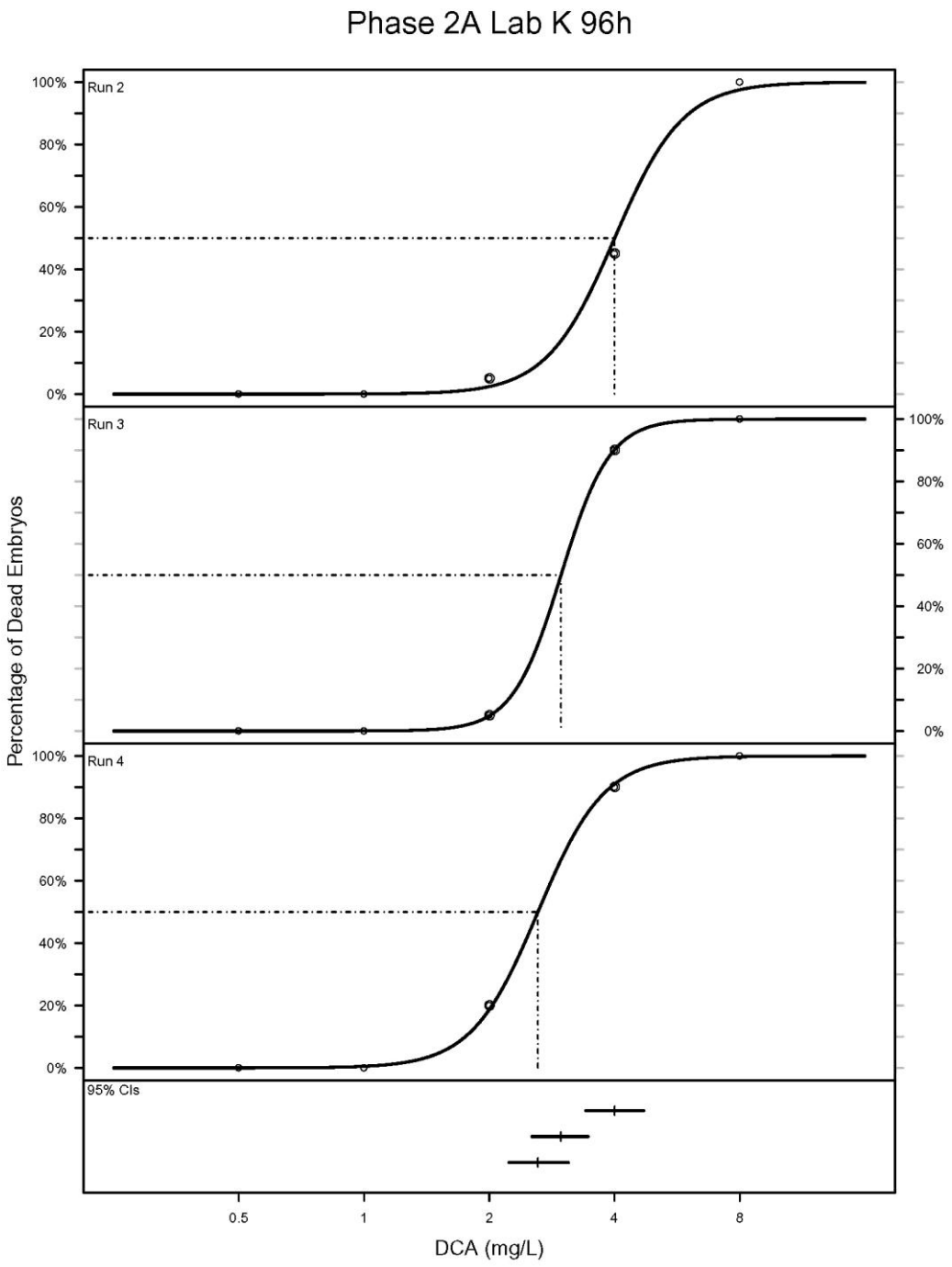

Phase 2A Lab I 48h
Run 2
Run 3
Run 4
95% CIs
Percentage of Dead Embryos
100%
80%
60%
40%
20%
0%
0.5
1
2
4
8
DCA (mg/L)


Phase 2A Lab I 96h
Run 2
Run 3
Run 4
95% CIs
Percentage of Dead Embryos
100%
80%
60%
40%
20%
0%
0.5
1
2
4
8
DCA (mg/L)


Phase 2A Lab J 48h
Run 2
Run 3
Run 5
95% CIs
Percentage of Dead Embryos
100%
80%
60%
40%
20%
0%
0.5
1
2
4
8
DCA (mg/L)


Phase 2A Lab J 96h
Run 2
Run 3
Run 5
95% CIs
Percentage of Dead Embryos
100%
80%
60%
40%
20%
0%
0.5
1
2
4
8
DCA (mg/L)


Phase 2A Lab K 48h
Run 2
Run 3
Run 4
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.5
1
2
4
8
DCA (mg/L)

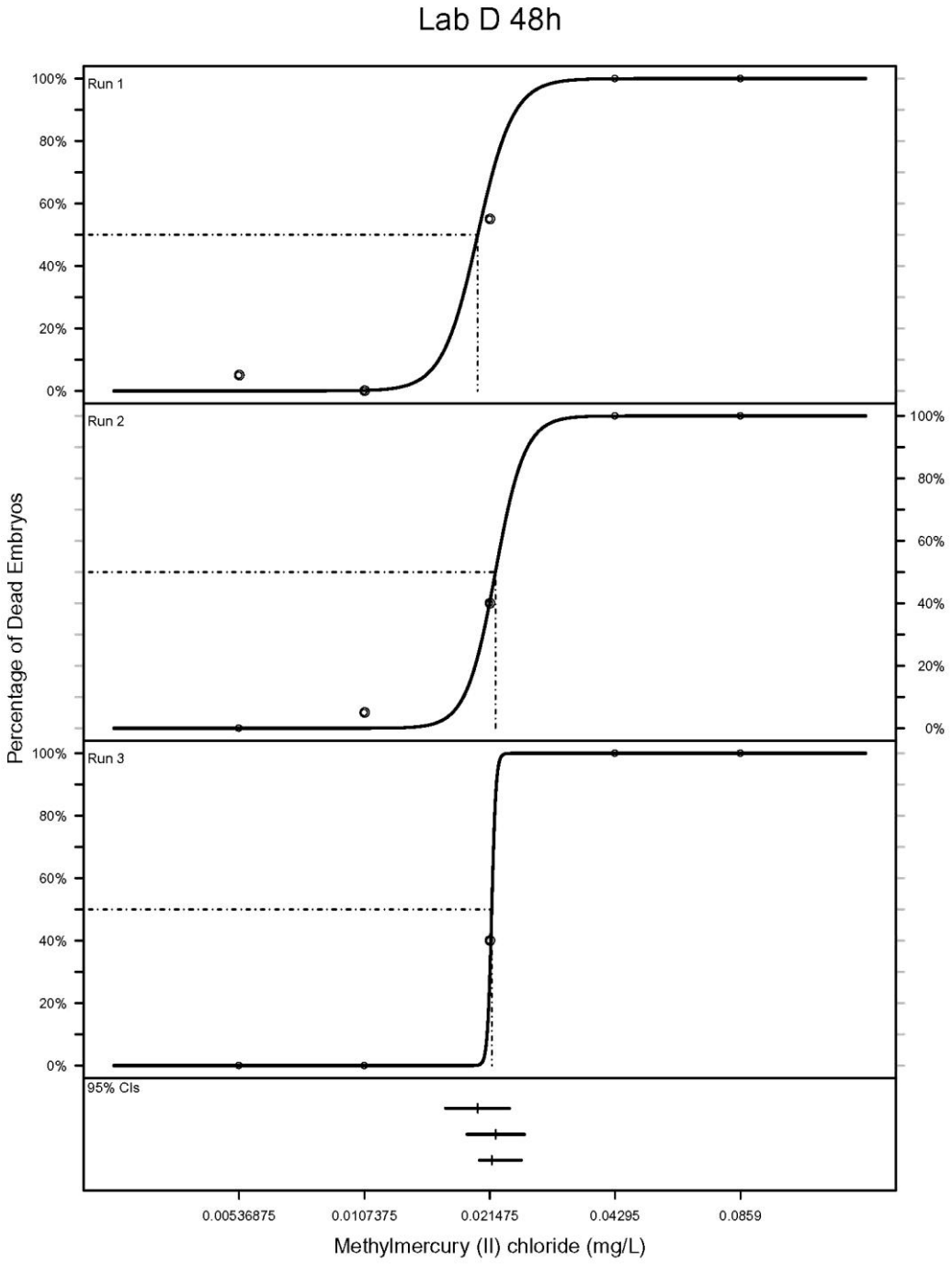

Phase 2A Lab K 96h
Run 2
Run 3
Run 4
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.5
1
2
4
8
DCA (mg/L)

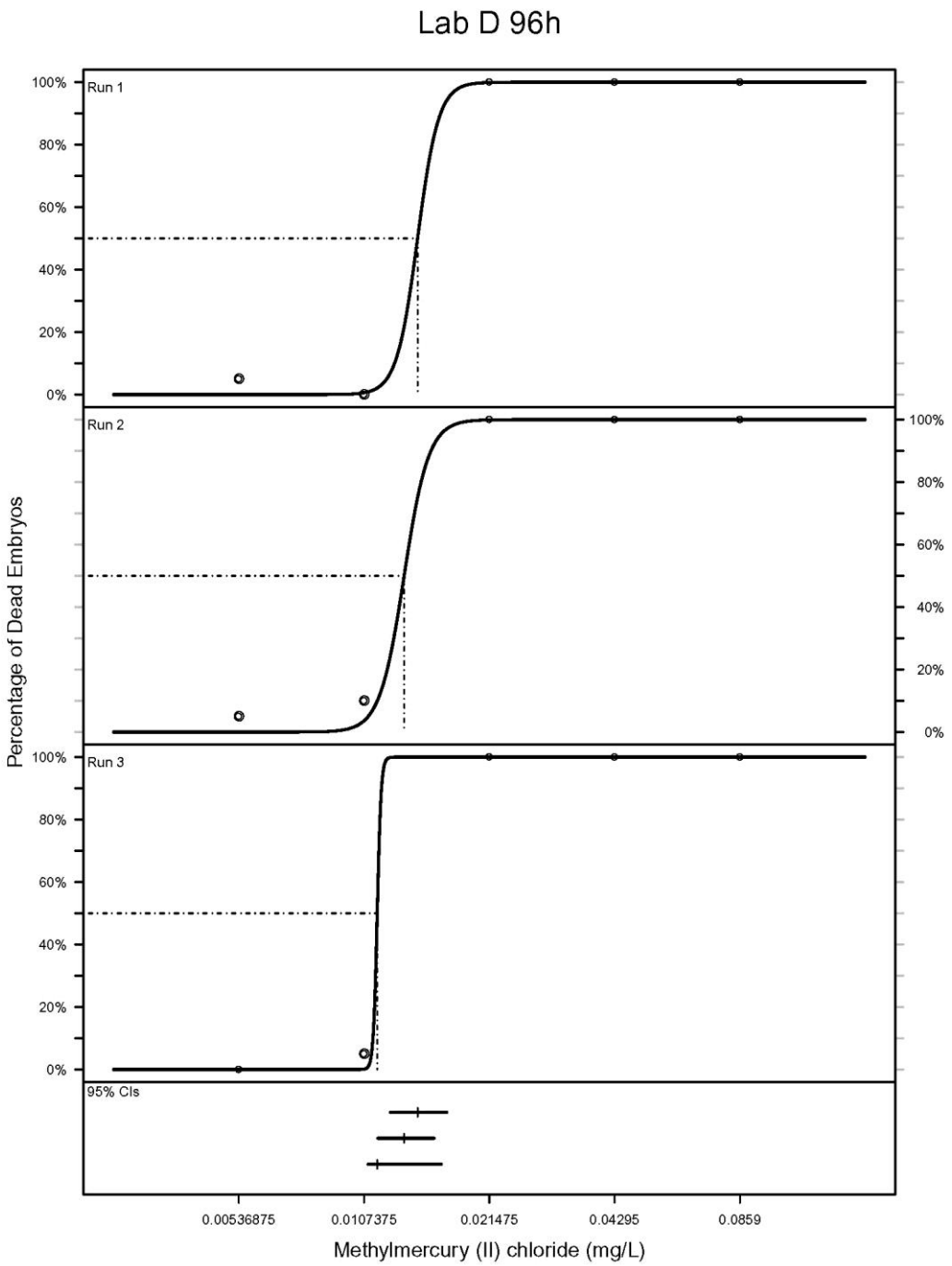
**Appendix B: Phase 2 – Three Runs with Thirteen Chemicals**

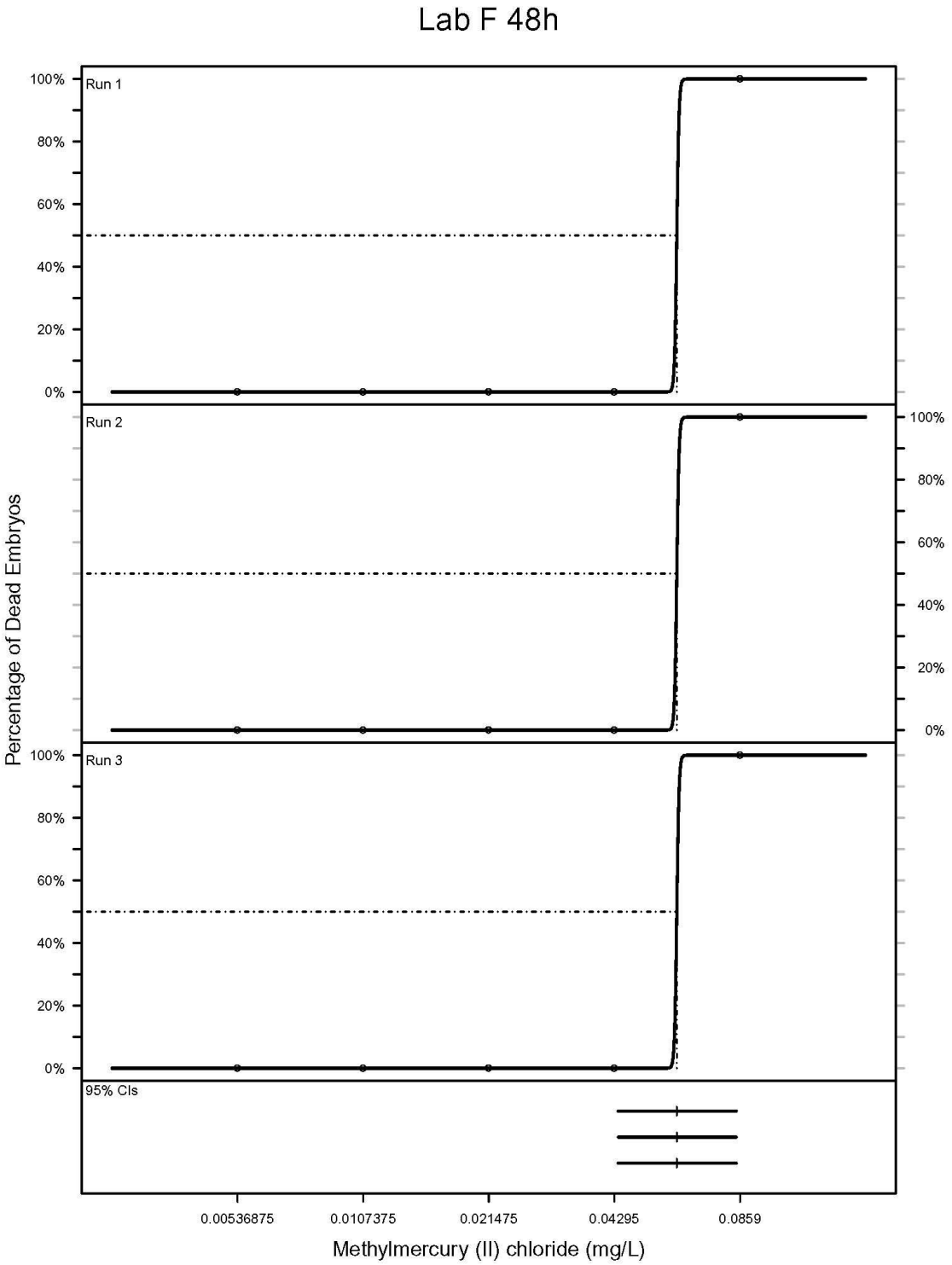
In each figure, the observed percentages of dead embryos are the points and the prediction model is the solid curve. Dashed lines represent the estimated $LC_{50}$, and associated confidence intervals are given in the lowest panel of each display. The larger points are the informative concentrations for fitting the curve. They are particularly important for estimating the slope of the prediction model. Ideally there will be two or more of these informative concentrations. When only one occurs, the slope will be arbitrarily steep very close to that concentration, and when none occur, the slope will be arbitrarily steep midway between the two concentrations that separate all 0% responses from all 100% responses.

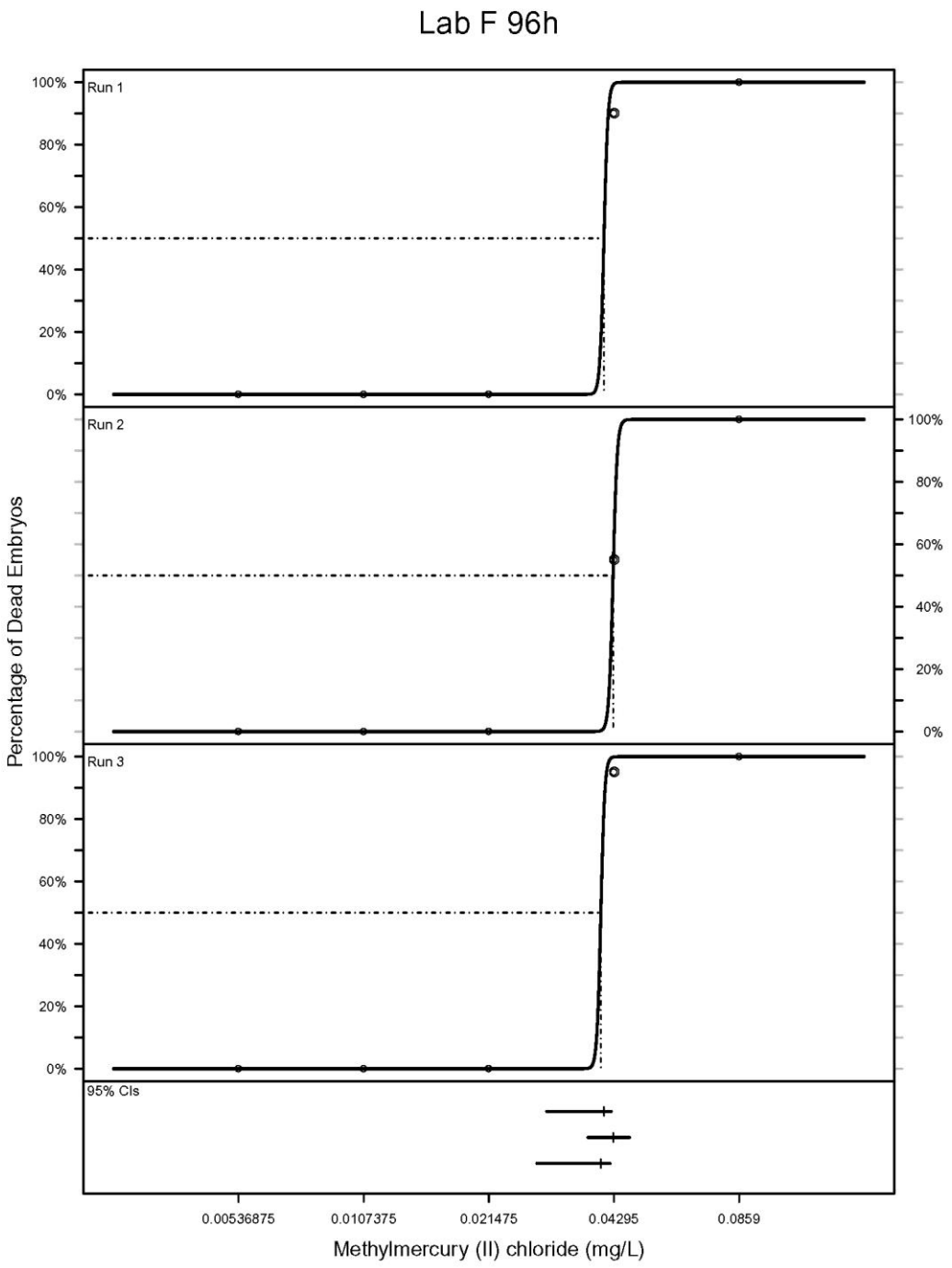
**Note that in figures for three chemicals, Methylmercury chloride, Sodium tetradecyl sulphate, and Copper chloride pentahydrate, the exposure axis reflects only the toxic part of ionic pair (e.g., chloride is not included for methylmercury chloride where only the cation methylmercury will contribute to toxicity).**

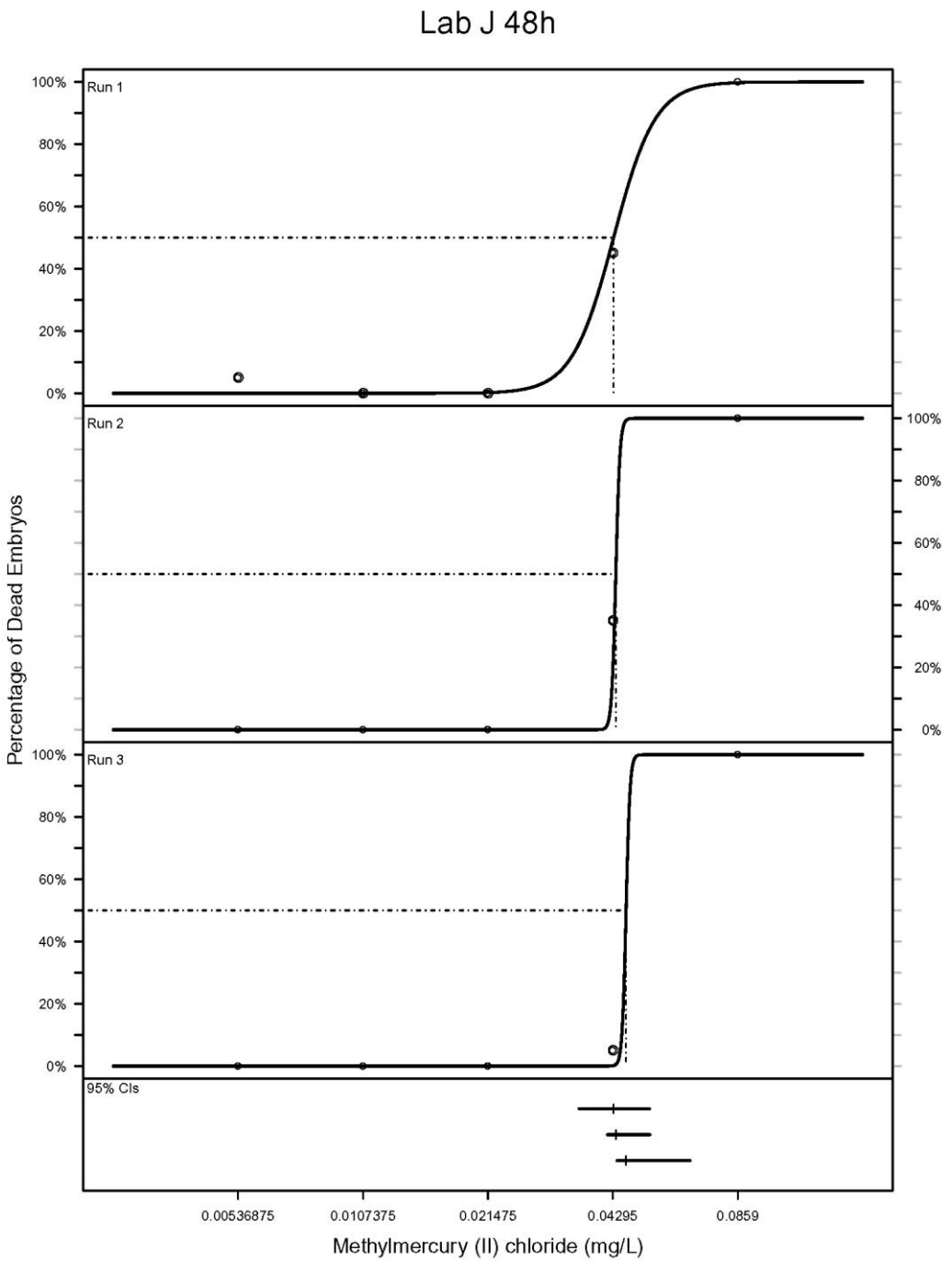
Methyl mercury (II) chloride (toxicity is expressed as mg/L methyl mercury)

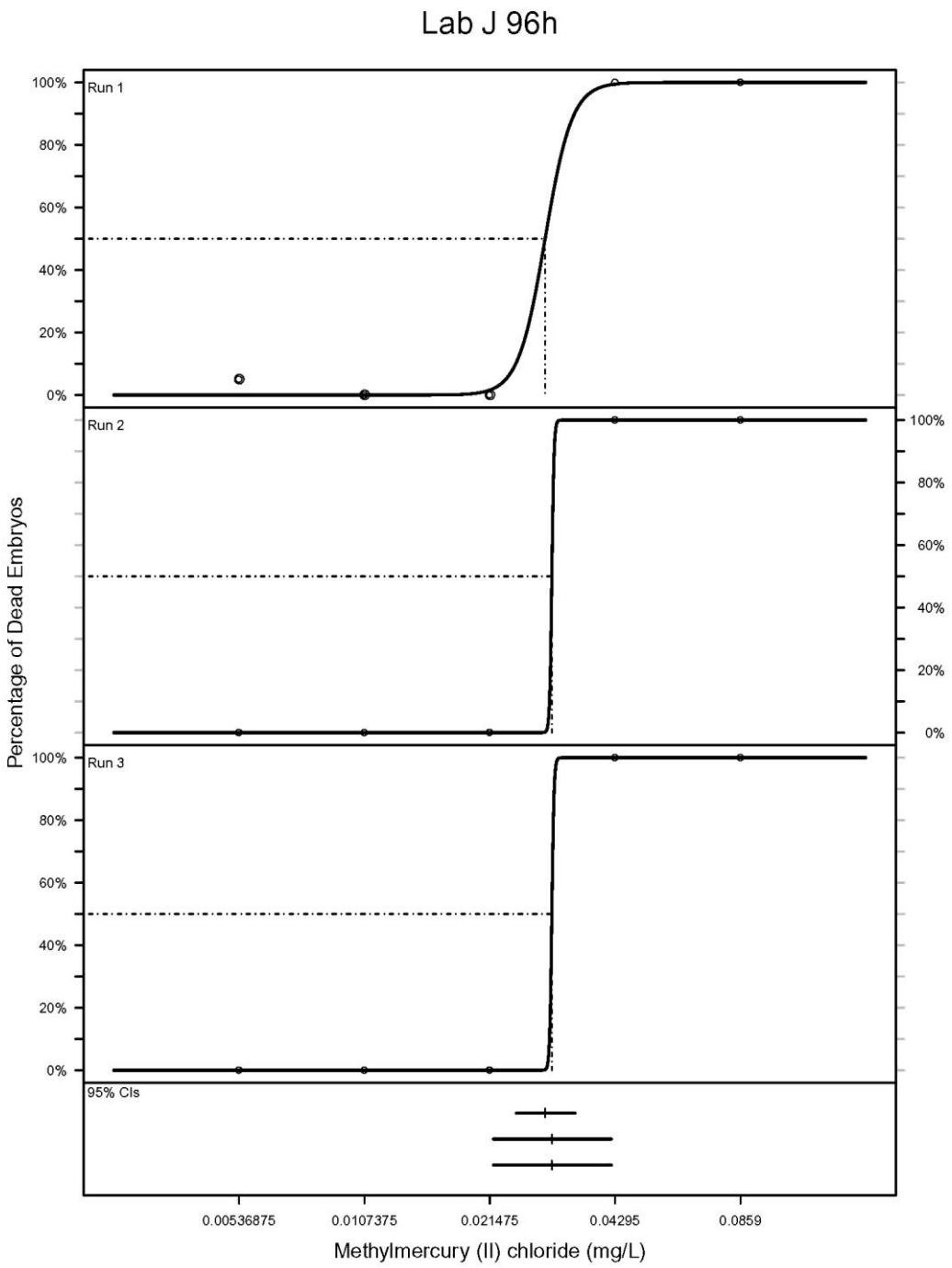



Lab D 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.00536875
0.0107375
0.021475
0.04295
0.0859
Methylmercury (II) chloride (mg/L)

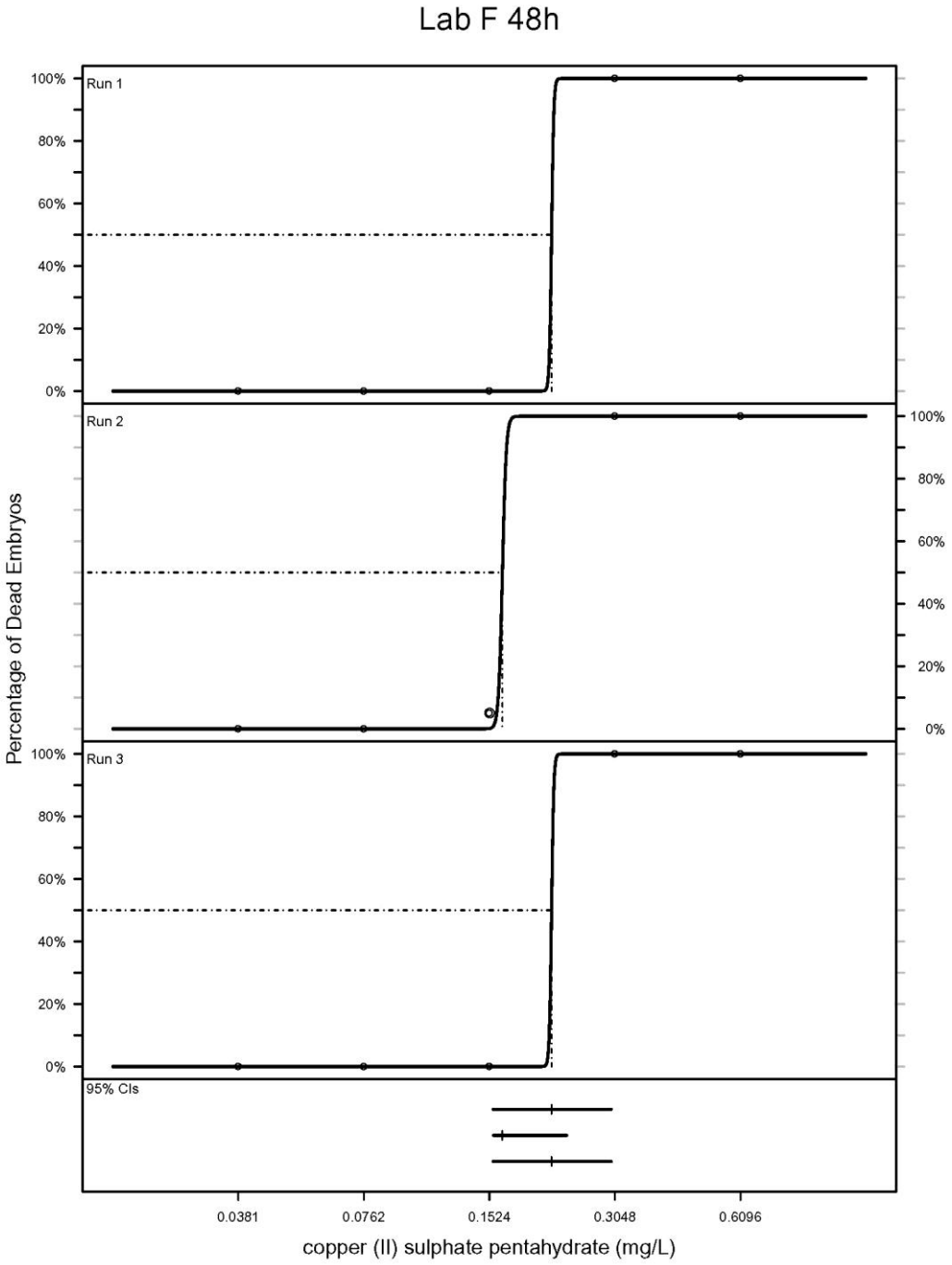

Lab F 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.00536875
0.0107375
0.021475
0.04295
0.0859
Methylmercury (II) chloride (mg/L)

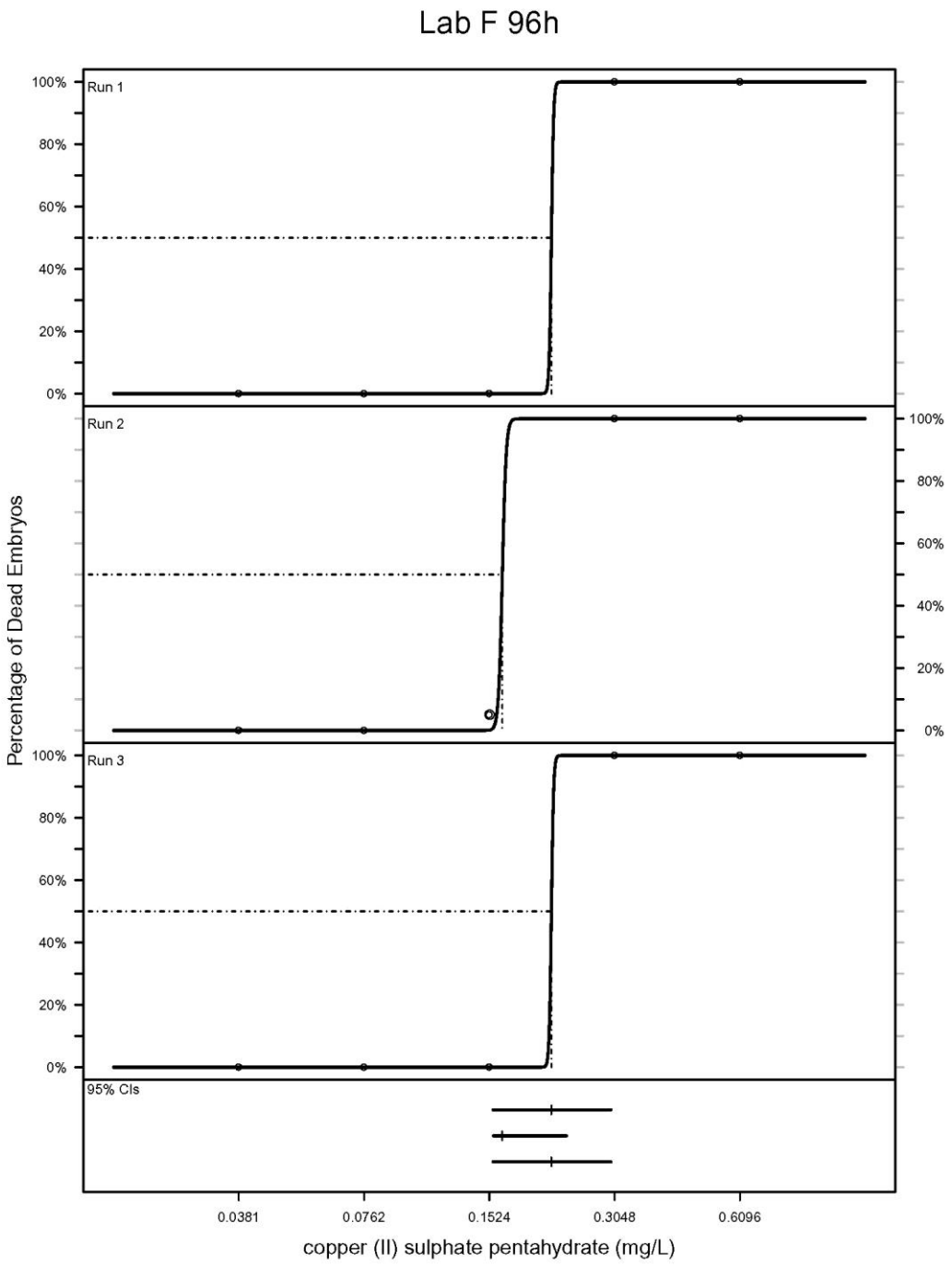

Lab F 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.00536875
0.0107375
0.021475
0.04295
0.0859
Methylmercury (II) chloride (mg/L)

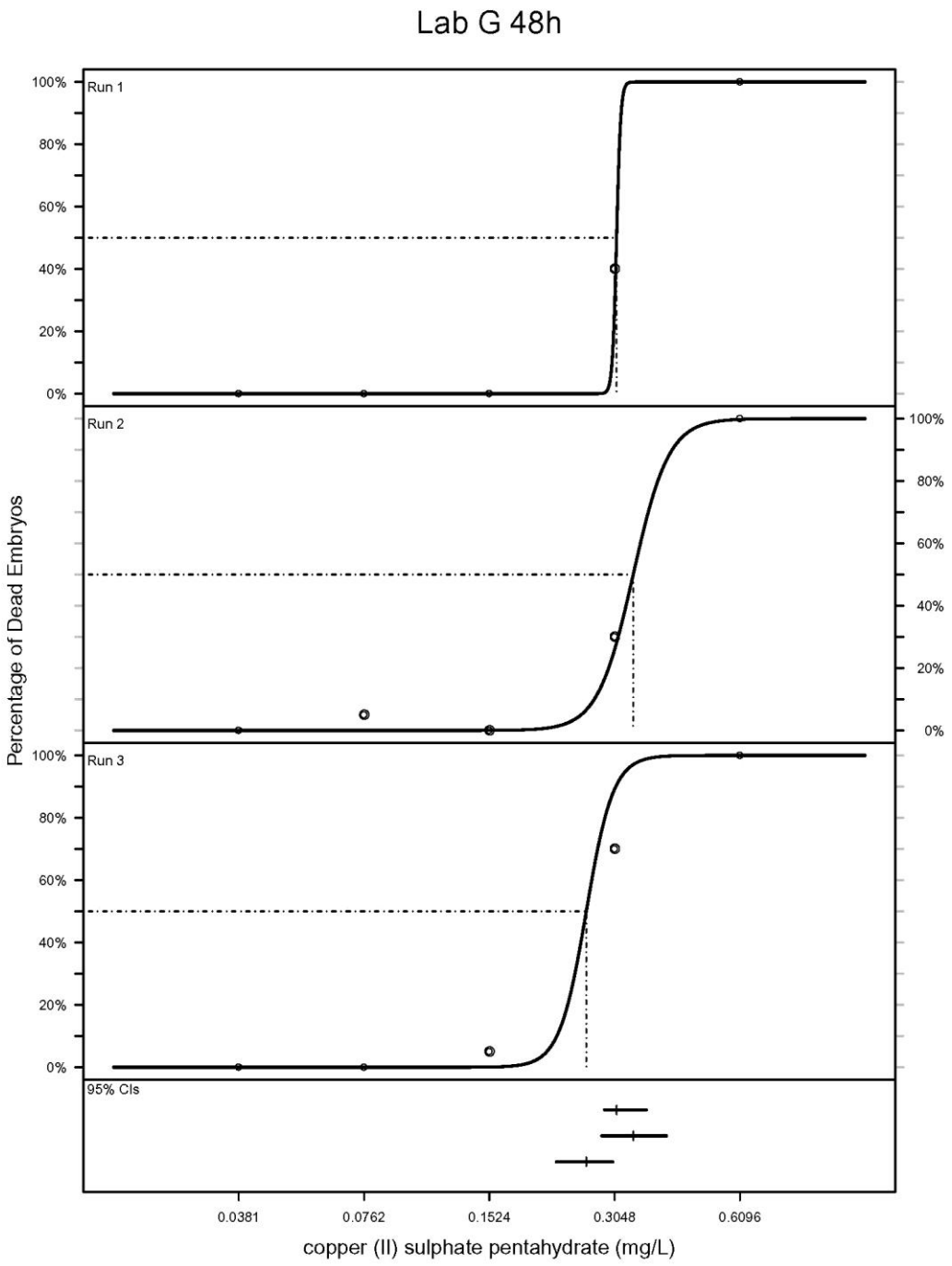

Lab J 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.00536875
0.0107375
0.021475
0.04295
0.0859
Methylmercury (II) chloride (mg/L)

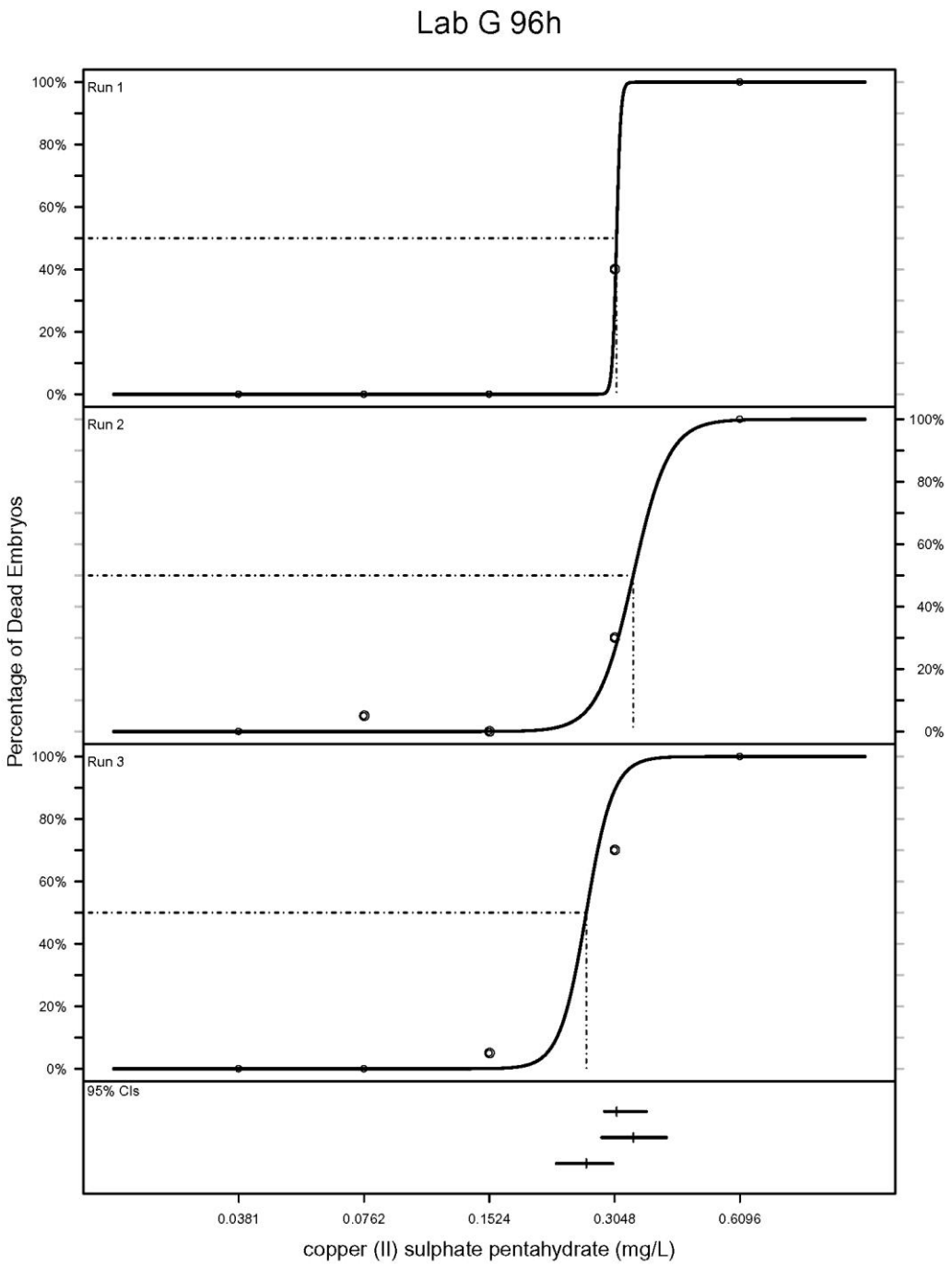

Lab J 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.00536875
0.0107375
0.021475
0.04295
0.0859
Methylmercury (II) chloride (mg/L)

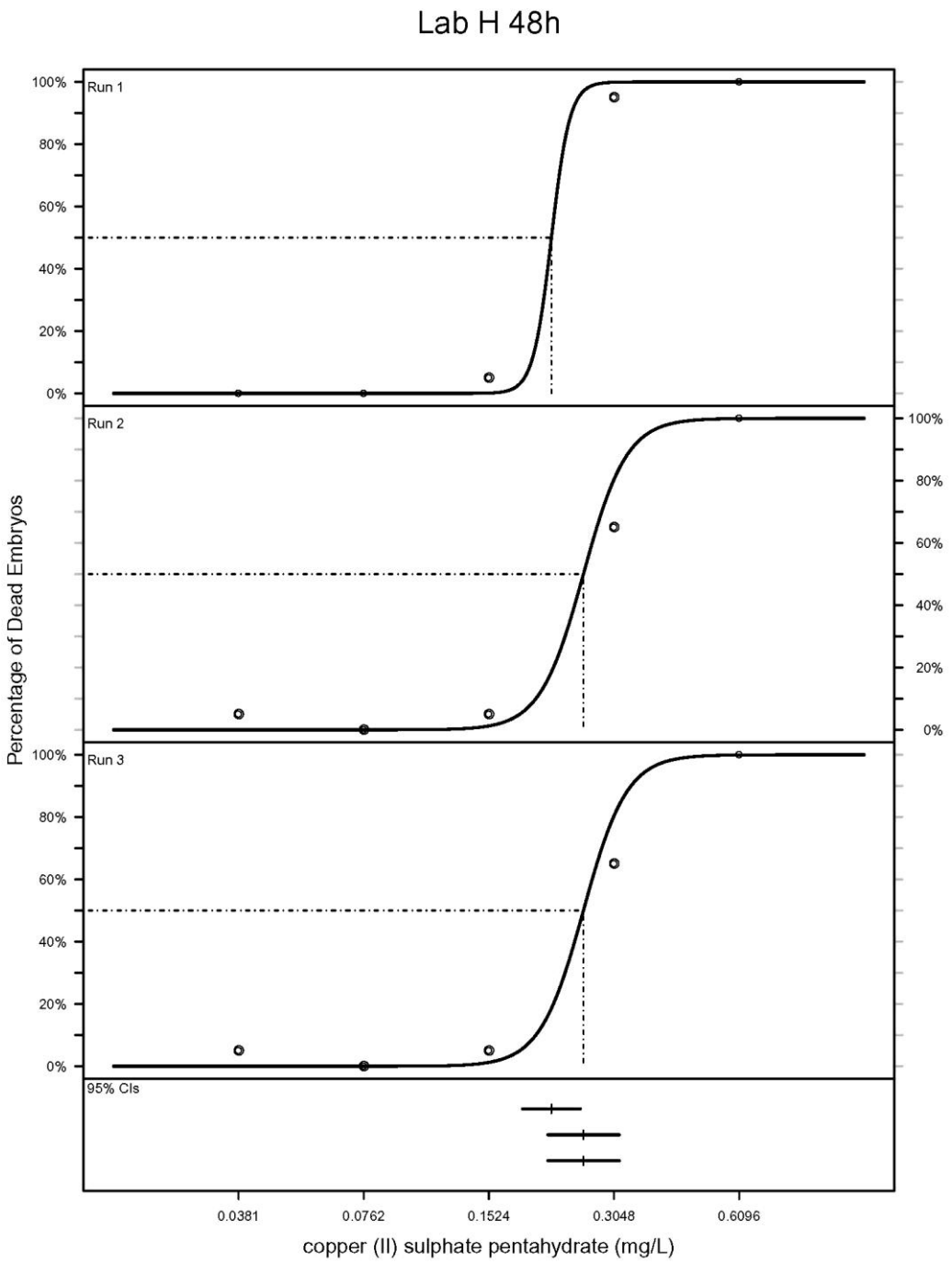
Copper (II) sulphate pentahydrate (toxicity is expressed as copper)

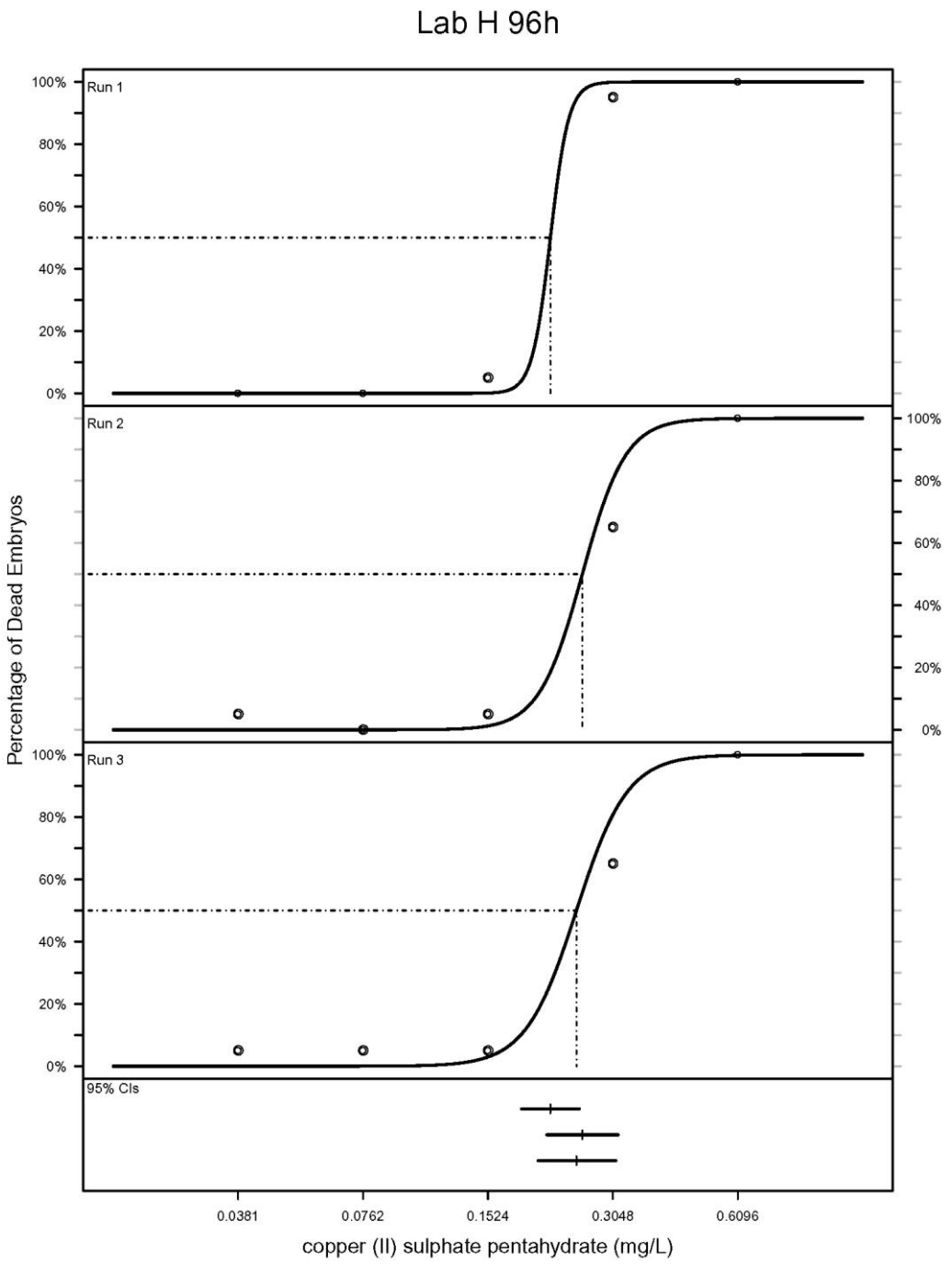


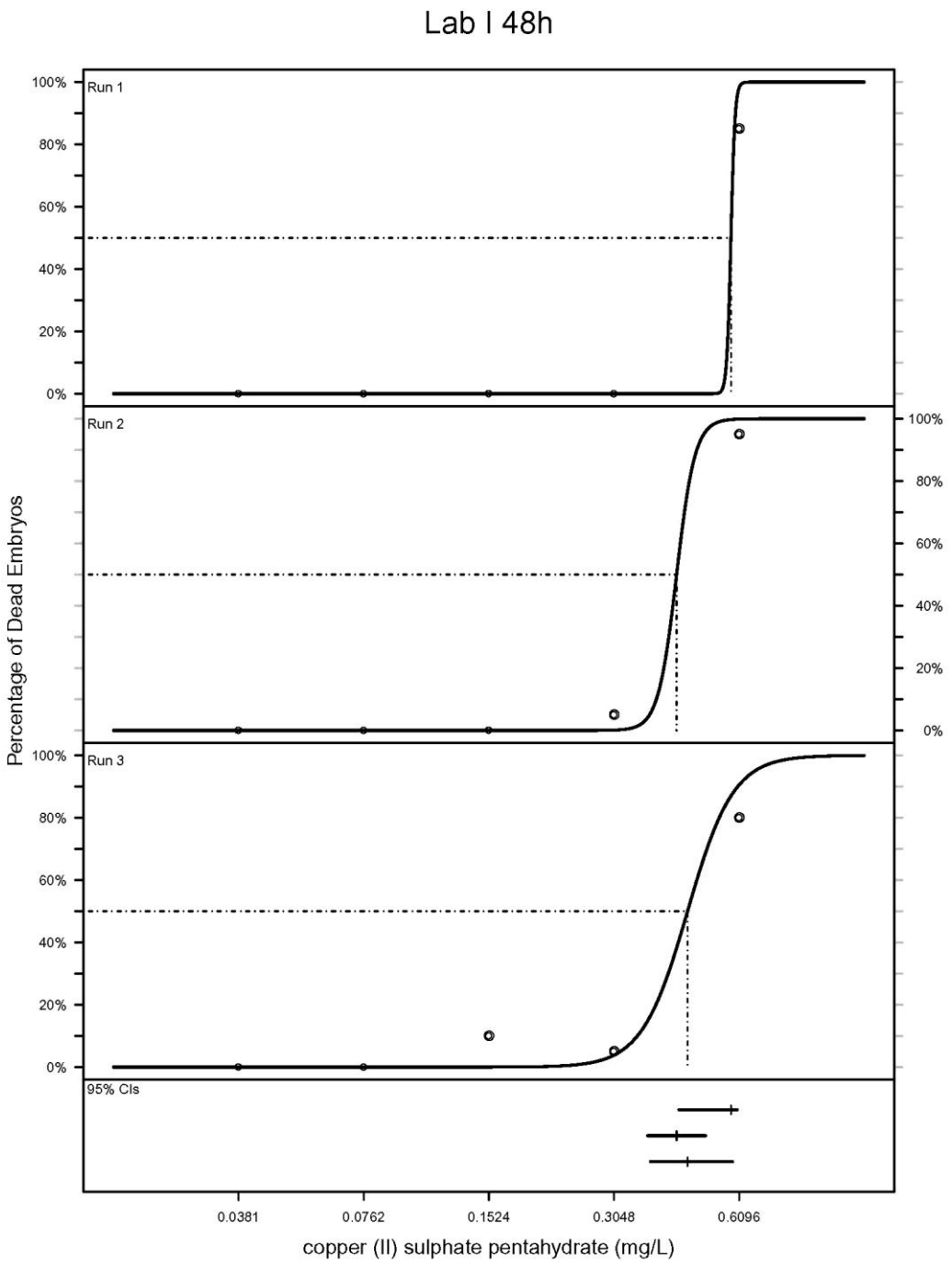

Lab F 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.0381
0.0762
0.1524
0.3048
0.6096
copper (II) sulphate pentahydrate (mg/L)

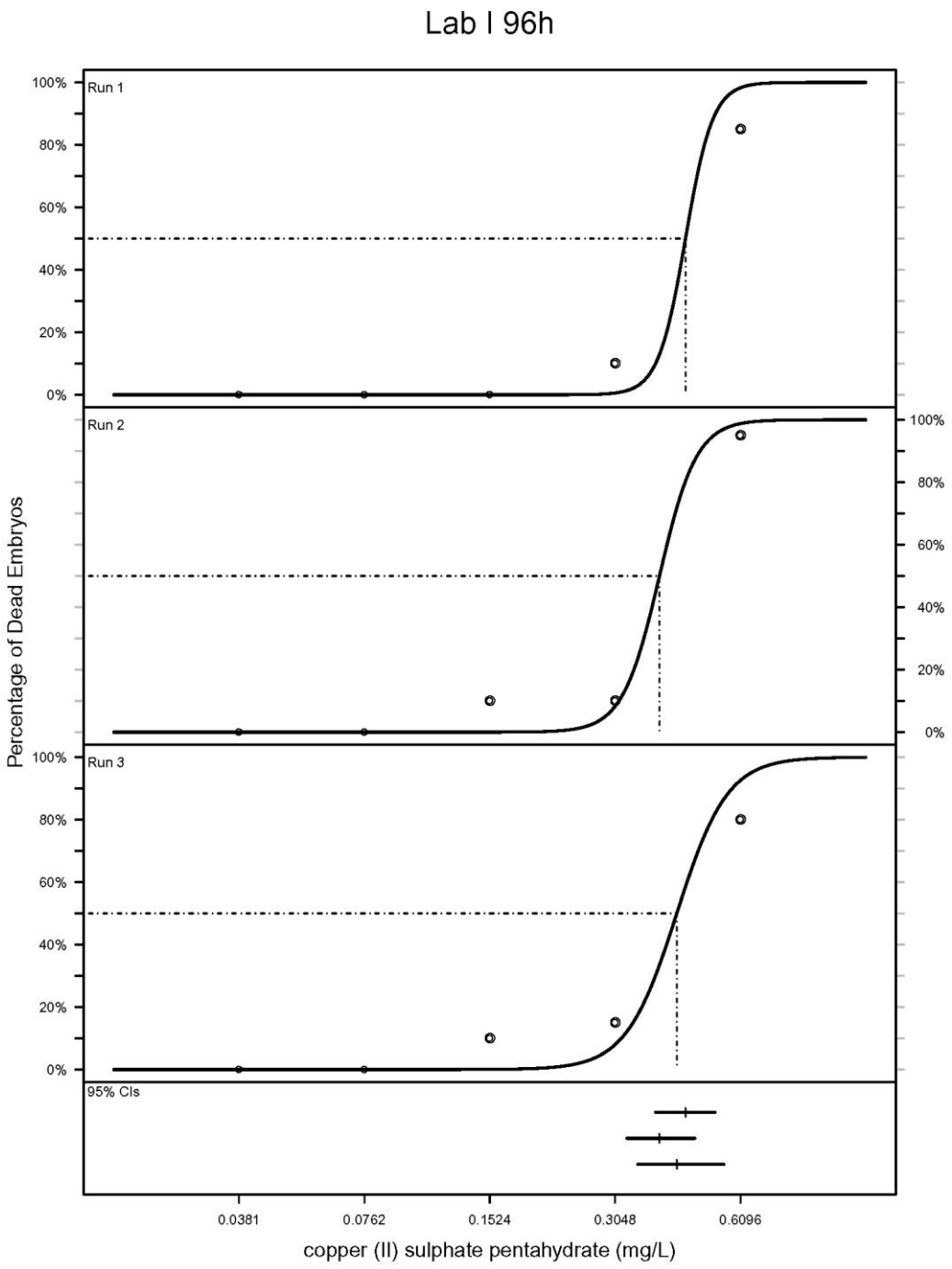

Lab G 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.0381
0.0762
0.1524
0.3048
0.6096
copper (II) sulphate pentahydrate (mg/L)


Lab G 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.0381
0.0762
0.1524
0.3048
0.6096
copper (II) sulphate pentahydrate (mg/L)

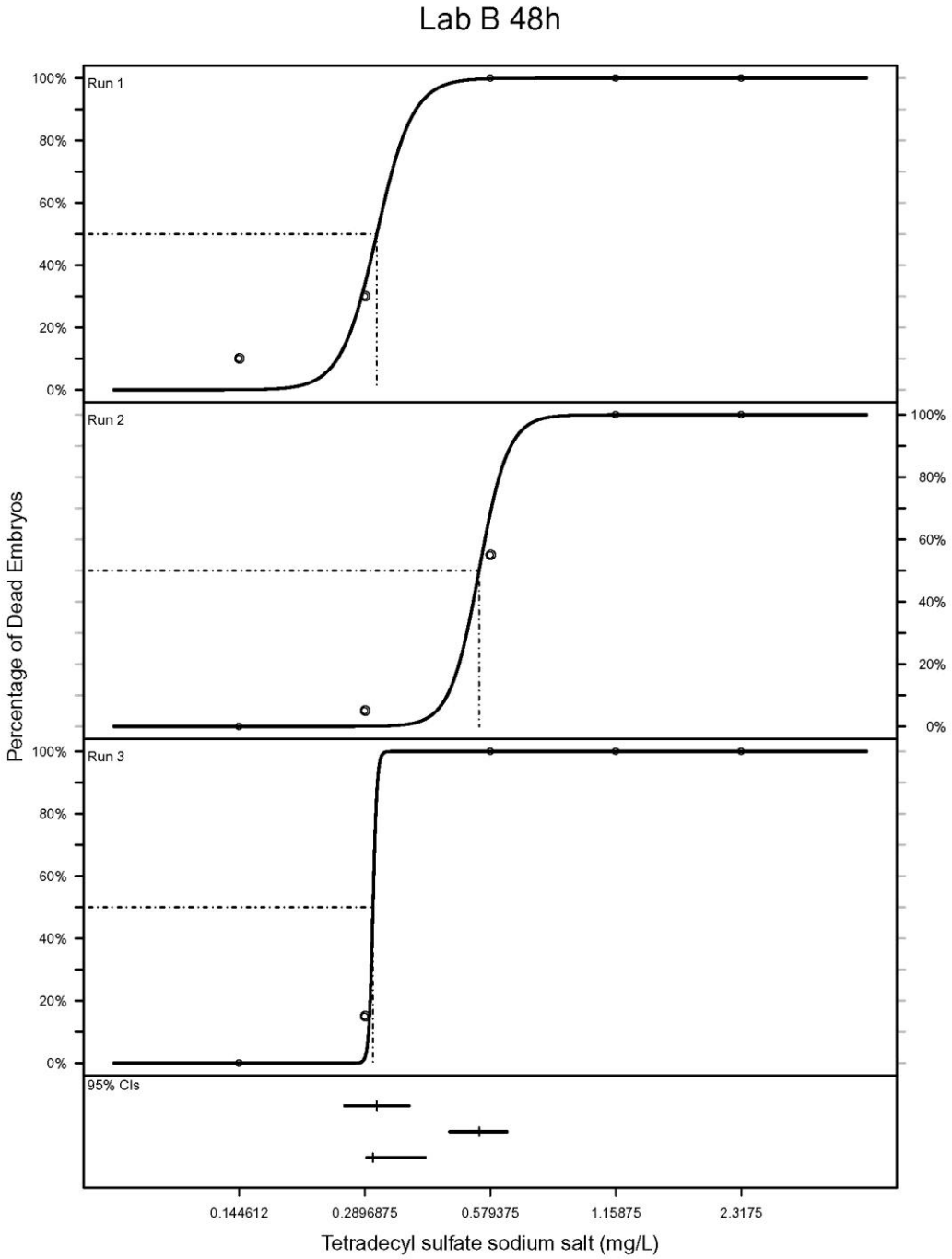

Lab H 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.0381
0.0762
0.1524
0.3048
0.6096
copper (II) sulphate pentahydrate (mg/L)

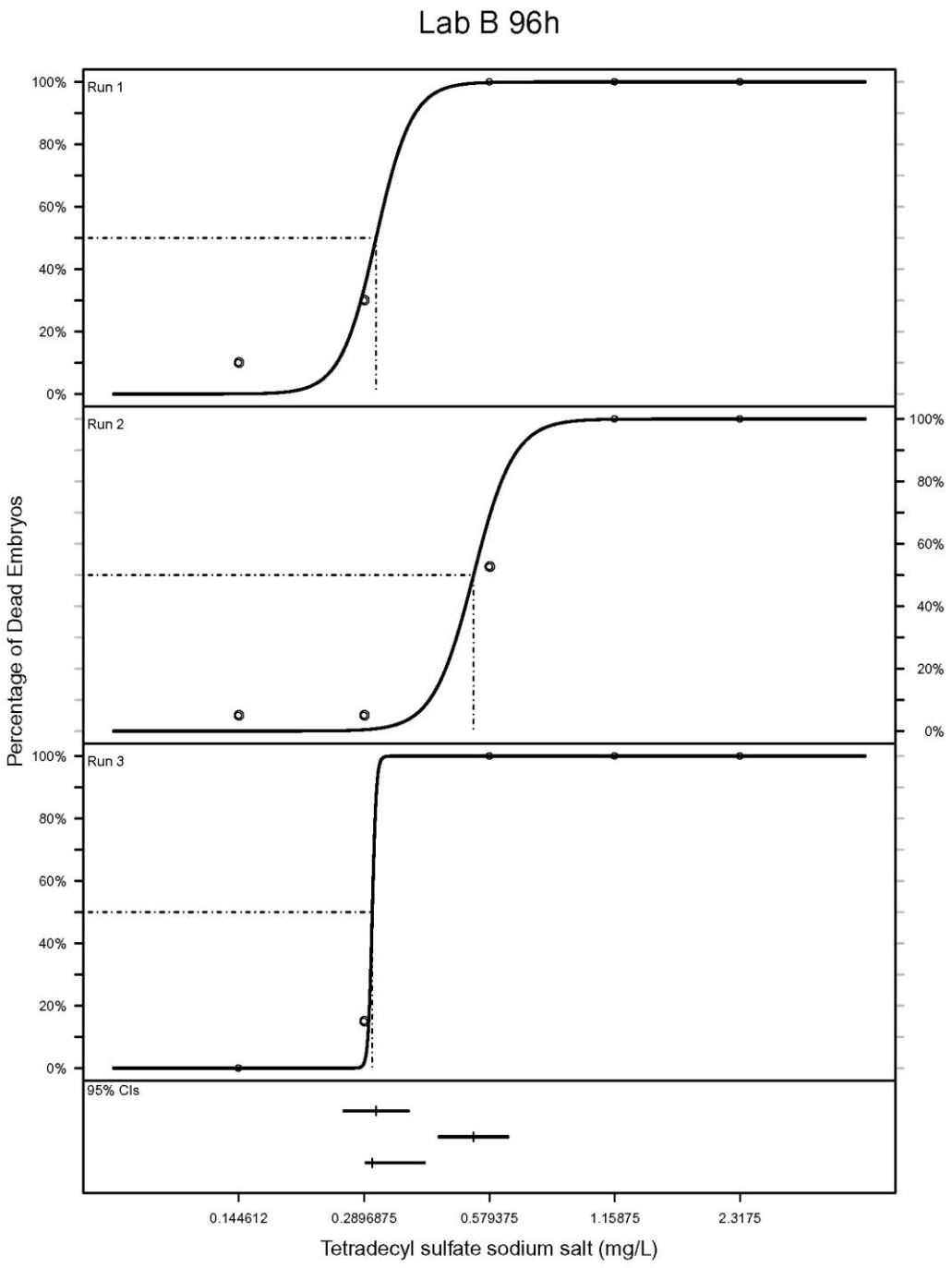

Lab H 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.0381
0.0762
0.1524
0.3048
0.6096
copper (II) sulphate pentahydrate (mg/L)

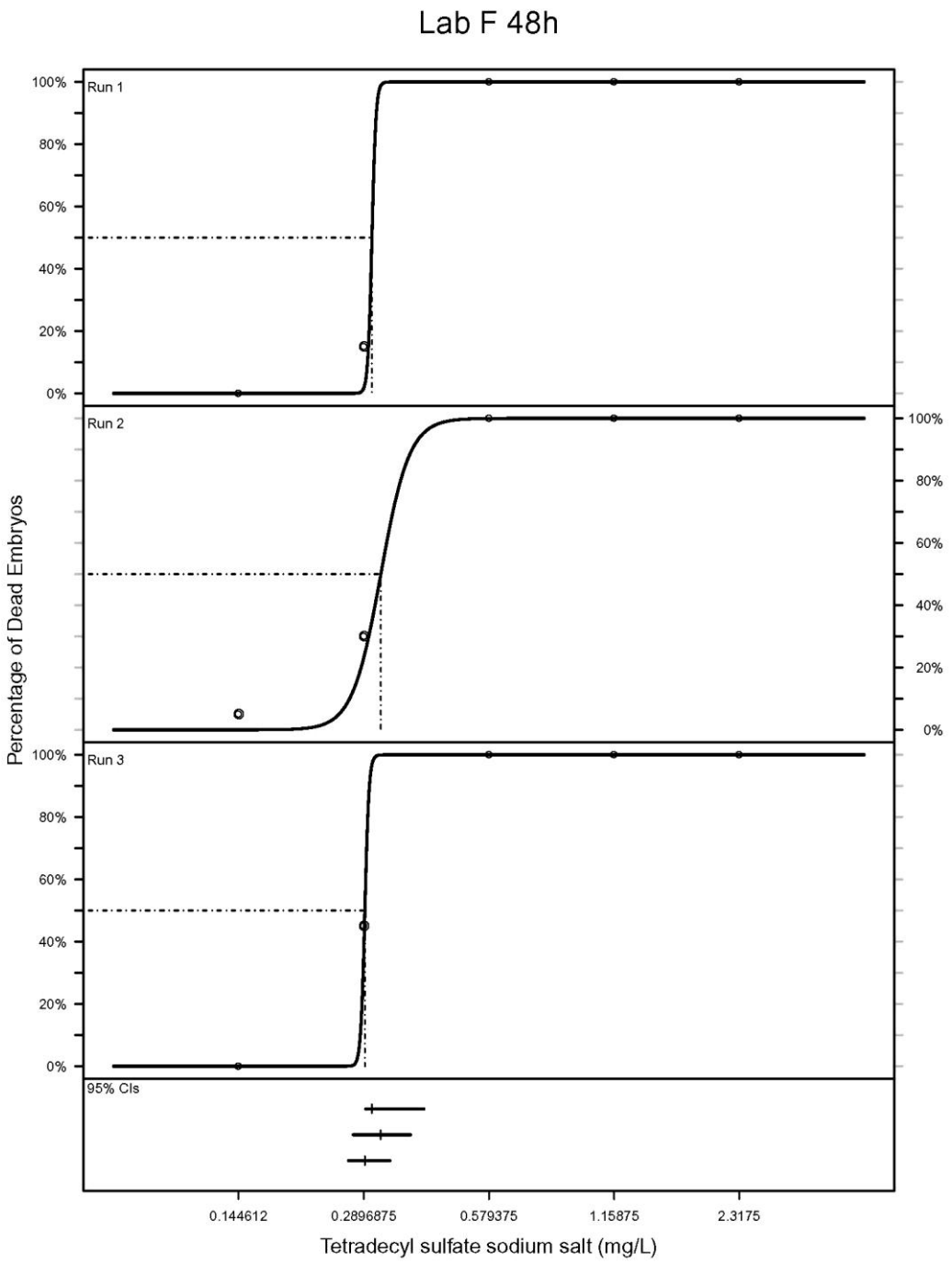

Lab I 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.0381
0.0762
0.1524
0.3048
0.6096
copper (II) sulphate pentahydrate (mg/L)

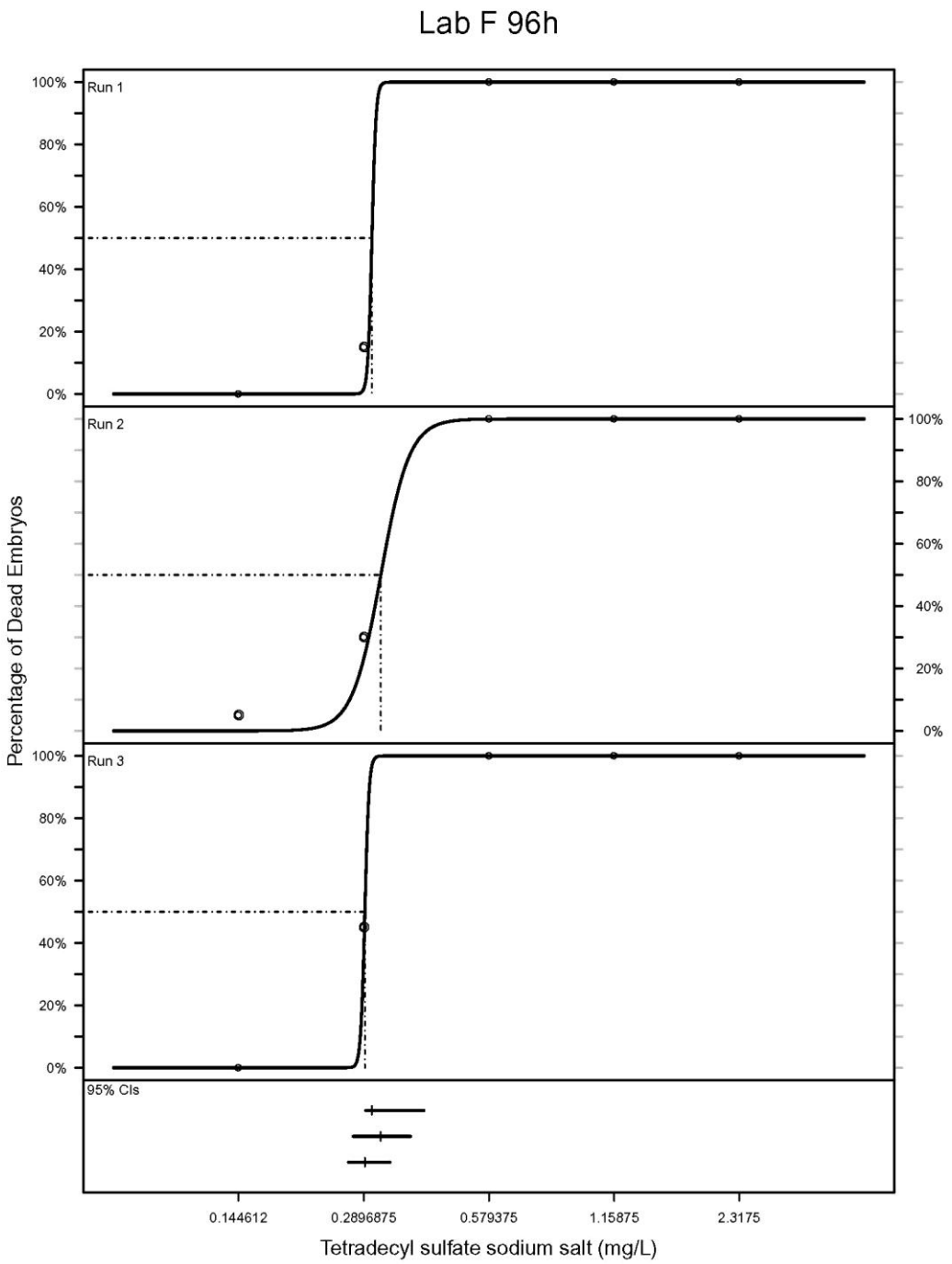

Lab I 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.0381
0.0762
0.1524
0.3048
0.6096
copper (II) sulphate pentahydrate (mg/L)

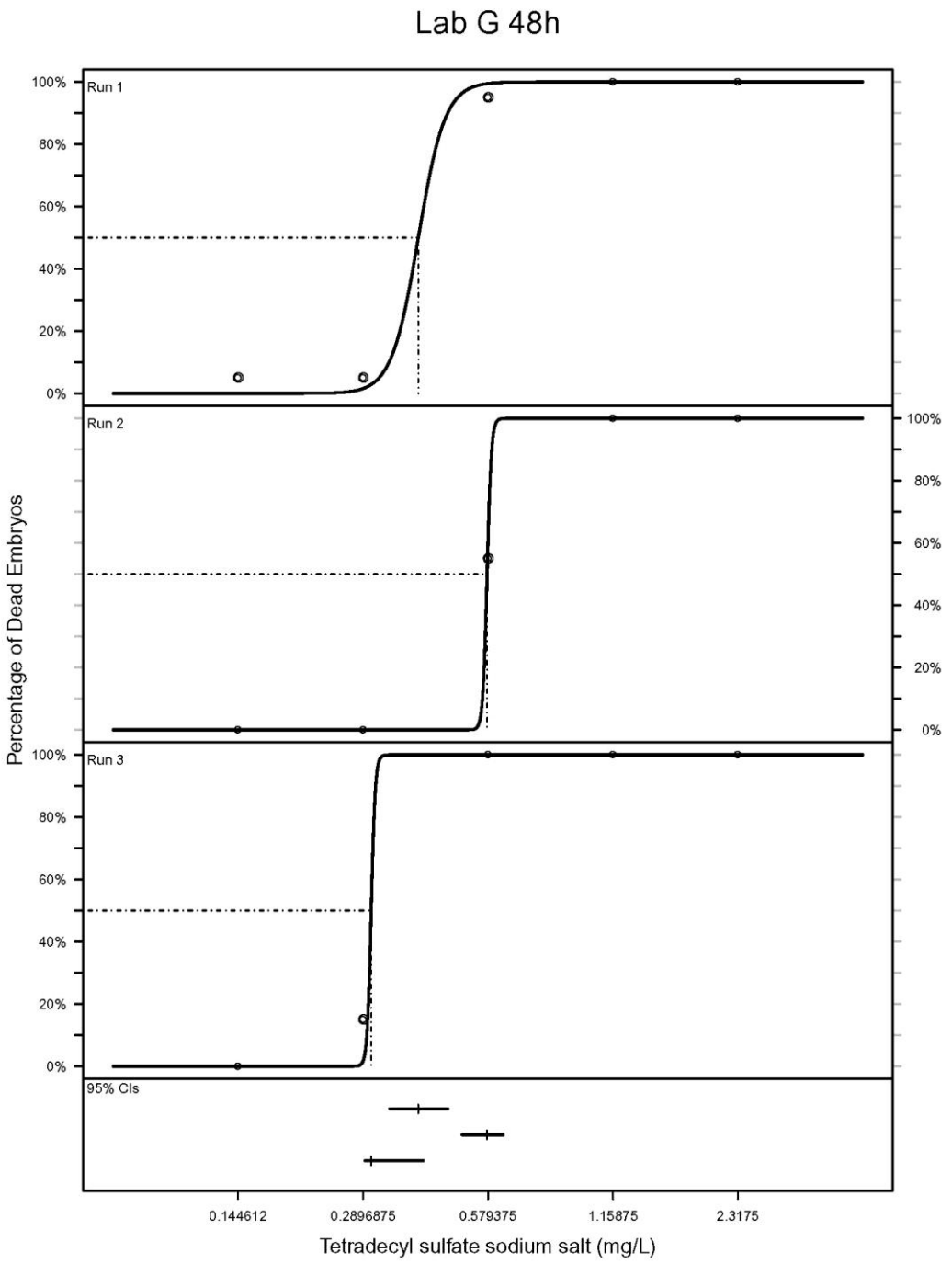
Tetradecyl sulphate sodium salt (toxicity is expressed as tetradecyl sulphate)

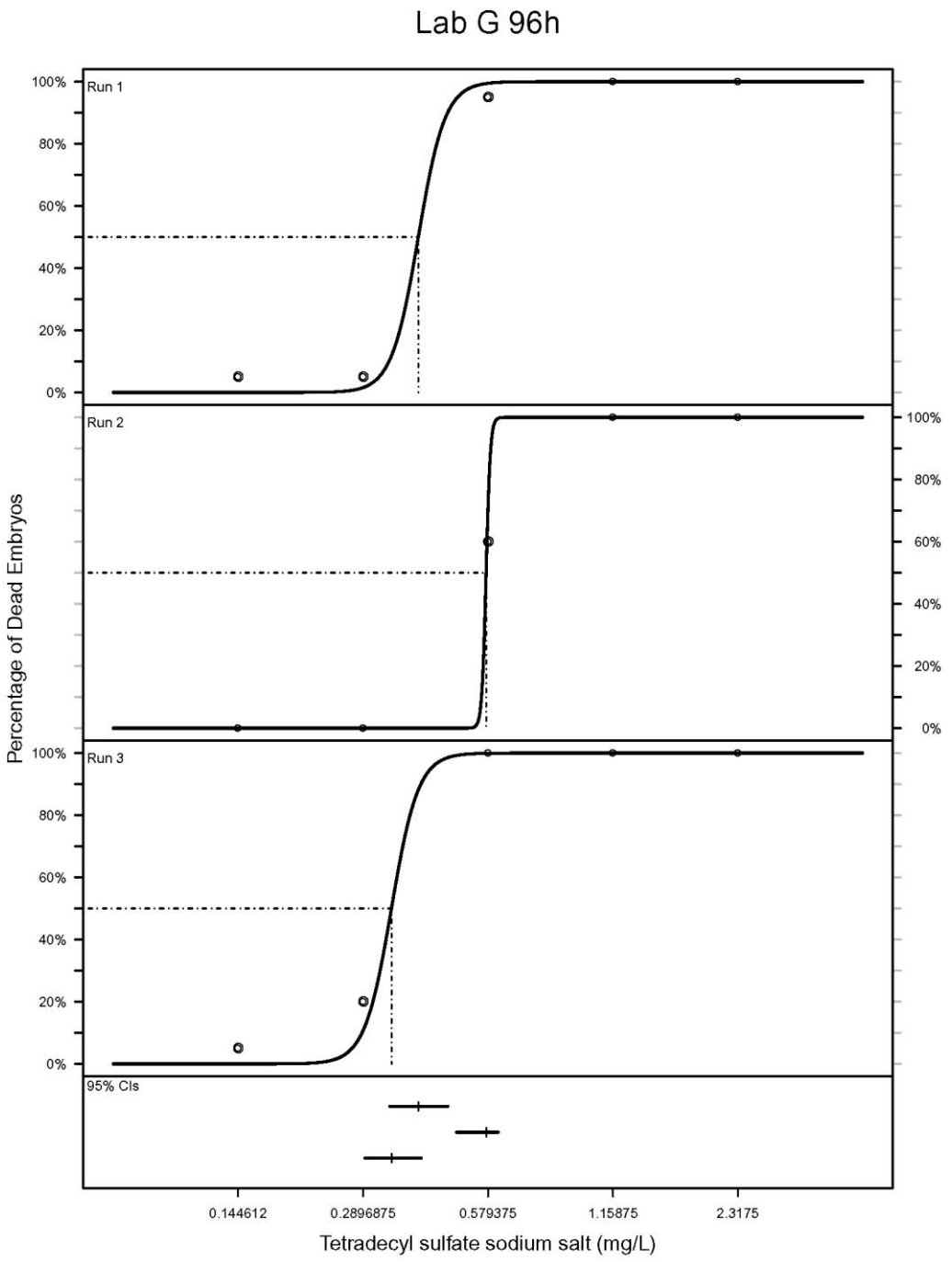


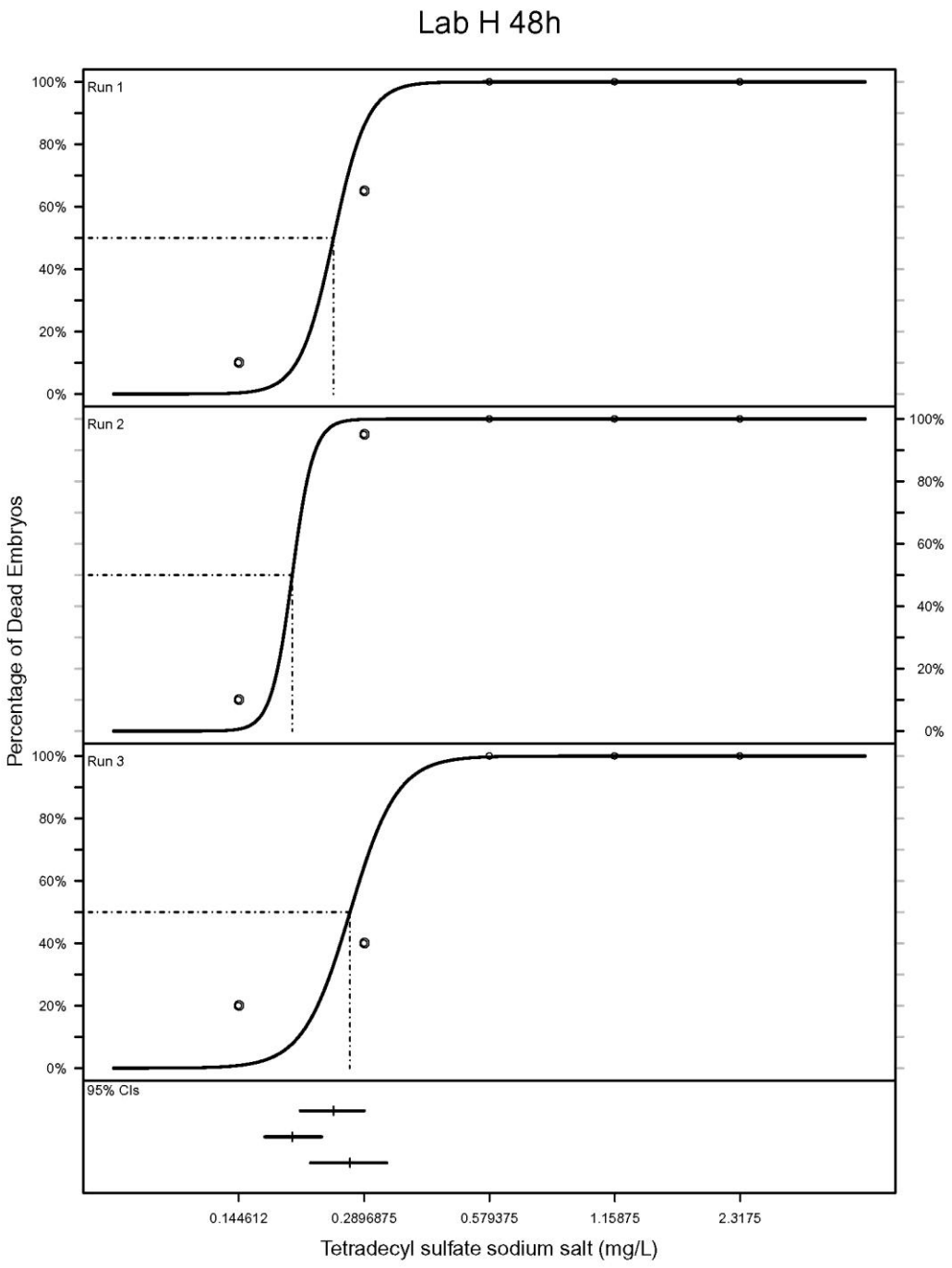

Lab B 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.144612
0.2896875
0.579375
1.15875
2.3175
Tetradecyl sulfate sodium salt (mg/L)

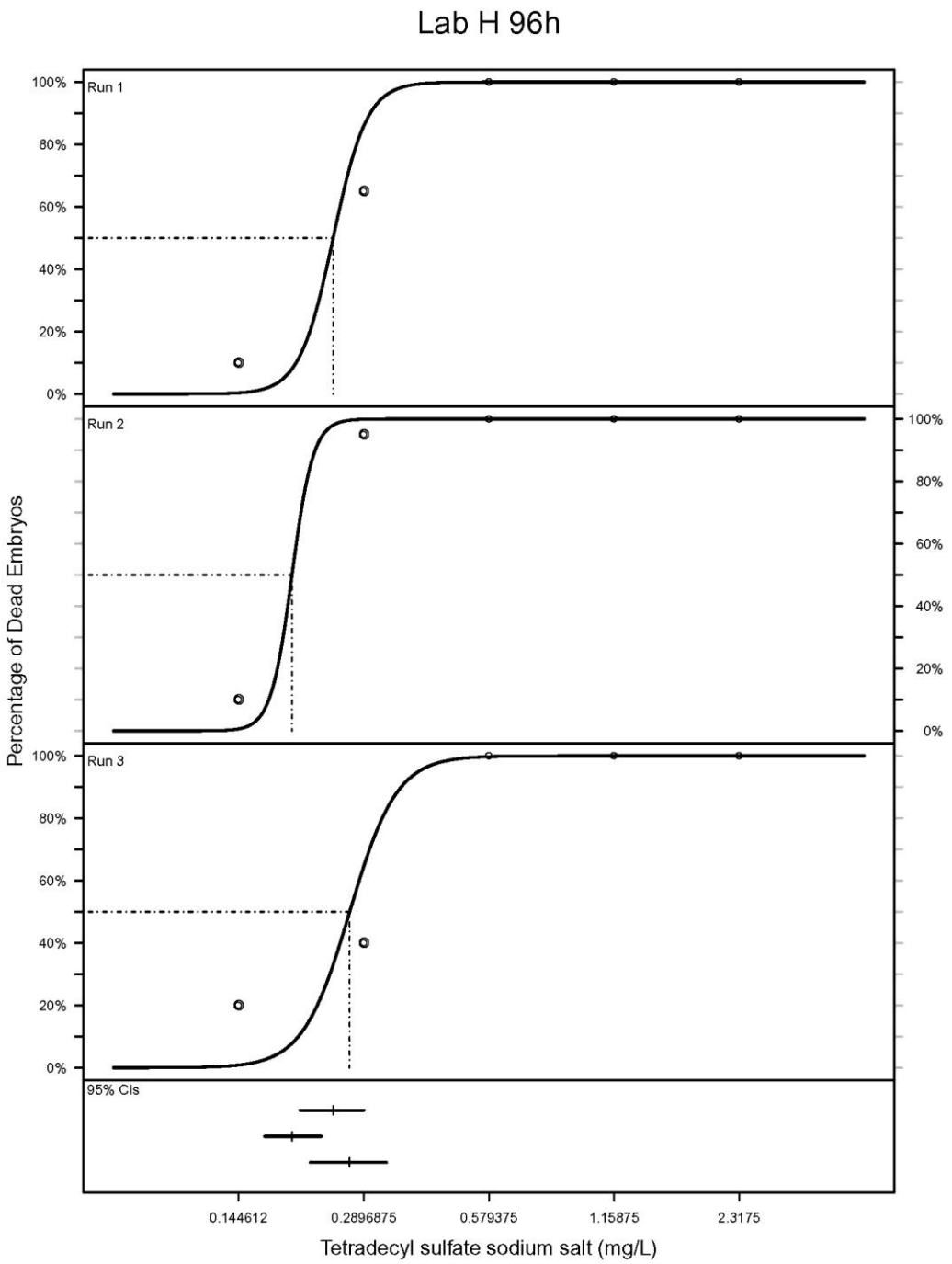

Lab F 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.144612
0.2896875
0.579375
1.15875
2.3175
Tetradecyl sulfate sodium salt (mg/L)


Lab F 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.144612
0.2896875
0.579375
1.15875
2.3175
Tetradecyl sulfate sodium salt (mg/L)

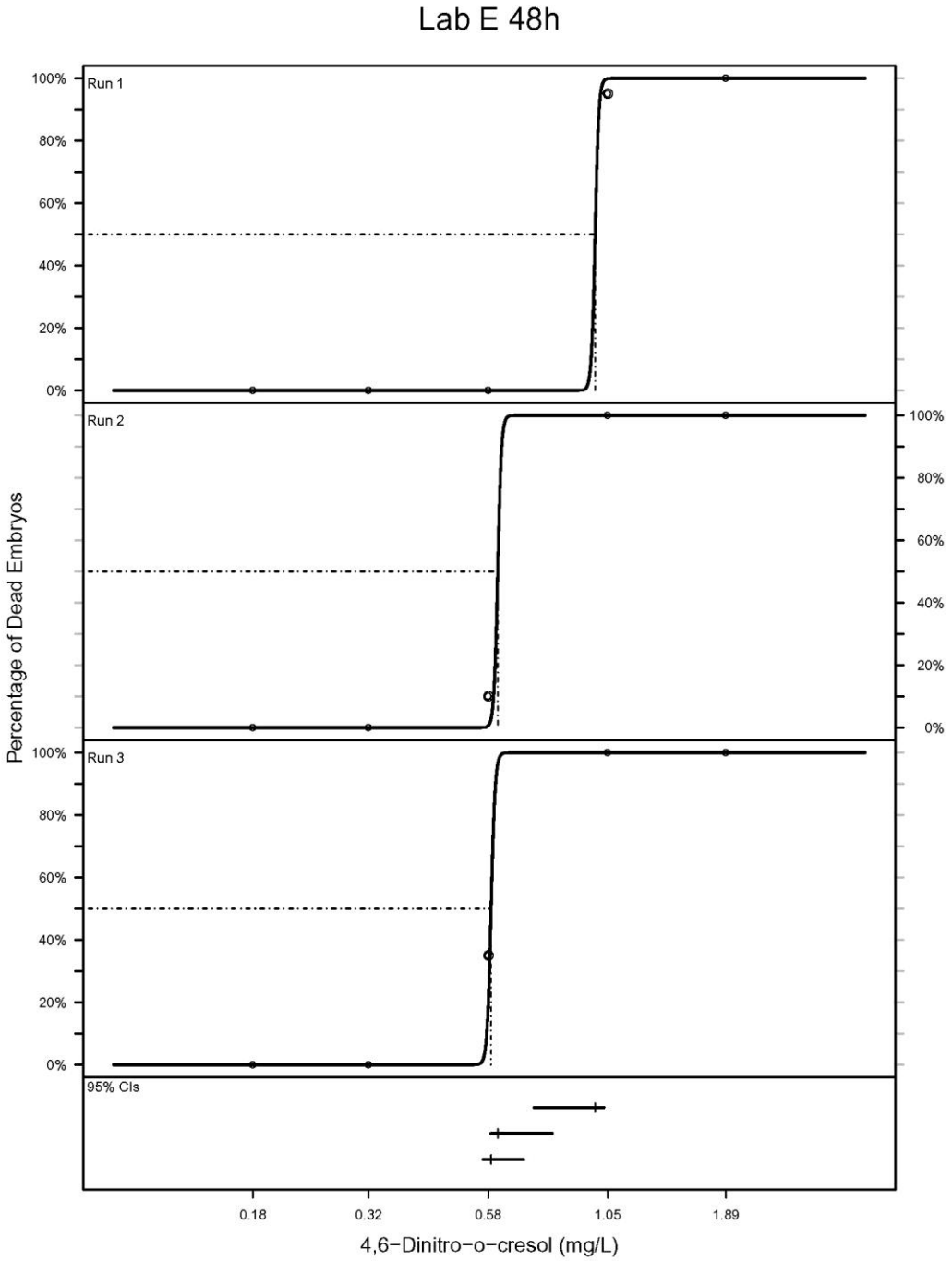

Lab G 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.144612
0.2896875
0.579375
1.15875
2.3175
Tetradecyl sulfate sodium salt (mg/L)

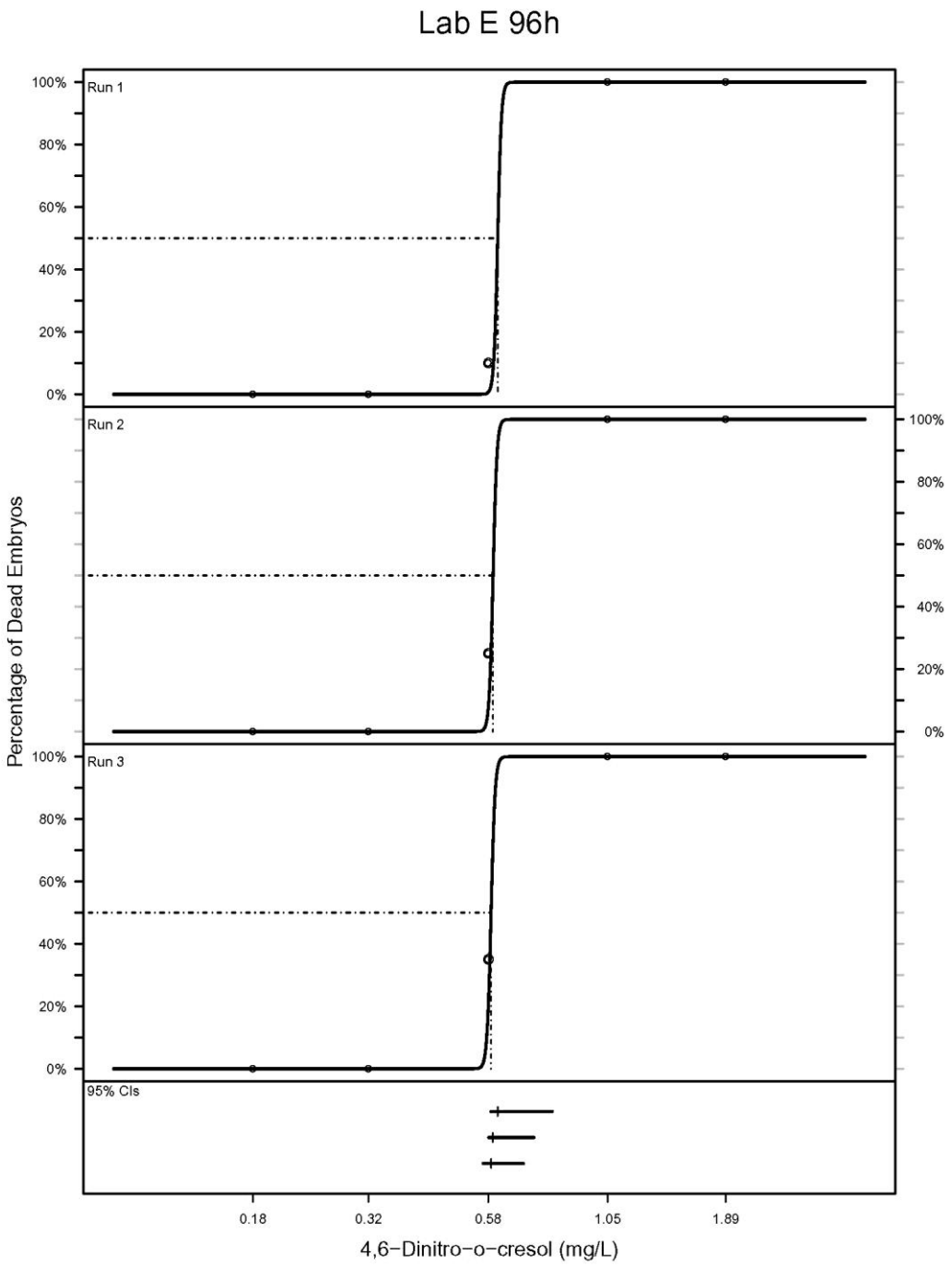

Lab G 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.144612
0.2896875
0.579375
1.15875
2.3175
Tetradecyl sulfate sodium salt (mg/L)

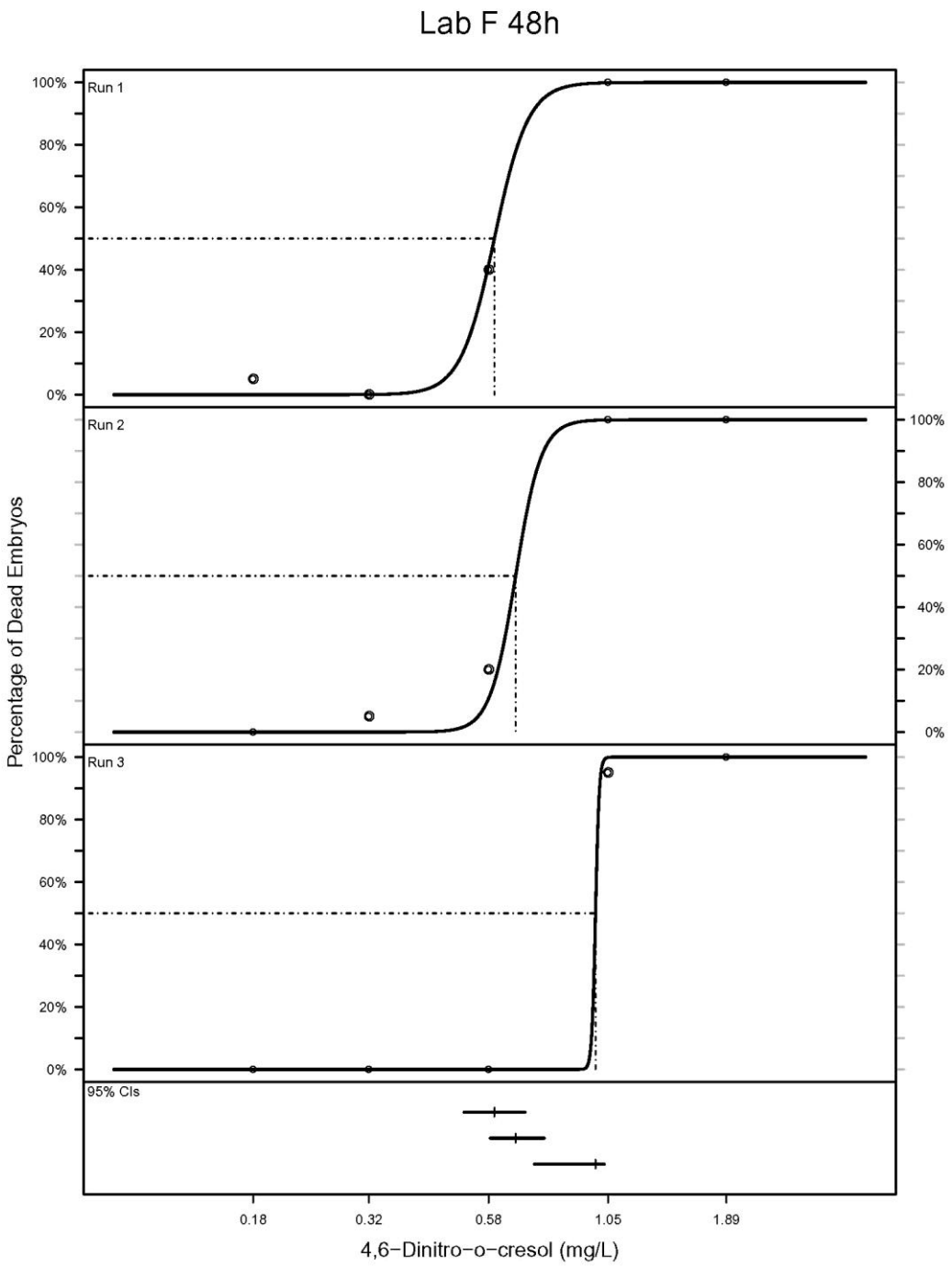

Lab H 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.144612
0.2896875
0.579375
1.15875
2.3175
Tetradecyl sulfate sodium salt (mg/L)

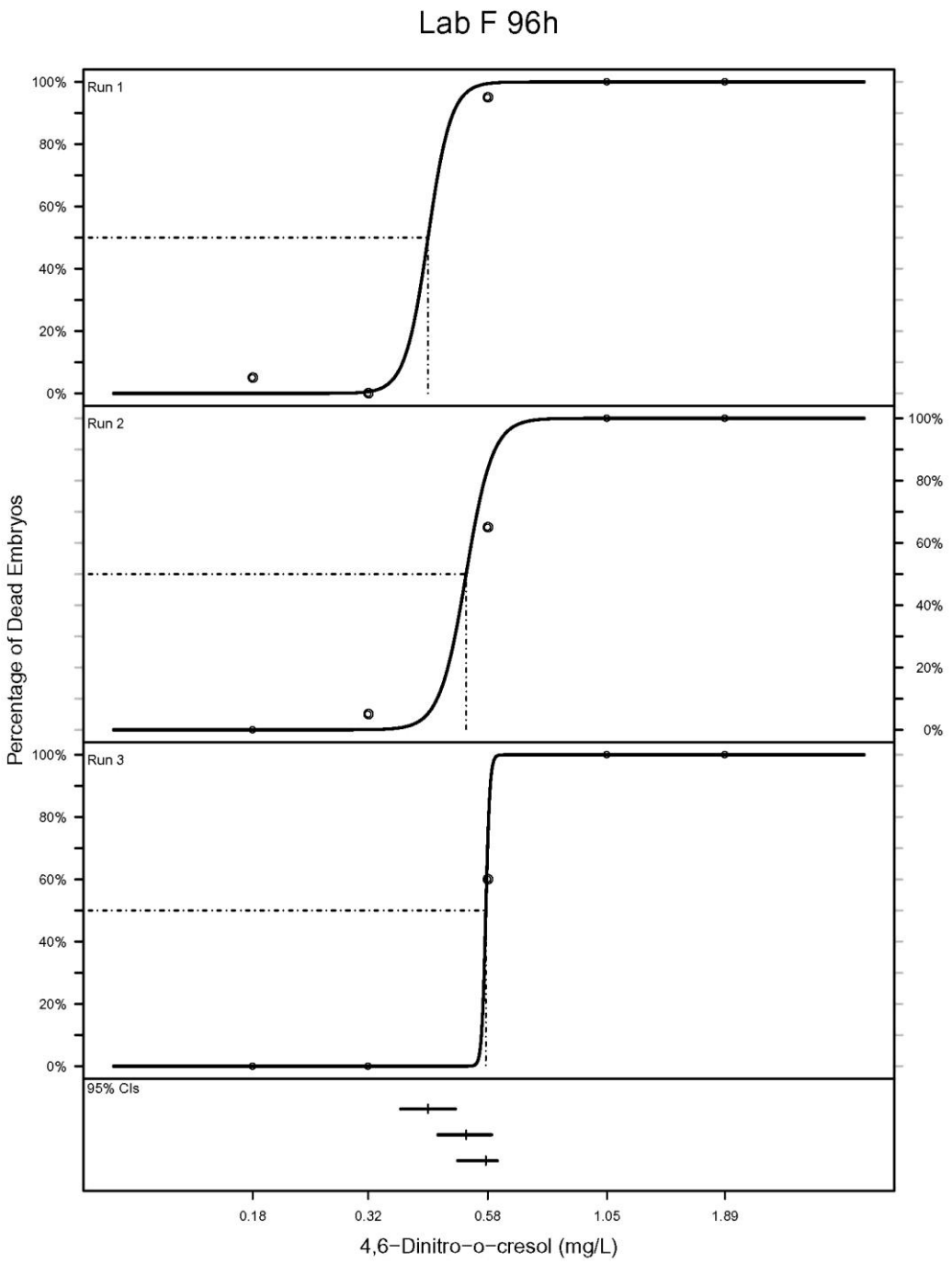

Lab H 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.144612
0.2896875
0.579375
1.15875
2.3175
Tetradecyl sulfate sodium salt (mg/L)

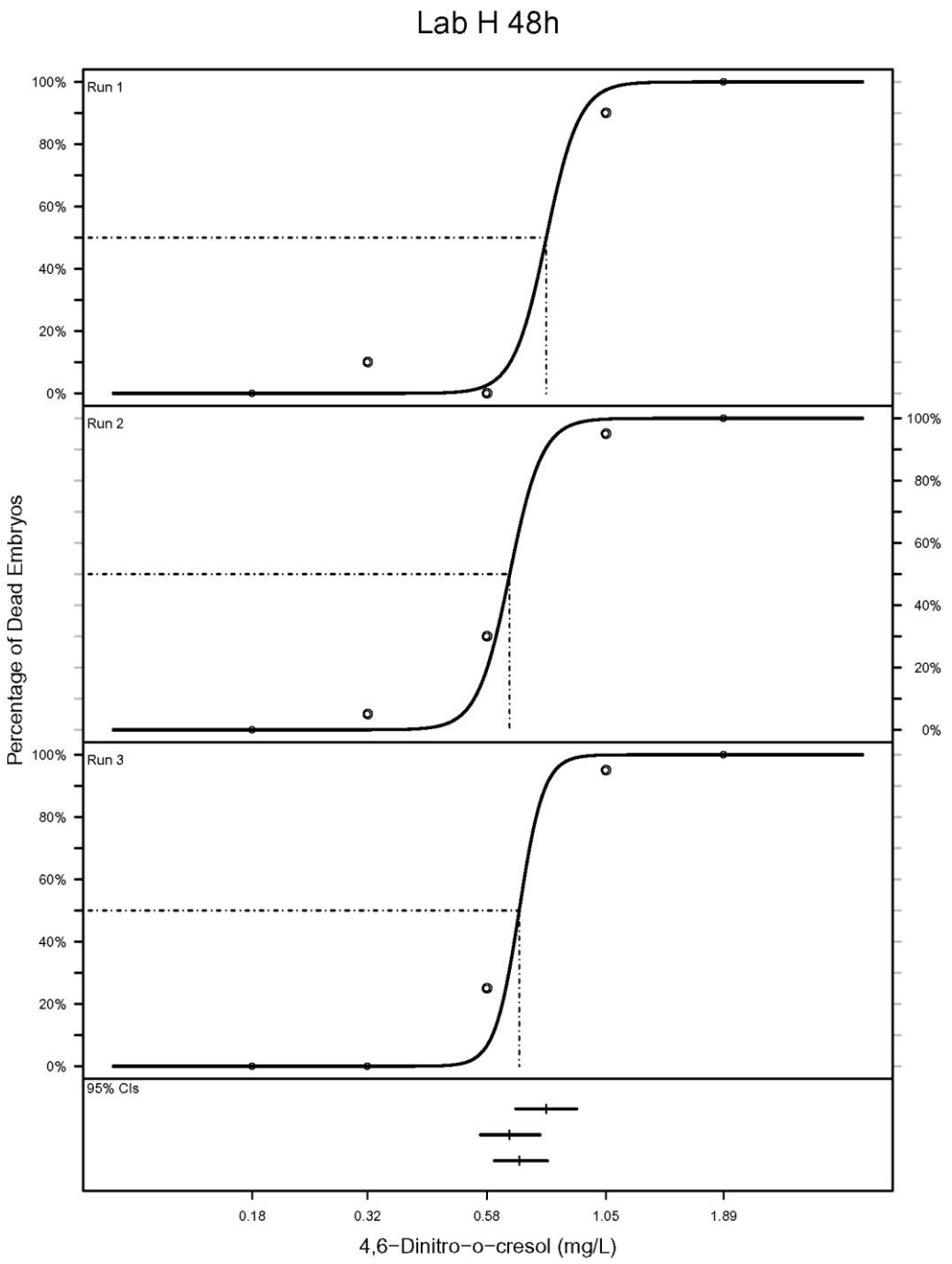
4,6-Dintro-*o*-cresol

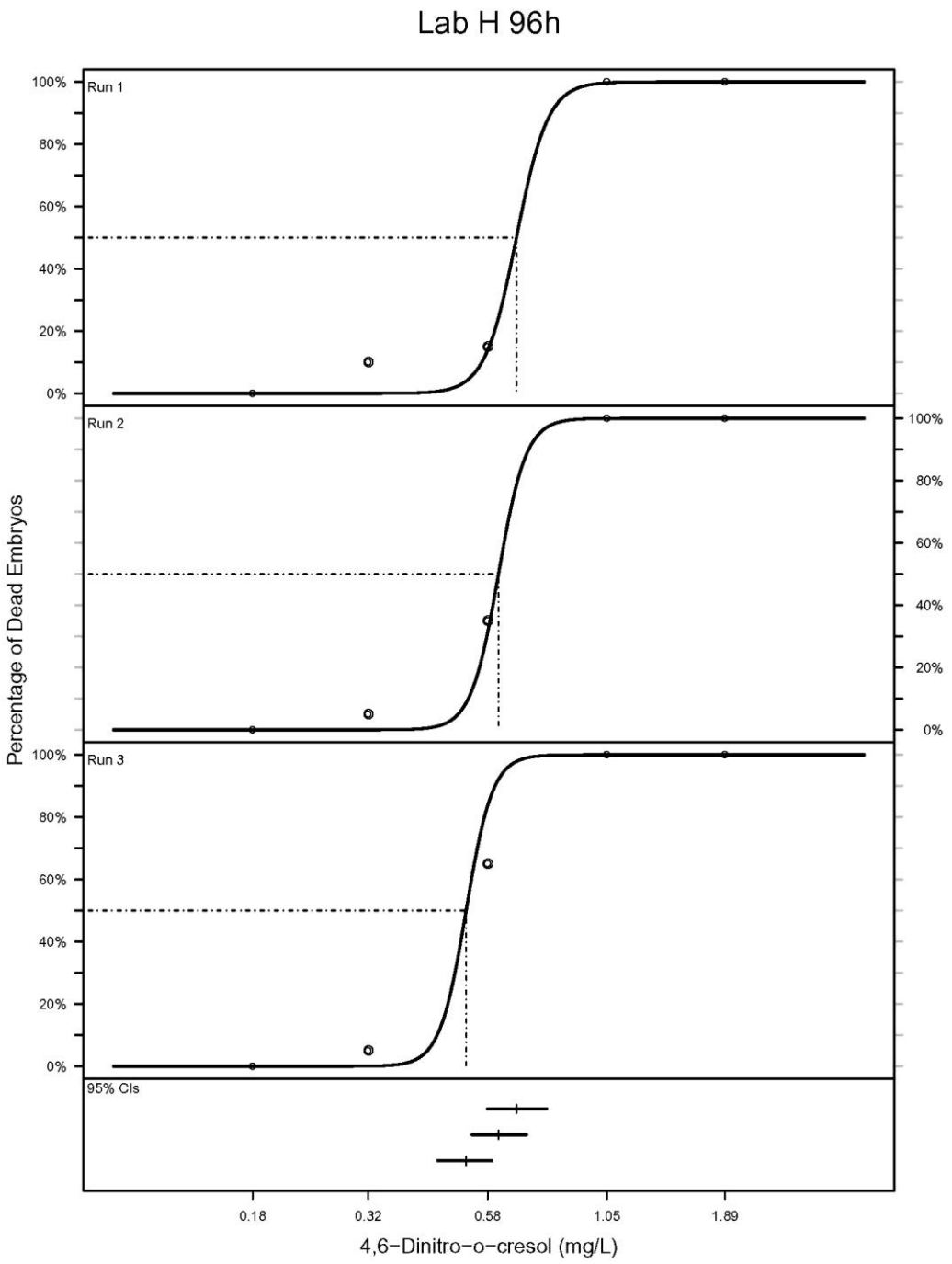


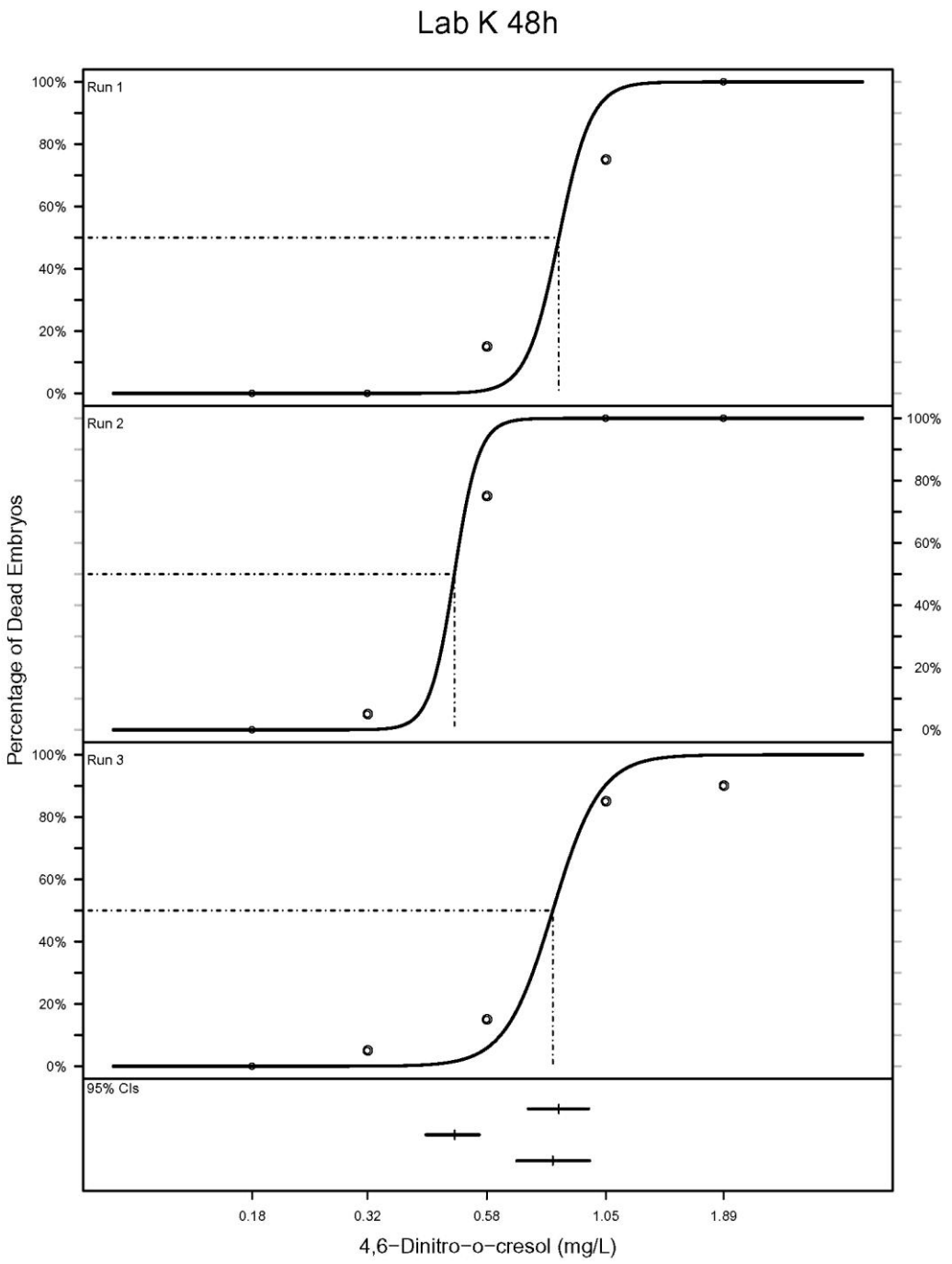

Lab E 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.18
0.32
0.58
1.05
1.89
4,6−Dinitro−o−cresol (mg/L)

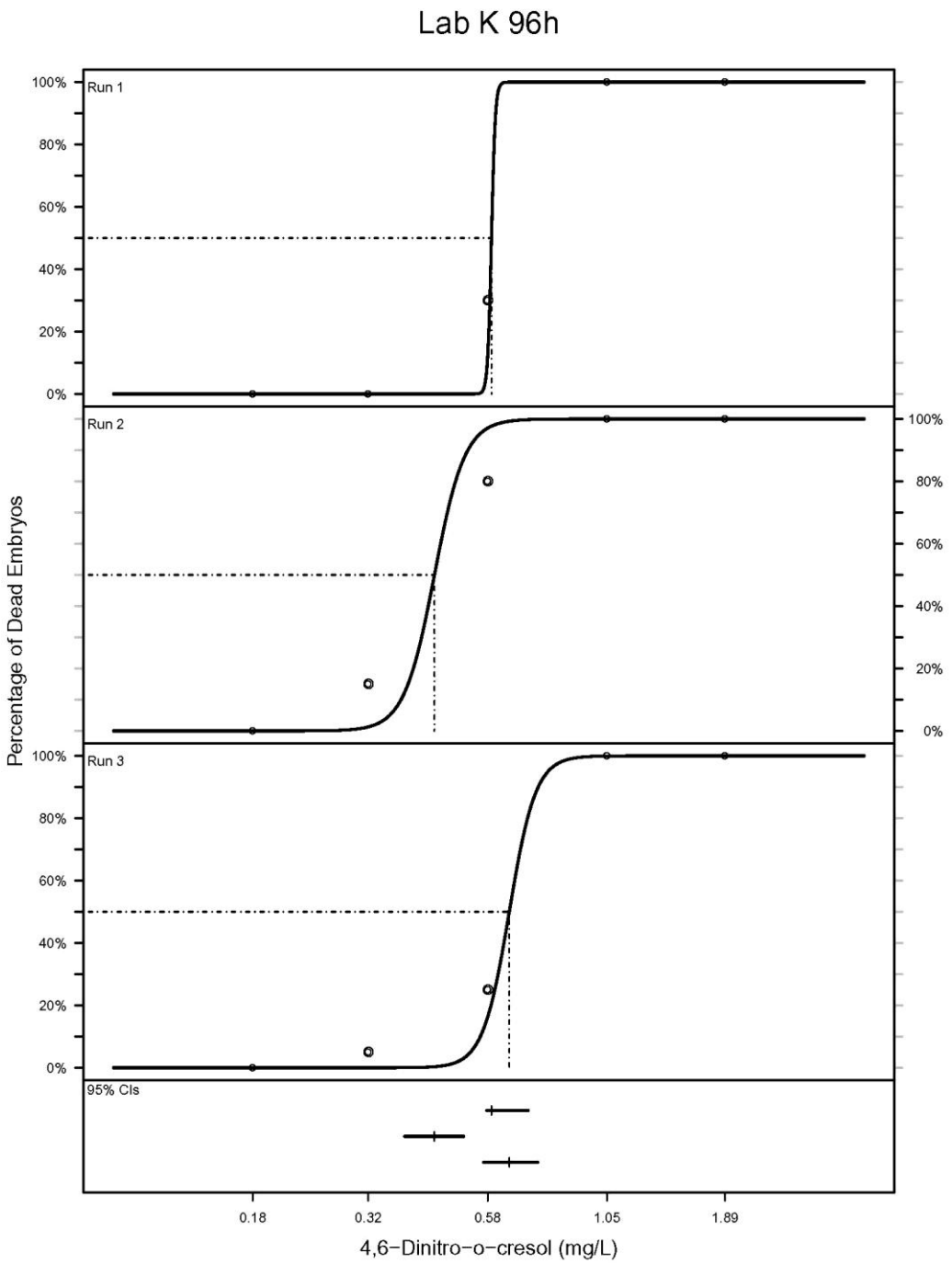

Lab F 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.18
0.32
0.58
1.05
1.89
4,6−Dinitro−o−cresol (mg/L)


Lab F 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.18
0.32
0.58
1.05
1.89
4,6-Dinitro-o-cresol (mg/L)

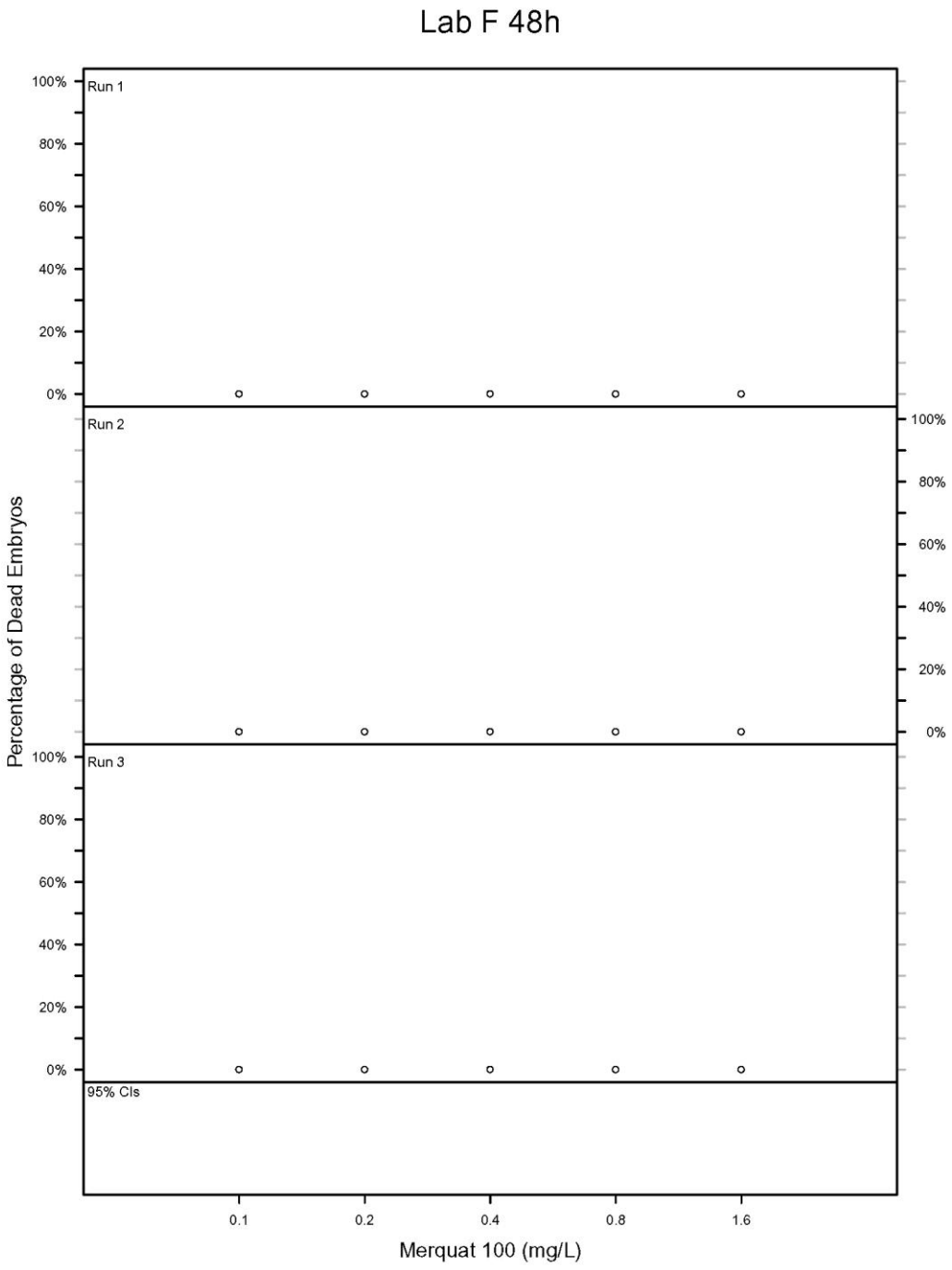

Lab H 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.18
0.32
0.58
1.05
1.89
4,6−Dinitro−o−cresol (mg/L)

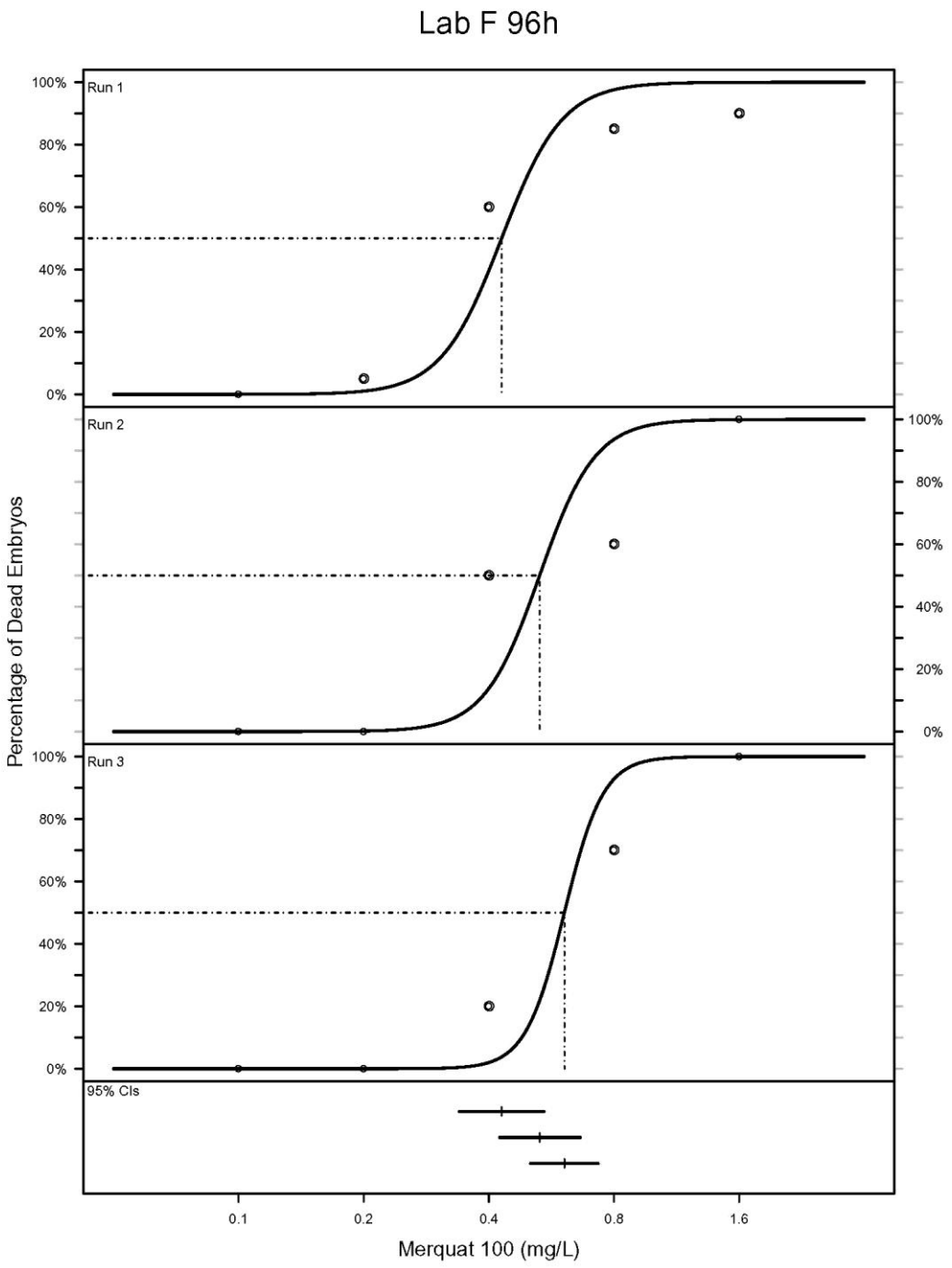

Lab H 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.18
0.32
0.58
1.05
1.89
4,6-Dinitro-o-cresol (mg/L)

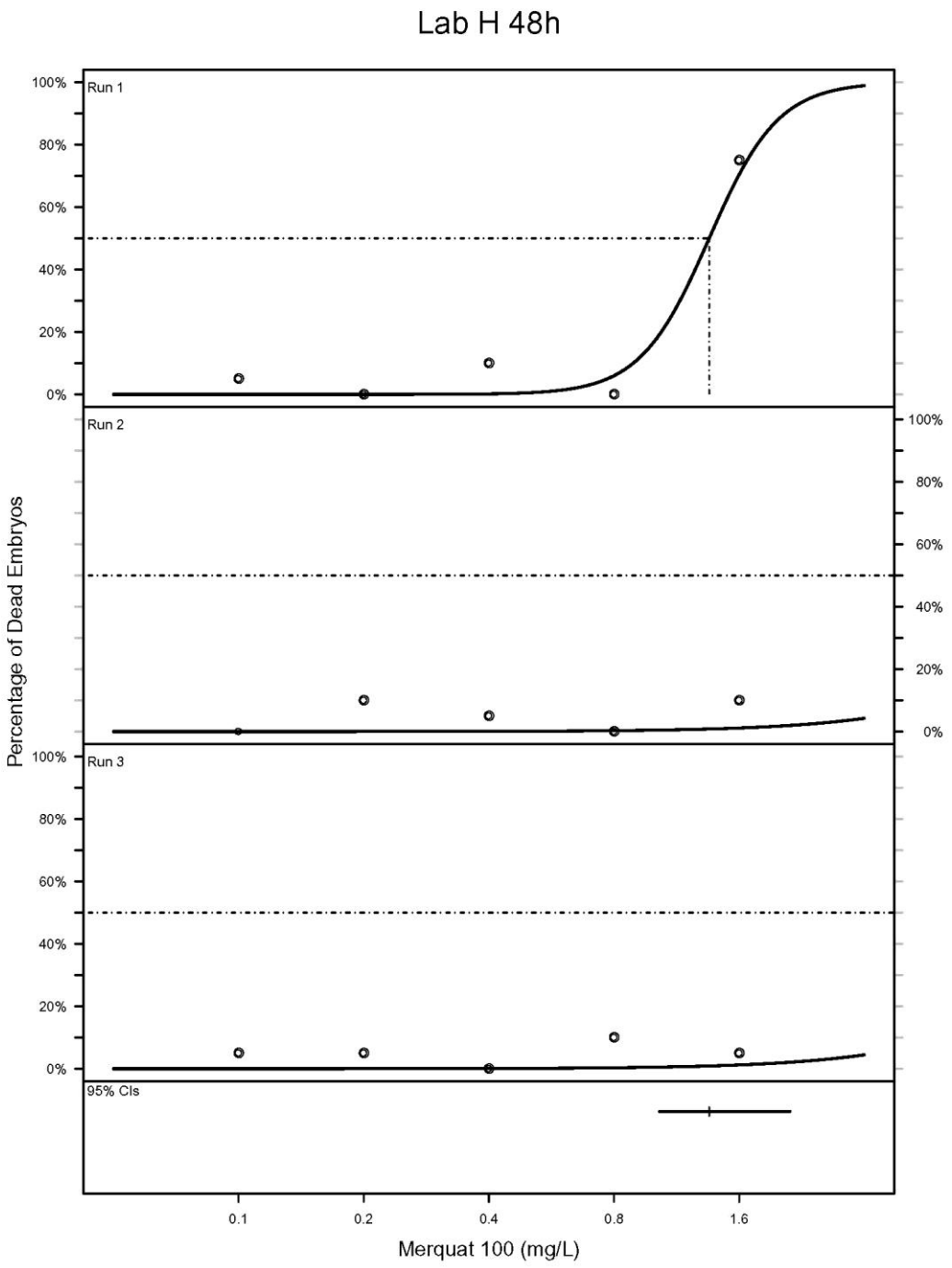

Lab K 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.18
0.32
0.58
1.05
1.89
4,6−Dinitro−o−cresol (mg/L)

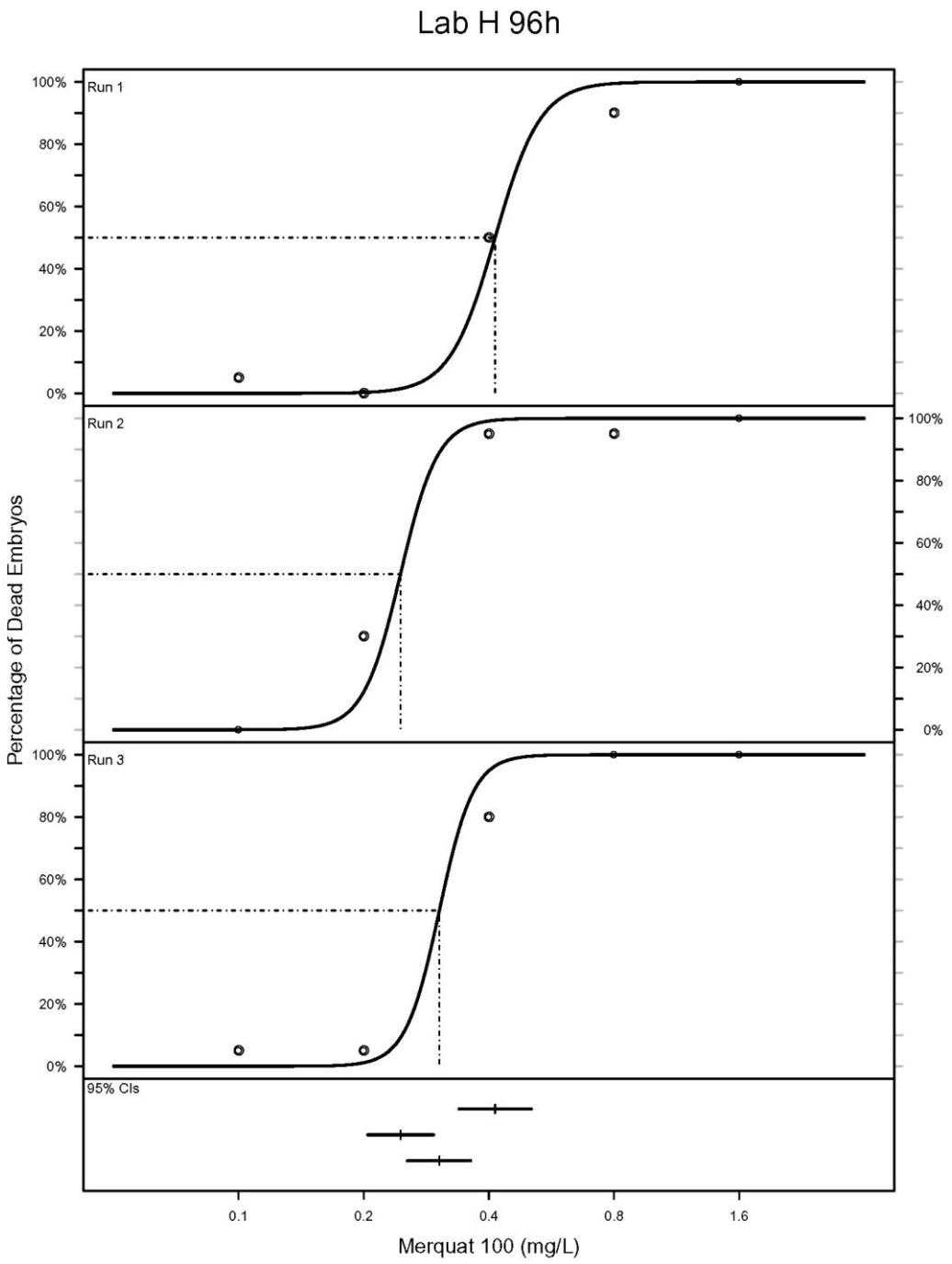

Lab K 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.18
0.32
0.58
1.05
1.89
4,6-Dinitro-o-cresol (mg/L)

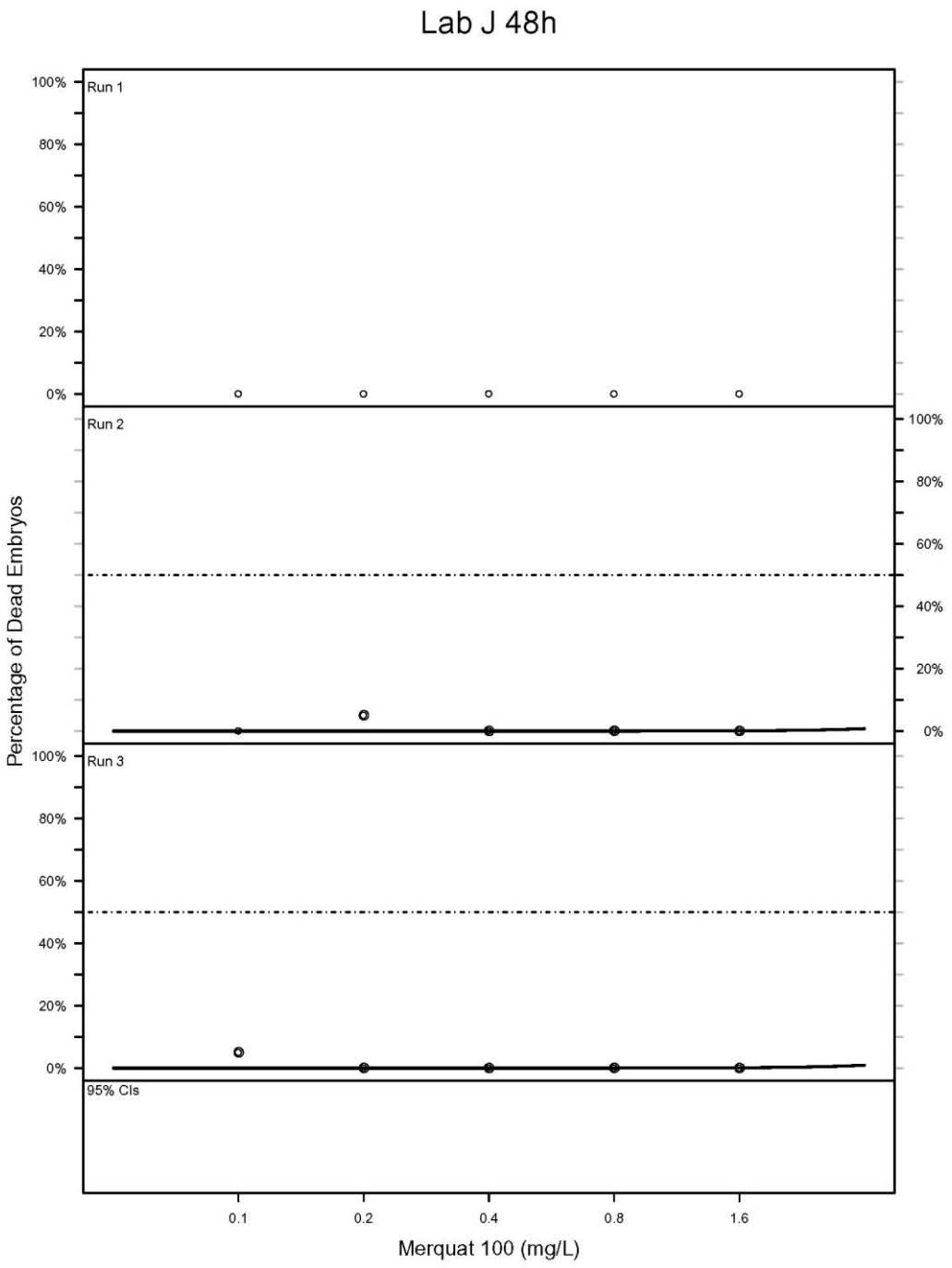
Merquat 100

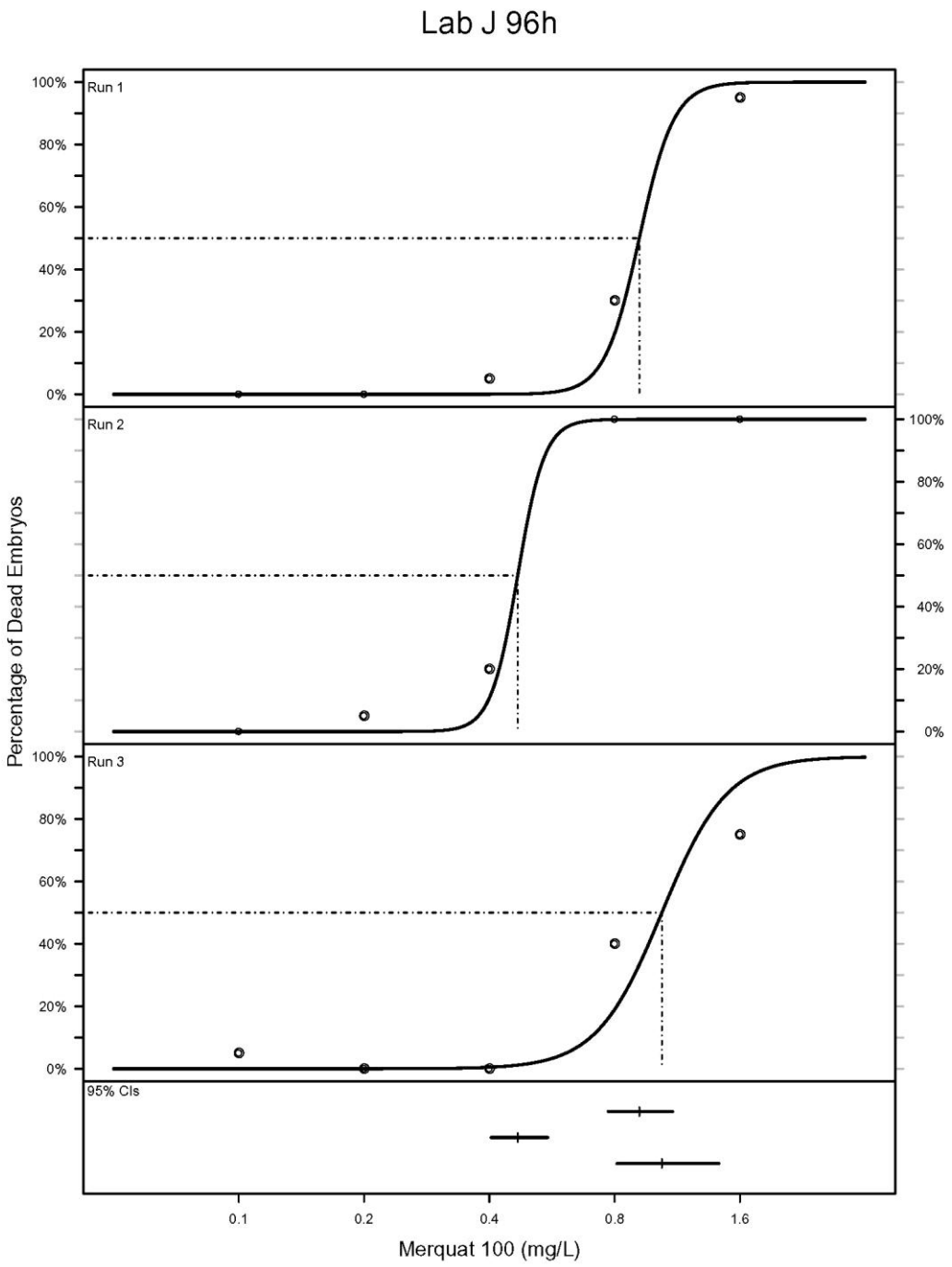

Lab F 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.1
0.2
0.4
0.8
1.6
Merquat 100 (mg/L)

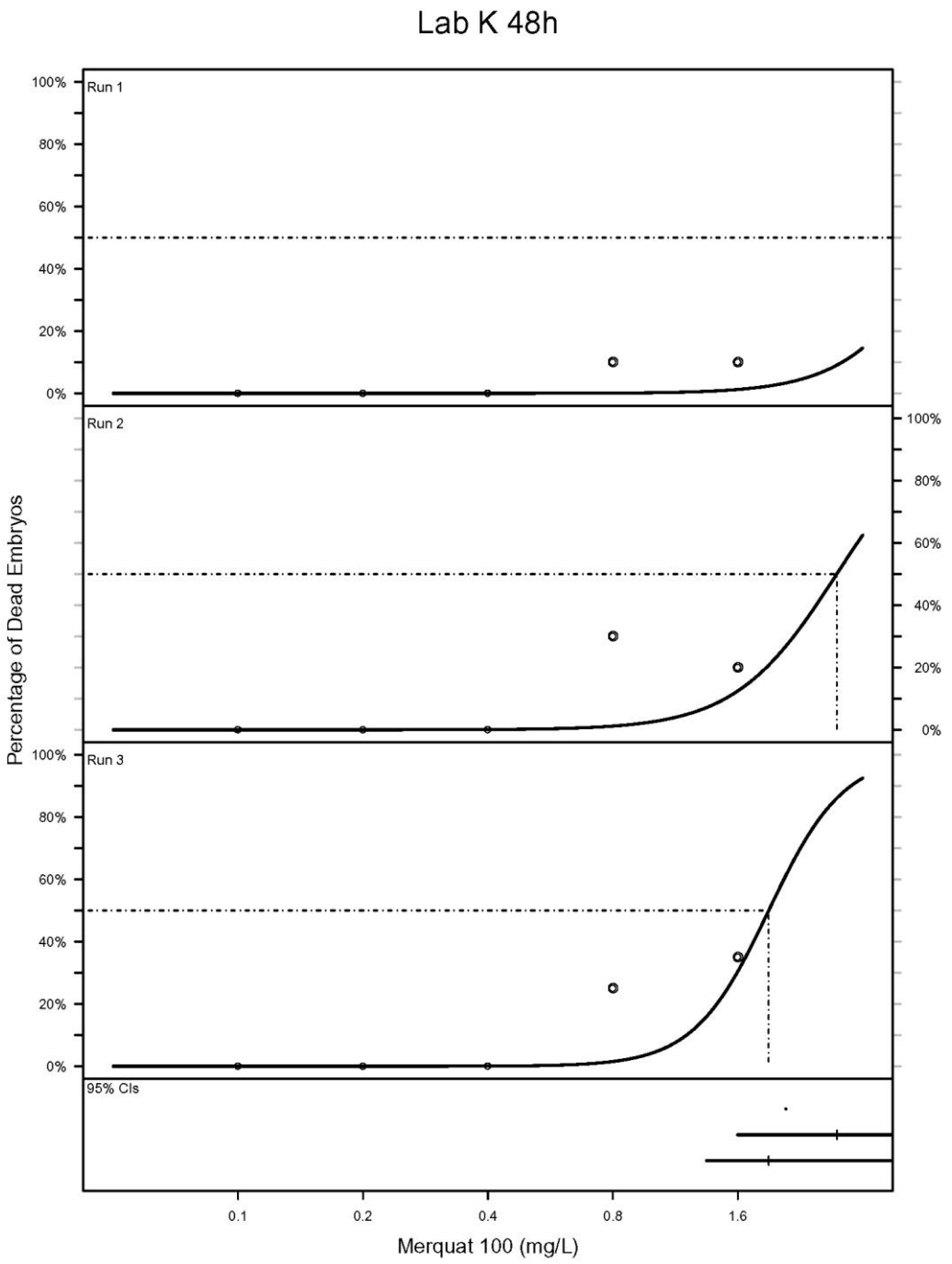

Lab F 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.1
0.2
0.4
0.8
1.6
Merquat 100 (mg/L)

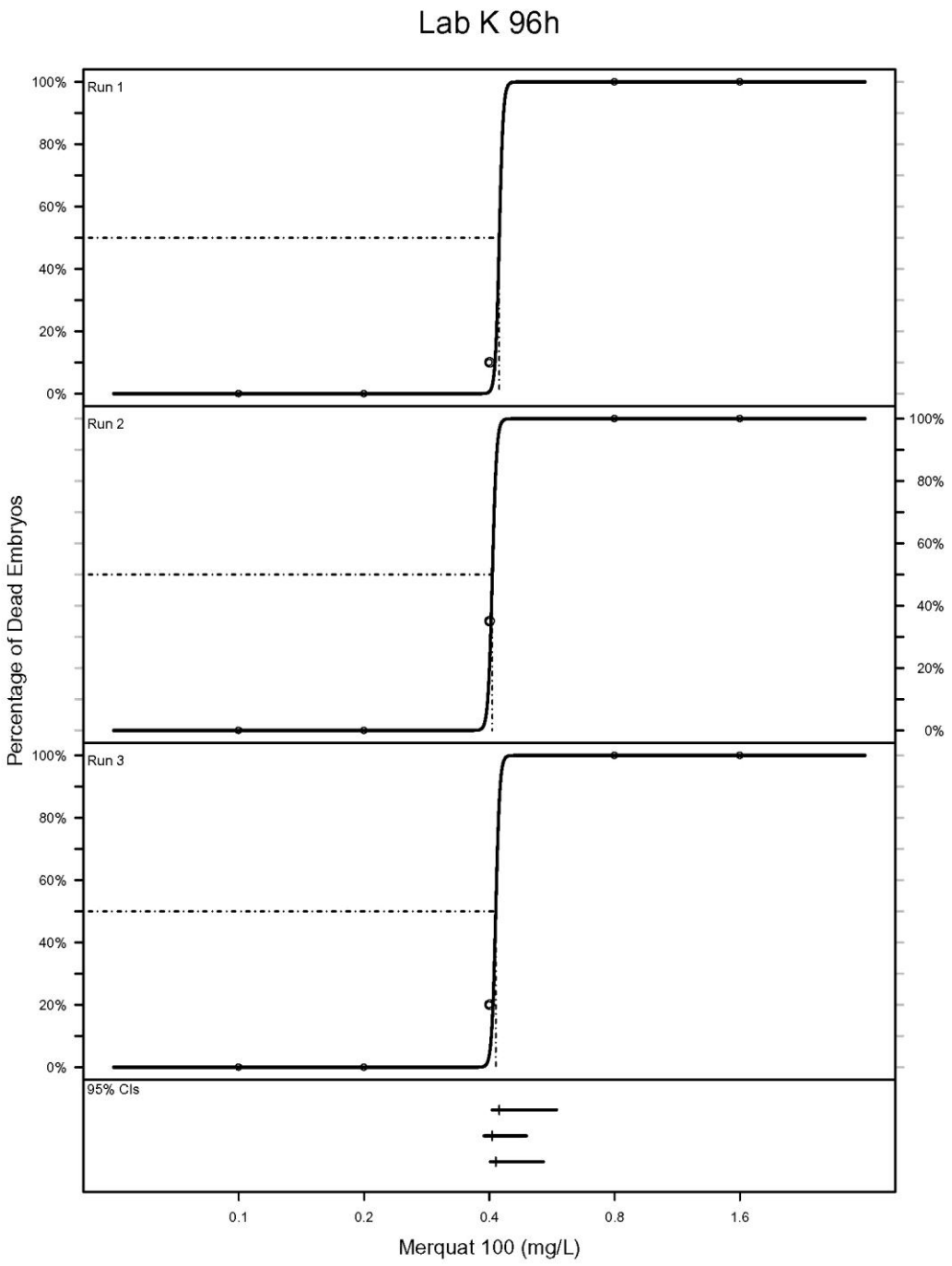

Lab H 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.1
0.2
0.4
0.8
1.6
Merquat 100 (mg/L)


Lab H 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.1
0.2
0.4
0.8
1.6
Merquat 100 (mg/L)

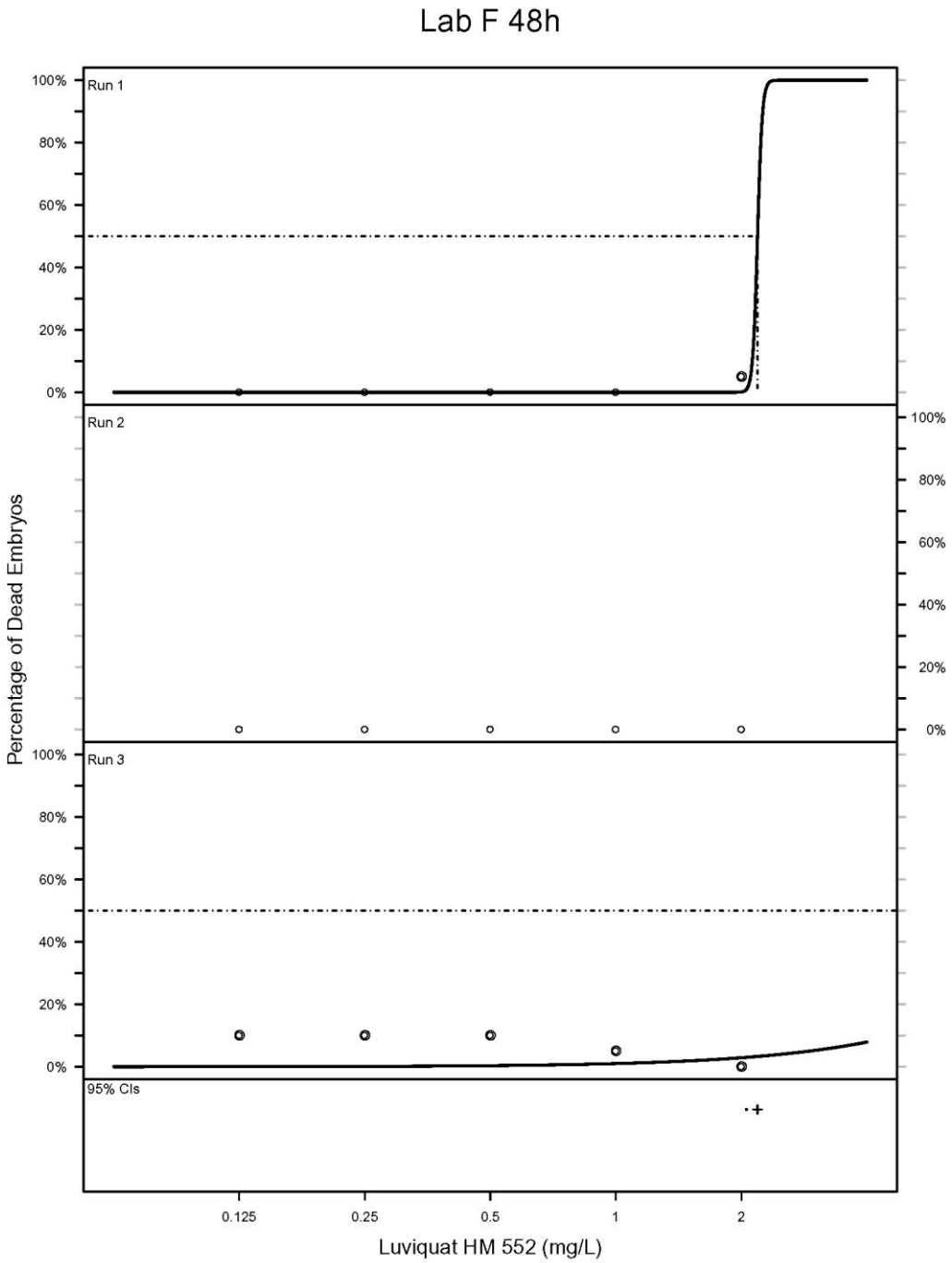

Lab J 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.1
0.2
0.4
0.8
1.6
Merquat 100 (mg/L)

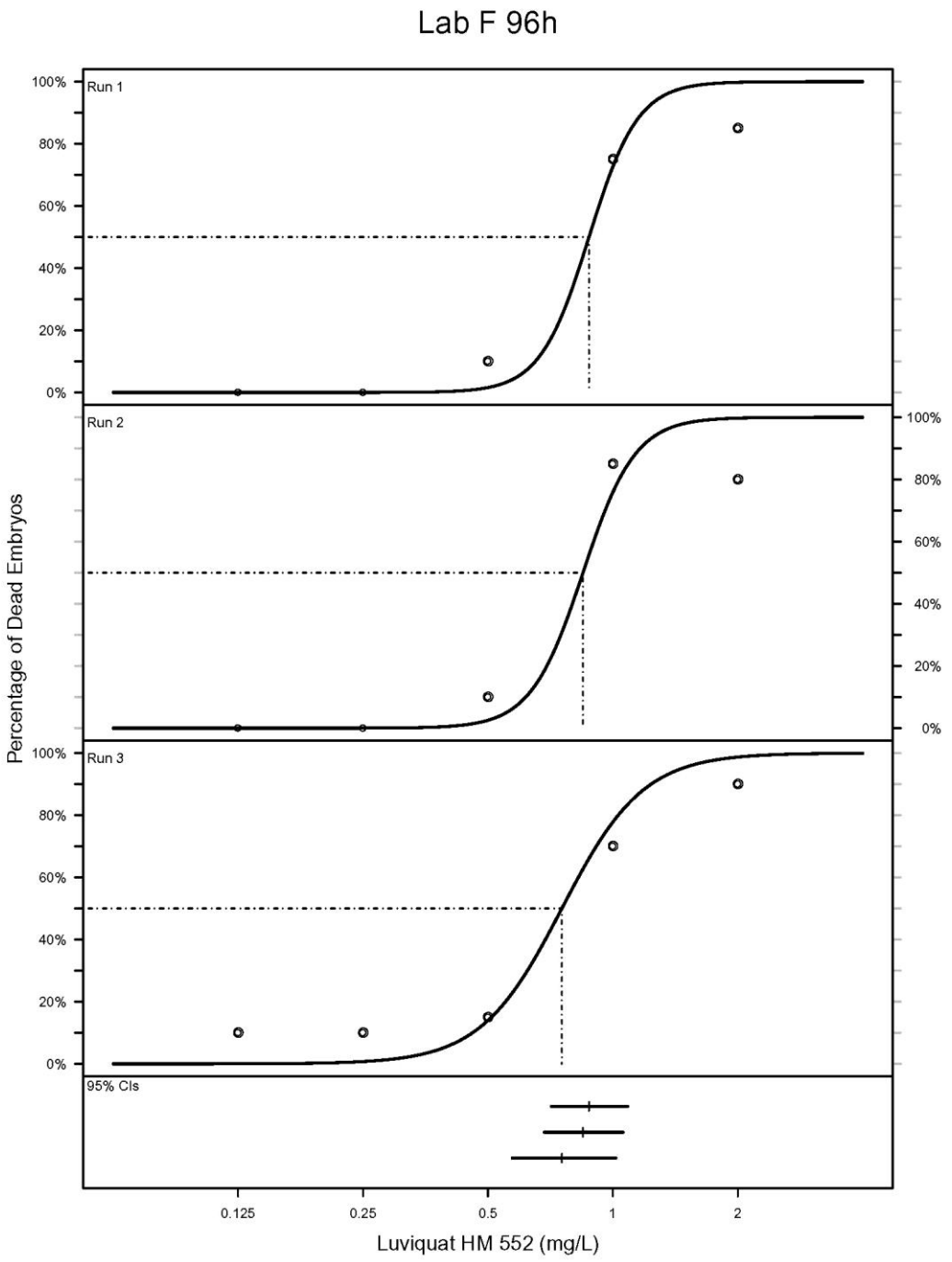

Lab J 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.1
0.2
0.4
0.8
1.6
Merquat 100 (mg/L)

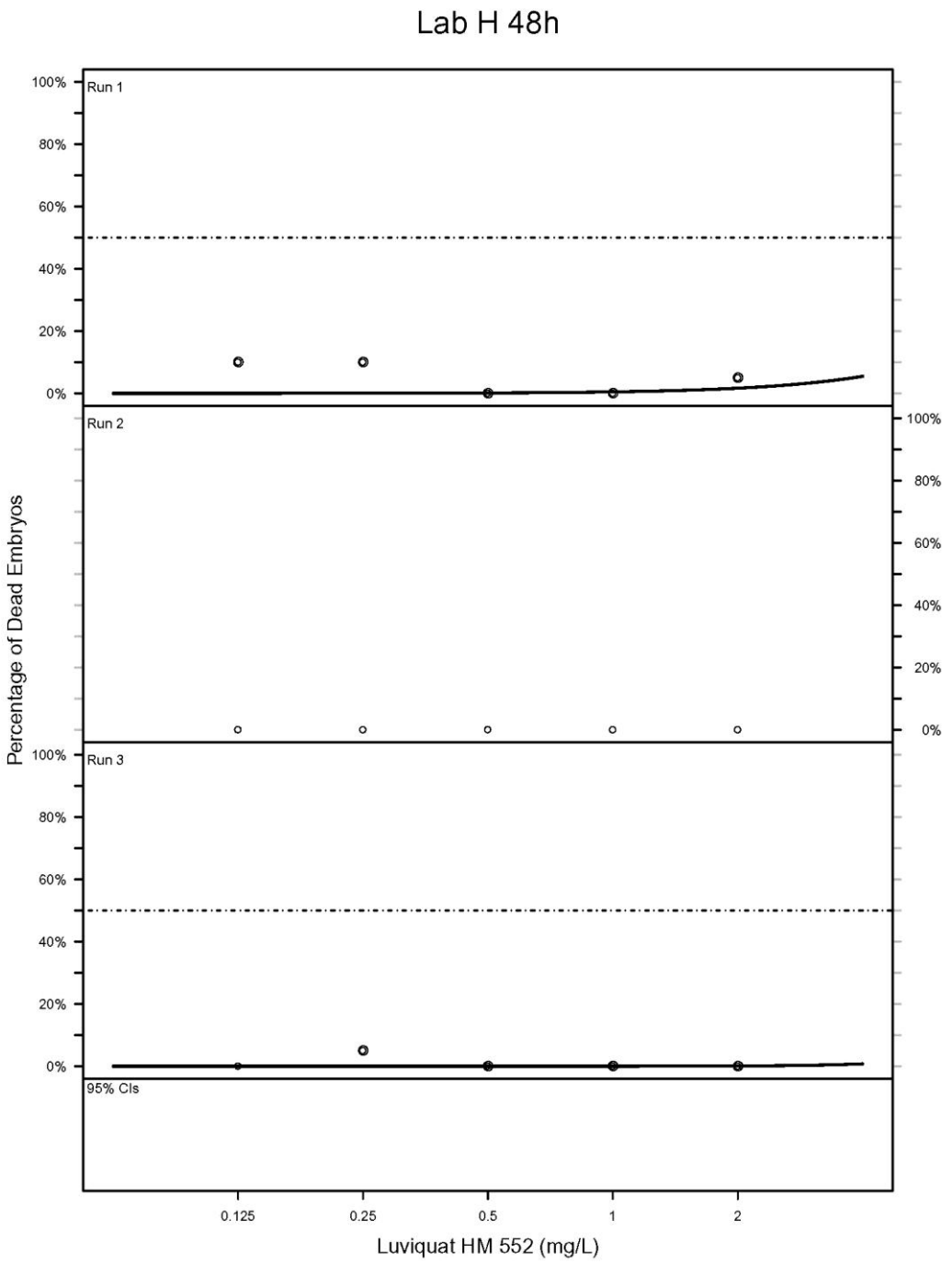

Lab K 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.1
0.2
0.4
0.8
1.6
Merquat 100 (mg/L)

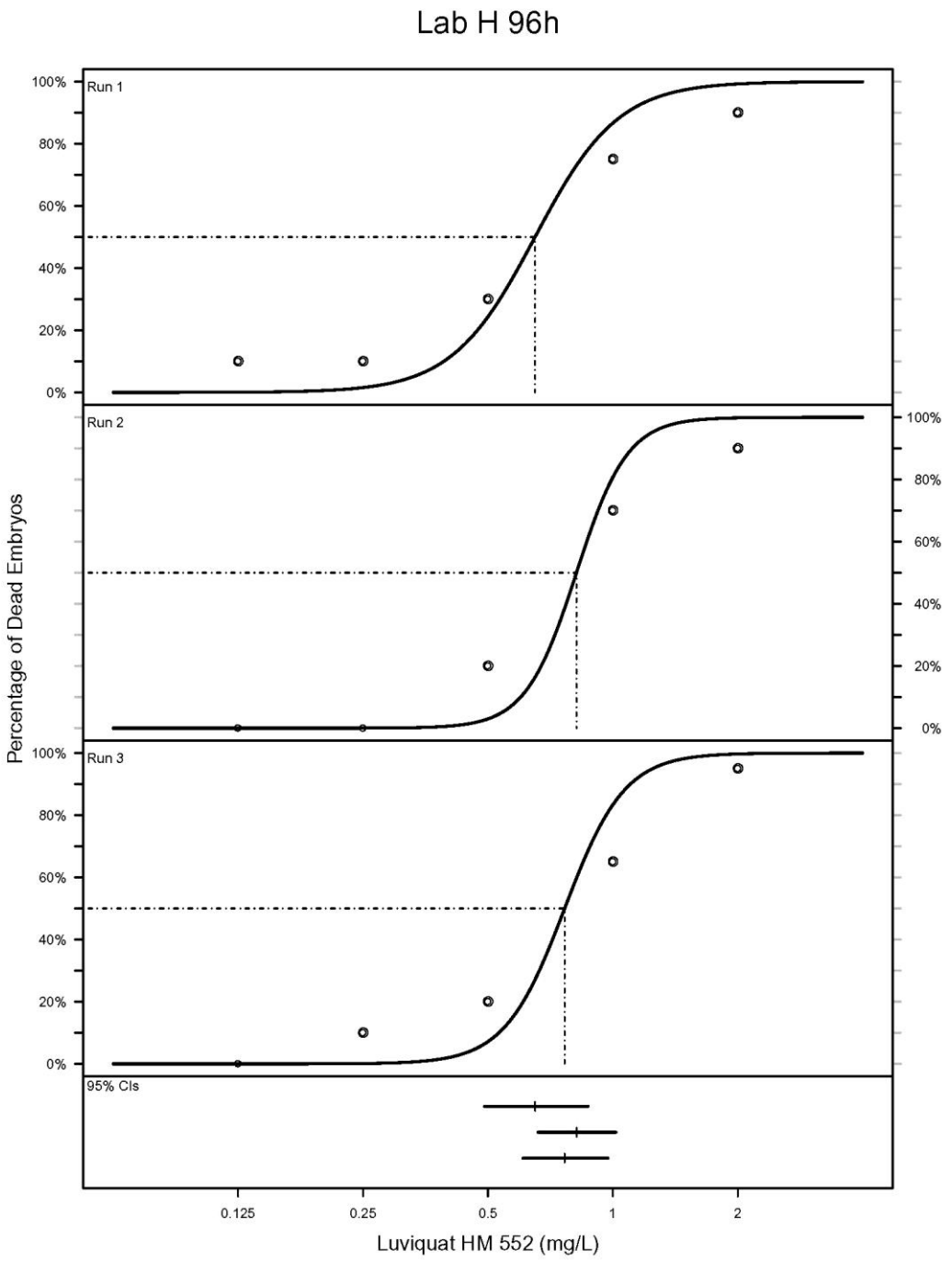

Lab K 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.1
0.2
0.4
0.8
1.6
Merquat 100 (mg/L)

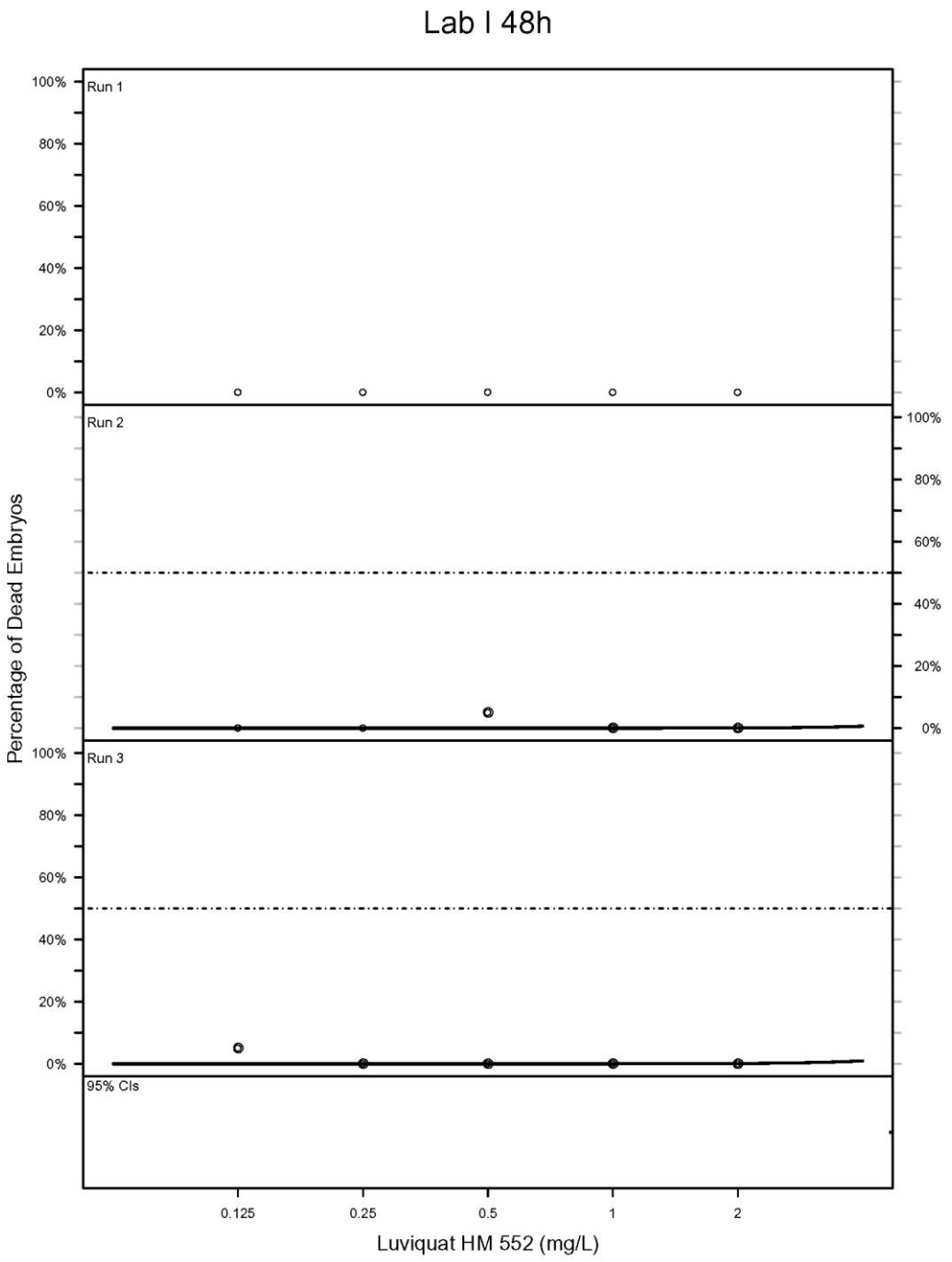
Luviquat HM 552

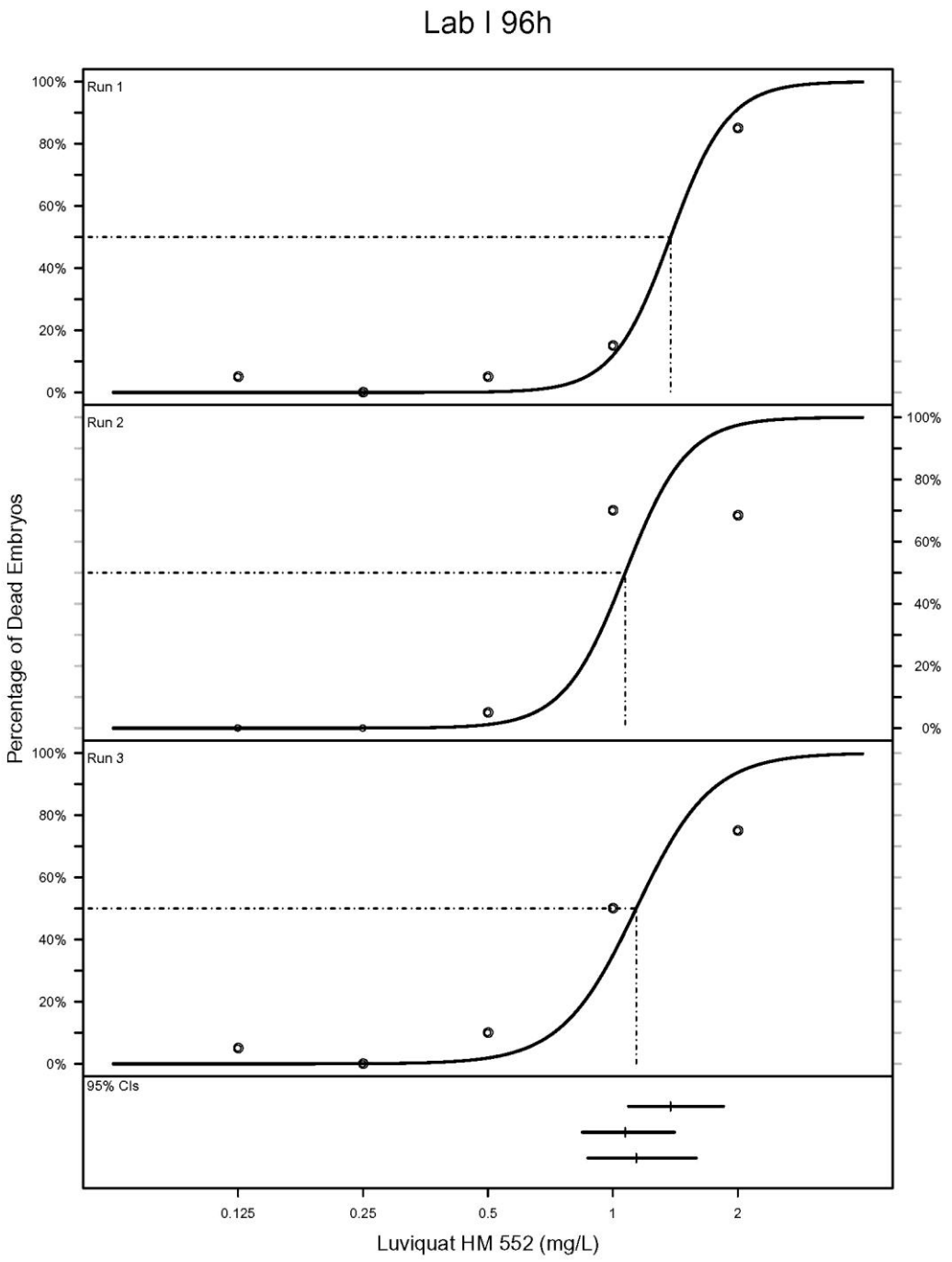


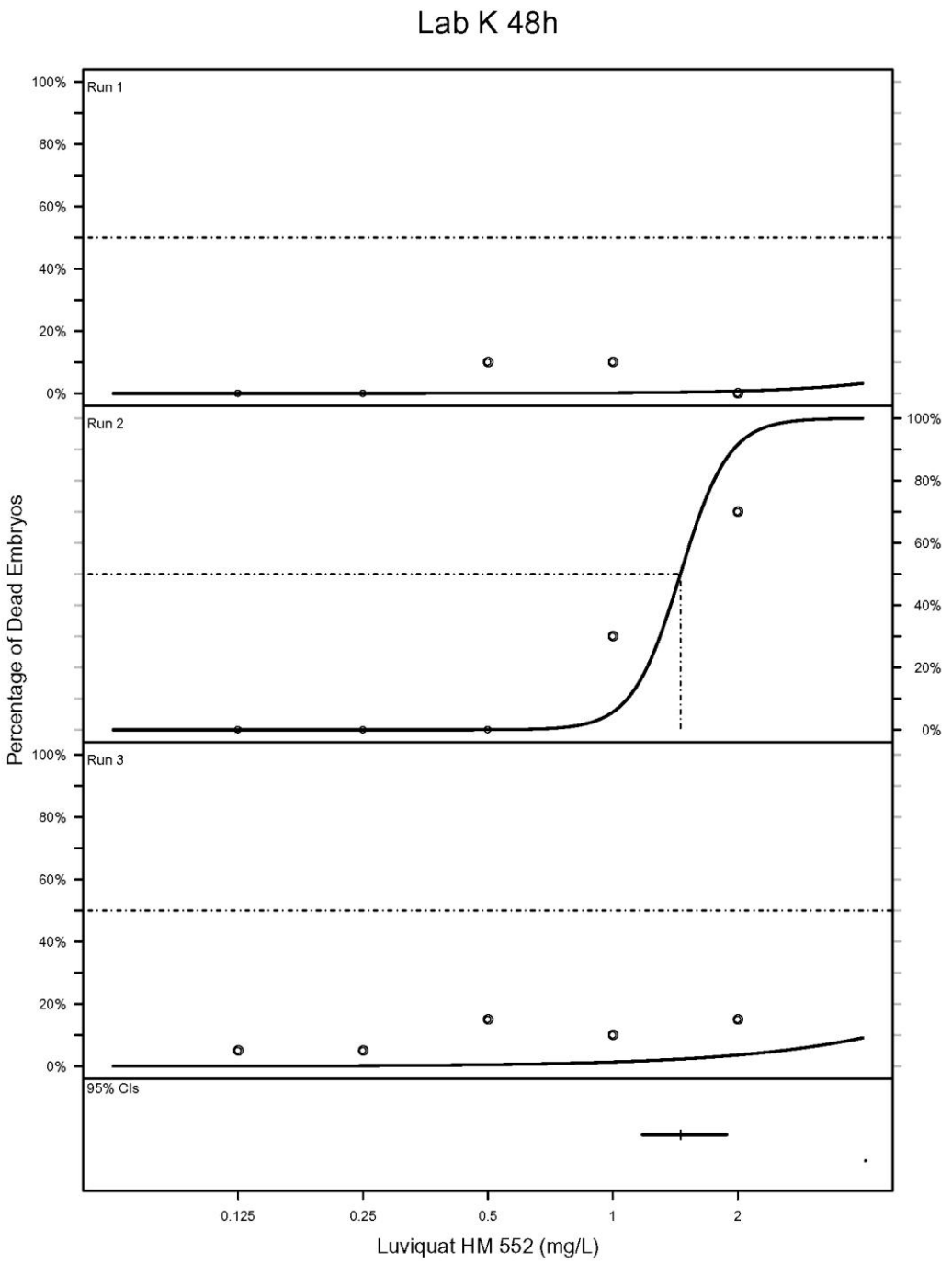

Lab F 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.125
0.25
0.5
1
2
Luviquat HM 552 (mg/L)

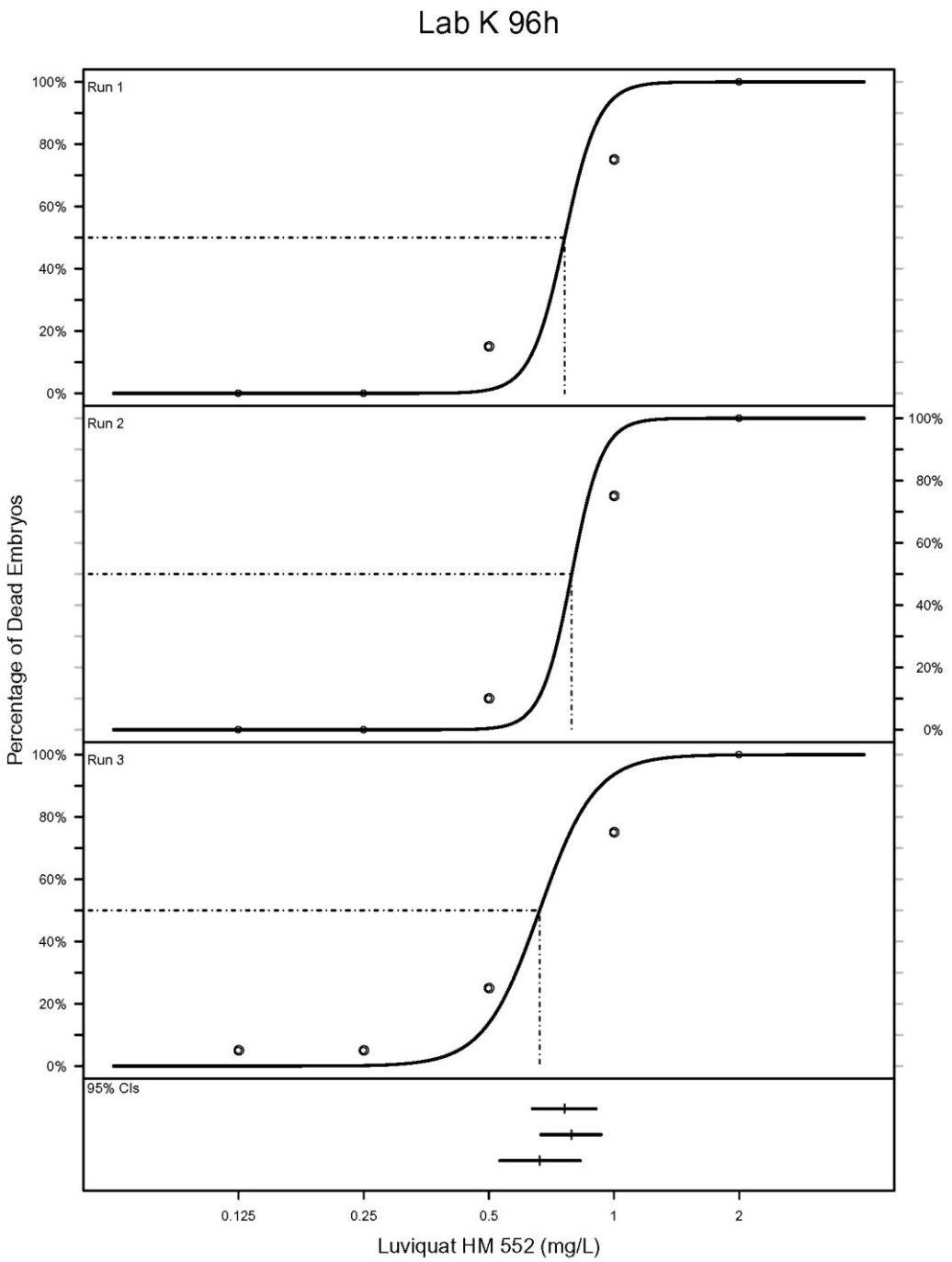

Lab H 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.125
0.25
0.5
1
2
Luviquat HM 552 (mg/L)


Lab H 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.125
0.25
0.5
1
2
Luviquat HM 552 (mg/L)

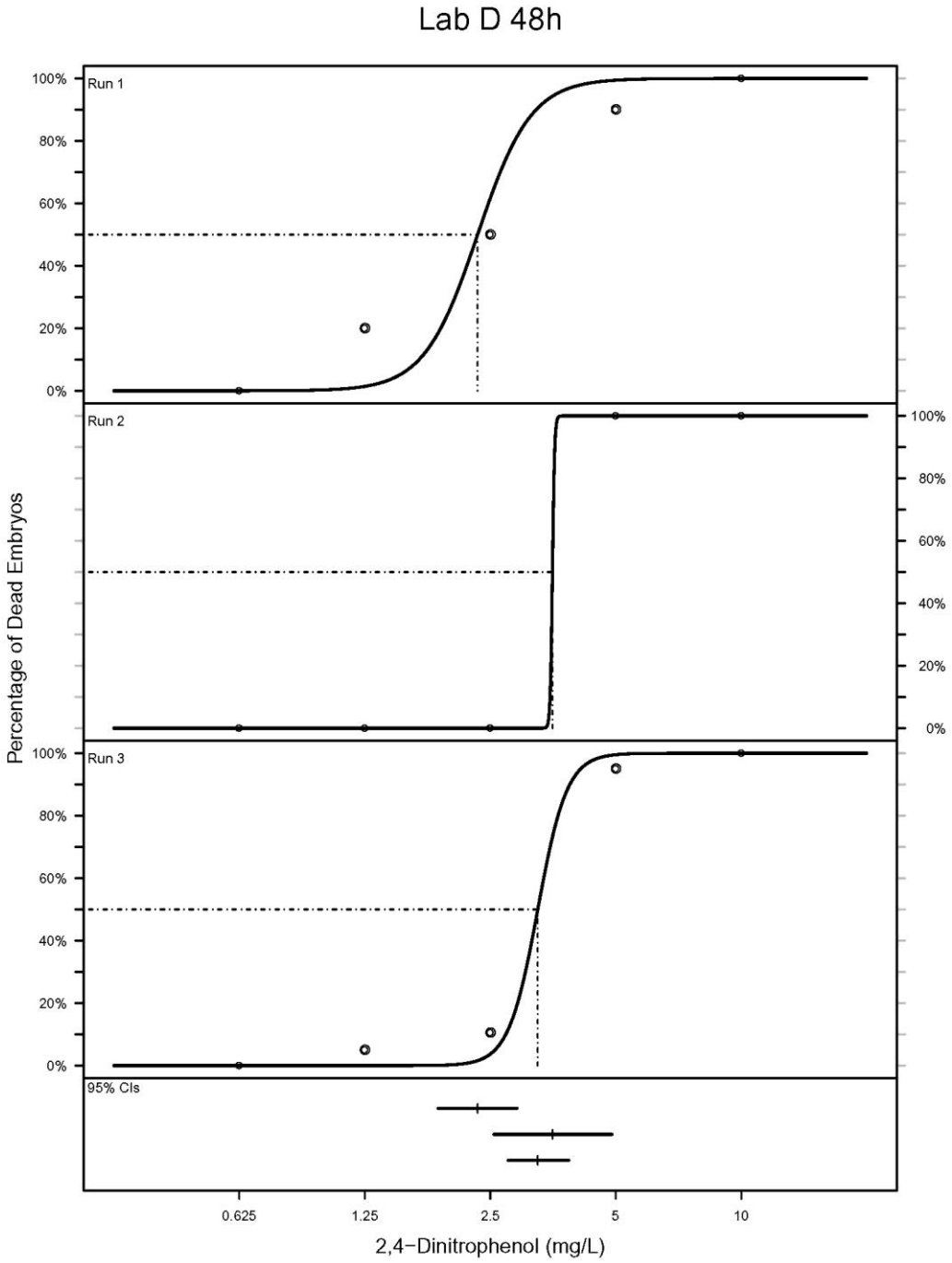

Lab I 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.125
0.25
0.5
1
2
Luviquat HM 552 (mg/L)

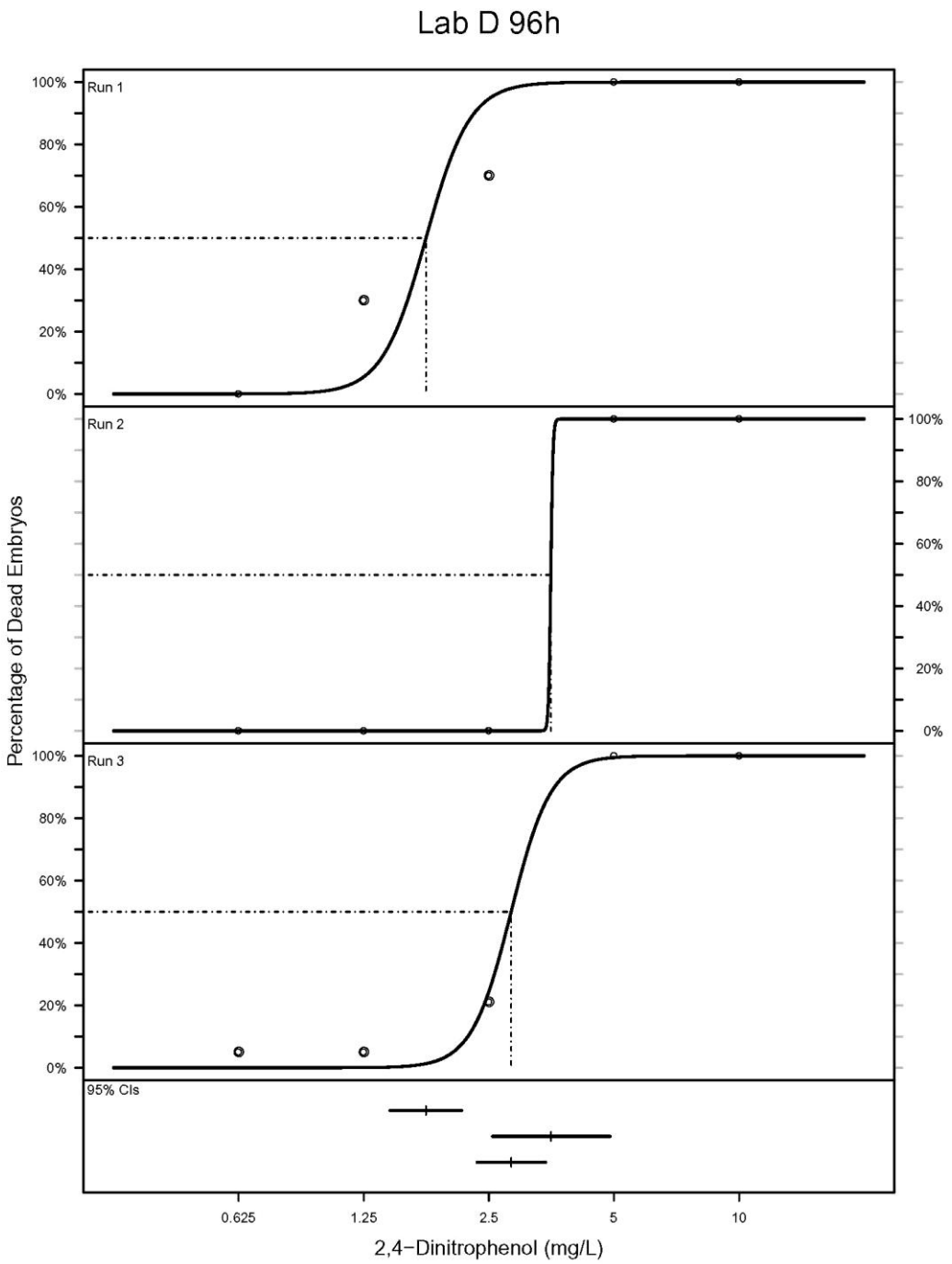

Lab I 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.125
0.25
0.5
1
2
Luviquat HM 552 (mg/L)

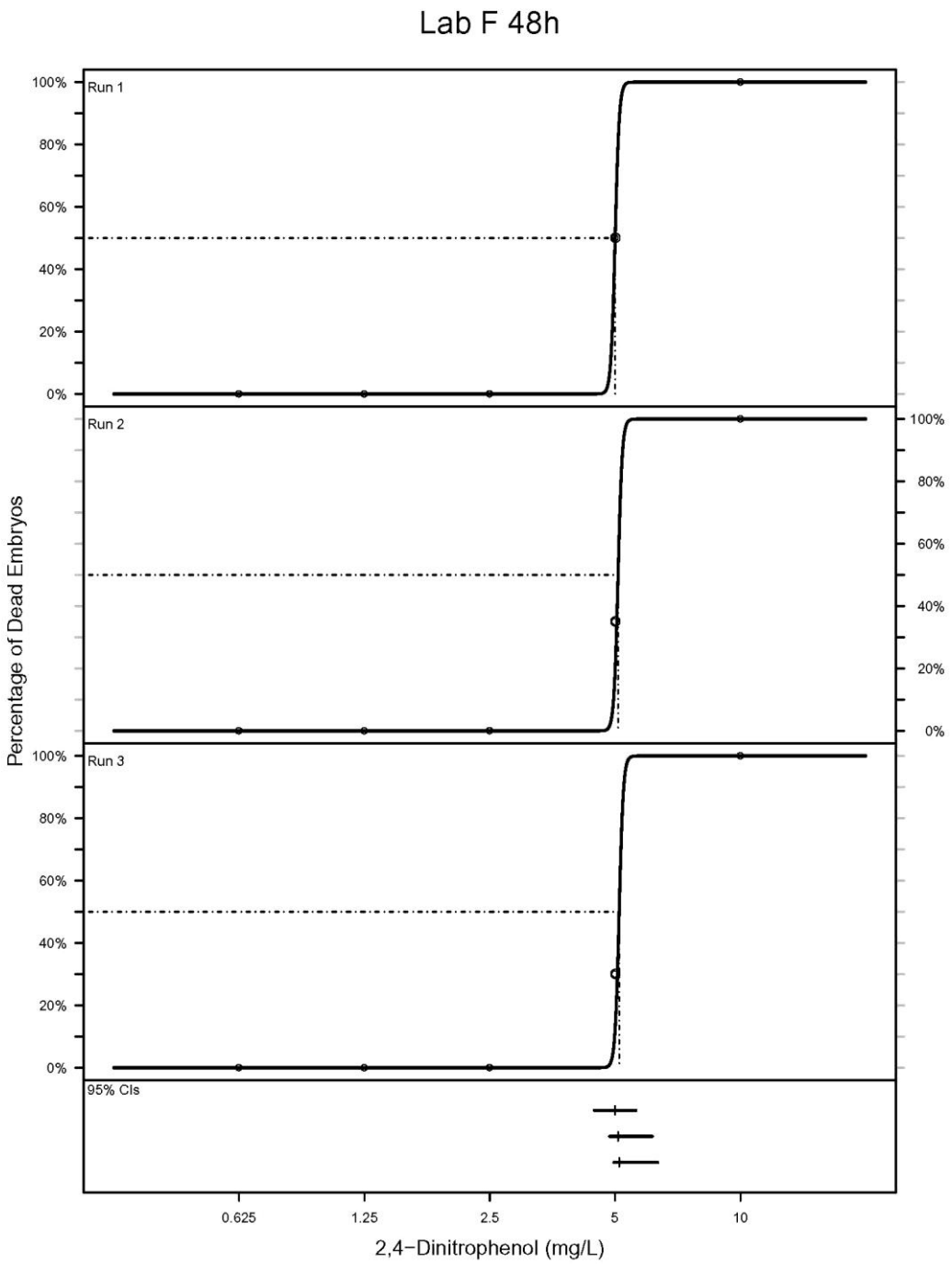

Lab K 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.125
0.25
0.5
1
2
Luviquat HM 552 (mg/L)

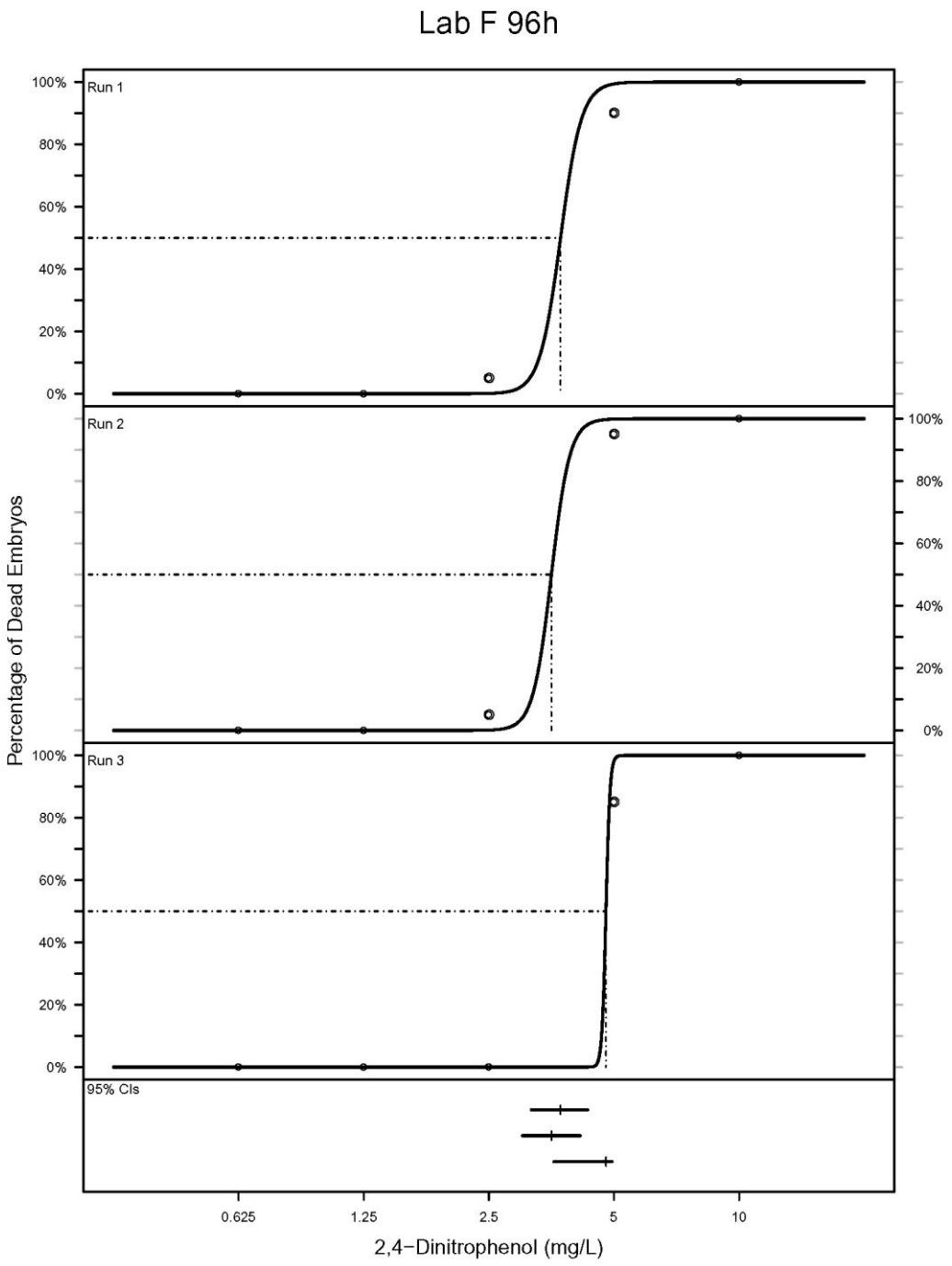

Lab K 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.125
0.25
0.5
1
2
Luviquat HM 552 (mg/L)

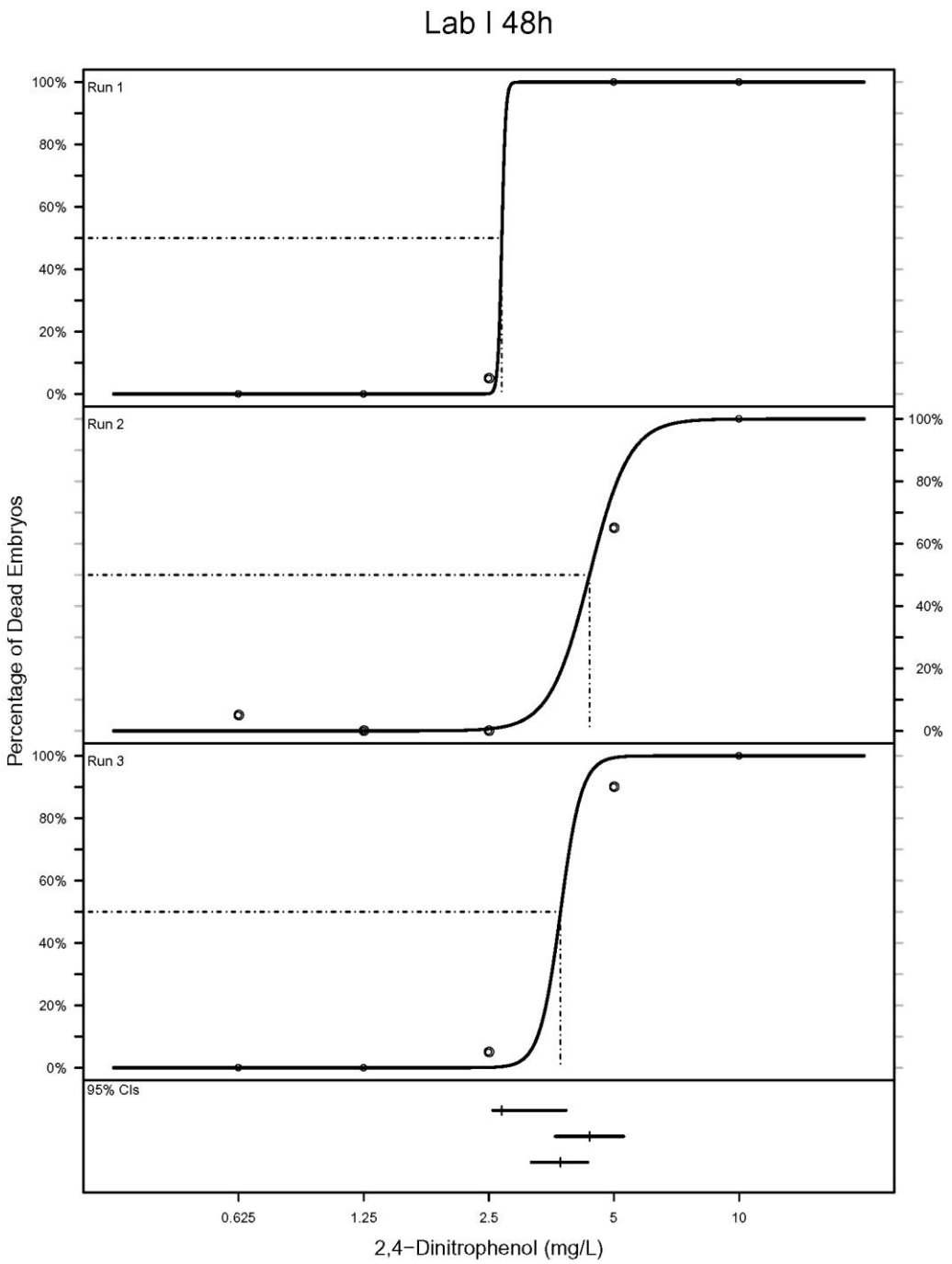
2,4-Dintrophenol

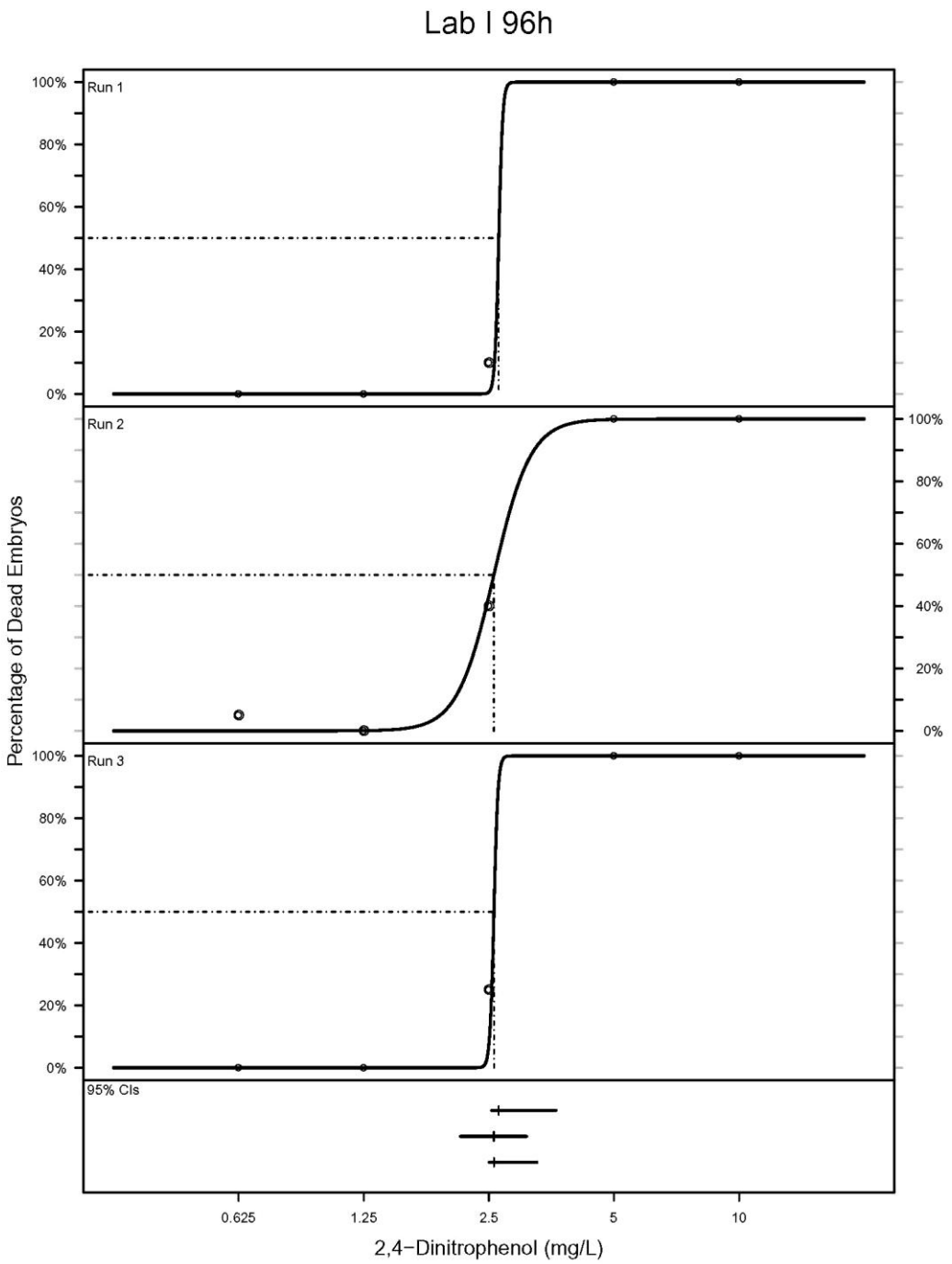


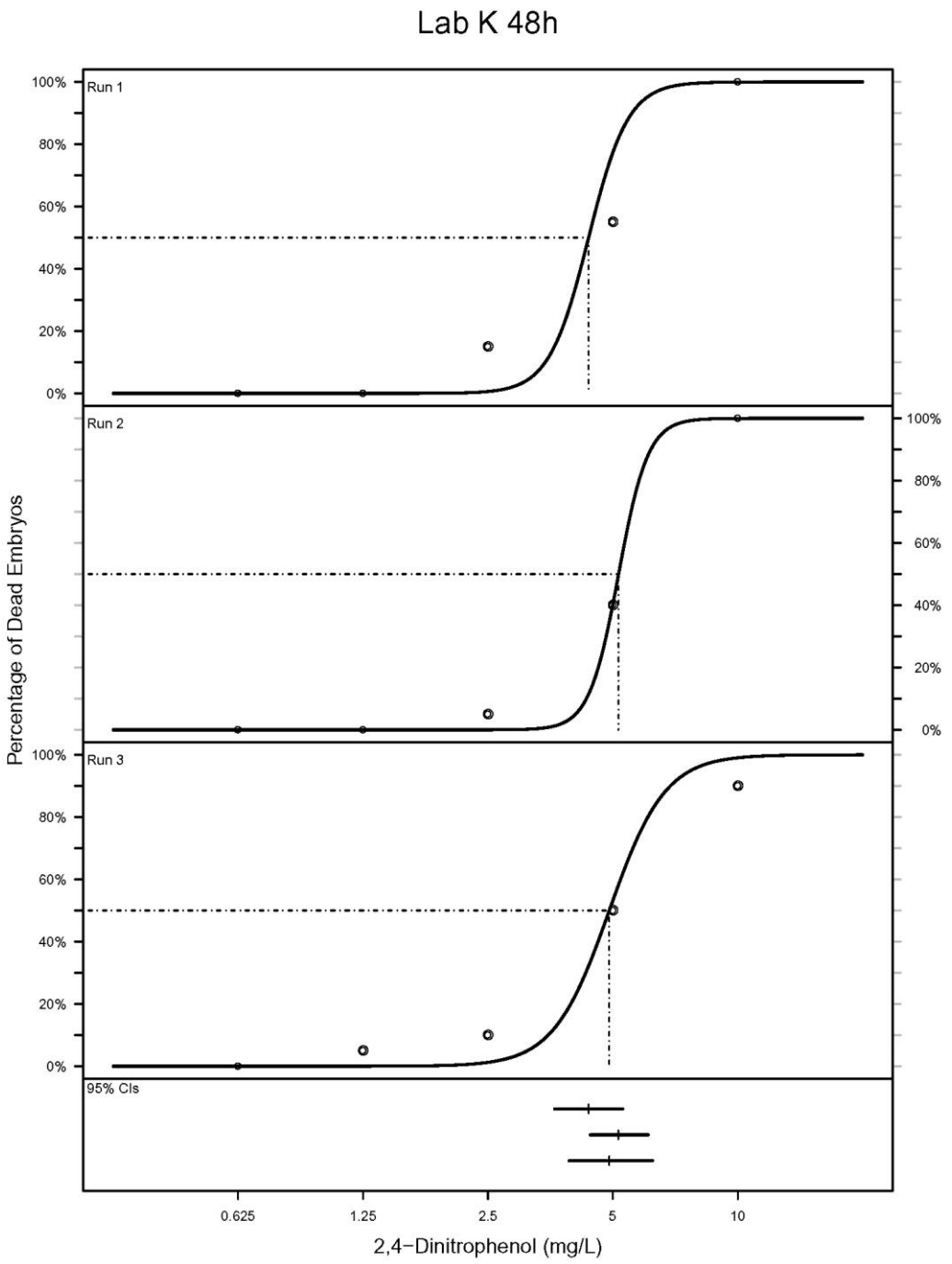

Lab D 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.625
1.25
2.5
5
10
2,4−Dinitrophenol (mg/L)

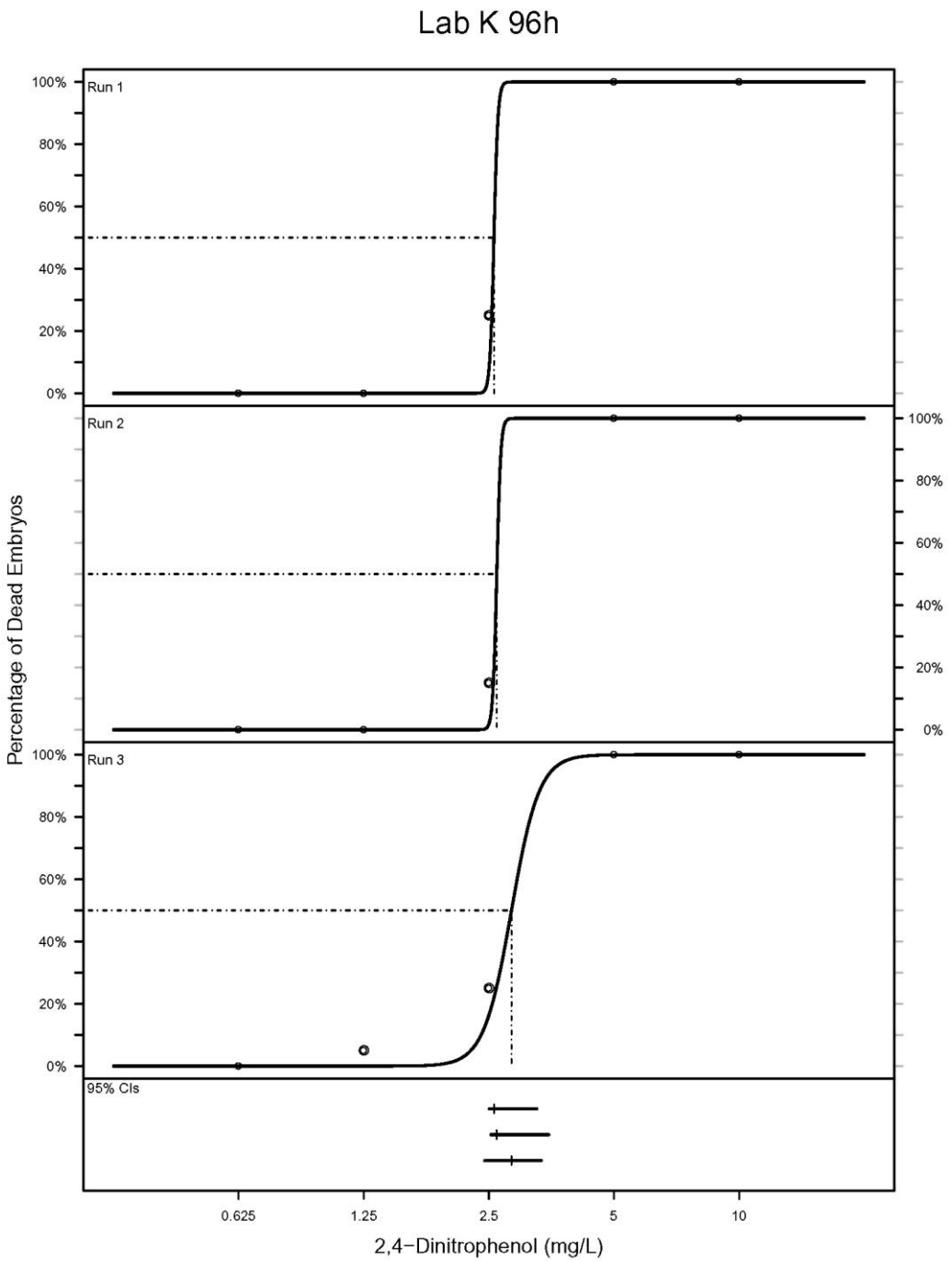

Lab F 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.625
1.25
2.5
5
10
2,4−Dinitrophenol (mg/L)


Lab F 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.625
1.25
2.5
5
10
2,4−Dinitrophenol (mg/L)

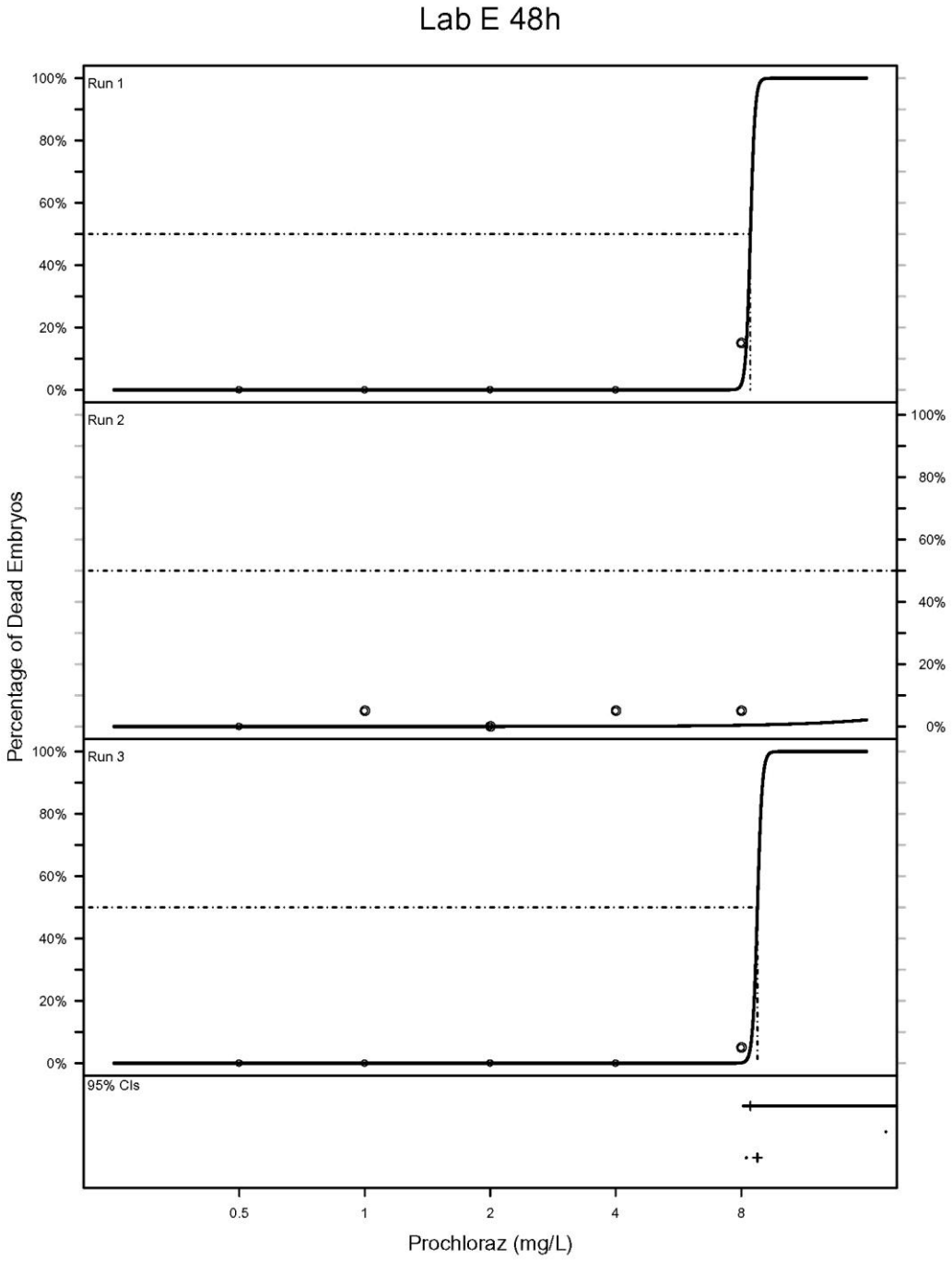

Lab I 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.625
1.25
2.5
5
10
2,4−Dinitrophenol (mg/L)

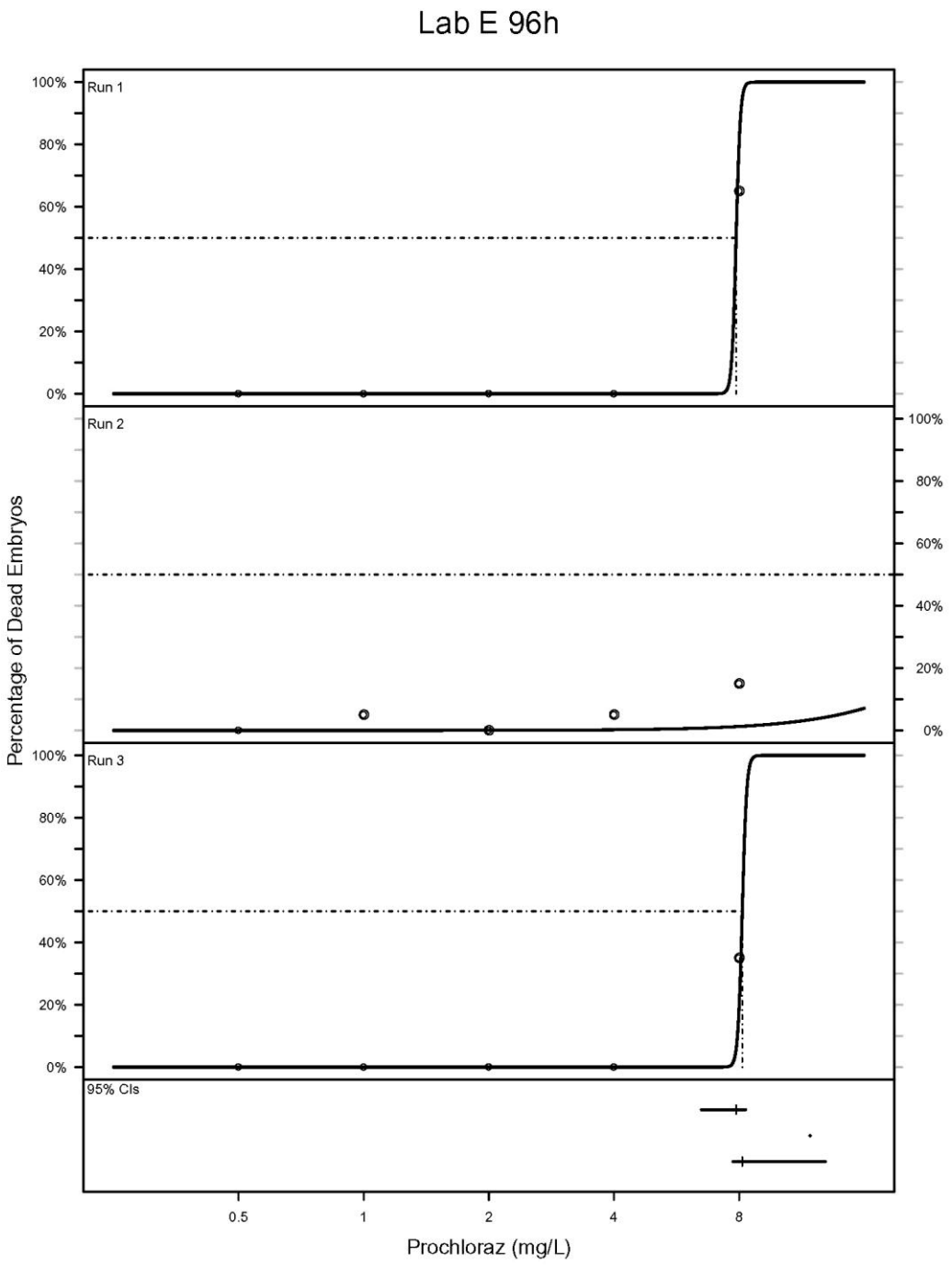

Lab I 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.625
1.25
2.5
5
10
2,4−Dinitrophenol (mg/L)

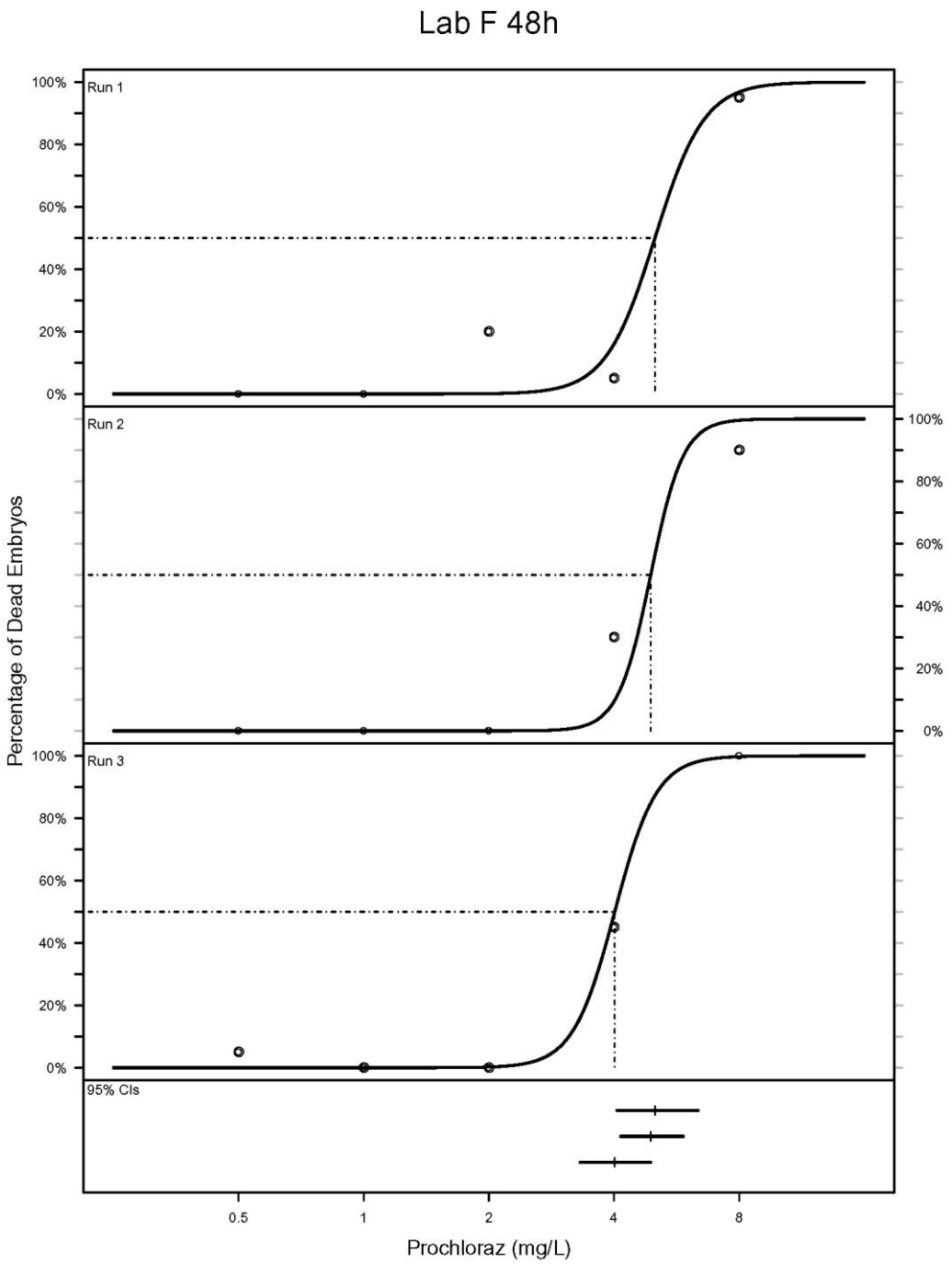

Lab K 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.625
1.25
2.5
5
10
2,4−Dinitrophenol (mg/L)

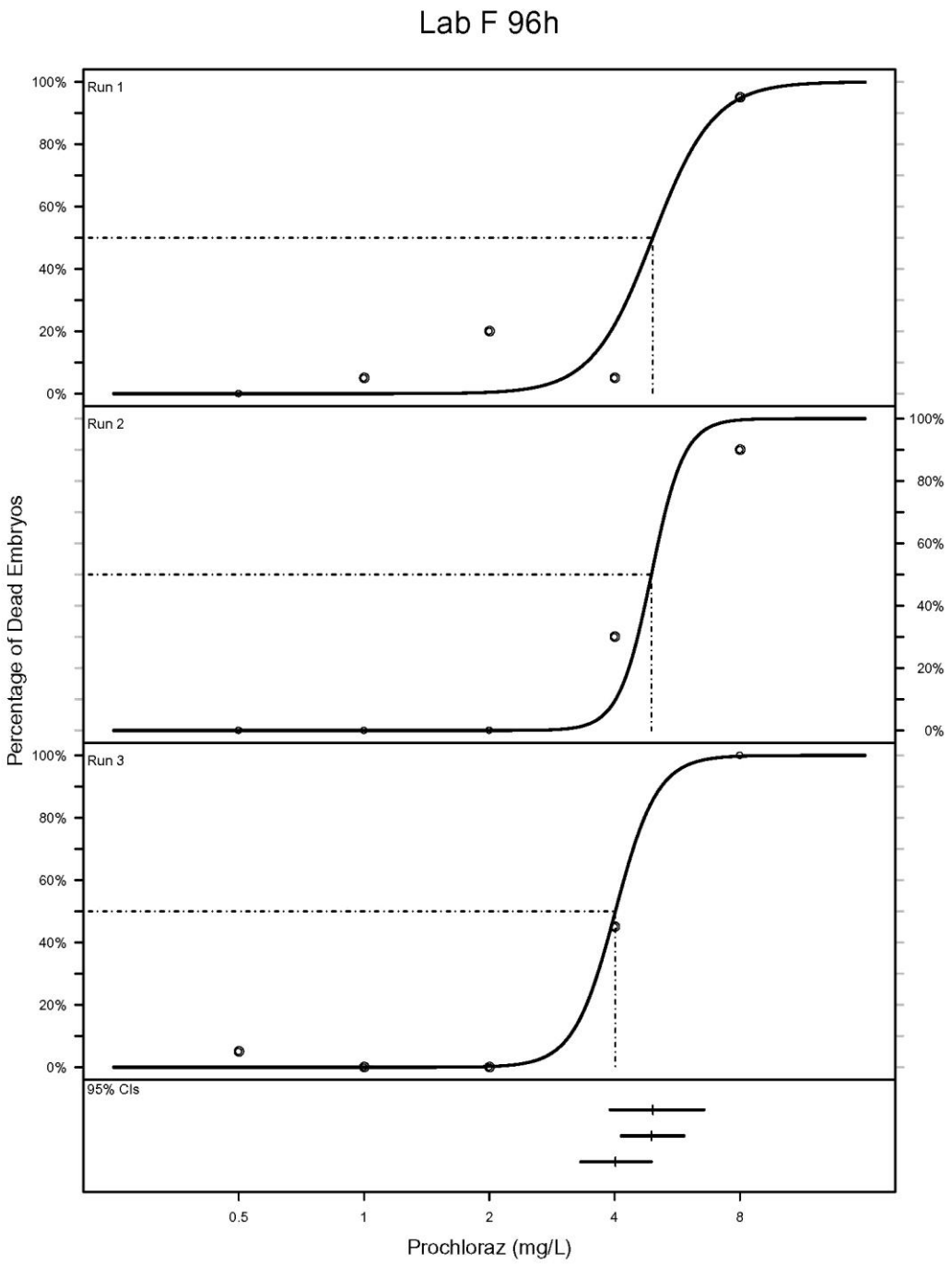

Lab K 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.625
1.25
2.5
5
10
2,4−Dinitrophenol (mg/L)

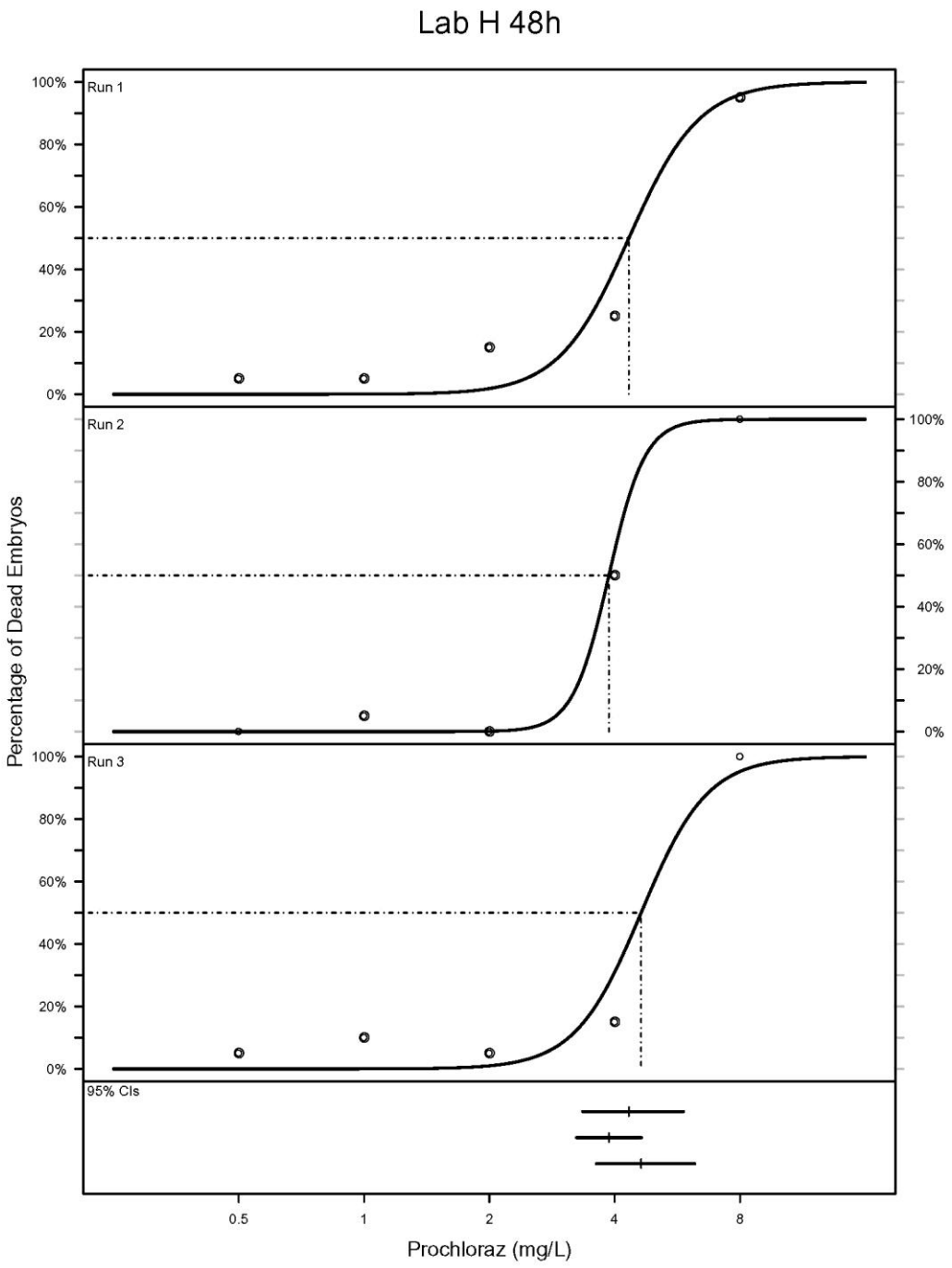
Prochloraz

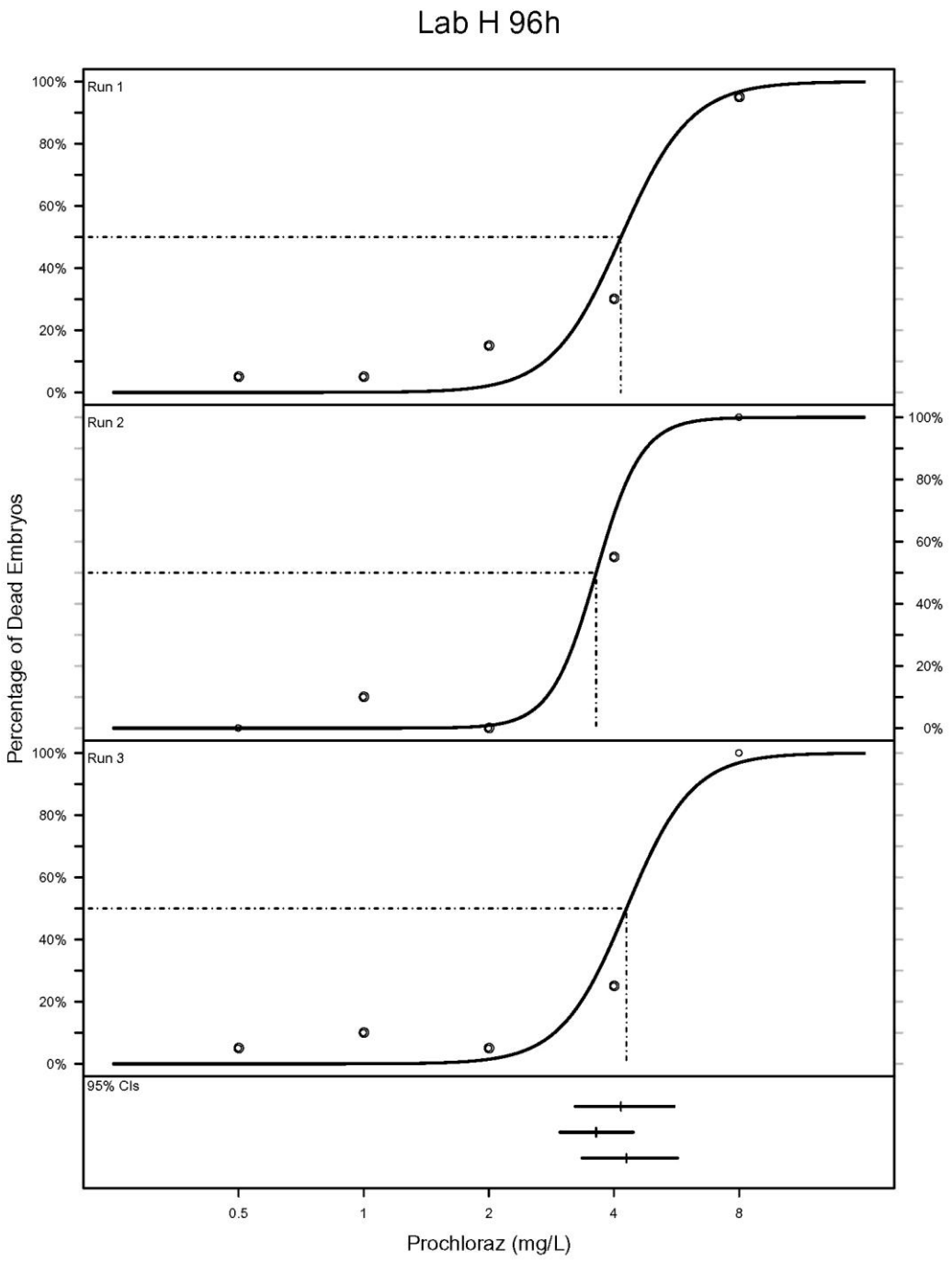

Lab E 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.5
1
2
4
8
Prochloraz (mg/L)

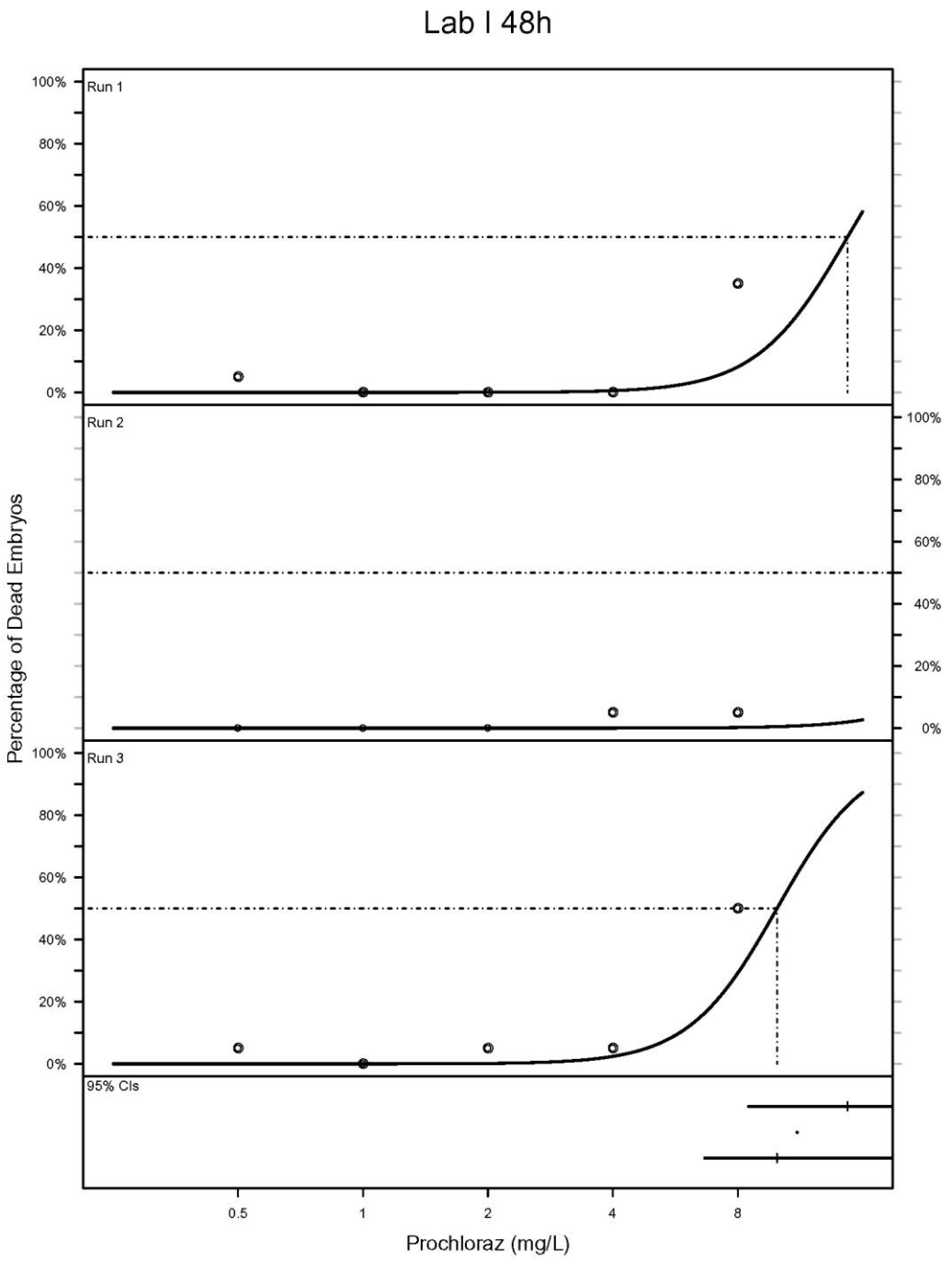

Lab E 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.5
1
2
4
8
Prochloraz (mg/L)

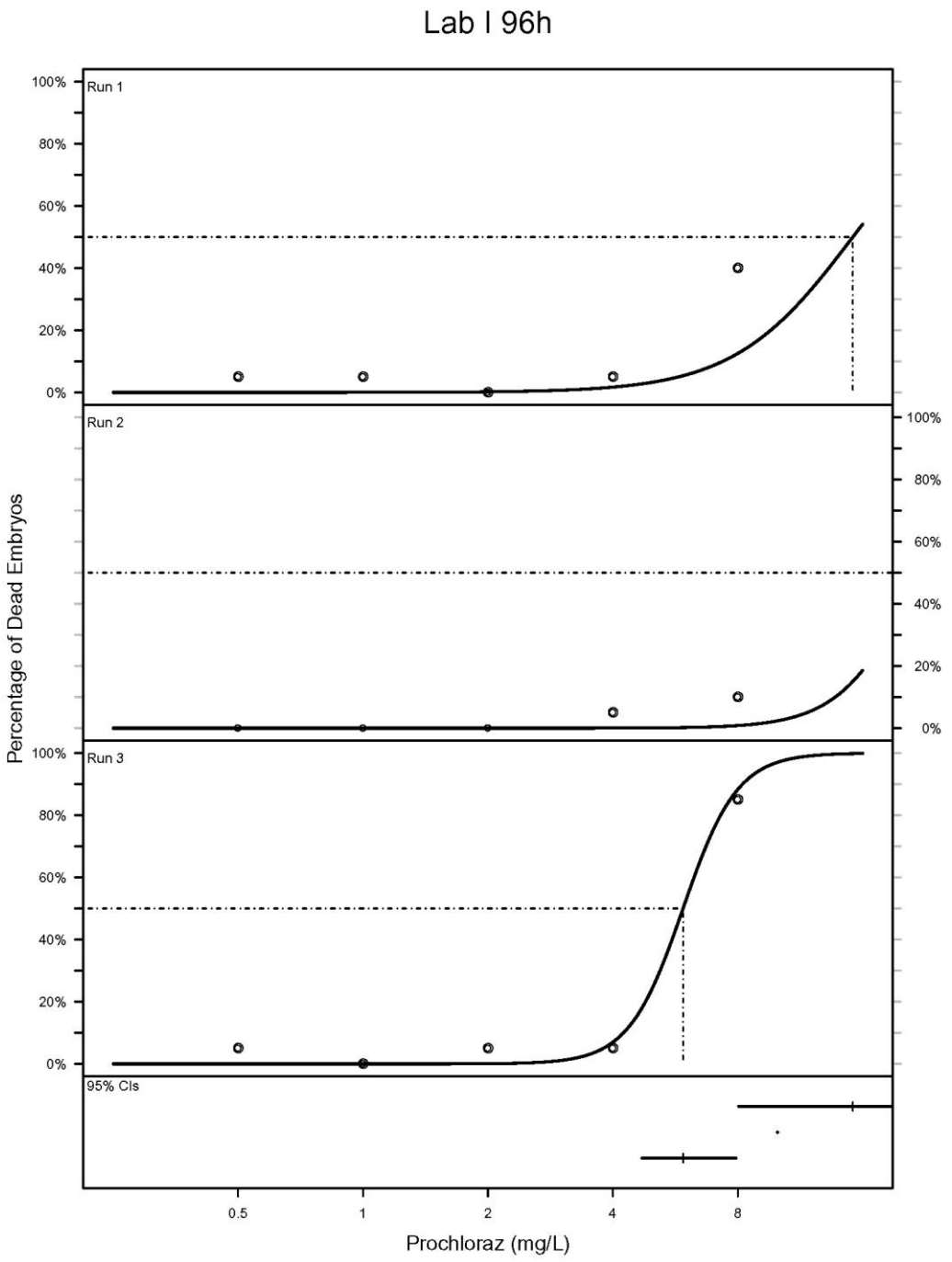

Lab F 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.5
1
2
4
8
Prochloraz (mg/L)


Lab F 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.5
1
2
4
8
Prochloraz (mg/L)

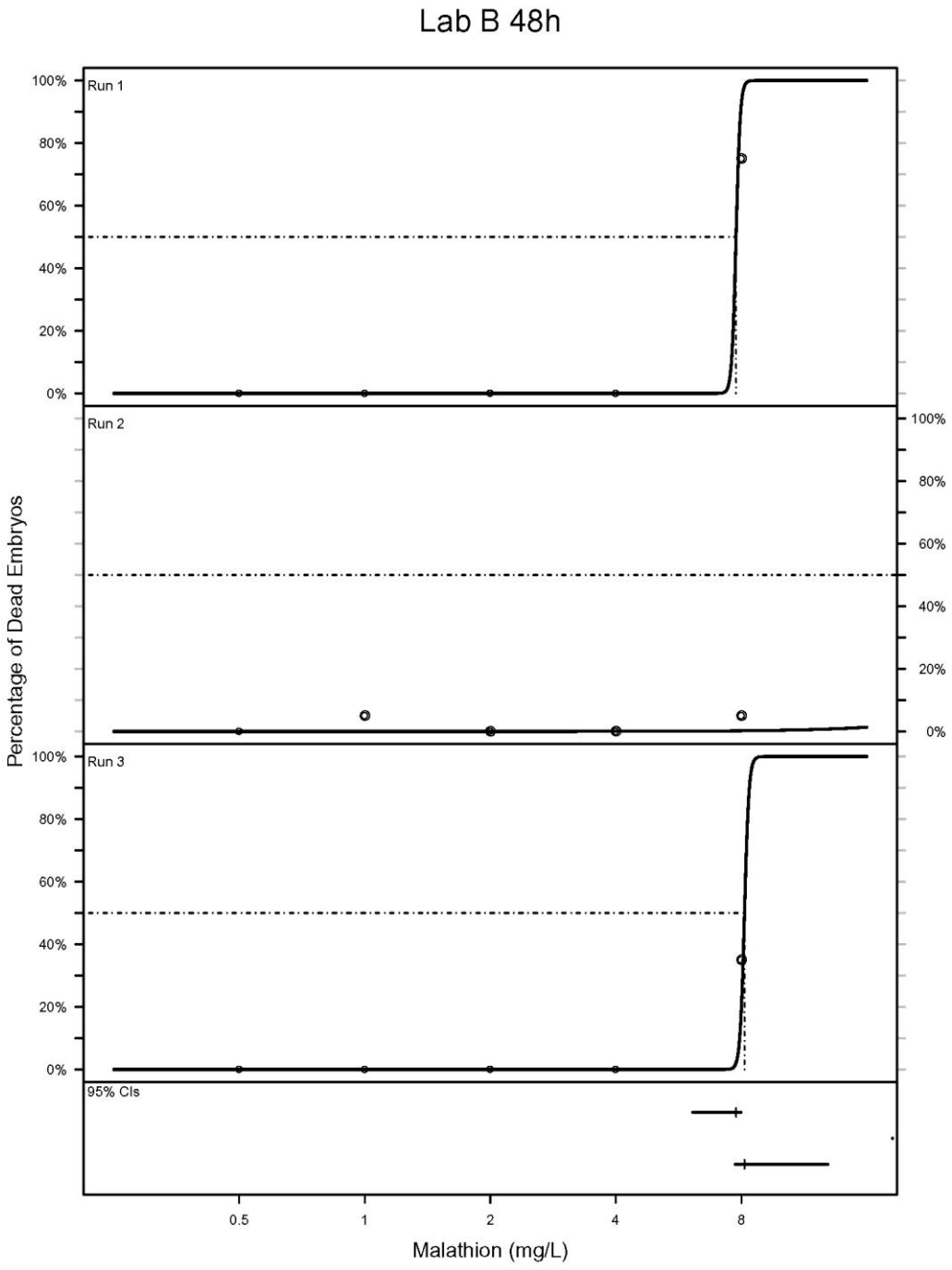

Lab H 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.5
1
2
4
8
Prochloraz (mg/L)

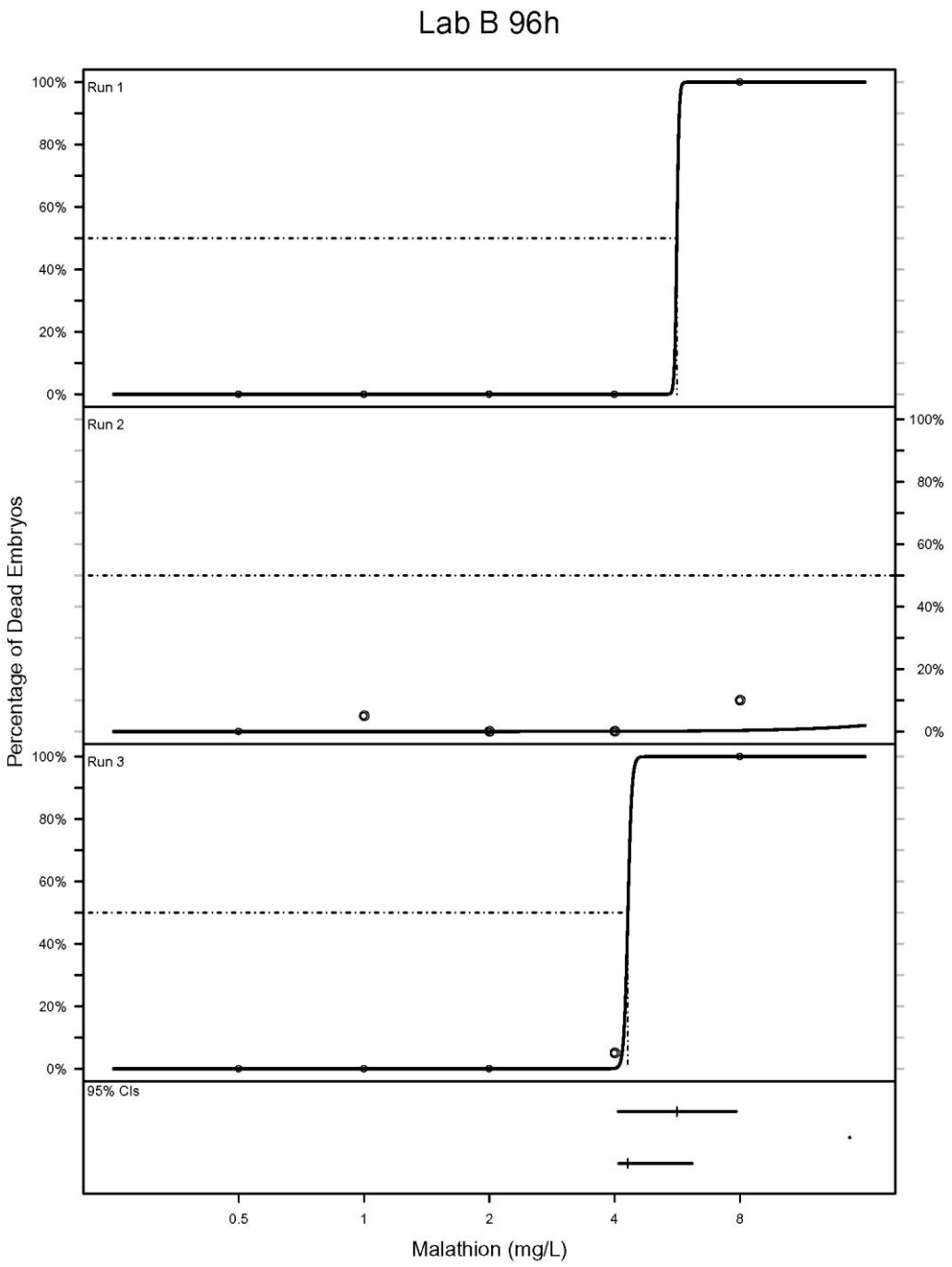

Lab H 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.5
1
2
4
8
Prochloraz (mg/L)

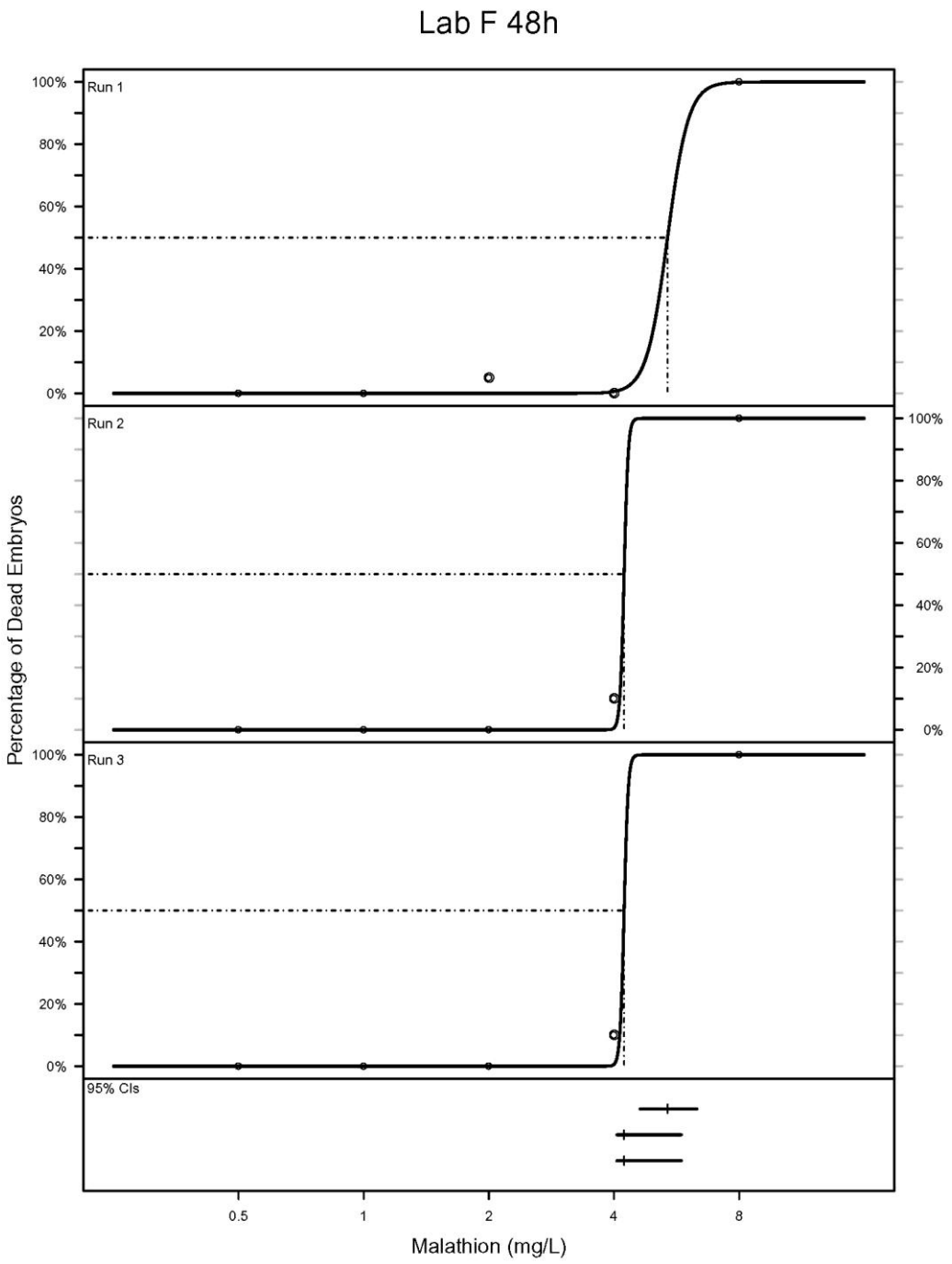

Lab I 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.5
1
2
4
8
Prochloraz (mg/L)

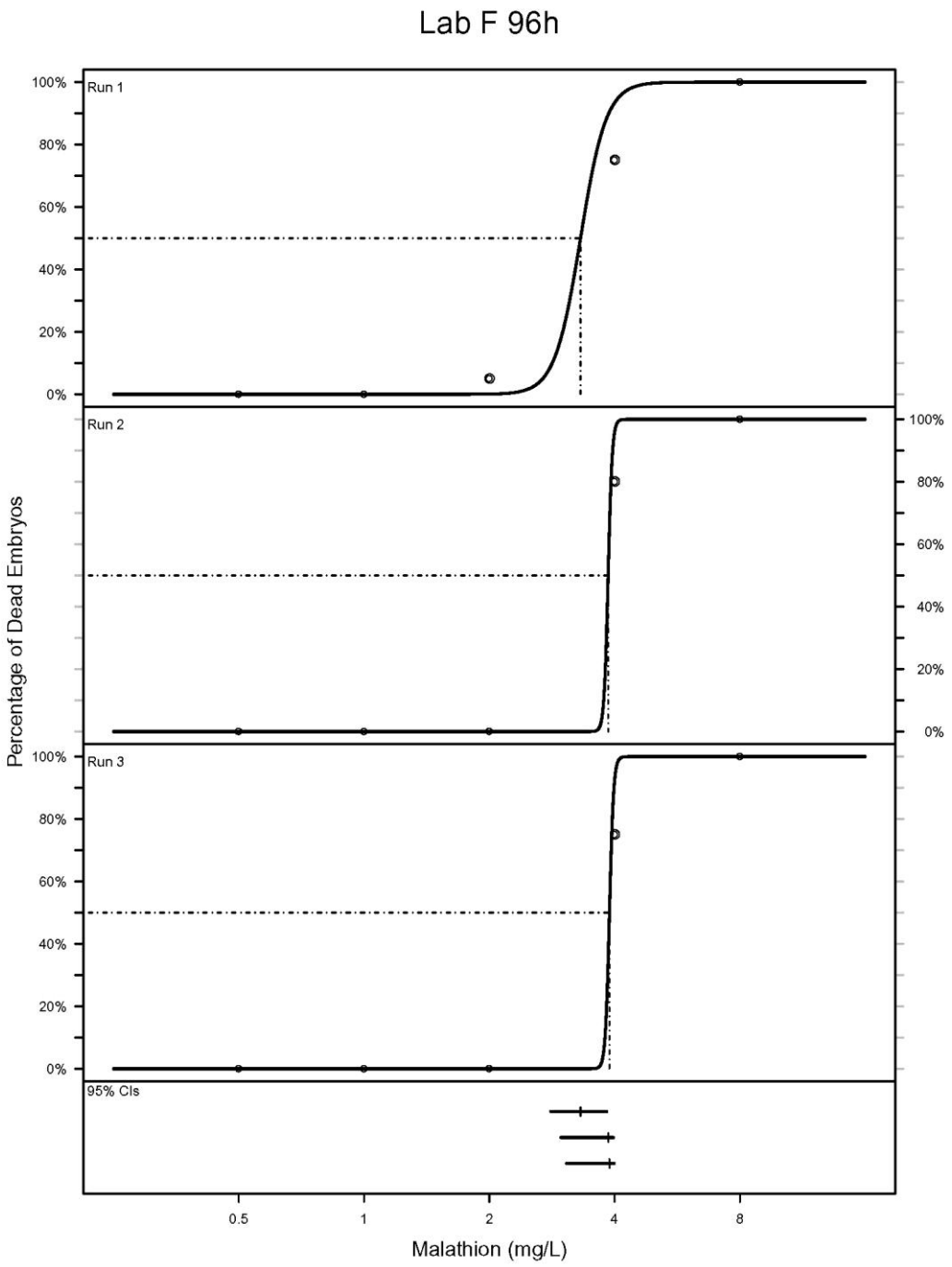

Lab I 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.5
1
2
4
8
Prochloraz (mg/L)

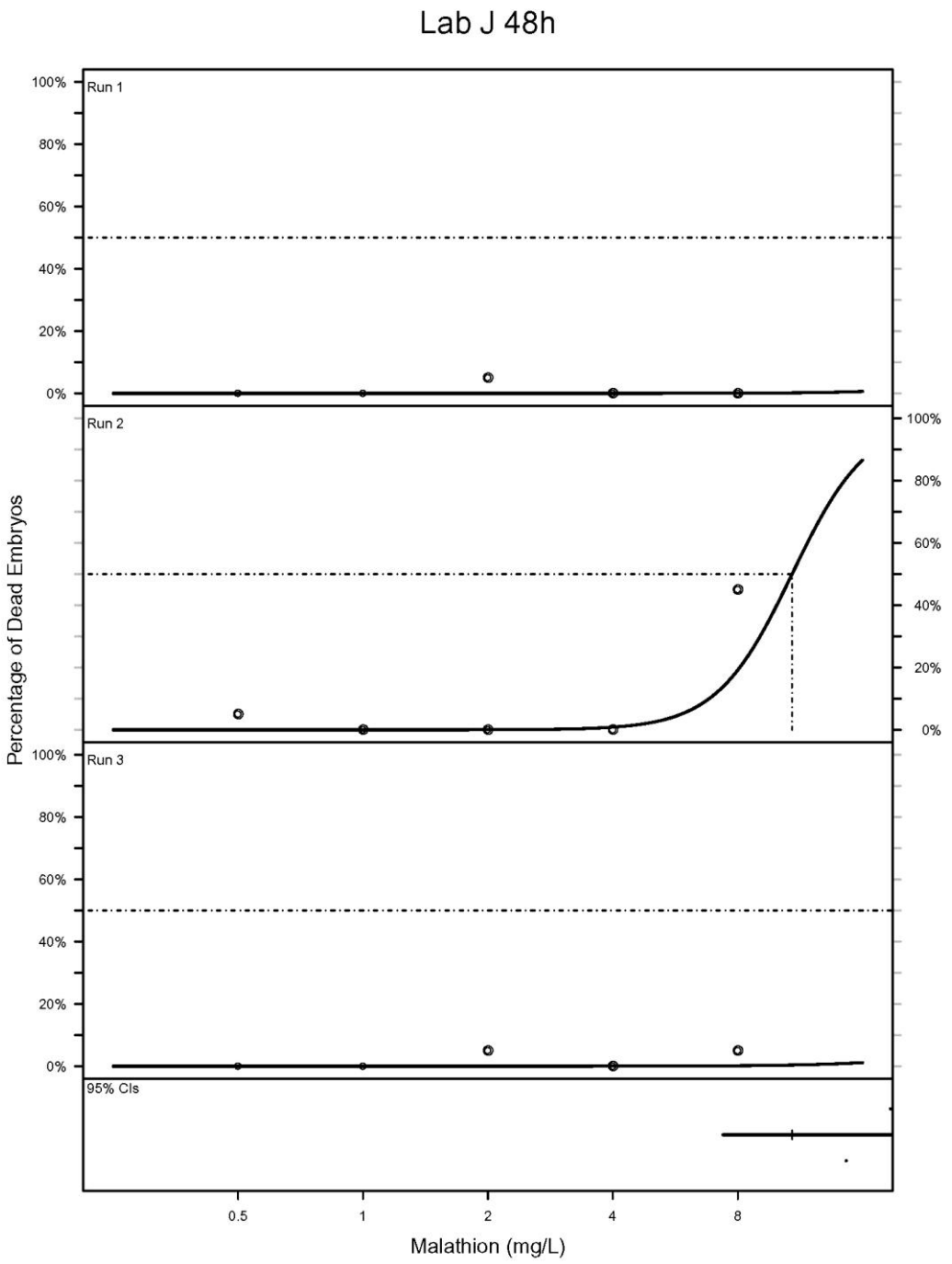
Malathion

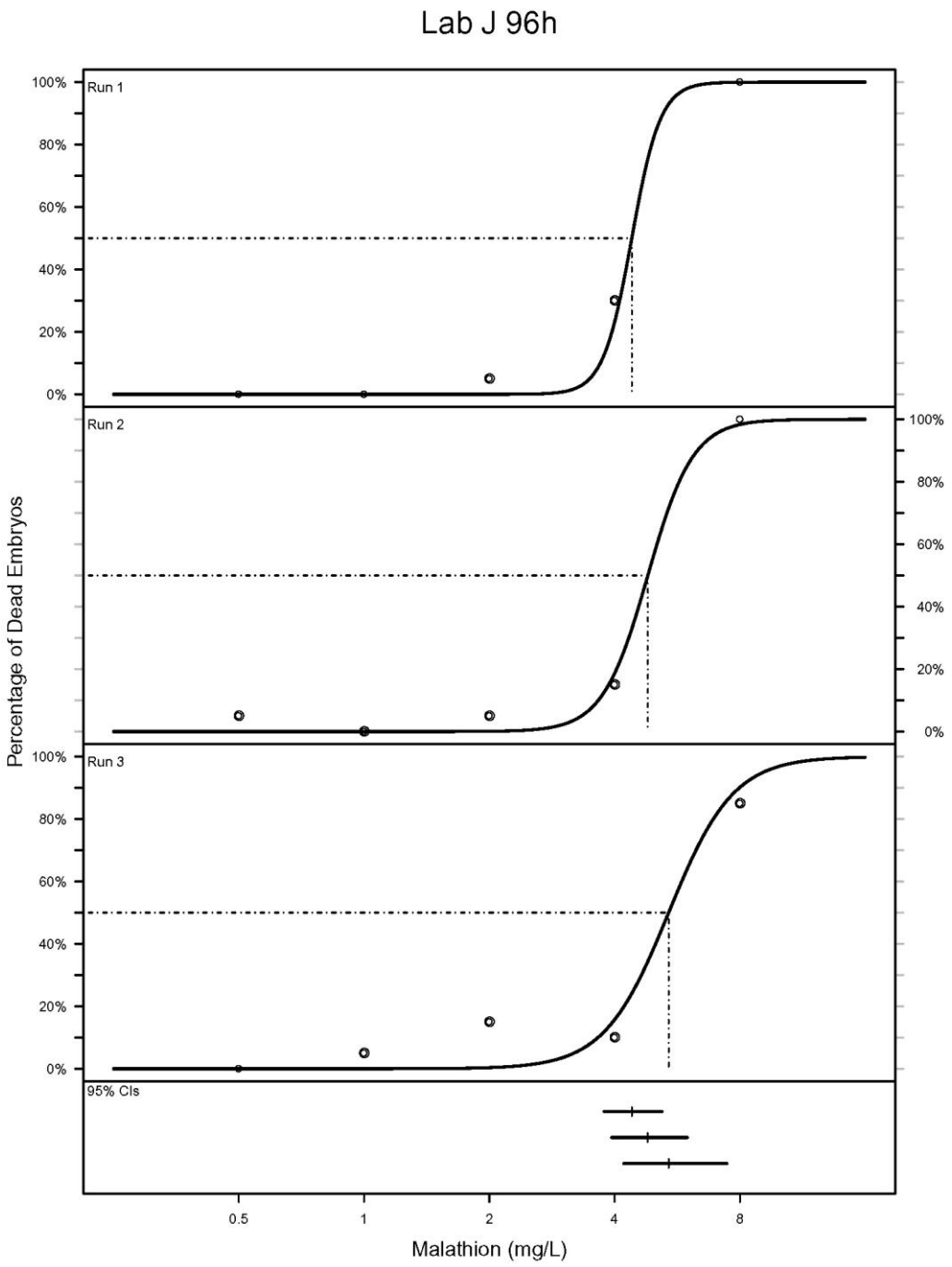

Lab B 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.5
1
2
4
8
Malathion (mg/L)

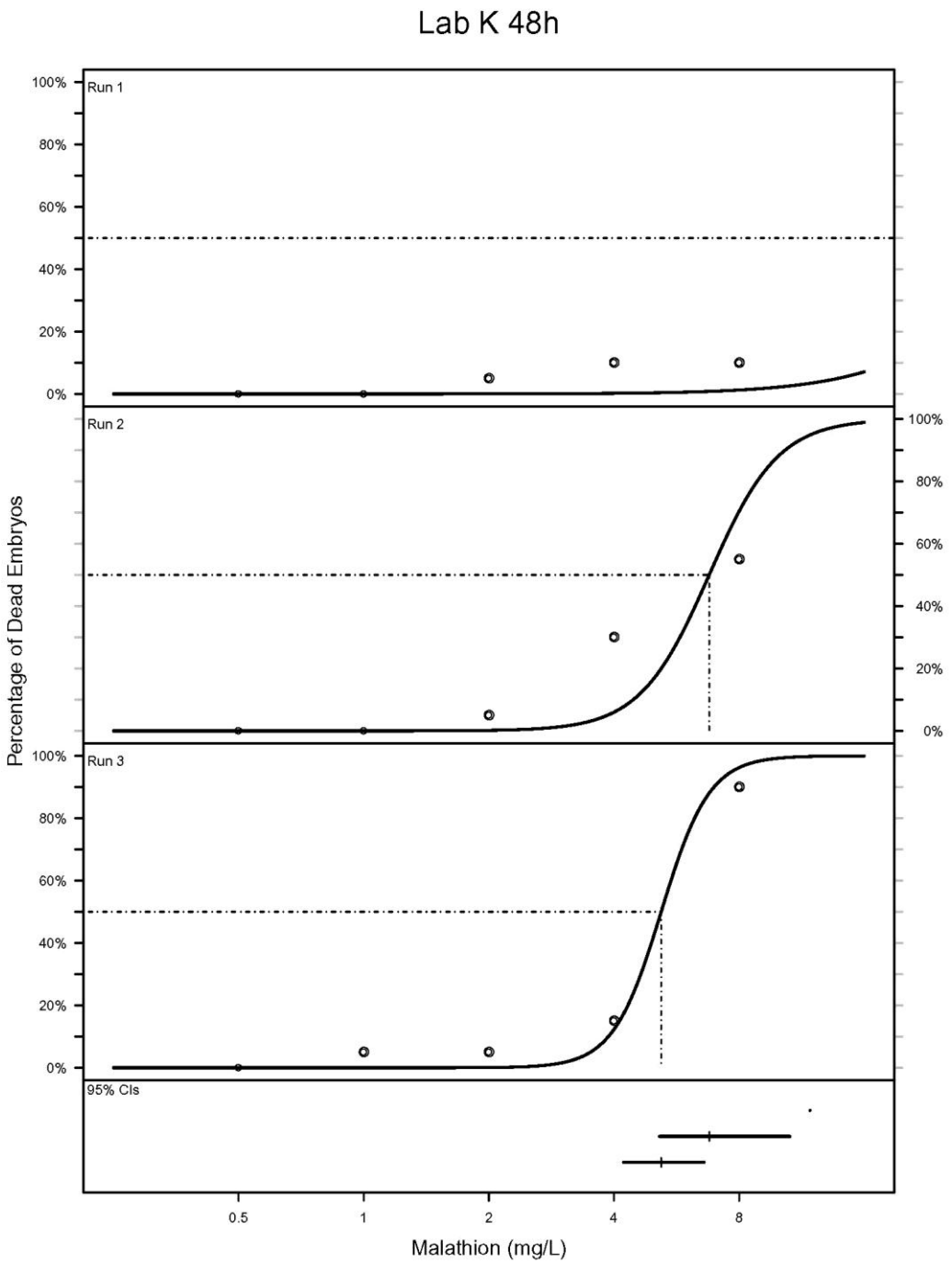

Lab B 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.5
1
2
4
8
Malathion (mg/L)

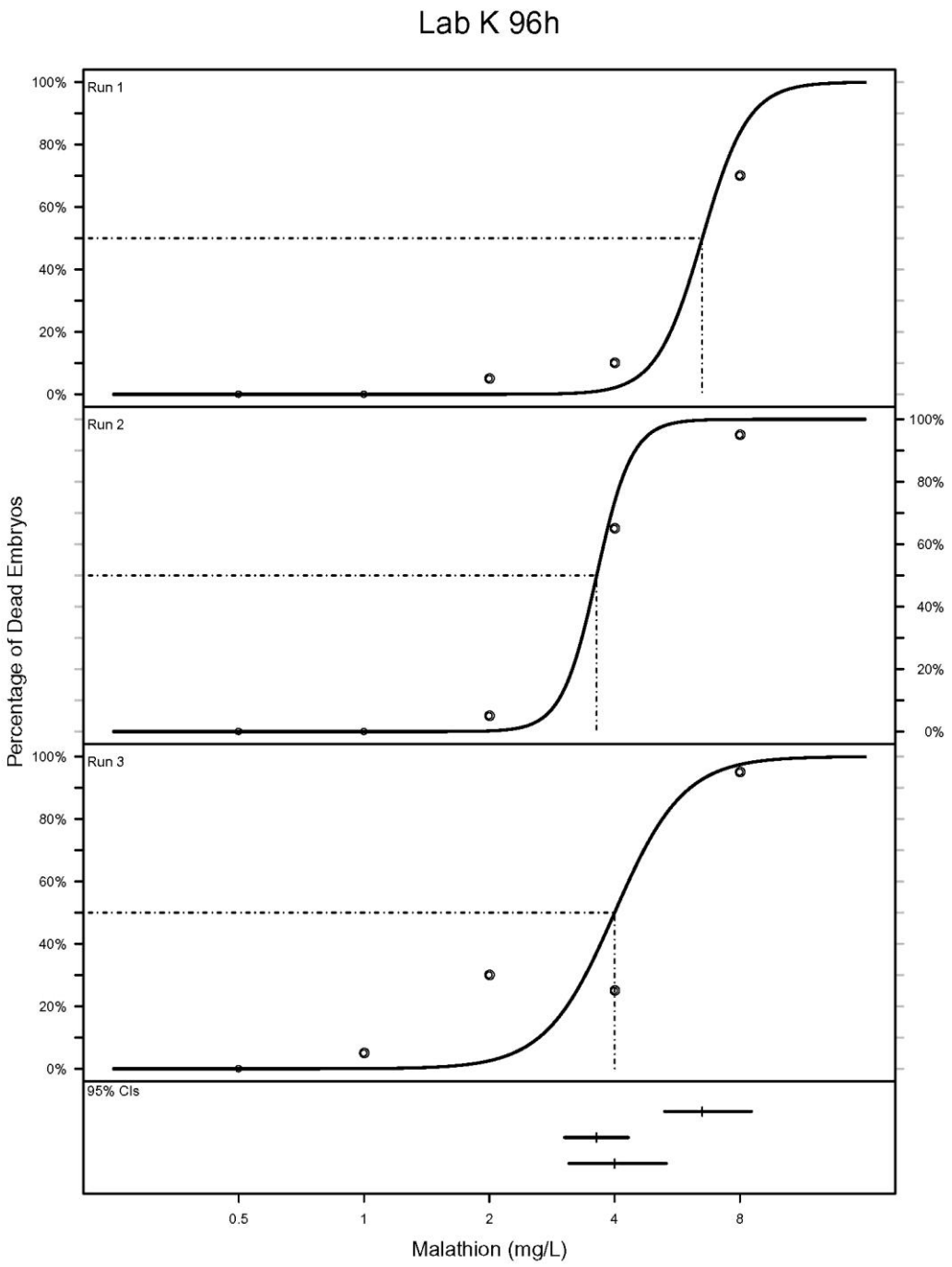

Lab F 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.5
1
2
4
8
Malathion (mg/L)


Lab F 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.5
1
2
4
8
Malathion (mg/L)

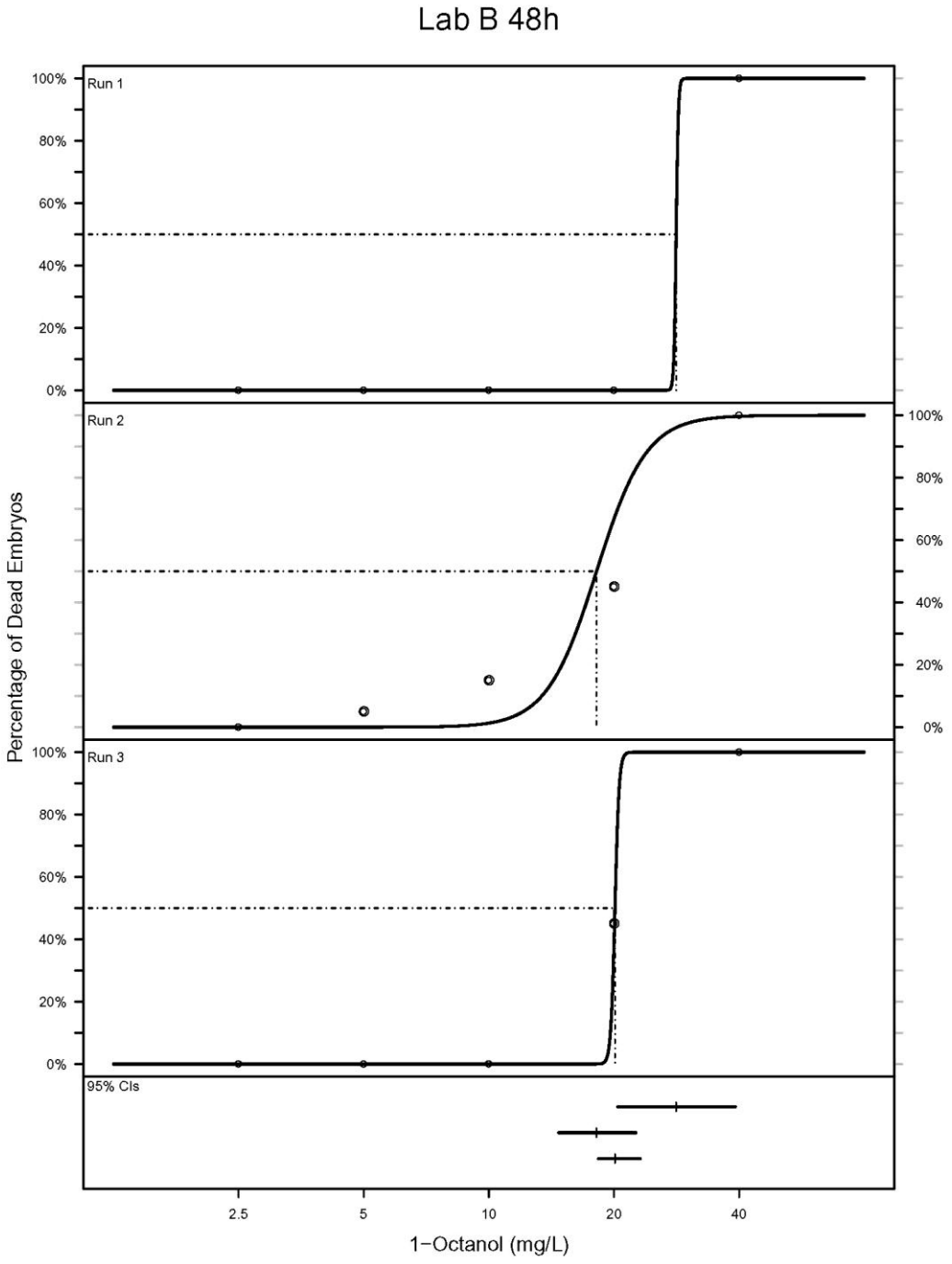

Lab J 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.5
1
2
4
8
Malathion (mg/L)

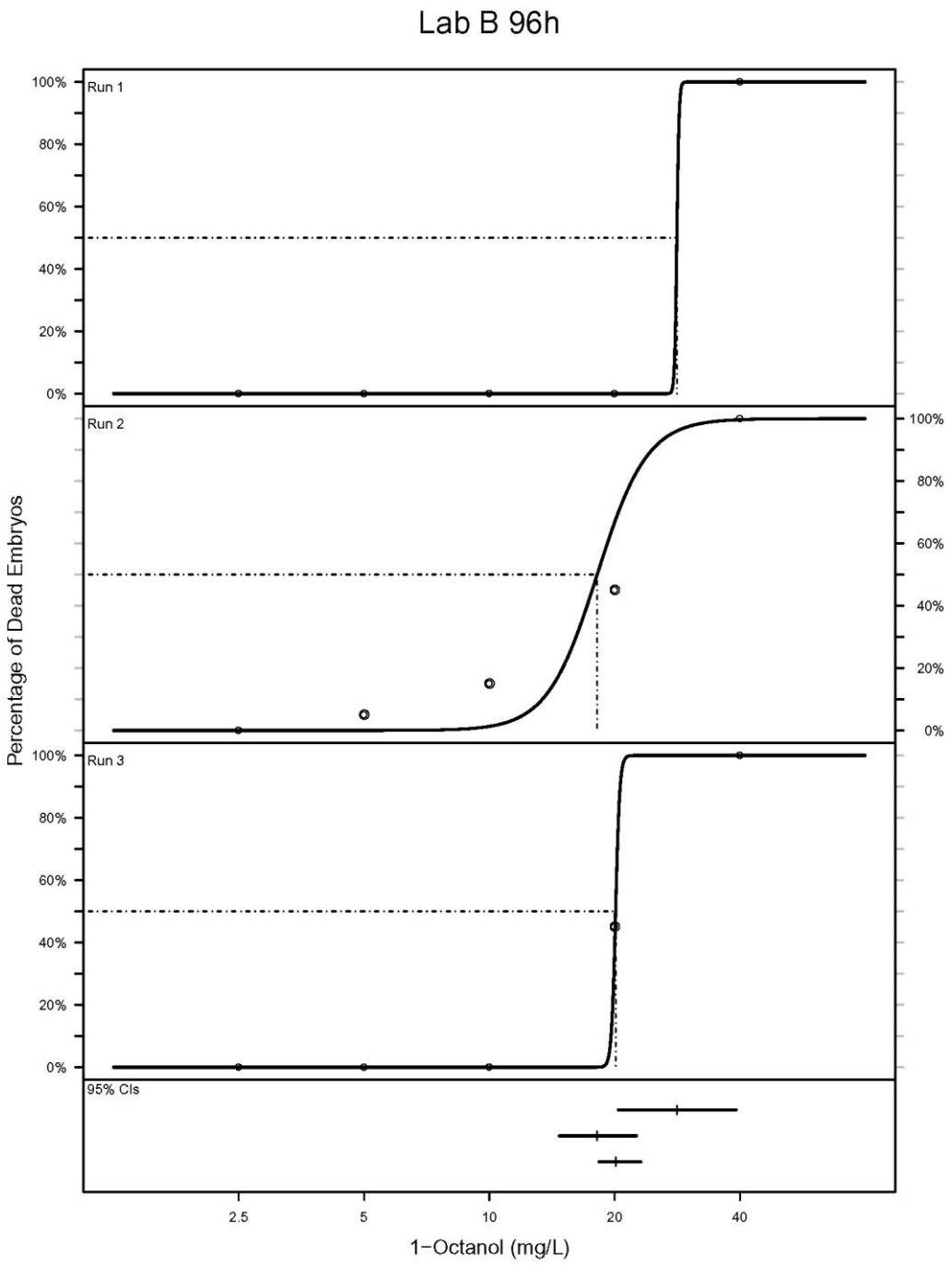

Lab J 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.5
1
2
4
8
Malathion (mg/L)

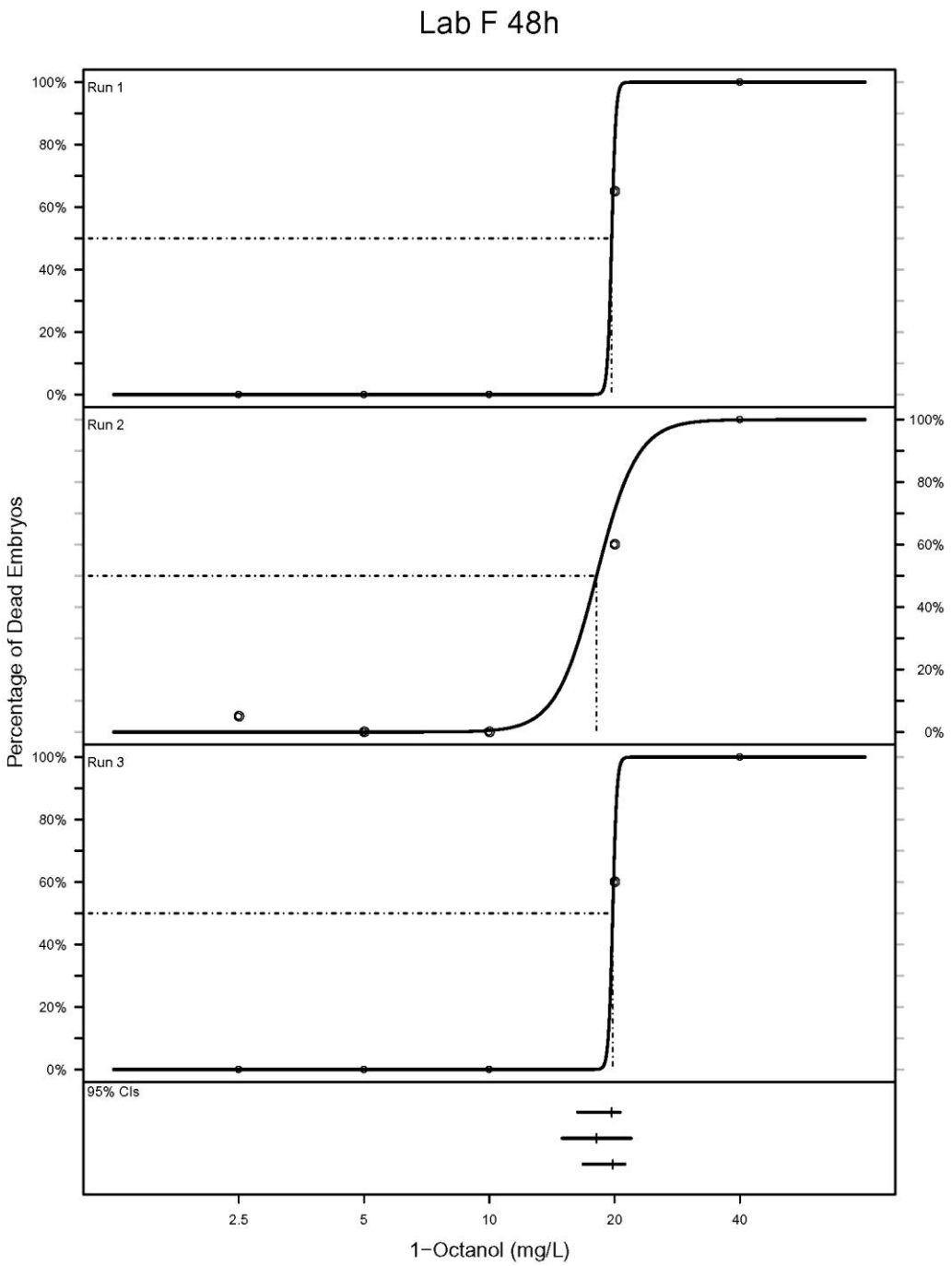

Lab K 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.5
1
2
4
8
Malathion (mg/L)

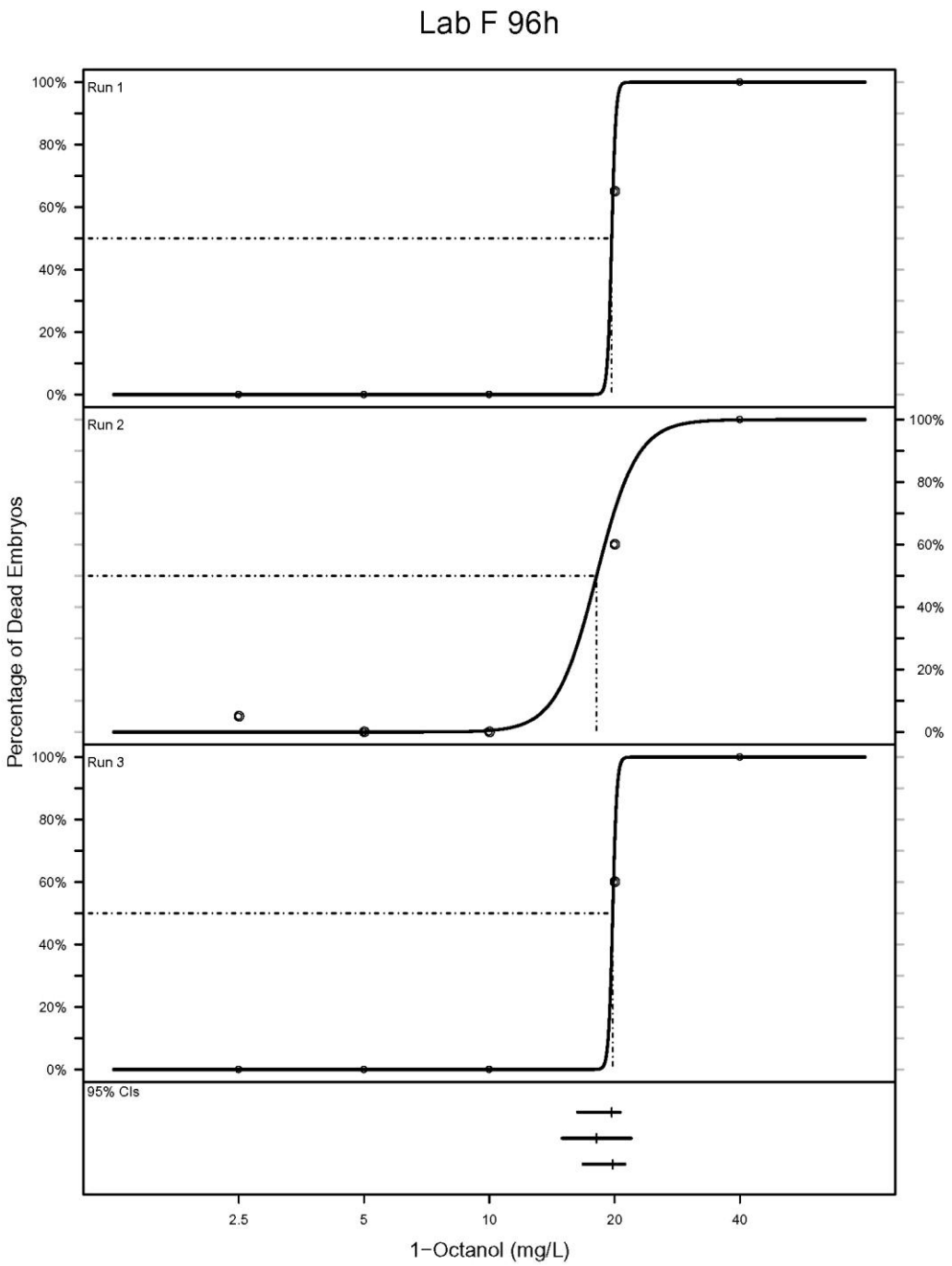

Lab K 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.5
1
2
4
8
Malathion (mg/L)

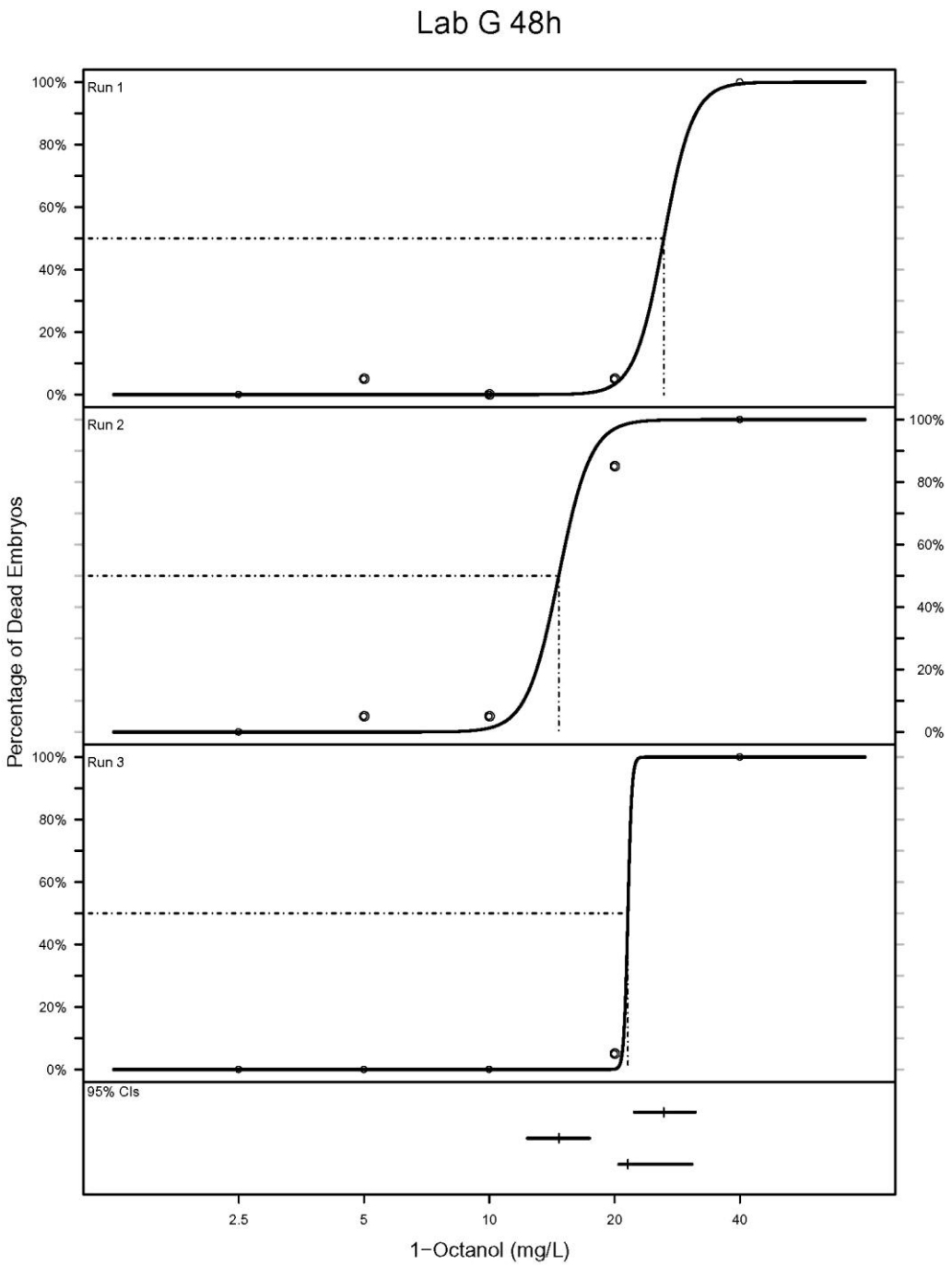
1-Octanol

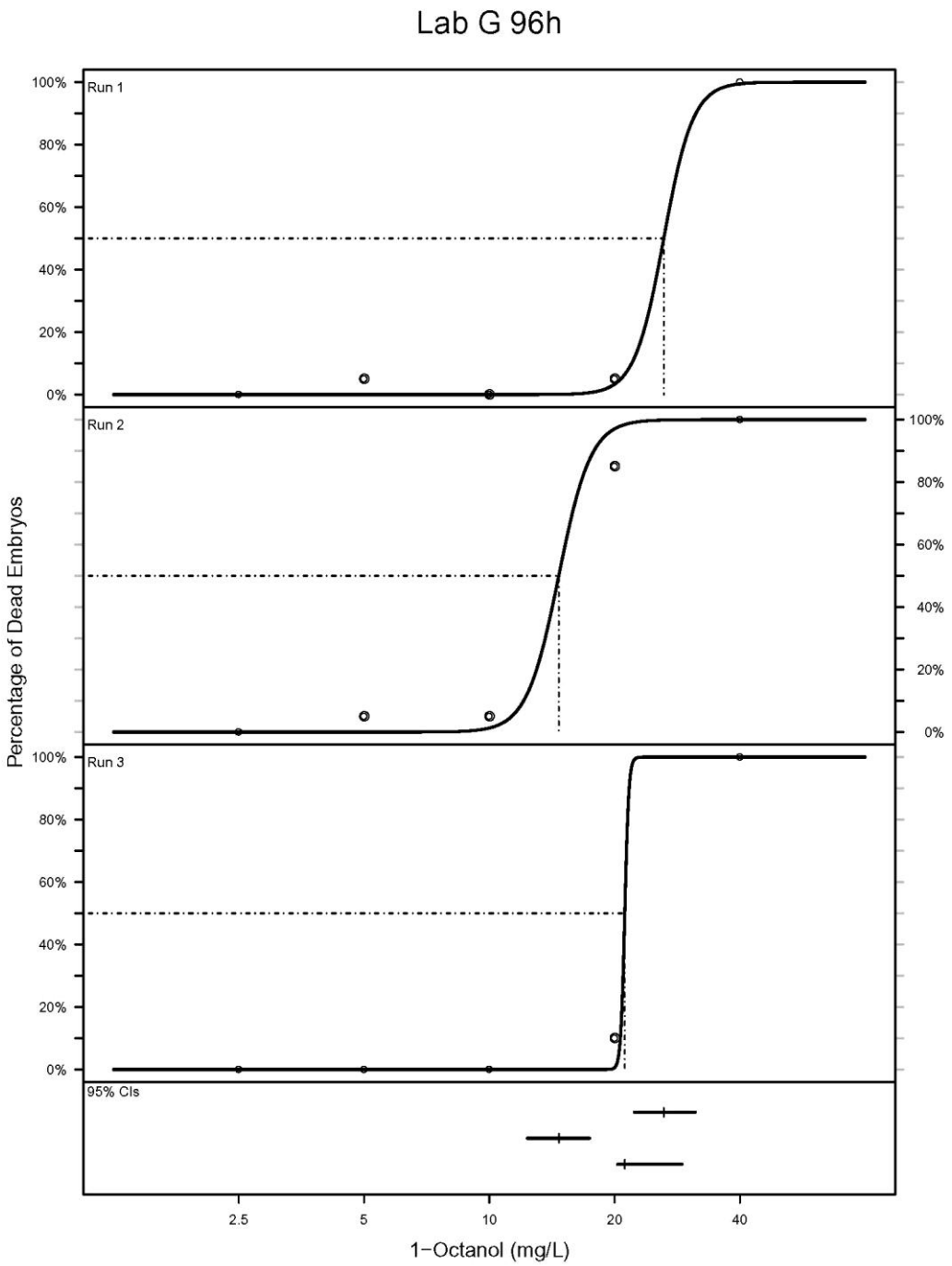

Lab B 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
2.5
5
10
20
40
1−Octanol (mg/L)

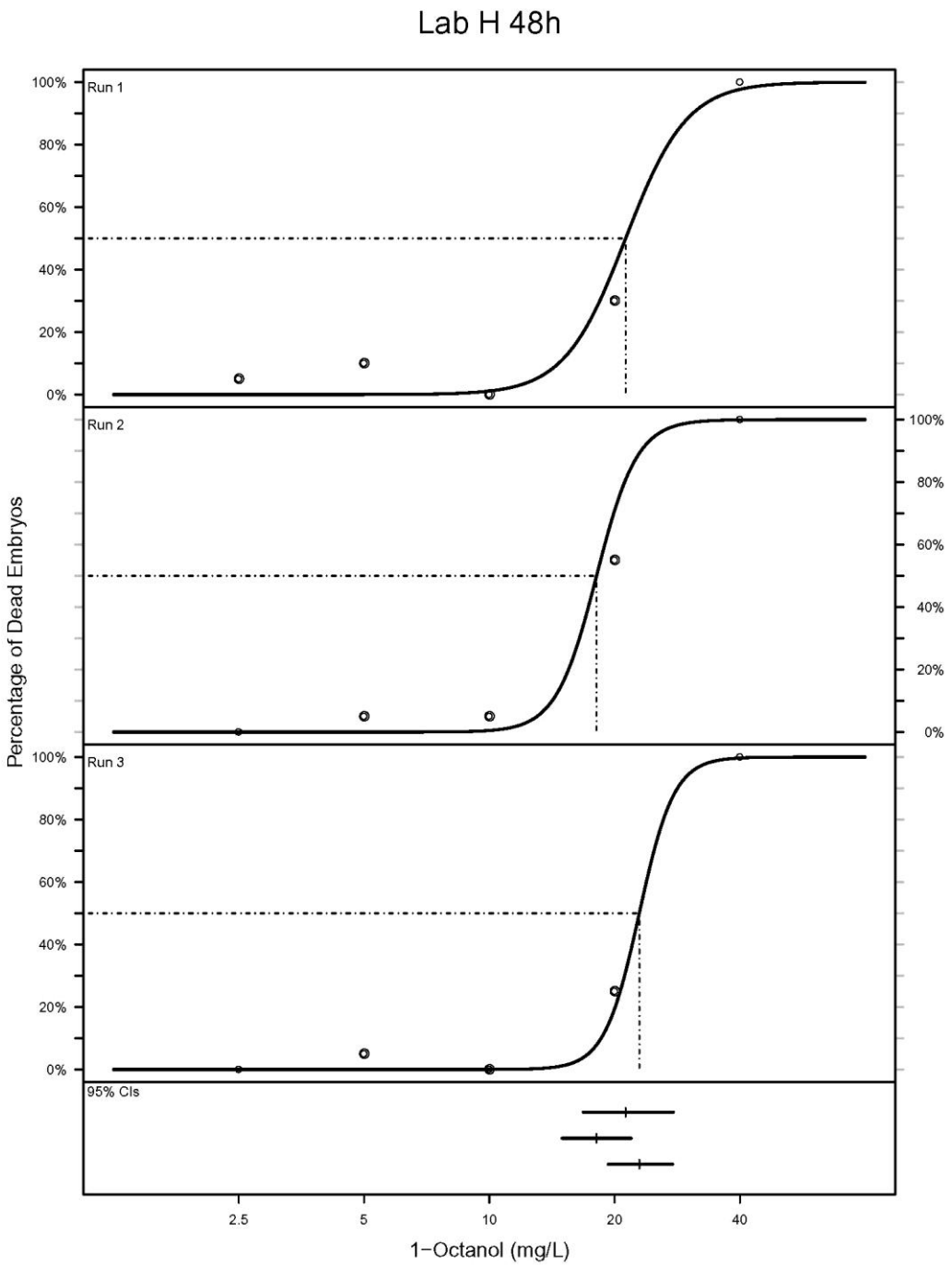

Lab B 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
2.5
5
10
20
40
1−Octanol (mg/L)

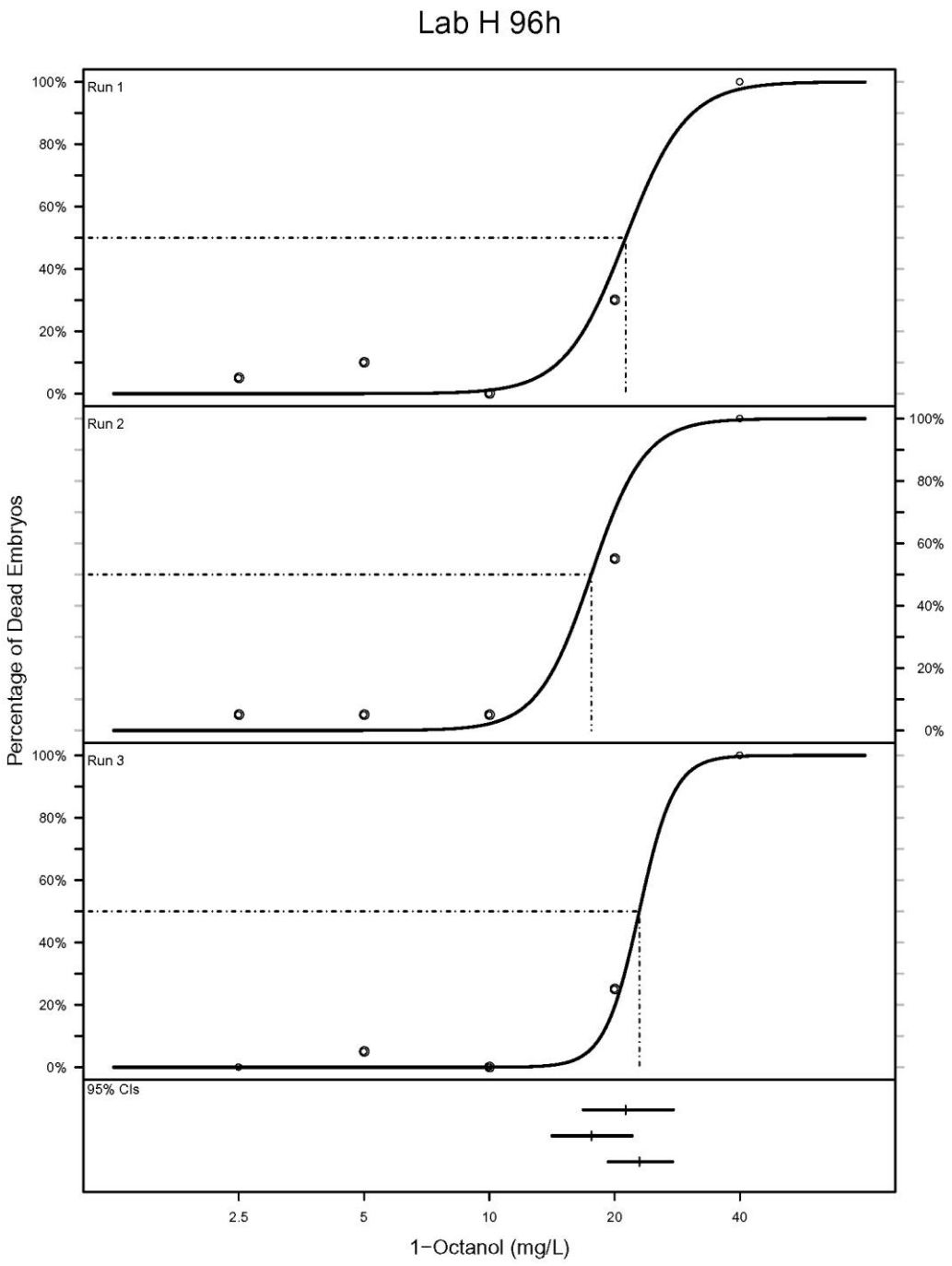

Lab F 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
2.5
5
10
20
40
1−Octanol (mg/L)


Lab F 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
2.5
5
10
20
40
1−Octanol (mg/L)

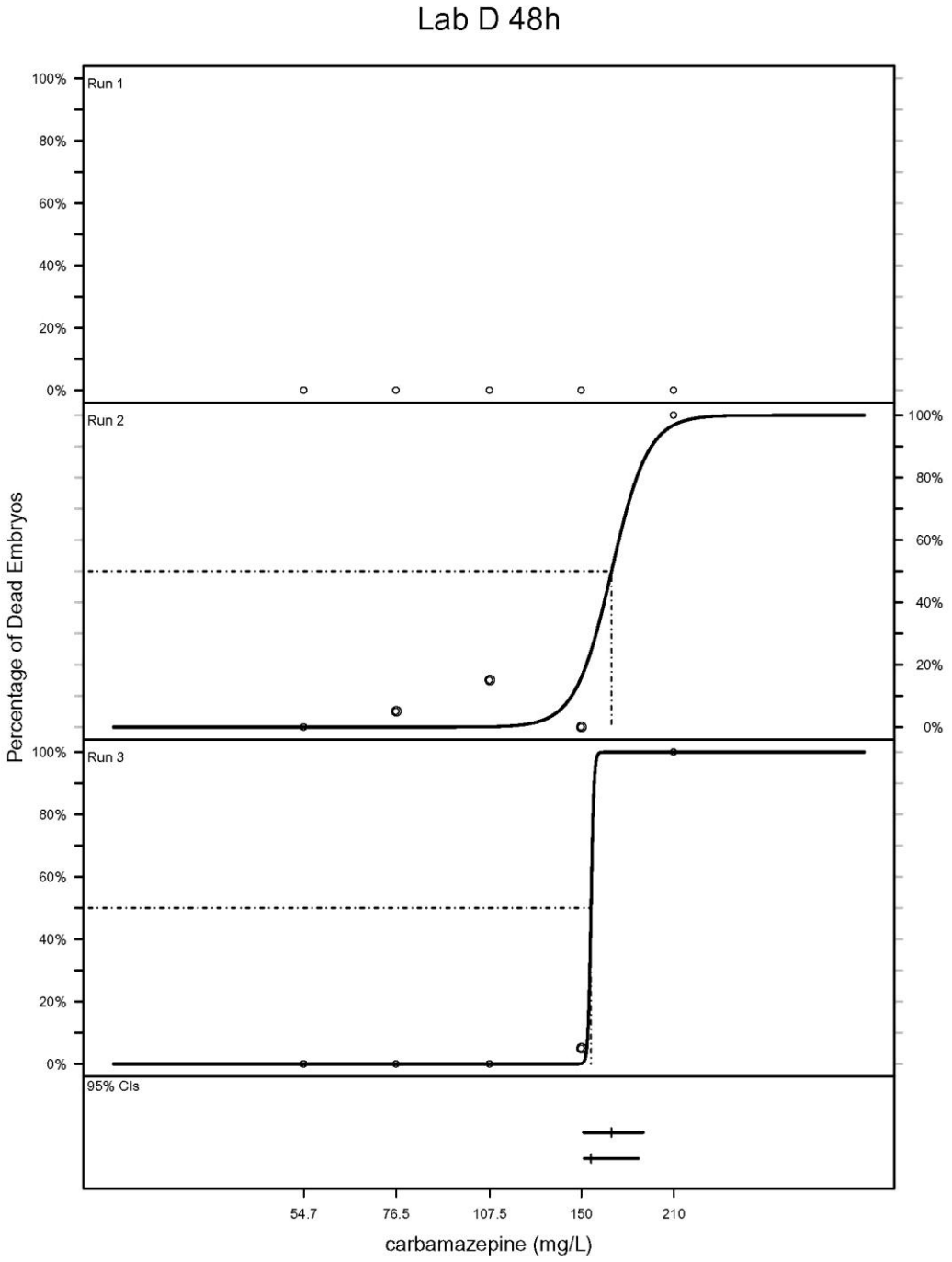

Lab G 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
2.5
5
10
20
40
1−Octanol (mg/L)

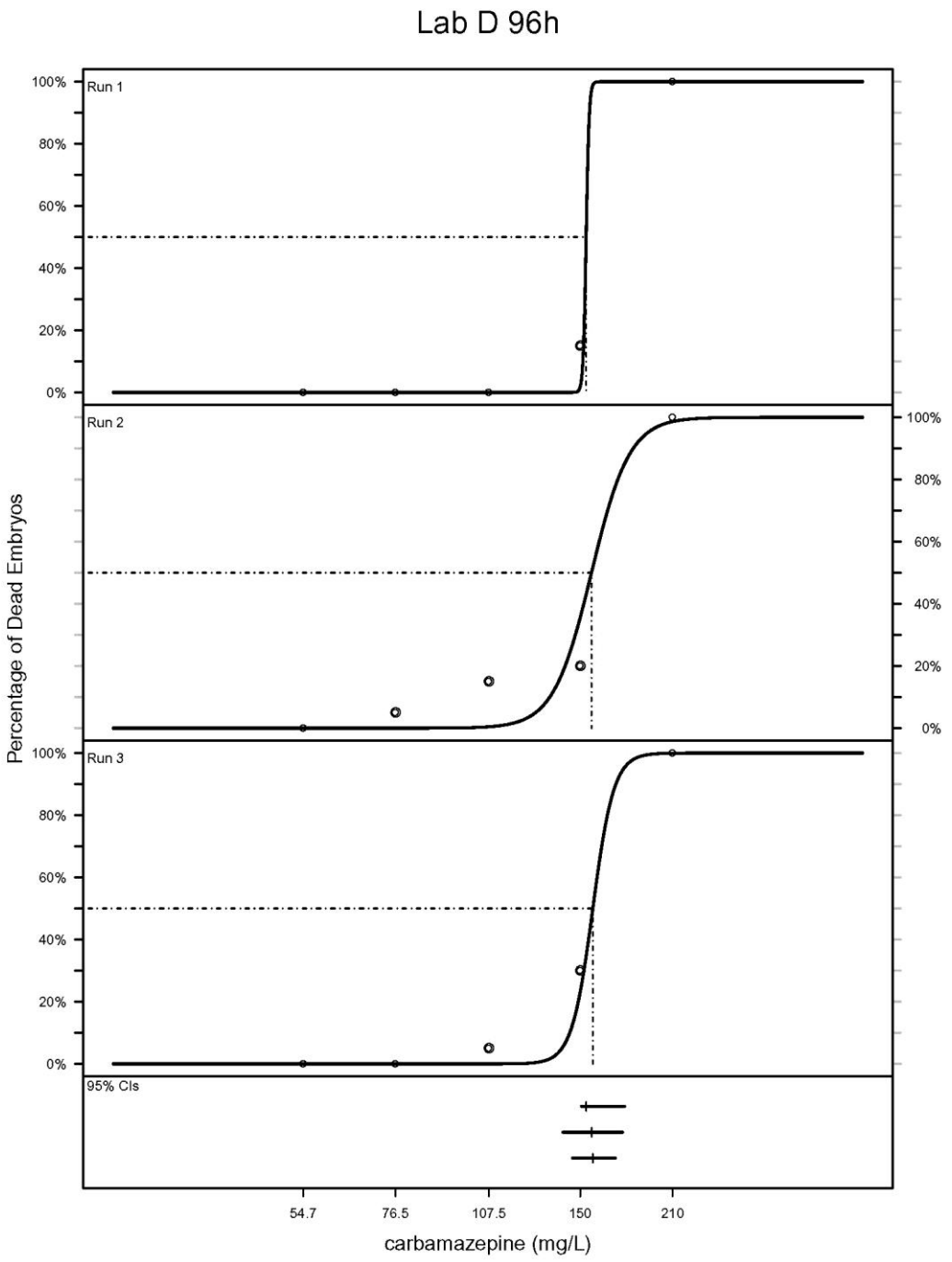

Lab G 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
2.5
5
10
20
40
1−Octanol (mg/L)

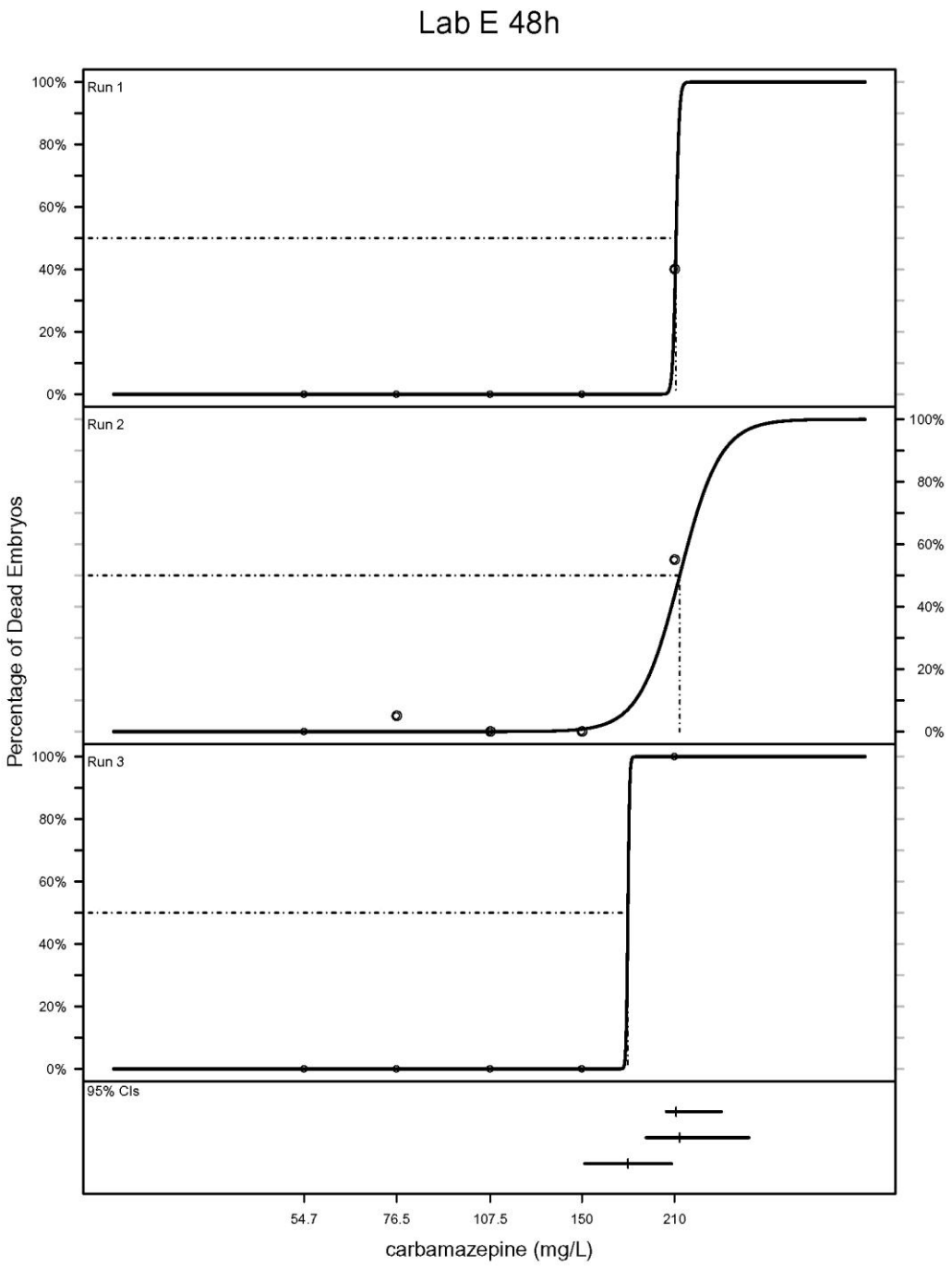

Lab H 48h
Run 1
Run 2
Run 3
95% CIs
Percentage of Dead Embryos
100%
80%
60%
40%
20%
0%
2.5
5
10
20
40
1−Octanol (mg/L)

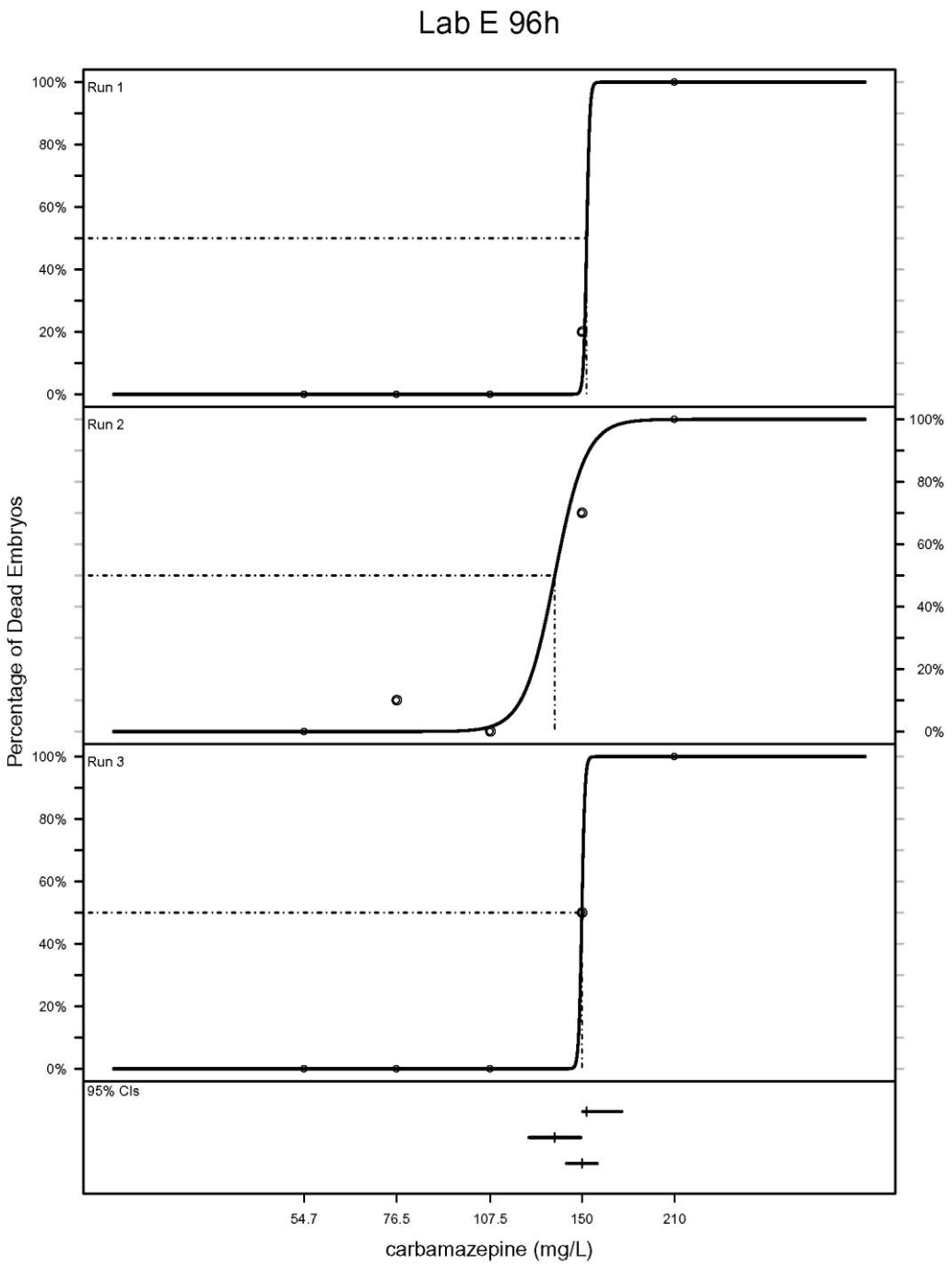

Lab H 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
2.5
5
10
20
40
1−Octanol (mg/L)

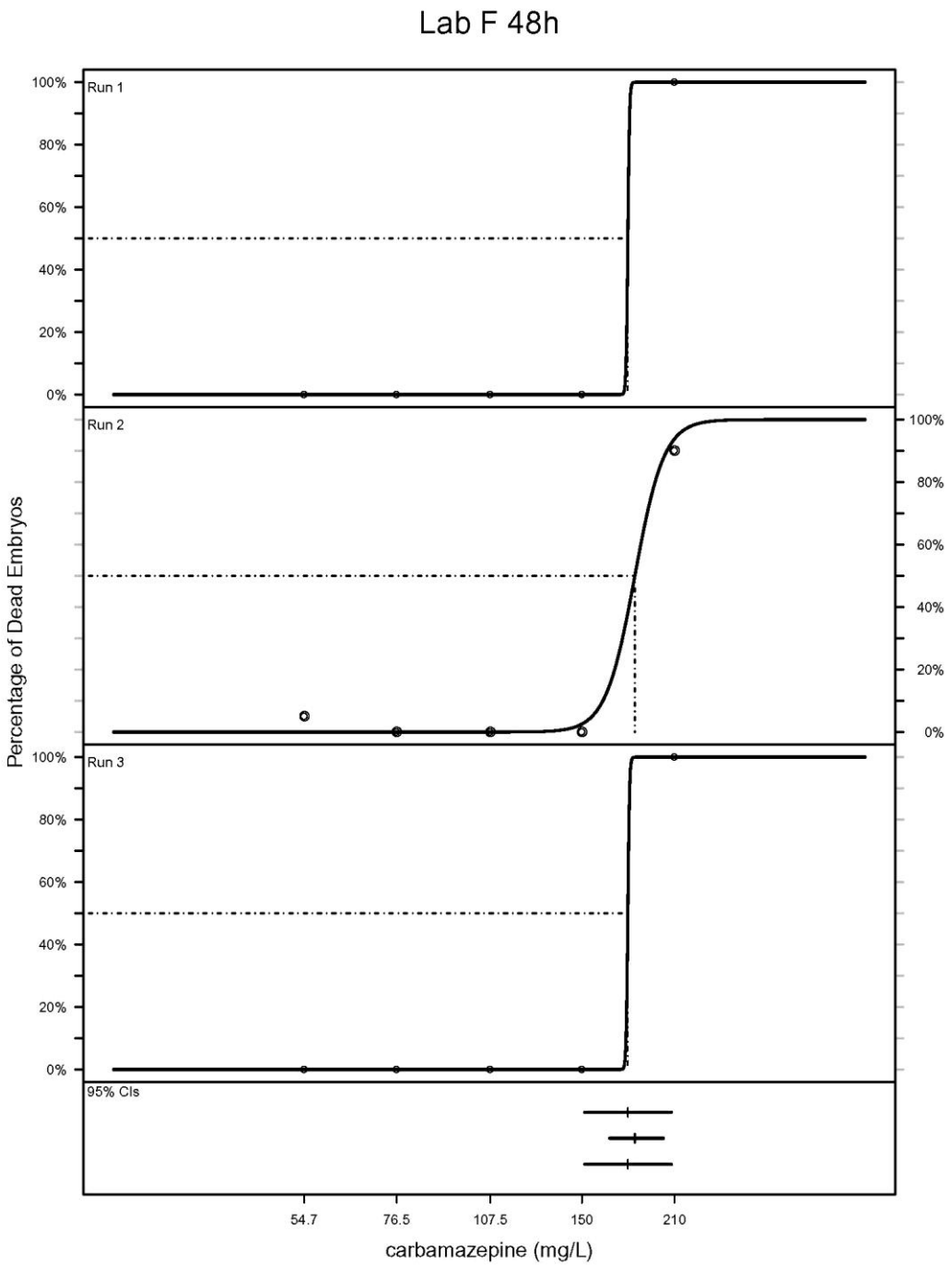
Carbamazepine

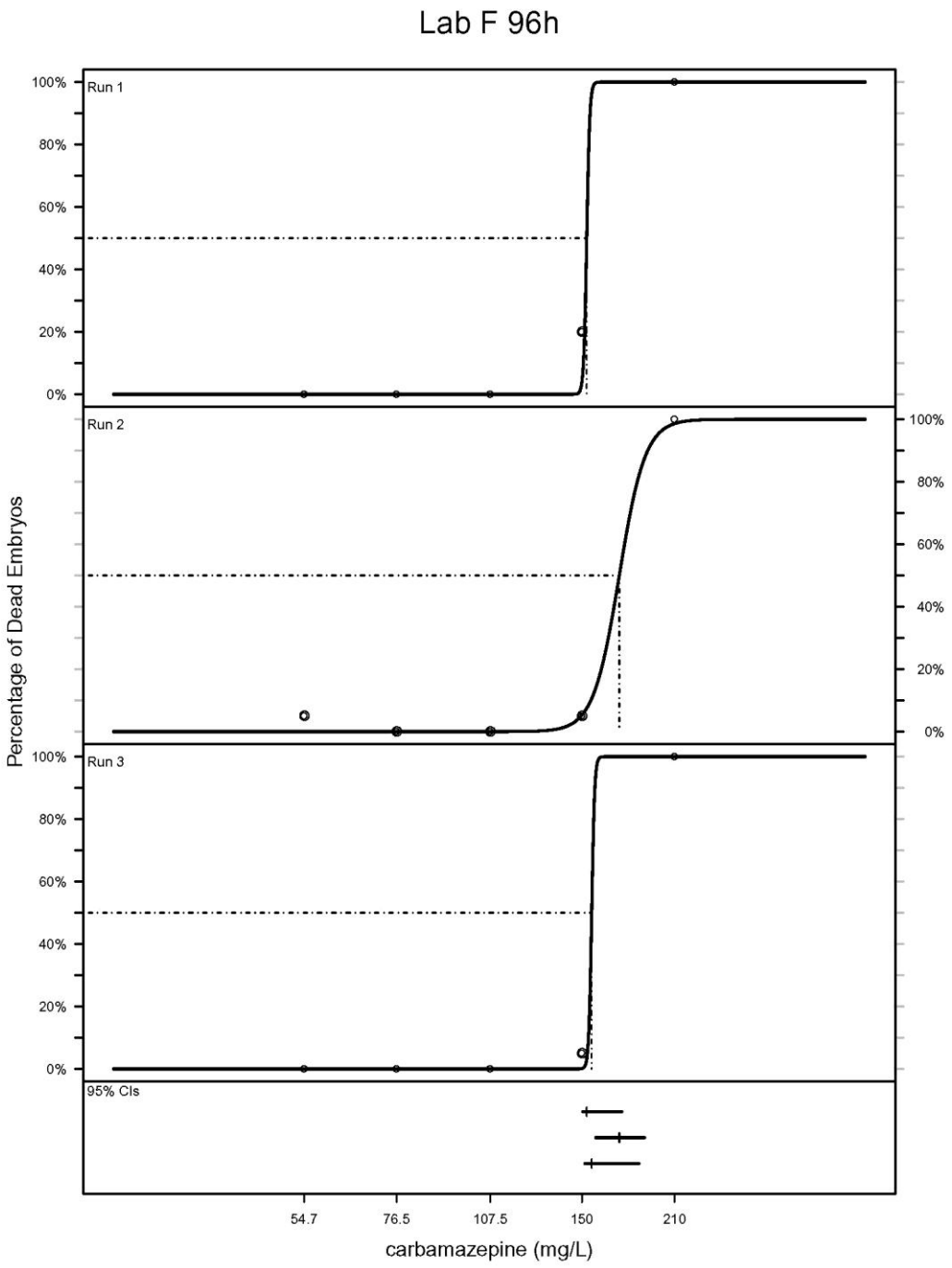


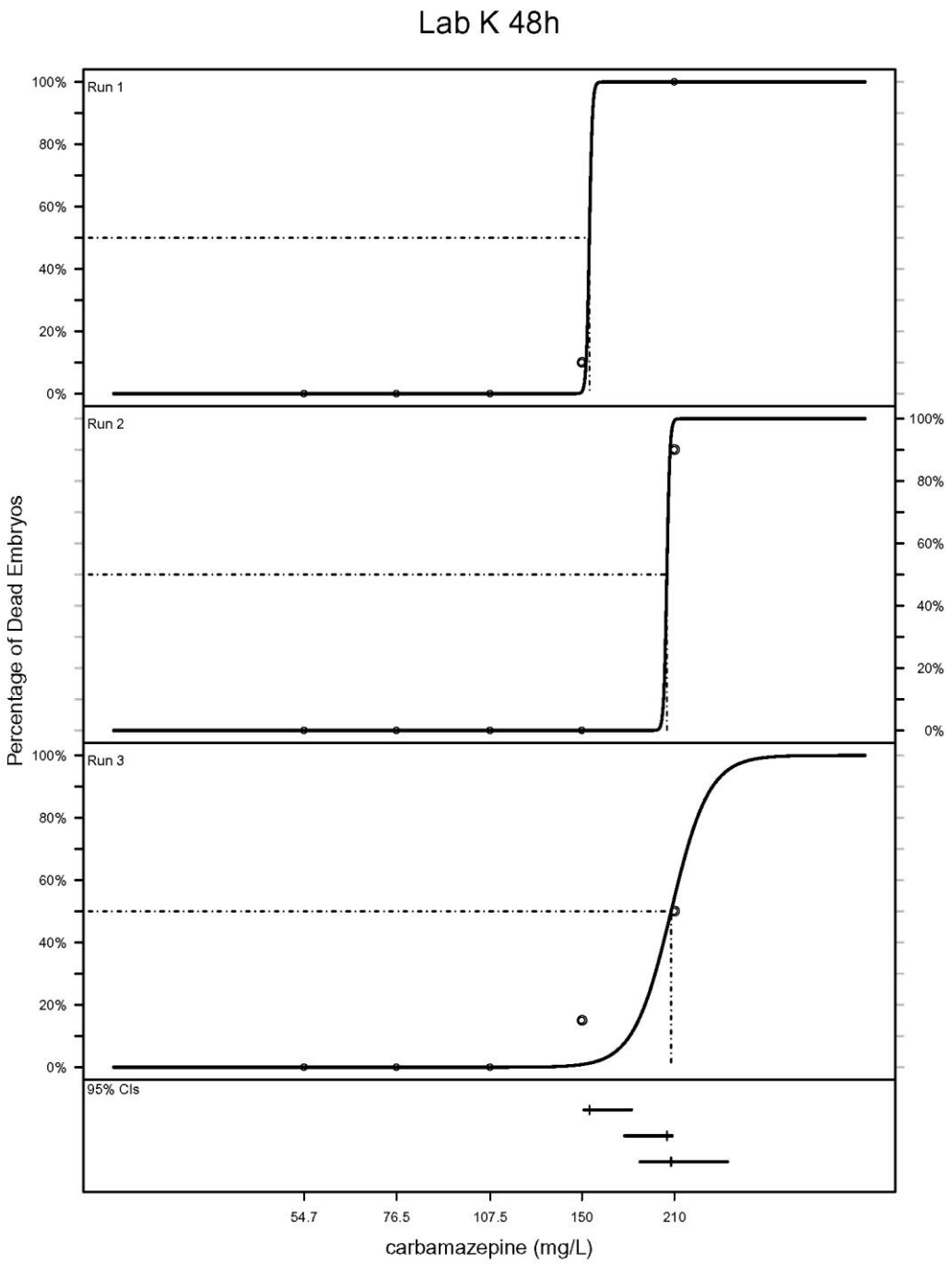

Lab D 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
54.7
76.5
107.5
150
210
carbamazepine (mg/L)

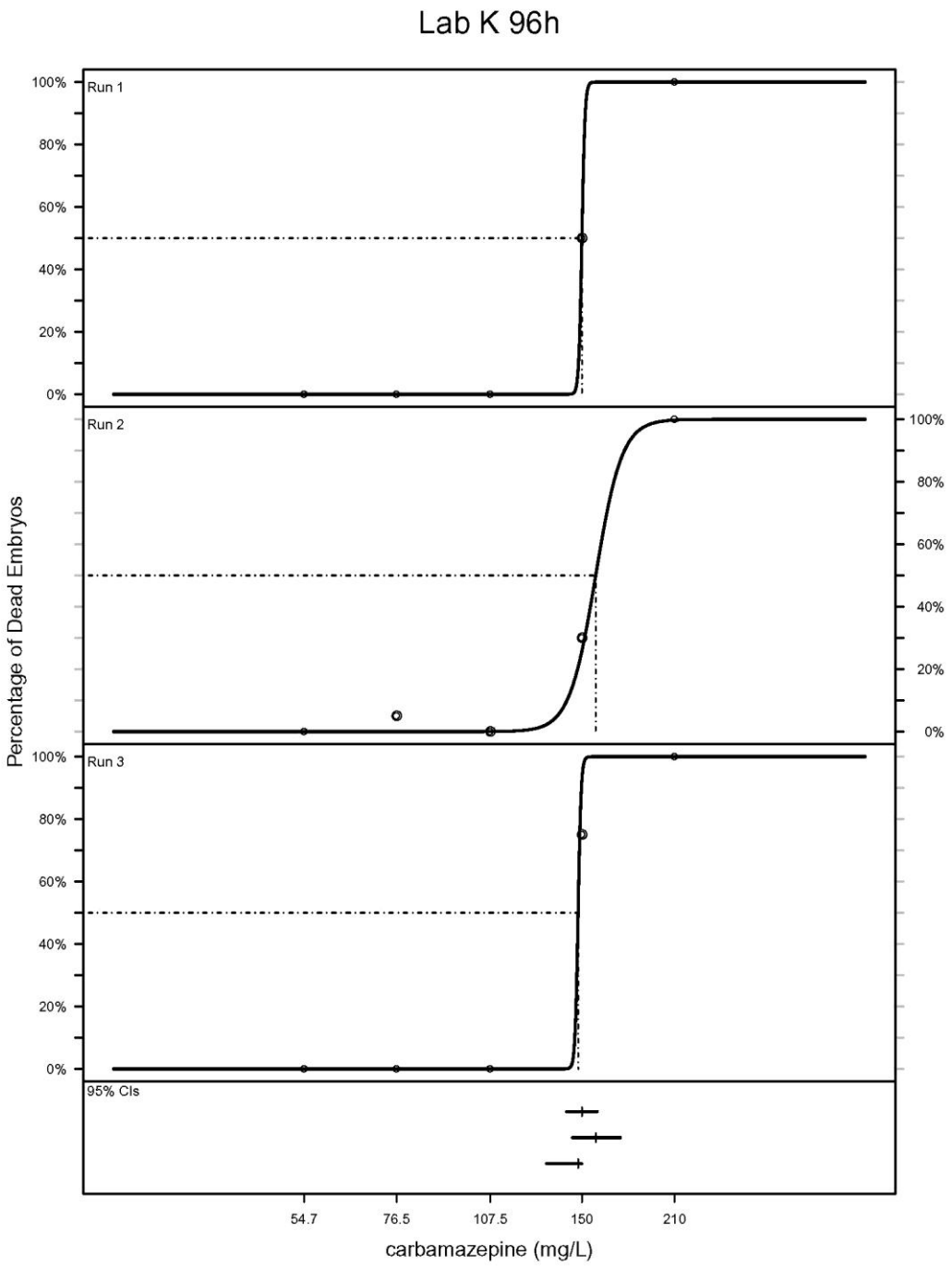

Lab E 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
54.7
76.5
107.5
150
210
carbamazepine (mg/L)


Lab E 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
54.7
76.5
107.5
150
210
carbamazepine (mg/L)

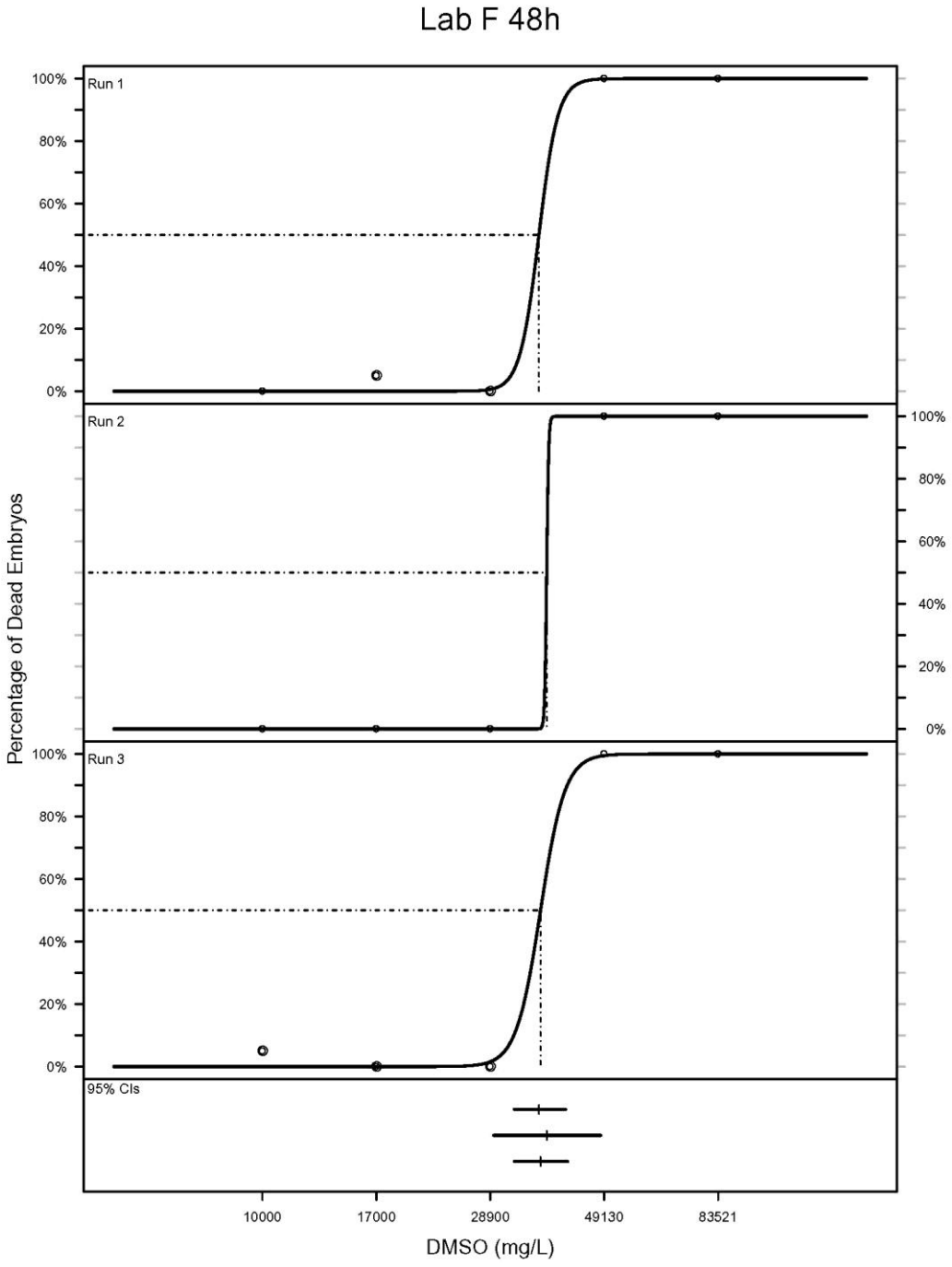

Lab F 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
54.7
76.5
107.5
150
210
carbamazepine (mg/L)

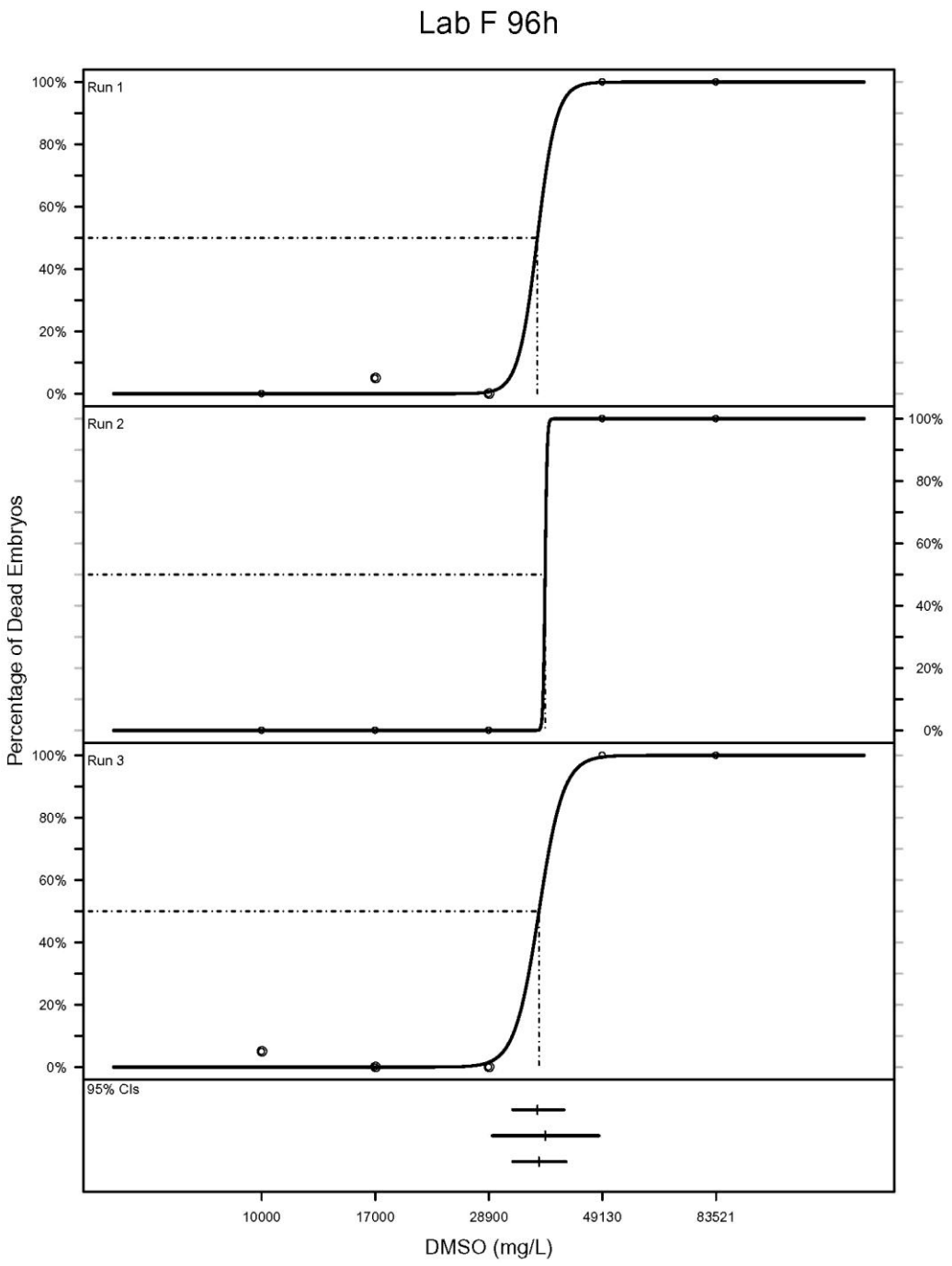

Lab F 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
54.7
76.5
107.5
150
210
carbamazepine (mg/L)

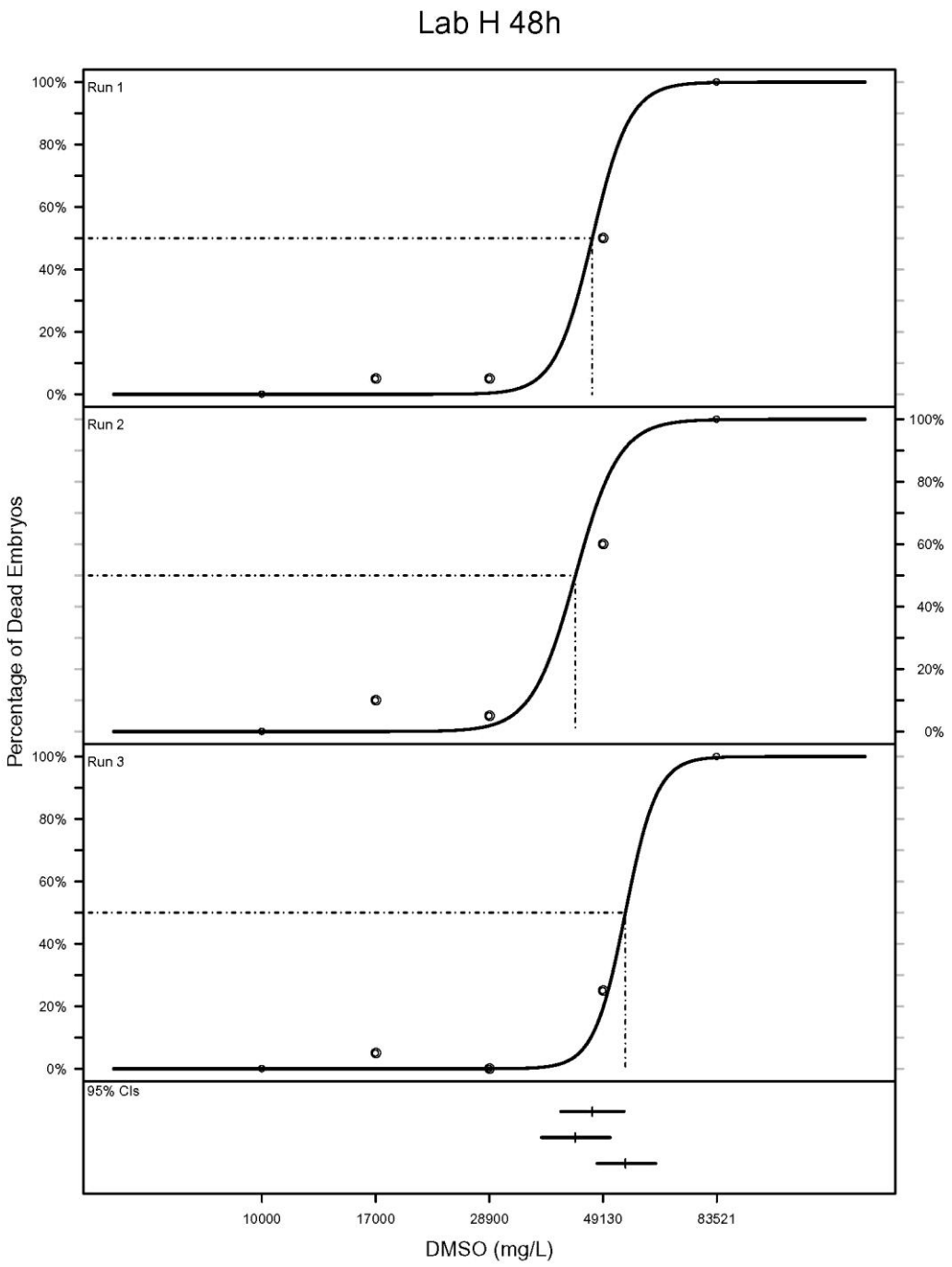

Lab K 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
54.7
76.5
107.5
150
210
carbamazepine (mg/L)

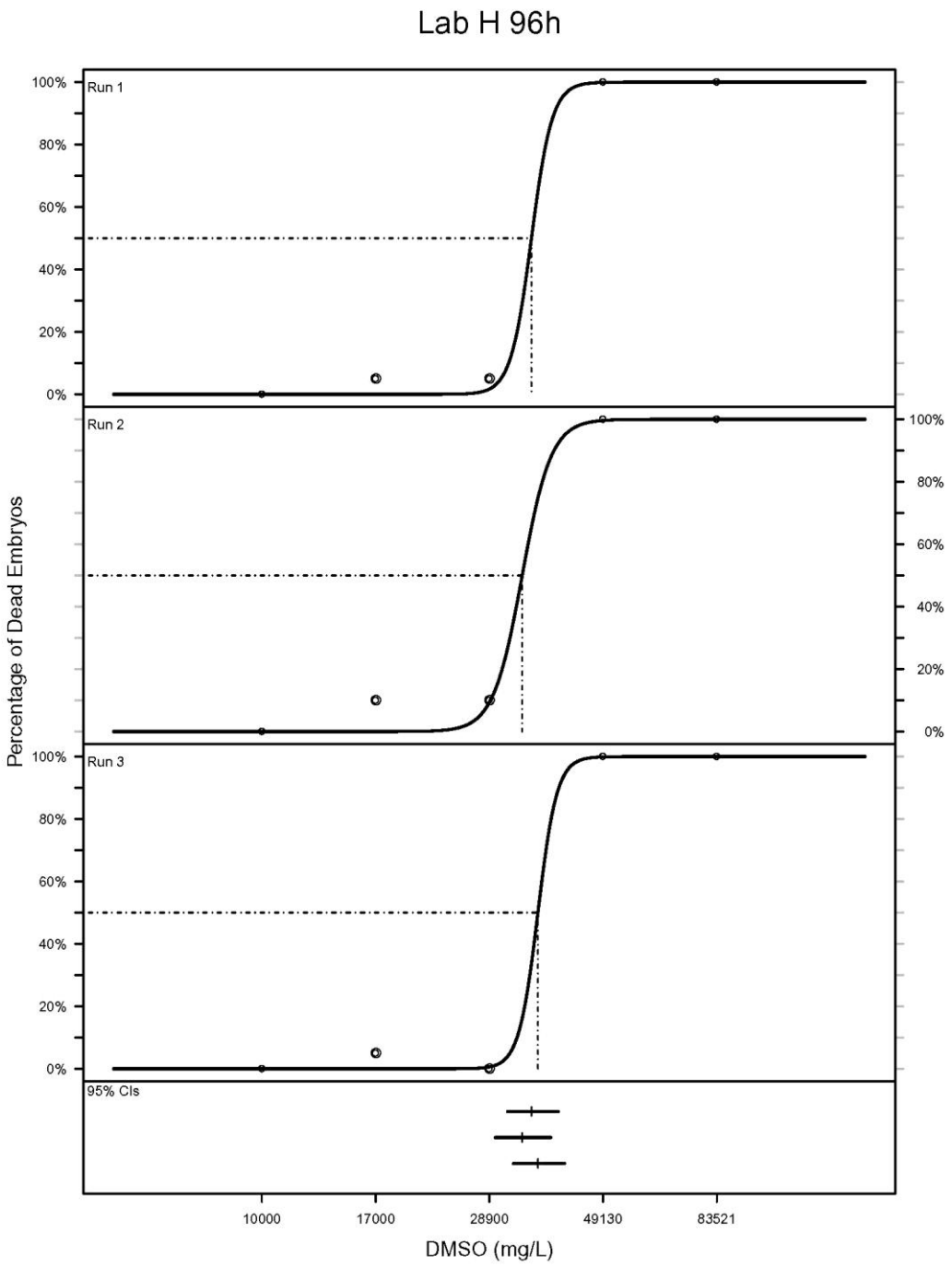

Lab K 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
54.7
76.5
107.5
150
210
carbamazepine (mg/L)

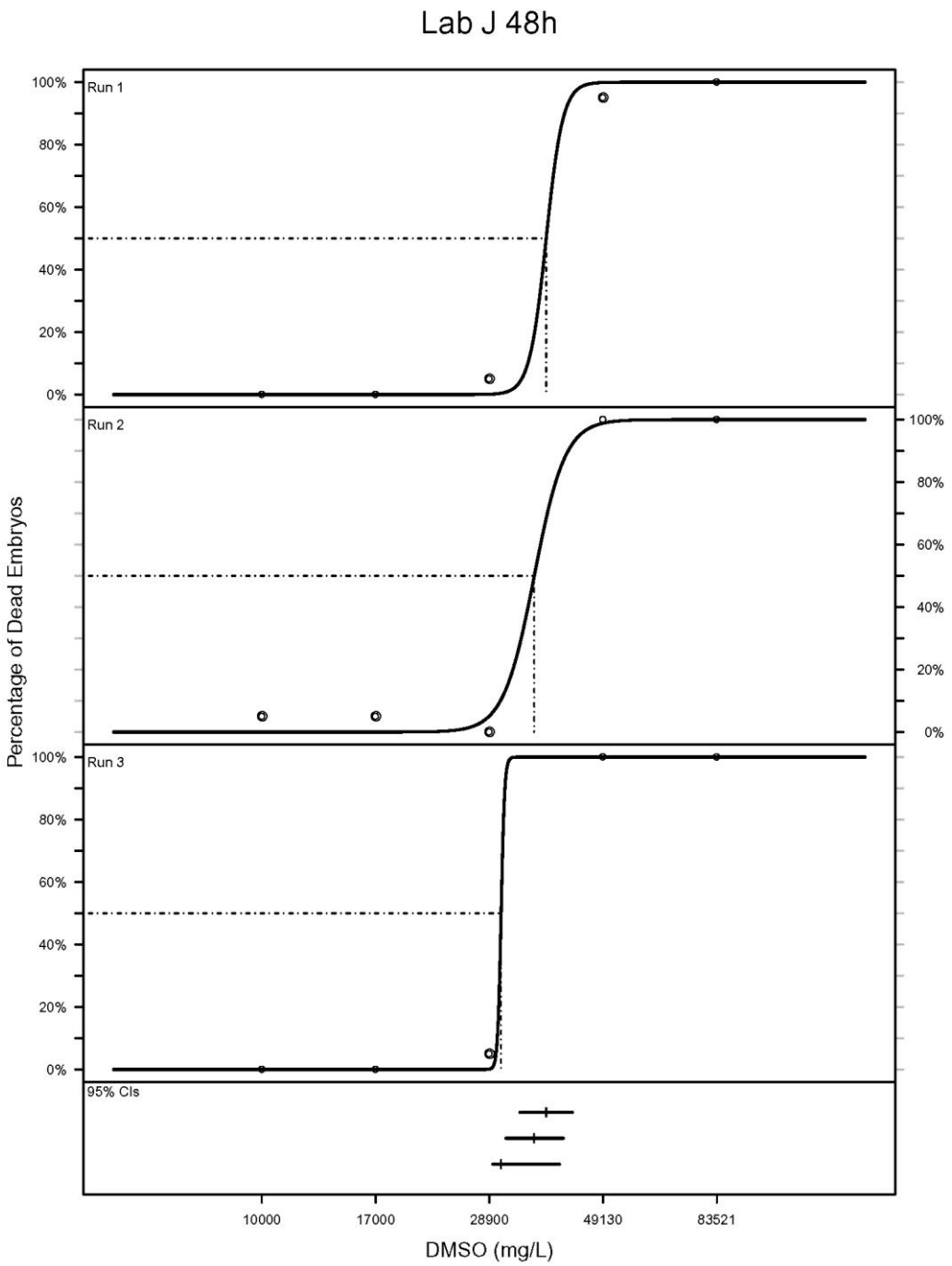
Dimethyl Sulfoxide

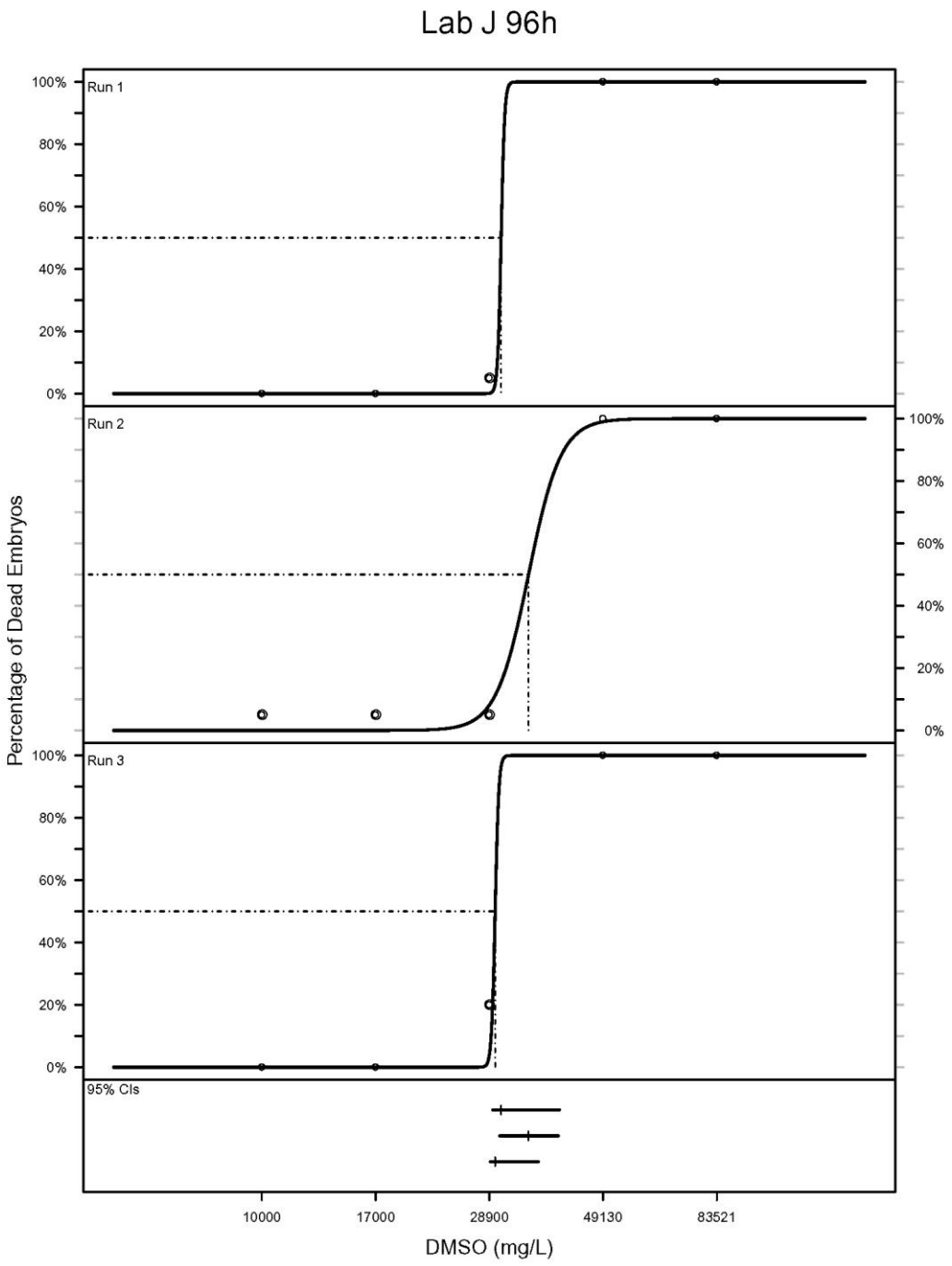


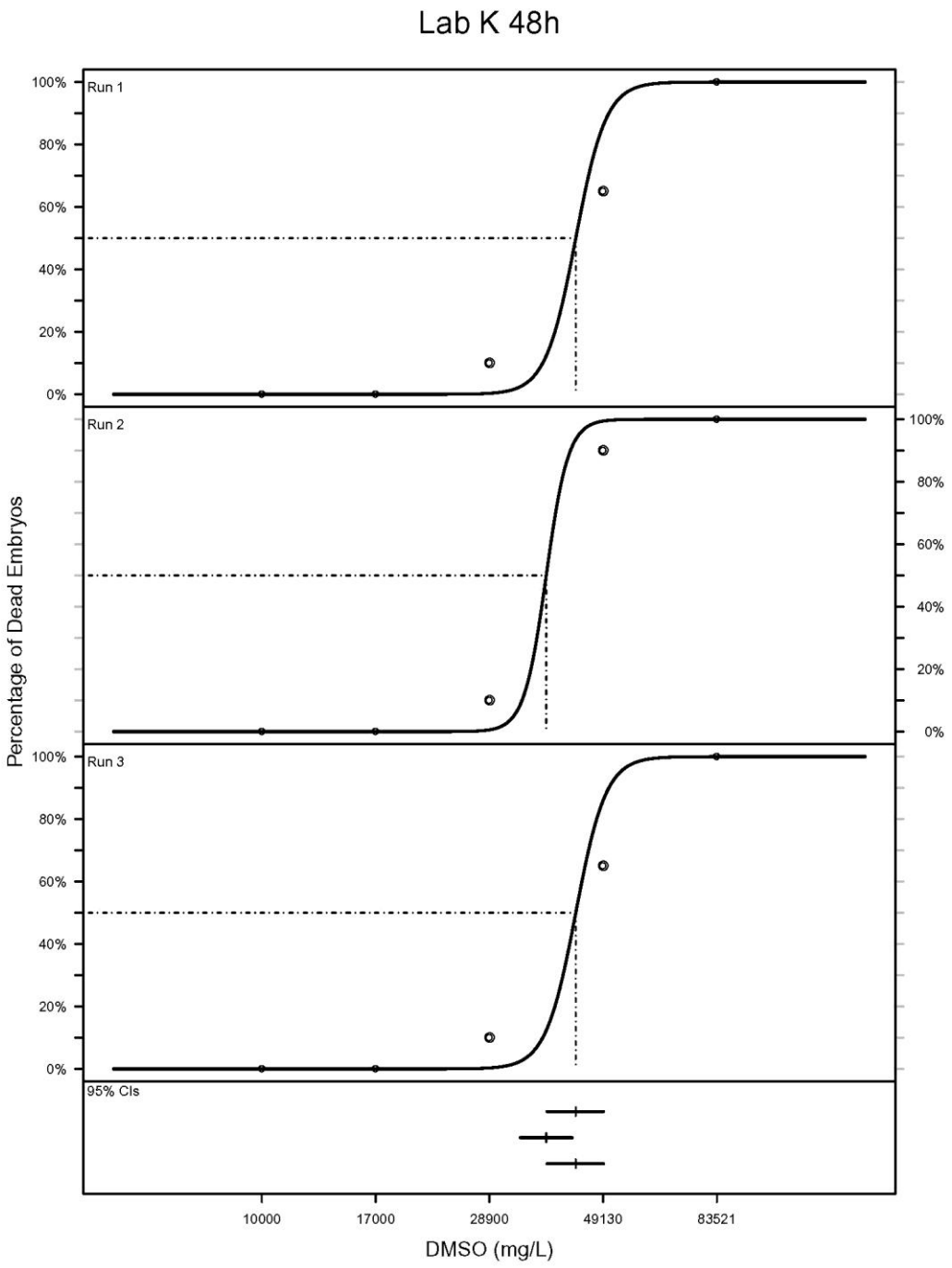

Lab F 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
10000
17000
28900
49130
83521
DMSO (mg/L)

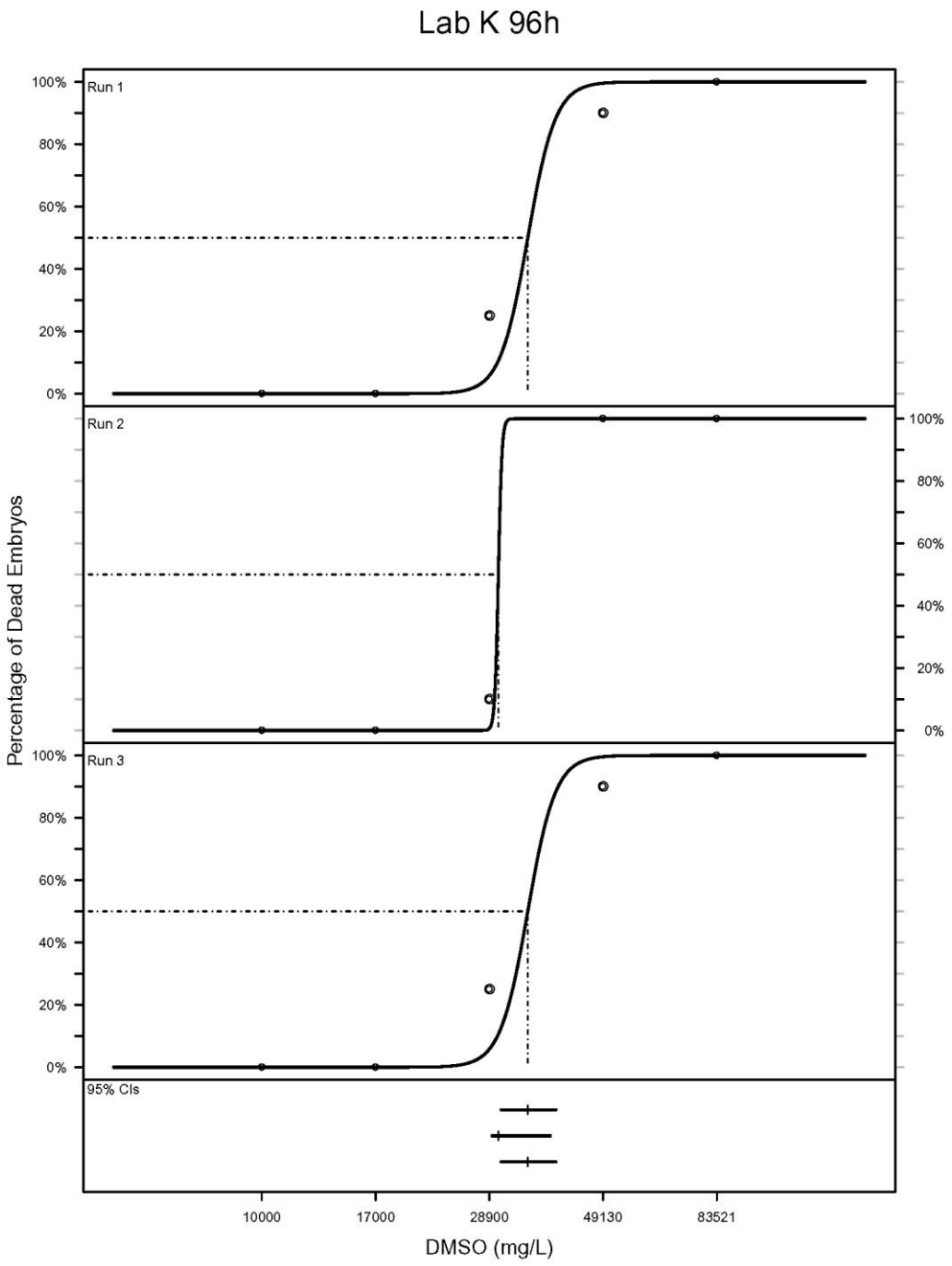

Lab H 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
10000
17000
28900
49130
83521
DMSO (mg/L)


Lab H 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
10000
17000
28900
49130
83521
DMSO (mg/L)

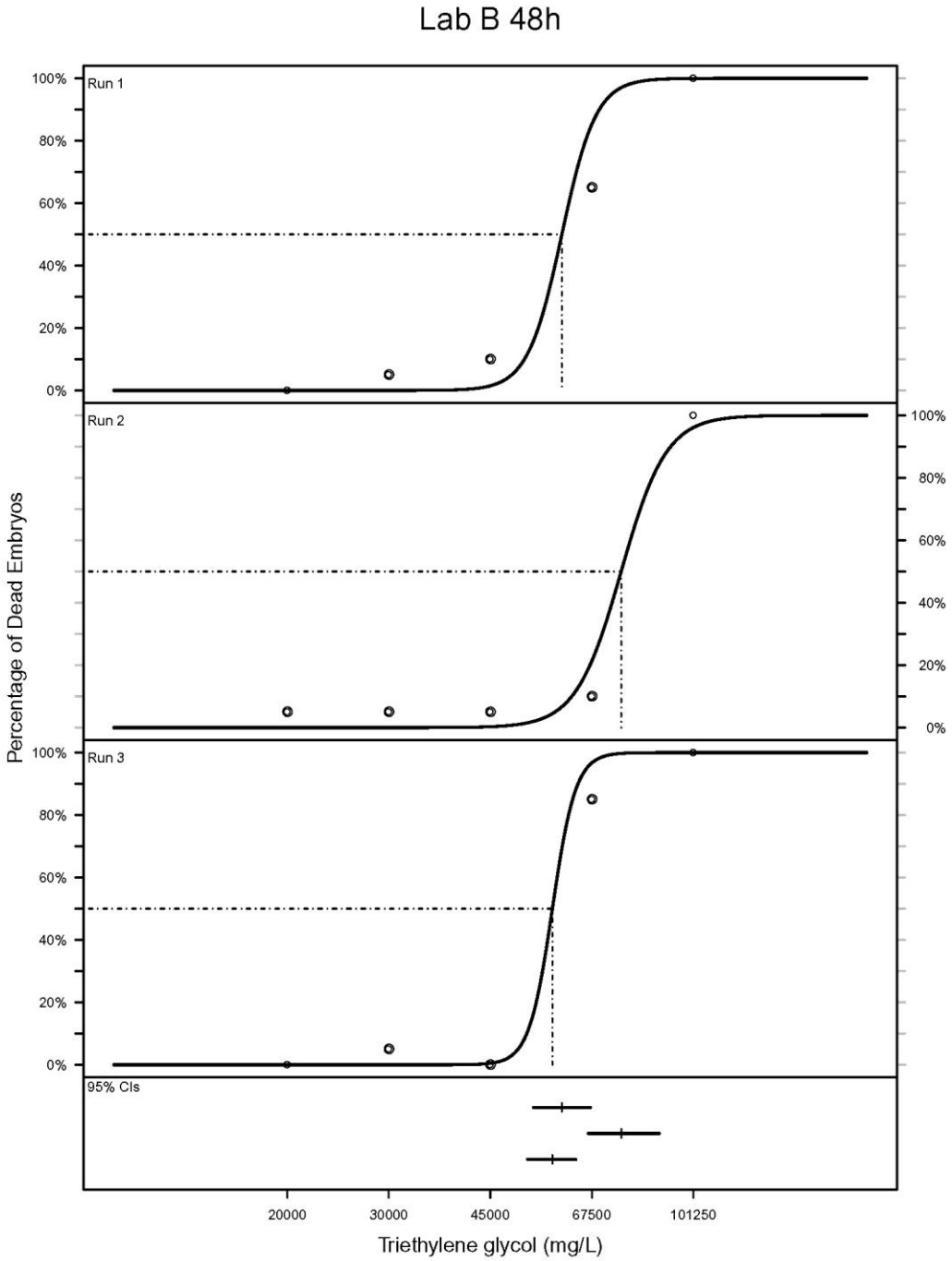

Lab J 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
10000
17000
28900
49130
83521
DMSO (mg/L)

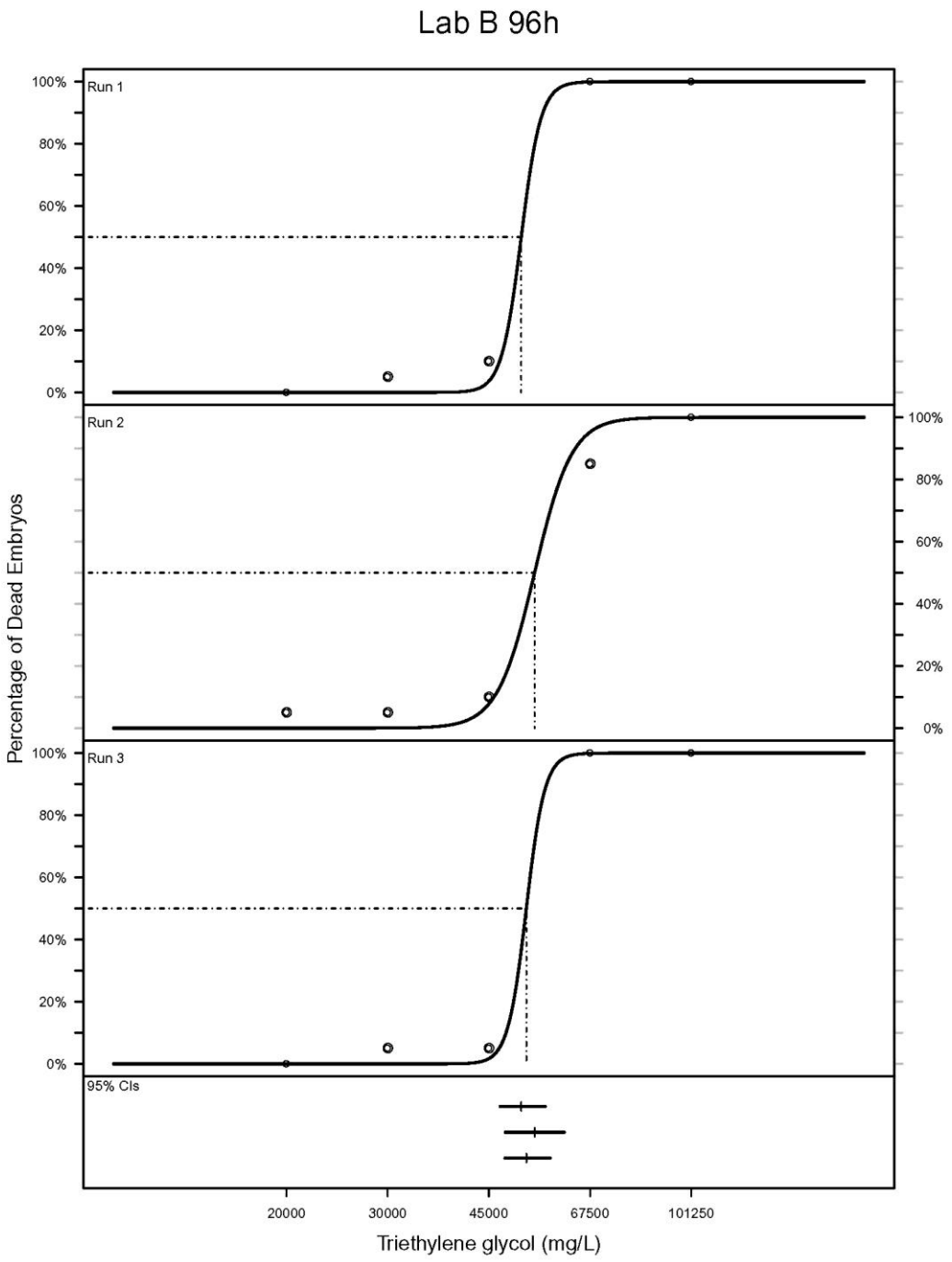

Lab J 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
10000
17000
28900
49130
83521
DMSO (mg/L)

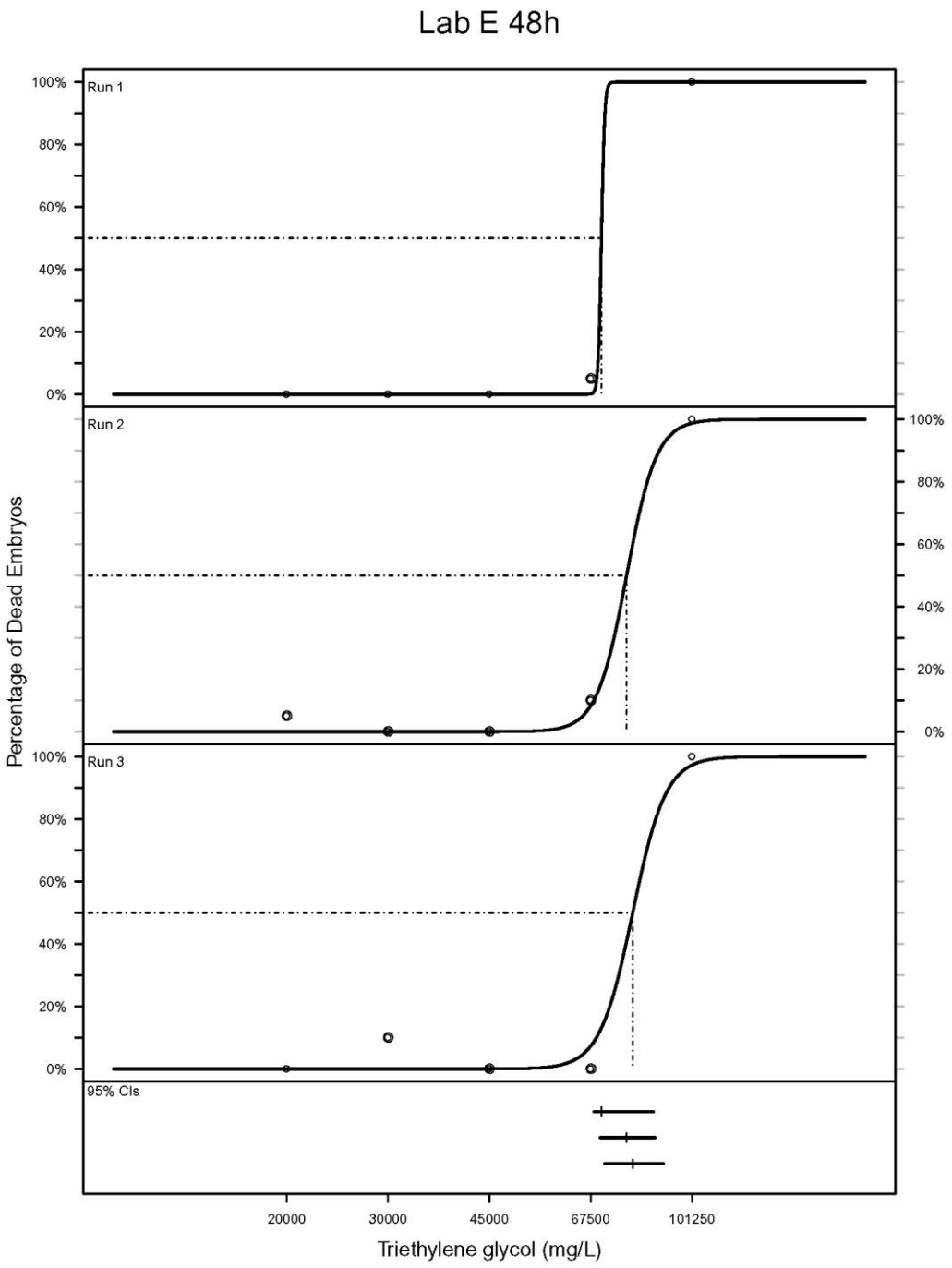

Lab K 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
10000
17000
28900
49130
83521
DMSO (mg/L)

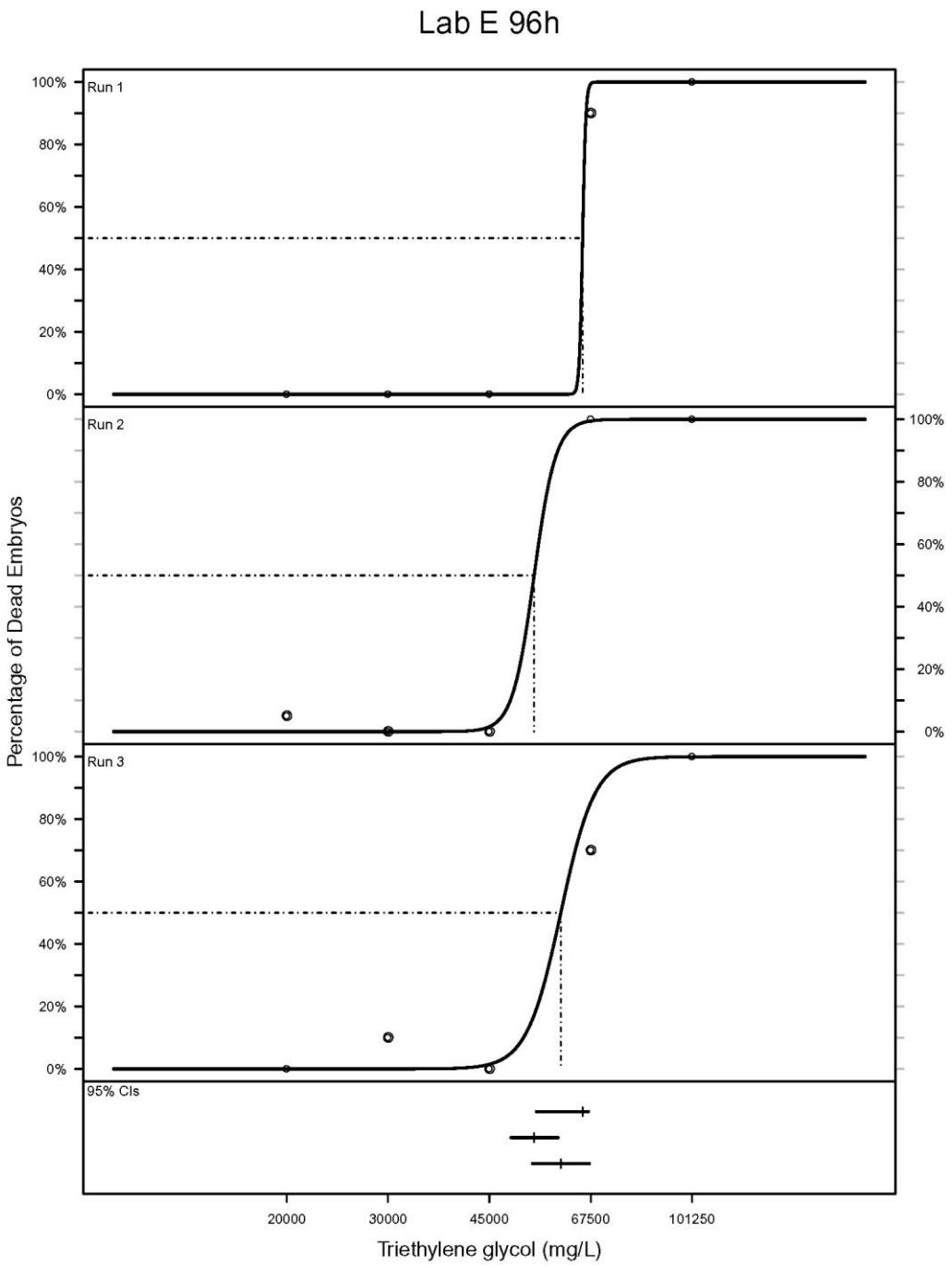

Lab K 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
10000
17000
28900
49130
83521
DMSO (mg/L)

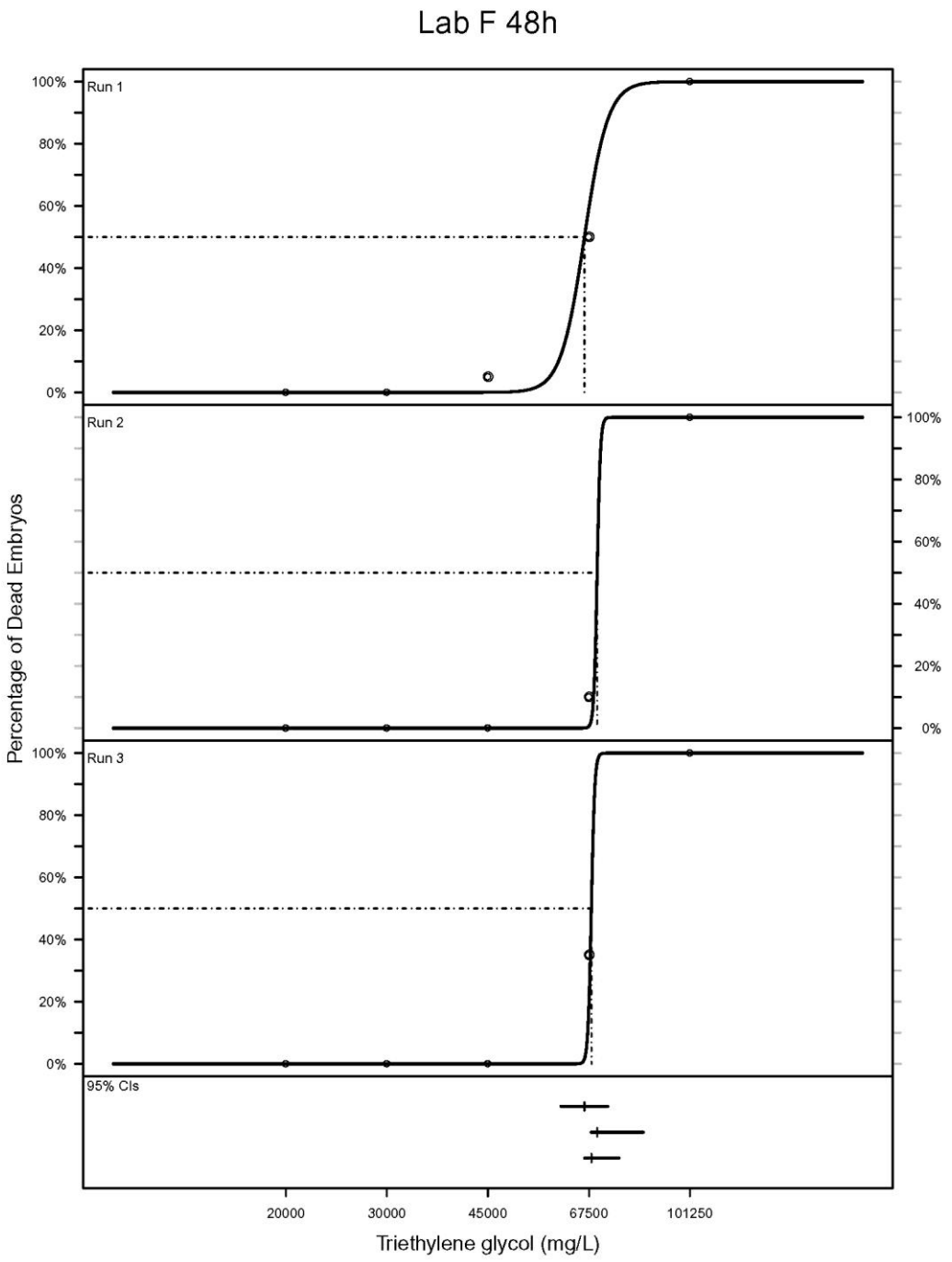
Triethylene glycol




Lab B 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
20000
30000
45000
67500
101250
Triethylene glycol (mg/L)


Lab E 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
20000
30000
45000
67500
101250
Triethylene glycol (mg/L)


Lab E 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
20000
30000
45000
67500
101250
Triethylene glycol (mg/L)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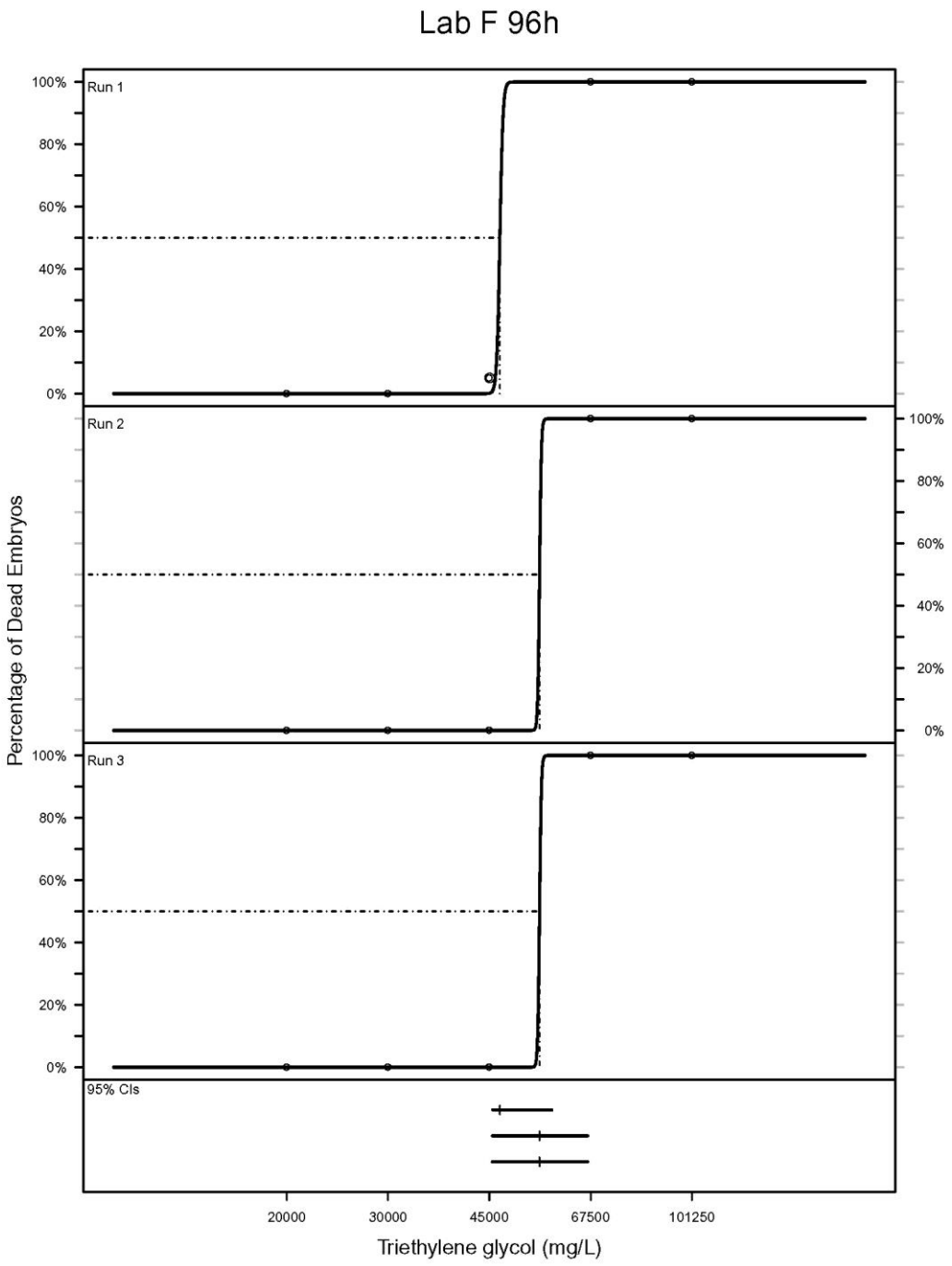
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
20000
30000
45000
67500
101250
Triethylene glycol (mg/L)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Run 2
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100%
80%
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40%
20%
0%
Percentage of Dead Embryos
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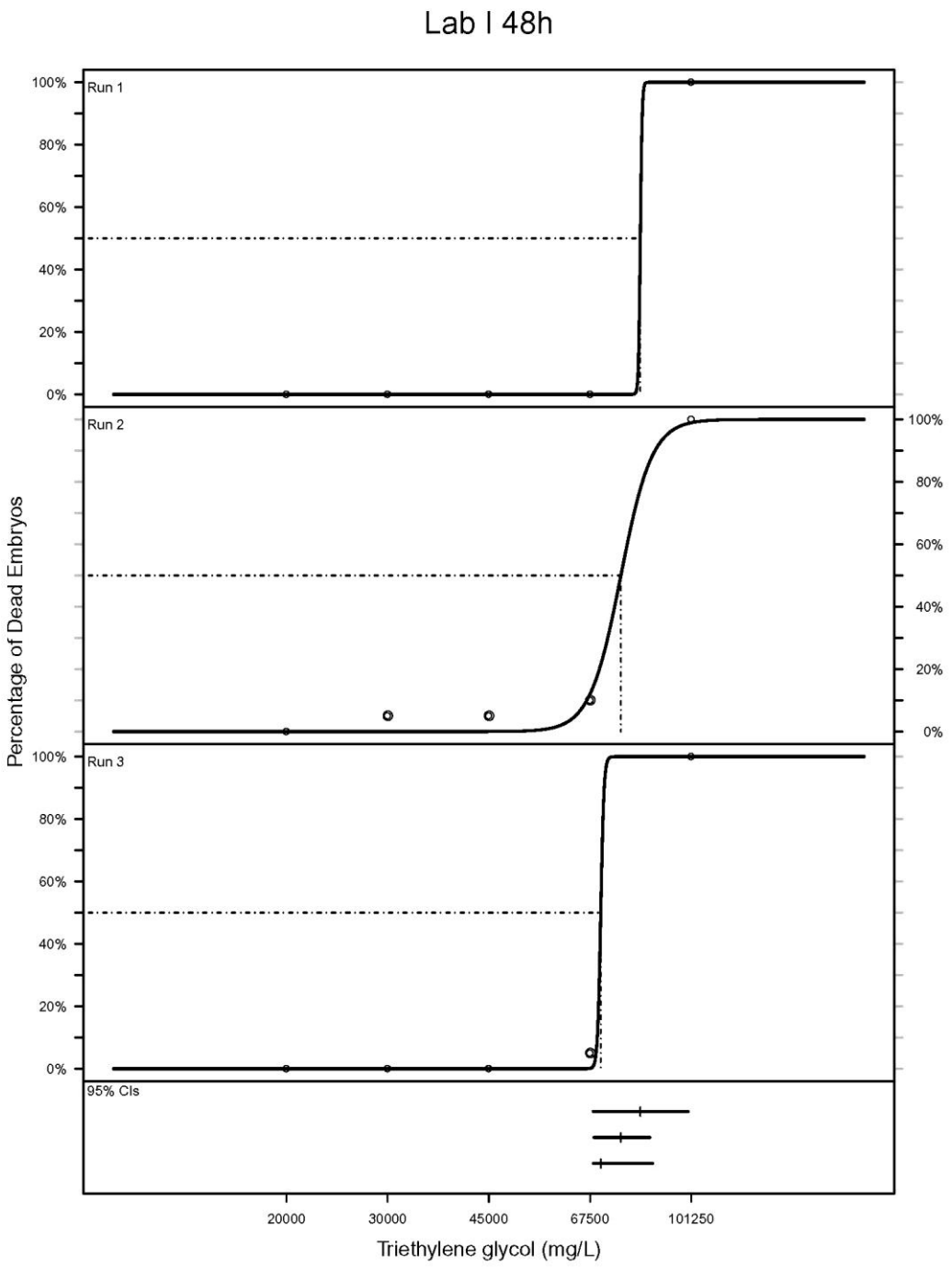
30000
45000
67500
101250
Triethylene glycol (mg/L)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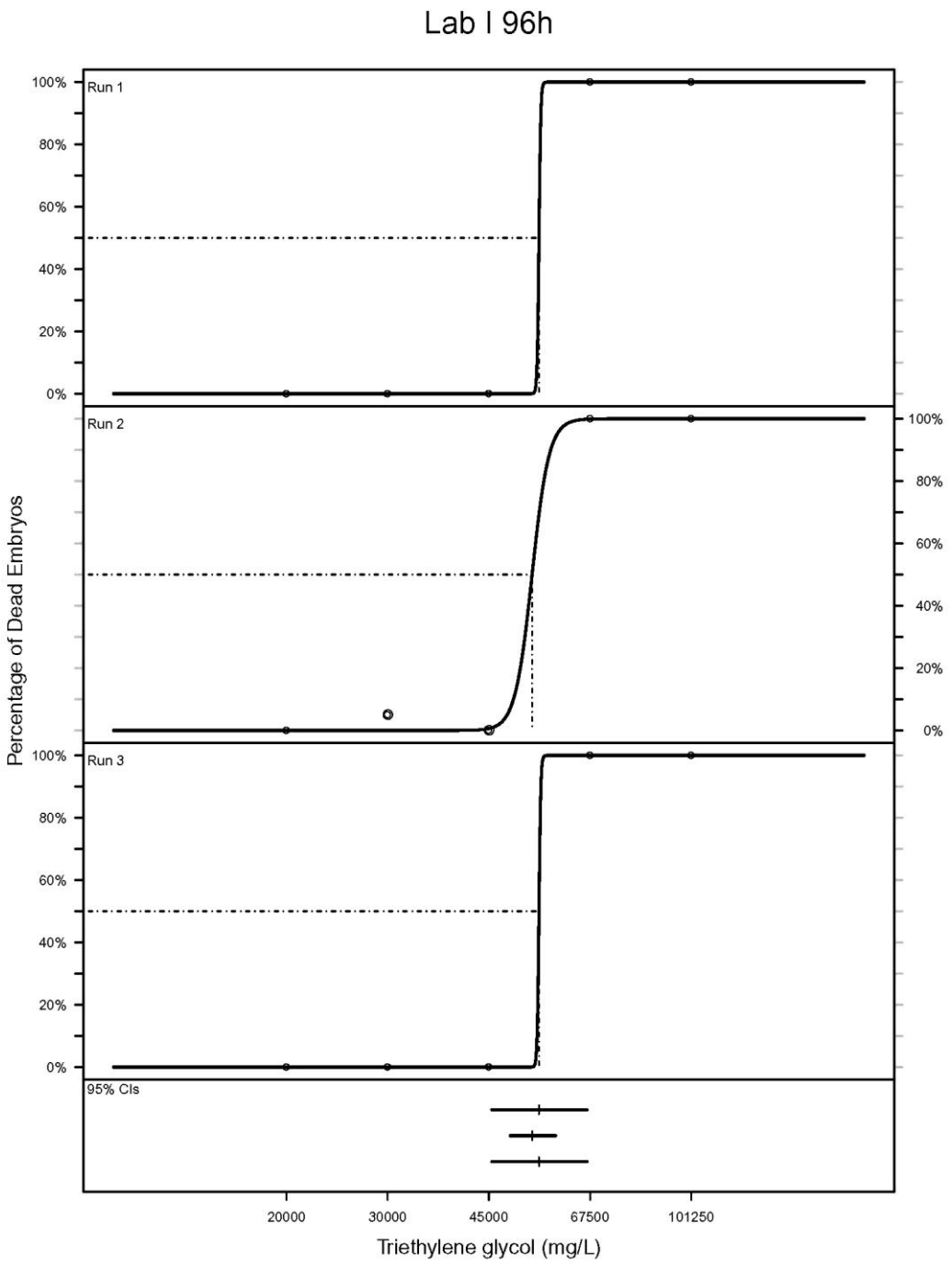
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Triethylene glycol (mg/L)

# *Zebrafish Embryo Toxicity Test*

# Standard Operation Procedure

**SOP ZFET OECD V02.10**

**January 7th, 2011**

# *Zebrafish Embryo Toxicity Test – ZFET*

| | | |
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## PAGE OF CHANGES

| Date of change | Version number | Changed pages/sections | Summary of the change(s) | Changed by/sign |
|---|---|---|---|---|
| 16/06/2009 | V02.8 | 4, 7, 8, 12, 13 | Minor editorial changes | M. Halder on behalf the VMG |
| | | 7 | 5.1:<br>- Vacuum pump was added<br>- Lids to cover 24-well plates were added | M. Halder on behalf the VMG |
| | | 8 | 5.3:<br>- Hardness $CaCO_3$ amended to total hardness | M. Halder on behalf the VMG |
| | | 12 | 6.3.2:<br>- Presaturation of 24-well plates and glass vessels becomes mandatory<br>- Freshly prepared test solutions = prepared on the day of the test | M. Halder on behalf the VMG |
| | | 15 | New section 6.5 Semi-static renewal procedure added | M. Halder on behalf the VMG |
| | | 15 | 6.6 renamed and revised, i.e. new instructions for measurements of test conditions | M. Halder on behalf the VMG |
| | | 16 | 7.1 Acceptance criteria, see bullet point (6) | M. Halder on behalf the VMG |
| | | | | |
| 06/11/2009 | V02.9 | 5, 11, 13, 16 | Minor editorial changes | M. Halder on behalf the VMG |
| | | 8 | Table 1 list of chemicals | M. Halder on behalf the VMG |
| | | 14, Annex 2 | 6.4<br>a minimum magnification 80x is used when scoring heart beat | M. Halder on behalf the VMG |
| | | 15 | | M. Halder on behalf the VMG |
| | | 16 | 7.1<br>An acceptance criteria for the positive control (3.4 DCA) is now defined | M. Halder on behalf the VMG |
| | | | | |
| 07/01/2011 | V02.10 | 9, 11, 13 | Minor editorial changes | M. Halder on behalf the VMG |
| | | 14 | 6.3.2.2 Clarification of distribution of eggs on the 24-well plates | M. Halder on behalf the VMG |
| | | 17 | 7.1 Note on acceptance criteria for internal control added | M. Halder on behalf the VMG |
| | | 17 | 7.3 Greg Carr is now responsible for the statistical analysis | M. Halder on behalf the VMG |

| | | | | |
|---|---|---|---|---|
| | | 30 | Annex 2 – Fig A.2d; revision of the definition of lack of heart beat and harmonisation with 6.4 | M. Halder on behalf the VMG |

**TABLE OF CONTENTS**

**1 PURPOSE ..... 6**
**2 SCOPE / LIMITATIONS ..... 6**
**3 METHOD OUTLINE ..... 6**
**4 LIST OF TERMS ..... 7**
4.1 Abbreviations ..... 7
4.2 Definitions ..... 7
**5 MATERIALS ..... 8**
5.1 Equipment, glass and plastic ware ..... 8
5.2 Chemicals ..... 9
5.3 Dilution water for the zebrafish embryo toxicity test ..... 9
**6 METHODS ..... 10**
6.1 Maintenance of zebrafish broodstock ..... 10
6.2 Egg production ..... 10
6.2.1 Background ..... 10
6.2.2 Egg production *via* spawning groups ..... 11
6.2.3 Egg production *via* mass spawning ..... 11
6.3 Zebrafish Embryo Toxicity Test ..... 12
6.3.1 Test concentrations and controls ..... 12
6.3.2 Exposure of fish embryos ..... 13
6.4 Determination of chemical toxicity (toxicological endpoints) ..... 15
6.5 Semi-static renewal procedure ..... 16
6.6 Measurements of test conditions ..... 16
**7 DATA ANALYSIS AND REPORTING ..... 17**
7.1 Acceptance Criteria ..... 17
7.2 Reporting ..... 17
7.3 Statistical analysis ..... 17
**8 REFERENCES ..... 18**
**ANNEX 1 ..... 20**
**ANNEX 2 ..... 28**
**ANNEX 3 ..... 31**

# 1 PURPOSE

This Standard Operation Procedure describes a Fish Embryo Toxicity test with the zebrafish (*Danio rerio*; Braunbeck *et al.,* 2005). This test is designed to determine the lethal effects of chemicals on embryonic stages of fish and constitutes an alternative test method to the acute toxicity tests with juvenile and adult fish, i.e., the OECD Test Guideline 203 (OECD TG 203, 1992), thus providing a reduction in fish usage.

# 2 SCOPE / LIMITATIONS

The method described below is for the evaluation of the Zebrafish Embryo Toxicity test (ZFET), which has been designed as an alternative to the acute fish toxicity test for chemical substances according to OECD TG 203 (OECD TG 203, 1992).

Some substances may cause delayed hatch beyond 96 hours, which will preclude the exposure of eleutheroembryos. In cases, when chemical exposure after hatch seems indispensable, other tests, e.g. OECD TG 203 (OECD TG 203, 1992), might be performed. Known examples of substances requiring prolonged exposure to the eleutheroembryos stage are quaternary ammonium salts.

# 3 METHOD OUTLINE

Zebrafish embryos are individually exposed in, e.g., 24-well microtiter plates or crystallization dishes. The main criteria for selecting the test vessels should be (a) their inertness (OECD TG 215, 2000) and (b) their volume, since the volume of test solution has to be sufficient for chemical analysis. The test is initiated immediately after fertilization and is continued for 96 hours. Lethal effects, as described by four apical observations (coagulation of the embryo, non-detachment of the tail, non-formation of somites and non-detection of the heart beat), are determined by comparison with controls to identify the $LC_{50}$ value. In addition, non-hatch will be recorded. The test method is based on using a minimum of five test concentrations as well as appropriate negative and positive controls. Each chemical is tested with 20 embryos per test concentration and controls.

# 4 LIST OF TERMS

## 4.1 Abbreviations

| | |
|---|---|
| cm | centimeters |
| °C | degree Celsius |
| d | day(s) |
| DMSO | dimethyl sulfoxide |
| Fig | figure |
| g | gram |
| h (hrs) | hour(s) |
| ISO | International Organization for Standardization |
| $LC_{50}$ | test concentration causing 50 % mortality in test organisms |
| L | liter |
| m | meter |
| M | molar |
| mg | milligram |
| min | minute |
| ml | milliliter |
| mm | millimeter |
| mM | millimolar |
| p.a. | per analysis |
| µl | microliter |
| OECD | Organisation for Economic Co-operation and Development |
| REACH | Registration, Evaluation, Authorisation and Restriction of Chemicals |
| SOP | standard operation procedure |
| tbd | to be determined |
| TG | test guideline |
| ZFET | Zebrafish fish embryo toxicity test |
| % | per cent |

## 4.2 Definitions

| | |
|---|---|
| Lethal endpoints | Lethal endpoints indicate acute toxicity to the zebrafish embryo and, consequently, death of the embryos. These are: coagulation of the embryo, non-detachment of the tail, non-formation of somites and non-detection of the heart beat. |
| Mortality | Observation of one of the above mentioned lethal endpoints indicates mortality. |
| Survival | Lethal endpoints are not observed. |

# 5 MATERIALS

## 5.1 Equipment, glass and plastic ware

- Fish maintenance tanks made of chemically inert material and of a suitable capacity in relation to the recommended loading[1];
- pH-meter;
- Oxygen meter;
- Equipment for determination of hardness of water and conductivity;
- Spawn trap:
  - instrument trays of glass, stainless steel or other inert material (e.g., L×W×H = 30 cm × 18 cm × 6 cm);
  - wire mesh of stainless steel or other inert material (e.g. grid size 2 mm) indented about 1 cm into the tray;
  - spawning substrate (e.g., plant imitates of inert material);
- Glass vessels to prepare different test concentrations and dilution water (e.g. beakers, graduated flasks, graduated cylinders, crystallisation dish) or to collect zebrafish embryos (e.g. beakers, crystallisation dish);
- Pipettes;
- Inverted microscope and/or binocular with at minimum 30-fold magnification. If the room cannot be adjusted to 26 ± 1 °C, a temperature-compensated cross movement stage is necessary (e.g. Minitüb HT 200, Tiefenbach, Germany);
- Test chambers; e.g., 24-well exposure plates (e.g. Nunc multidish Nunclon 144530; Renner TPP 92424);
- Self-adhesive foil to cover the 24-well plates (e.g. Nunc Sealing Tape SH, no. 236269) or lids provided with plates if available;
- Incubator or air-conditioned room maintained at 26 ± 1 °C;
- Pasteur pipettes to collect eggs.

[1] cf. section 6.1

## 5.2 Chemicals

Table 1. List of chemicals

| Name [formula] | CAS nº. | Purity | Supplier | Catalog nº. |
|---|---|---|---|---|
| 3,4-Dichloroaniline [$Cl_2C_6H_3NH_2$] | 95-76-1 | 99 | Sigma-Aldrich (Fluka Pestanal® analytical standard) | 35827 |
| Calcium chloride dehydrate [$CaCl_2 2\ H_2O$] | 10035-04-8 | p.a. | e.g., Merck | 1.02382.0500 |
| Magnesium sulfate heptahydrate [$MgSO_4 7\ H_2O$] | 10034-99-8 | p.a. | e.g., Merck | 1.05886.0500 |
| Sodium carbonate [$NaHCO_3$] | 144-55-8 | p.a. | e.g., Merck | 1.06329.0500 |
| Potassium chloride [KCl] | 7447-40-7 | p.a. | e.g., Merck | 1.04936.0500 |
| Hydrochloric acid [HCl] | 7647-01-0 | p.a. | e.g., Merck | 1.09063.1000 |
| Sodium hydroxyde [NaOH] | 1310-73-2 | p.a. | e.g., Merck | 1.09136.1000 |

## 5.3 Dilution water for the zebrafish embryo toxicity test

For the zebrafish embryo toxicity test (ZFET), dilution water is prepared according to OECD TG 203 Annex 2 (1992):

- 294.0 mg/L $CaCl_2 2\ H_2O$;
- 123.3 mg/L $MgSO_4 7\ H_2O$;
- 64.7 mg/L $NaHCO_3$;
- 5.7 mg/L KCl.

The resulting degree of total hardness should be equivalent to 10 - 250 mg/L. The water is aerated until oxygen saturation is achieved, then stored for about two days without further aeration before use. The pH should be adjusted to a range between pH 6.5 and 8.5. Use of HCl and NaOH is recommended. The conductivity of the distilled or deionized water used for preparing the dilution water should not excess 10 µS/cm.
Dilution water temperature should be 26.0 ± 1.0 °C when used for preparation of test concentrations/controls.

Table 2: Preparation of dilution water

| Stock solution | Compound | | Distilled or deionized water volume | Add | Final volume |
|---|---|---|---|---|---|
| 1 | $CaCl_2$ 2 $H_2O$ | 14.700 g | 500 ml | 10 ml | 1 L |
| 2 | $MgSO_4$7 $H_2O$ | 6.165 g | 500 ml | 10 ml | |
| 3 | $NaHCO_3$ | 3.235 g | 500 ml | 10 ml | |
| 4 | KCl | 0.285 g | 500 ml | 10 ml | |

Stock solutions are 100fold concentrated in comparison to concentrations finally used in the test; therefore solutions must be diluted by the factor 100. For 1 liter dilution water in the fish embryo test, 10 ml of each stock solution are required.

# 6 METHODS

## 6.1 Maintenance of zebrafish broodstock

A breeding stock of unexposed, mature zebrafish with an age between 4 and 18 months is used for egg production. Each laboratory should precisely specify strain, origin of the strain, duration of maintenance in the particular laboratory and reproductive performance (fecundity, standard fertilization rate). In any case, on a regular basis (at least, each 6 months), the $LC_{50}$ of the standard positive control 3,4-dichloroaniline (see Table 1) should be determined[2], and the $LC_{50}$ should range between 1.6 and 4.4 mg/L after 48 hpf (Braunbeck *et al.*, 2005; Lange *et al.*, 1995; Schulte, 1997).

Fish should be free of macroscopically discernable symptoms of infection or disease and should not have been treated with any pharmaceutical (acute or prophylactic) treatment for 2 months before spawning. Spawners are maintained in aquaria with a loading capacity of a minimum of 1 L water per fish and a fixed 12-16 hour light photoperiod (Braunbeck *et al.*, 2005; Nagel, 2002; Schulte and Nagel, 1994; Laale, 1977; Westerfield, 2000). Males and females are continuously held together. Oxygen saturation ≥ 80 % should always be maintained for keeping and breeding; water temperature should be adjusted to 26 ± 1 °C. Optimal filtering rates should be adjusted; excess filtering rates causing heavy perturbation of the water should be avoided. Alternatively, permanent flow-through or semi-static conditions may be used to guarantee that ammonia, nitrite, and nitrate levels are kept below the critical limit for toxicity (0 - 5, 0.025 - 1 and 0 - 140 mg/L, respectively). Fish are fed with commercially available artificial diets (e.g., TetraMin™ flakes; Tetra, Melle, Germany) at regular intervals (e.g. 3 to 5 times daily would be optimal), occasionally supplemented with brine shrimp (*Artemia* spec.) nauplii or small daphnids of appropriate size obtained from an uncontaminated source. Overfeeding should be strictly avoided to ensure optimal water quality; remaining food and feces should be removed daily. From three days before spawning, feeding with brine shrimp (*Artemia* spec.) twice daily (*ad libitum*) is recommended to achieve optimal mating.

## 6.2 Egg production

### 6.2.1 Background

Under spawning conditions, male zebrafish can easily be distinguished from females by their more slender body shape and an orange to reddish tint in the silvery bands along the body. Due to the large number of eggs produced, females can be recognized by their swollen bellies (Fig. 1). Egg production can be performed via spawning groups (6.2.2) or mass spawning (6.2.3).

[2] Not applicable for phase 2b

A single mature female spawns at least 50 - 80 eggs per day. Depending on the strain, spawning rates may be considerably higher. The fertilization rate should be ≥70 %. In case first time spawning fish are used, fertilization rates may be lower in the first few spawns.


Fig. 1: Sexually mature zebrafish (*Danio rerio*). Adult zebrafish females (upper individual) can easily be differentiated from males (lower individual) by their extended bellies and the lack of reddish tint along the silvery longitudinal lines. Photo: Erik Leist, Heidelberg.

### 6.2.2 Egg production *via* spawning groups

*Note: Annex 3 describes egg production in spawning groups as used at the University of Heidelberg (Germany). A more general description is given in the following.*

The day before a test, males and females are placed in spawning tanks a few hours before the onset of darkness. Since spawning groups of zebrafish may occasionally fail to spawn, the parallel use of at least three spawning tanks is strongly recommended.

For collection of eggs, trays covered with a grid are placed into the spawning tanks before the onset of darkness. If considered necessary, artificial plants made of green plastic or glass can be fixed to the grid as spawning stimulus. Mating, spawning and fertilization take place within 30 min after the onset of light in the morning and the egg trays can be carefully removed.

For selection of fertilized eggs see 6.3.2.1.

### 6.2.3 Egg production *via* mass spawning

Alternatively, eggs may be collected with larger trays covered with a grid. They are placed at the bottom of the normal maintenance tanks before the onset of darkness. If considered necessary, artificial plants made of green plastic or glass can be fixed to the grid as spawning stimulus. Mating, spawning and fertilization take place within 30 min after the onset of light in the morning and the egg trays can be carefully removed.

For selection of fertilized eggs see 6.3.2.1.

## 6.3 Zebrafish Embryo Toxicity Test

### 6.3.1 Test concentrations and controls

*NOTE: The following describes the general procedures.* ***For the validation study, the procedures given in the trial plan should be followed, e.g. preparation of stock solutions, test concentrations, use of solvent, negative and positive controls, number of runs.***

Chemicals should be tested in 5 concentrations spaced by a constant factor not exceeding 2.2 and prepared as dilutions with standard dilution water (see 5.3). Test solutions of the selected concentrations can be prepared, e.g., by dilution of a stock solution. The stock solutions should preferably be prepared by simply mixing or agitating the test substance in the dilution water by mechanical means (e.g., stirring or ultrasonification). If the test substance is difficult to dissolve in water, procedures described in the OECD Guidance Document No. 23 for handling difficult substances should be followed (OECD GD 23, 2000). The use of solvents or dispersants (solubilizing agents) should, if ever possible, be avoided, but may be required in some cases in order to produce a suitably concentrated stock solution. Additionally to the examples of suitable solvents given in OECD (OECD TG 215, 2000), dimethyl sulfoxide (DMSO) might be useful. In case a solubilizing agent is required to assist in stock solution preparation, its final concentration should not exceed 1000 µl/L for most of the commonly used solvents. The solvent concentration should be the same in all test vessels. In case a solvent has to be used, a separate solvent control has to be run (see trial plan).

Justification should be provided if fewer than five concentrations are used. The highest concentration tested should preferably result in 100% mortality, and the lowest concentration tested should preferably give no observable effect. A range-finding test properly conducted before the definitive test enables the choice of the appropriate concentration range.

Pure dilution water is used as a negative control. Negative controls are required both as internal and as external controls. For localization of negative controls, see trial plan.

As a positive control, 3,4-dichloroaniline should be tested at a concentration of **4mg/L**.

Each chemical is tested with 20 eggs/embryos per test concentration and controls.

There should be evidence that the concentration of the substance being tested has been satisfactorily maintained, and preferably it should be at least 80 % of the nominal concentration throughout the test[3]. If the deviation from the nominal concentration is higher than 20 %, results should be based on the measured concentration.

[3] Not mandatory in Phase 2b.

### 6.3.2 Exposure of fish embryos

***NOTE: The 24-well plates and glass vessels must be pre-saturated with the respective concentrations of test substances and controls for <u>at least</u> 24 hrs before the day of the test. Glass vessels and 24-well plates are filled with the required quantity of freshly prepared test concentrations (freshly = prepared on the same day) and respective controls (see 6.3.2.1; 6.3.2.2 and trial plan).***

In order to start exposure with minimum delay, at least twice of the number of eggs needed per treatment group (see 6.3.2.2) are randomly selected and transferred not later than 1 h post fertilization, into glass vessels containing an appropriate volume (e.g. 50 ml; eggs should be fully covered) of the different test concentrations and respective controls. Viable fertilized eggs should be separated from unfertilized eggs (see 6.3.2.1) and be transferred to 24-well plates within 3 h post fertilization (Fig. 2).

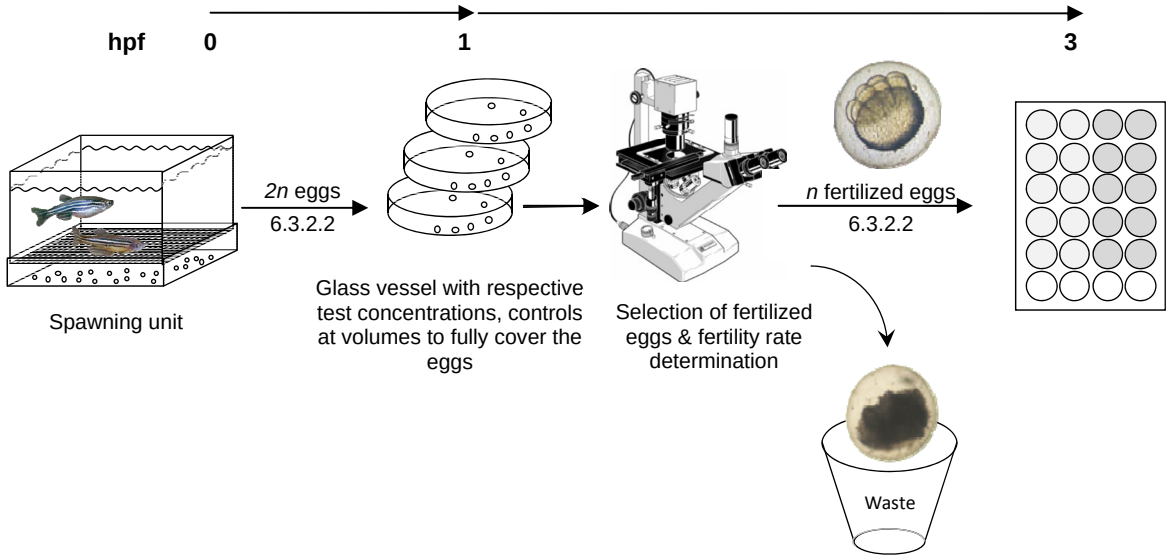


Fig. 2: Scheme of the ZFET test procedure (from left to right): collection of the eggs, pre-exposure immediately after fertilization in glass vessels, selection of fertilized eggs with an inverted microscope or binocular and distribution of fertilized eggs into prepared 24-well plates, n = number of eggs required for the test run.

#### 6.3.2.1 Selection of fertilized eggs

The glass vessels containing the eggs (as described in 6.3.2.) are placed under an inverted microscope or a binocular with a minimum magnification of 25× to identify fertilized eggs and determine the fertility rate. Fertilized eggs can easily be identified by their transparency (see Fig 3), at best by putting the glass vessels on a black pad and using flexible swan neck lights or transverse light under the binocular.

In the following, details on the appearance of developmental stages critical for the identification of fertilized eggs are given (see also Annex 1):

- Freshly spawned eggs are characterized by a fully transparent perivitelline space surrounded by the egg membrane and containing the yolk, and the germinal disc, which has already formed at the animal pole.
- After fertilization, the first cell division is initiated at 26 °C after about 15 min.
- From the 4-cell stage onwards, fertilized eggs can unambiguously be distinguished by their transparency from non-fertilized eggs.

- Eggs with overt anomalies (asymmetries, formation of vesicles) or damaged membranes should be discarded.
- Non-fertilized eggs can be identified by a lack of blastomer formation and, at later stages, by their non-transparency.

*NOTE: For the ZFET, only fertilized eggs between the 4- and 128-cell stages should be used.*

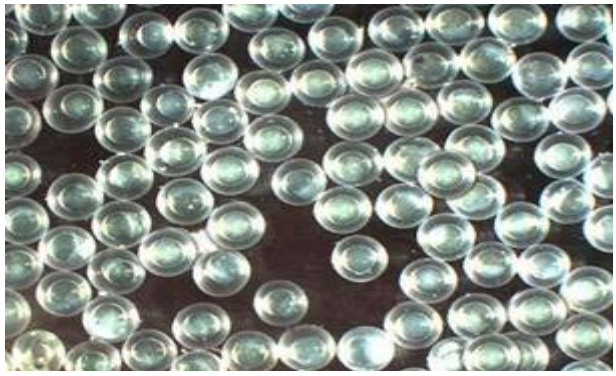

Fig. 3: Batch of newly spawned zebrafish (*Danio rerio*) eggs. Photo: Dr. T. Meinelt, Institute of Freshwater Ecology and Inland Fisheries, Berlin, FRG.

### 6.3.2.2 Distribution of eggs over 24-well plates

Fertilized eggs are individually transferred to the freshly prepared 24-well plates (final volume of 2 ml per well) and distributed as given in the trial plan (TP_ZFET_OECD_2b_V01):

- 20 eggs for each test concentration (for each concentration one plate)
- 20 eggs as solvent control on one plate;
- 20 eggs as positive control on one plate;
- 4 eggs as negative internal control on each of the above plates

- 24 eggs as negative external control on one plate

### 6.3.2.3 Incubation conditions

The 24-well plates are covered with self-adhesive foil or lids provided with plates and incubated at 26 ± 1 °C for 96 hrs. Control of the light cycle (12 – 16 hours) is achieved by keeping the eggs in either an incubator or separate room equipped with an automatic light control.

## 6.4 Determination of chemical toxicity (toxicological endpoints)

The following four endpoints indicate acute toxicity and, consequently, death of the embryos:

- coagulation of the embryo,
- non-detachment of the tail,
- non-formation of somites and
- non-detection of the heart beat.

These lethal endpoints are recorded after 24, 48, 72 and 96 hrs as listed in Table 3.

*NOTE: Observation of <u>one</u> of the above mentioned lethal endpoints indicates mortality.*

Table 3: Lethal endpoints and their recording in the Zebrafish Embryo Toxicity Test (ZFET)

| | Exposure time (h) | | | |
|---|---|---|---|---|
| | 24 | 48 | 72 | 96 |
| Coagulated embryos | + | + | + | + |
| Tail not detached | + | + | + | + |
| No somite formation | + | + | + | + |
| No heart beat | | + | + | + |

*Coagulation of the embryo:* Coagulated embryos are milky white and appear dark under the microscope (See Annex 2, Fig. A2a). The number of coagulated embryos is determined after 24, 48, 72 and 96 hrs.

*Tail not detached:* In a normal developing zebrafish embryo, detachment of the tail (see Annex 2, Fig. A2b) from the yolk is observed following posterior elongation of the embryonic body. Absence of tail detachment is recorded after 24, 48, 72 and 96 hrs.

*No somite formation:* At 26 ± 1°C, about 20 somites have formed after 24 hours (see Annex 2, Fig. A2c) in a normal developing zebrafish embryo; however, it is not possible to determine the exact number at this time (spontaneous movements indicate the formation of somites). A normally developed embryo shows spontaneous movements (side-to-side contractions). The absence of somites is recorded after 24, 48, 72 and 96 hrs.

*No heart beat:* In a normal developing zebrafish embryo at 26 ± 1 °C, the heart beat is visible after 48 hrs (see Annex 2, cf. Fig. A2d). Absence of heart beat is recorded after 48, 72 and 96 hrs. Particular care should be taken when recording this endpoint, since irregular (erratic) heart-beat should *not* be recorded as lethal. Moreover, visible heart beat without circulation in aorta abdominalis is considered non-lethal. The observation time to record an absence of heart beat should be at least of 1 min with a minimum magnification of 80×.

*Hatching rate and post-hatch mortality:* Since zebrafish embryos usually hatch after 72 hrs, non-hatching may represent an important toxic effect. However, since the time to hatch may differ between test concentrations, controls may have already hatched, whereas embryos exposed to the test concentration may still have not. Hatching rates will not be used for the calculation of $LC_{50}$ values. In case of abnormal hatching time, hatching rates should be recorded until 96 hrs. Post-hatch mortality will be covered by observing the above defined lethal endpoints until 96 hours.

*NOTE: In addition to the lethal endpoints, other observations should be recorded in the reporting template under "remarks".*

## 6.5 Semi-static renewal procedure

*NOTE: The following steps are carried out **after** the daily recording of the lethal effects (see 6.4)*

Renewal of the test solutions **and** the controls must be performed after 24, 48, 72 hours:

- Test concentrations are freshly prepared from the stock solution (see *NOTE* 6.3.2)
- Solutions are removed by using an appropriate pipette or vacuum suction (cell culture-fitted vacuum pump plus suction bottle). For the removal of *each* test concentration, separate pipette tips must be used.

*NOTE: In any case, contact with the eggs must be avoided!*

- At least 90% of the volume of each well must be removed and immediately replaced with the corresponding volume of freshly prepared test solutions/controls.

## 6.6 Measurements of test conditions

Measurements of test conditions should be performed at least on the following time points:

- 0 hour
- 24 hours (old solution)
- 72 hours (fresh renewal solution)
- 96 hours

for the controls and the highest concentration.

The following parameters should be measured by using microprobes or carefully pooling test solutions (e.g., by pipetting):

- The dissolved oxygen concentration should be in compliance with the test requirements (see 7.1).
- The pH should normally be within a range of pH 6.5 and 8.5.
- The total hardness should be within 10 to 250 mg/l (OECD TG 203, 1992).
- The temperature and the conductivity

If the equipment is available, the light intensity can be measured at least once.

The results must be recorded in the corresponding section of reporting template (see 7.2*).*

# 7 DATA ANALYSIS AND REPORTING

## 7.1 Acceptance Criteria

For a test to be considered to fulfill the performance requirements, the following conditions should apply:

(1) The fertility rate of the parent generation should be ≥ 70%.
(2) The dissolved oxygen concentration should be ≥ 80 % of the air saturation value at the beginning of the test.
(3) The water temperature should be maintained at 26 ± 1 °C in test chambers at any time during the test.
(4) Overall survival of embryos in the negative external control and, where relevant, in the solvent control should be ≥ 90% until the end of exposure.
(5) Exposure to the positive control (e.g., 4.0 mg/L 3,4-dichloroaniline) should result in a minimum mortality of 30 % at the end of the exposure.
(6) Test solutions must be renewed on a daily basis (see 6.5).

**Note: If acceptance criteria are not met, the test is considered to be failed and needs to be repeated.**

**Note: If more than 1 dead embryo is observed in the internal negative control the plate might be rejected.**

## 7.2 Reporting

A template for reporting will be provided for each phase of the study by the study coordinator: *RT_ZFET_OECD_2b_V01.0_Laboratory code_Chemical_Run*

Each laboratory should use the template to report the results of valid and failed experiments. The file should be returned to the study coordinator. Printed and signed originals should be archived by the laboratories.

## 7.3 Statistical analysis

The $LC_{50}$ determination and statistical evaluation will be carried out by Greg Carr.

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# – ANNEXES –

## ANNEX 1:

## Table and atlas of normal zebrafish development

## ANNEX 2:

## Atlas of lethal endpoints for the Zebrafish Embryo Toxicity Test

## ANNEX 3:

## Egg production in spawning groups as performed at University of Heidelberg

# ANNEX 1

## Table and atlas of normal zebrafish development

Freshly spawned eggs are characterized by a fully transparent perivitelline space surrounded by the egg membrane and containing the yolk, and the germinal disc, which has already formed at the animal pole. After fertilization, the first cell division is initiated at 26 °C after about 15 min. Subsequently, the germinal disc is divided synchronously into 4, 8, 16 and 32 blastomers after 1 h, 1,25 h, 1,5 h and 1,75 h (Table A1; Figs. A1a, A1c; Kimmel *et al*., 1995). From the 4-cell stage onwards, fertilized eggs can unambiguously be distinguished by their transparency from non-fertilized eggs. For the ZFET, only fertilized eggs between the 4- and 128-cell stages should be used. Eggs with overt anomalies (asymmetries, formation of vesicles) or damaged membranes should be discarded. Non-fertilized eggs can be identified by a lack of blastomer formation and, at later stages, by their non-transparency.

Table A1: Stages of embryonic development of zebrafish (*Danio rerio*) at 26 ± 1 °C (Nagel, 2002)

| **Time (h)** | **Stage** | **Characterization (after Kimmel *et al.*, 1995)** |
|---|---|---|
| 0 | Fertilization | Zygote |
| 0 | Zygote period | Cytoplasm accumulates at the animal pole, one-cell stage |
| 0.75 | Cleavage period | Discoidal partial cleavage: |
| | | 1. (median vertical) division: two-cell-stage |
| 1 | | 2. (vertical) division: four-cell-stage |
| 1.25 | | 3. (vertical and parallel to the plane of the first) division: 8-cell-stage |
| 1.5 | | 4. (vertical and parallel to the second) division: 16-cell-stage |
| 2 | Blastula period | Start of blastula stage |
| 3 | | Late cleavage; blastodisc contains approximately 256 blastomers |
| 4 | | Flat interface between blastoderm and yolk |
| 5.25 | Gastrula period | 50 % of epibolic movements; blastoderm thins and interface between periblast and blastoderm become curved |
| 8 | | 75 % of epibolic movement |
| 10 | | Epibolic movement ends, blastopore is nearly closed |
| 10.5 | Segmentation period | First somite furrow |
| 12 | | Somites are developed, undifferentiated mesodermal component of the early trunk, tail segmented or metameric |
| 20 | | Muscular twitches; sacculus; tail well extended |
| 22 | | Side to side flexures; otoliths |
| 24 | Pharyngula period | Phylotypic stage, spontaneous movements, tail is detached from the yolk; early pigmentation |
| 30 | | Reduced spontaneous movement; retina pigmented, cellular degeneration of the tail end; circulation in the aortic arch 1 visible |
| 36 | | Tail pigmentation; strong circulation; single aortic arch pair, early motility; heart beating |
| 72 - 96 | Hatching period | Heart-beat regular; yolk extension beginning to taper; dorsal and ventral pigmentation stripes meet at tail; segmental blood vessels detectable: thickened sacculus with two chambers visible; foregut development; neuromasts |

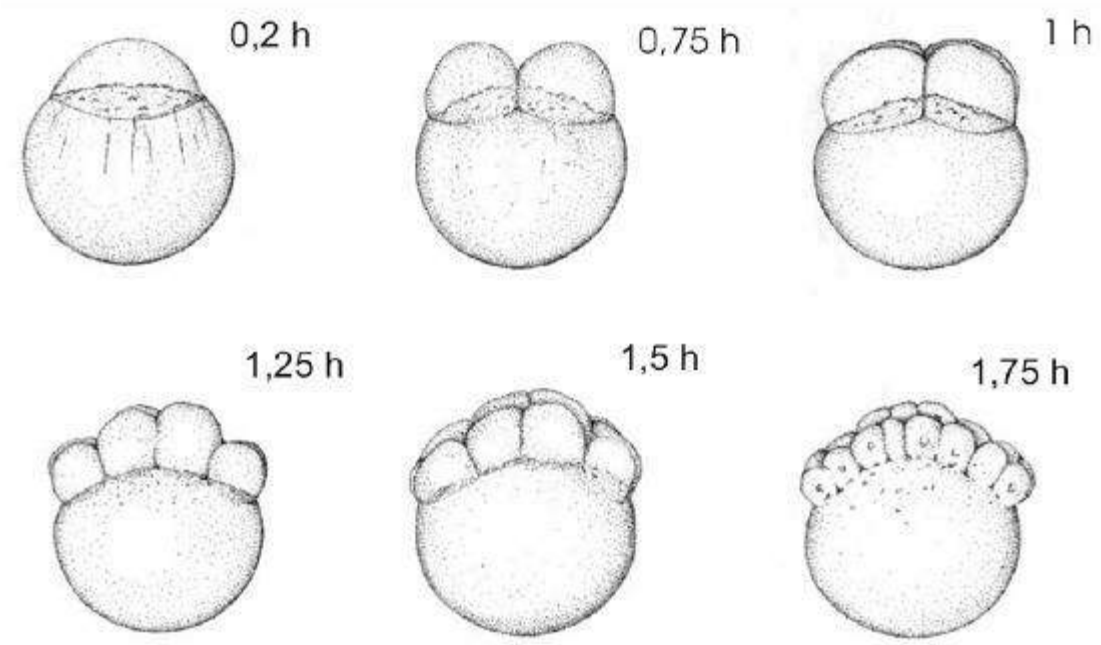


Fig. A1a: **Selected stages of early zebrafish (*Danio rerio*) development**: 0.2 – 1.75 h post-fertilization (from Kimmel *et al.*, 1995). The time sequence of normal development may be taken to diagnose both fertilization and viability of eggs (see paragraph 6.3.2.1: Selection of fertilized eggs).

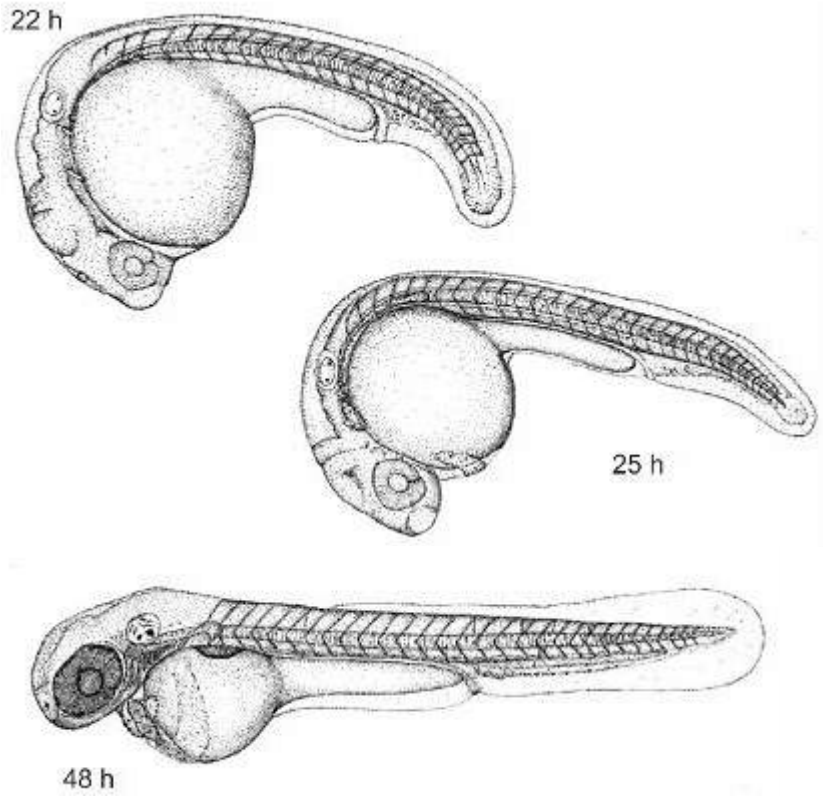


Fig. A1b: **Selected stages of late zebrafish (Danio rerio) development**: 22 - 48 h after fertilization (from Kimmel *et al.*, 1995).

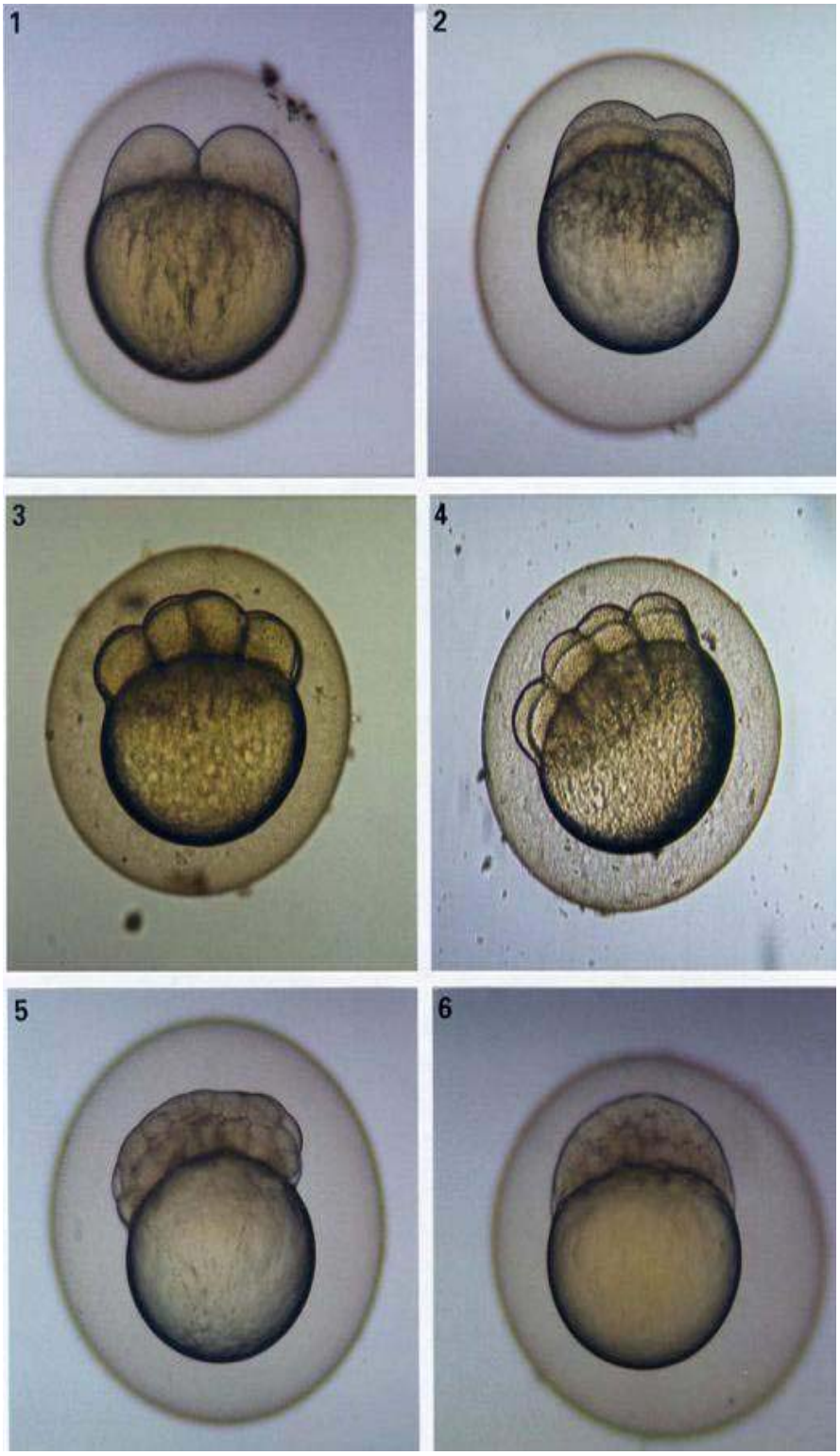


Fig. A1c: **Normal development of zebrafish (*Danio rerio*) embryos** I: (1) 0.75 h, 2-cell stage; (2) 1 h, 4-cell stage; (3) 1.2 h, 8-cell stage; (4) 1.5 h, 16-cell stage; (5) 4.7 h, beginning epiboly; (6) 5.3 h, approx. 50 % epiboly (from Braunbeck & Lammer 2005).

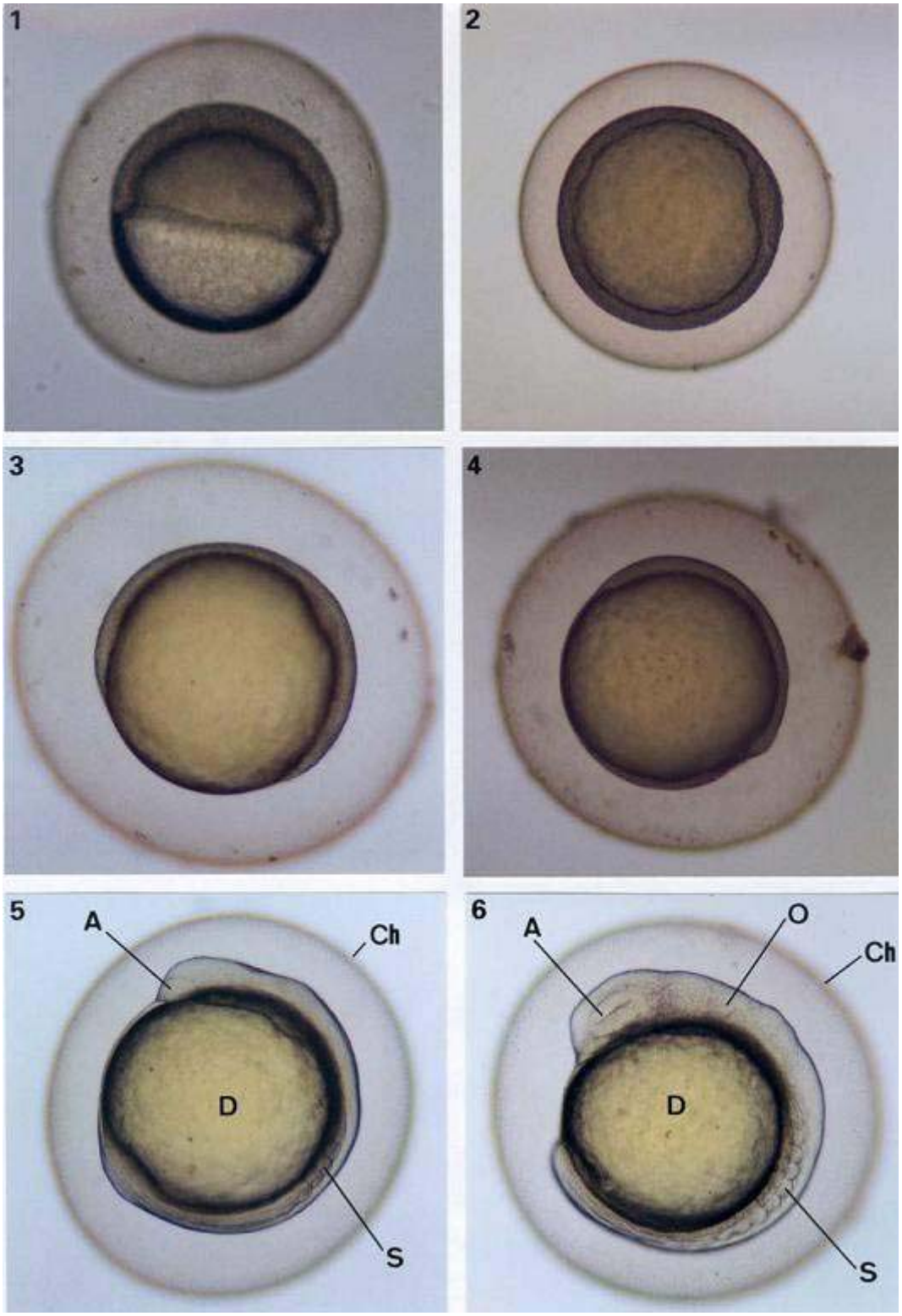


Fig. A1d: **Normal development of zebrafish (*Danio rerio*) embryos** II: (1) 6 h; (2) 6 h; (3) 8 h; (4) 9 h; (5) 12 h; (6) 14 h. A – eye bud; Ch – chorion; D – yolk; O – ear bud; S – somites (muscle segments; from Braunbeck & Lammer 2005).

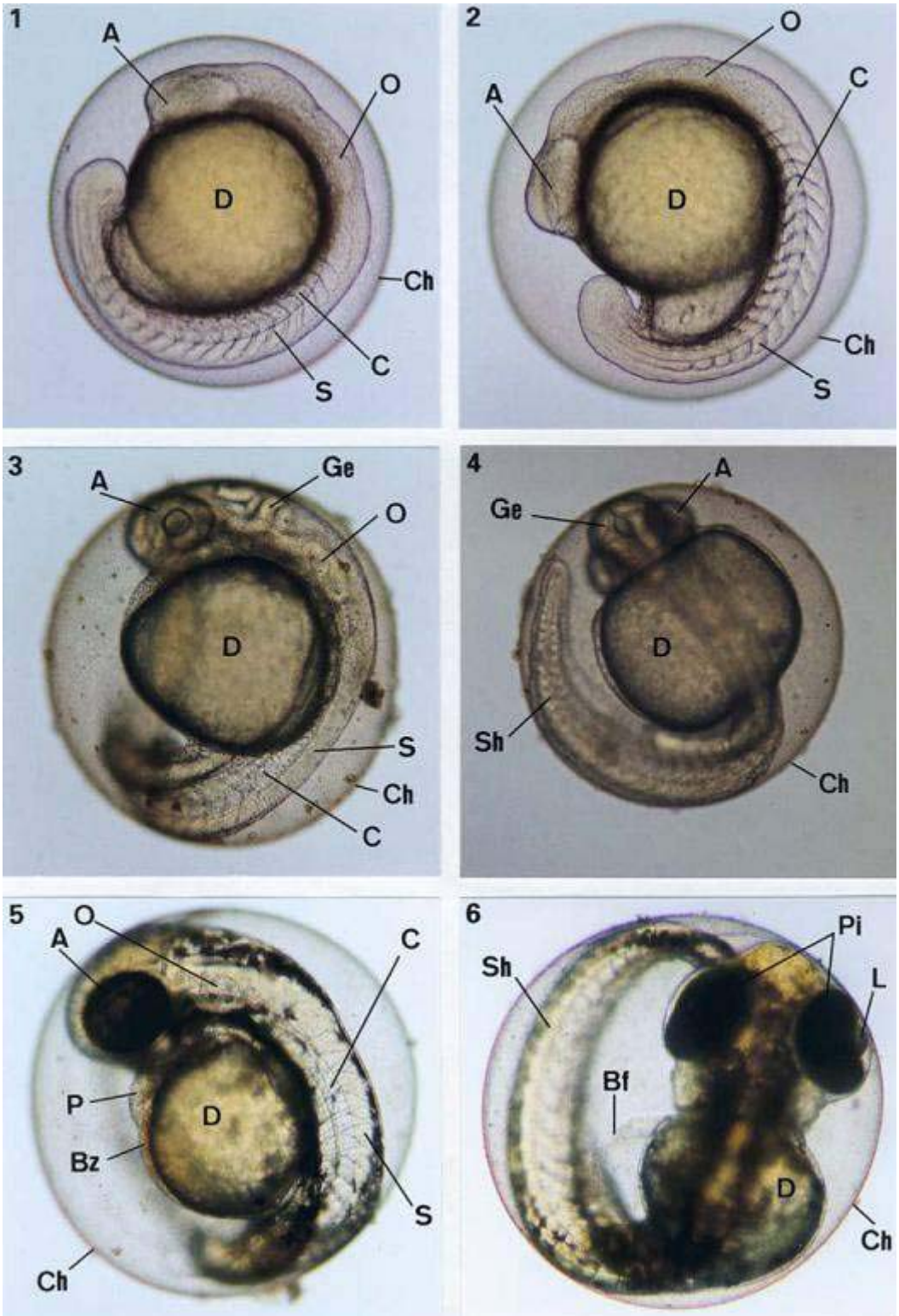


Fig. A1e: **Normal development of zebrafish (*Danio rerio*) embryos** III: (1) 16 h; (2) 18 h; (3) 25 h; (4) 25 h; (5) 48 h; (6) 72 h. A – eye bud; Bf – pectoral fin; Bz – blood cells; C – chorda; Ch – chorion; D – yolk; Ge – brain; L – lens; P – pericardium; Pi – ocular pigment layer; S – Somites; Sh – tail; O – ear bud (from Braunbeck & Lammer 2005).

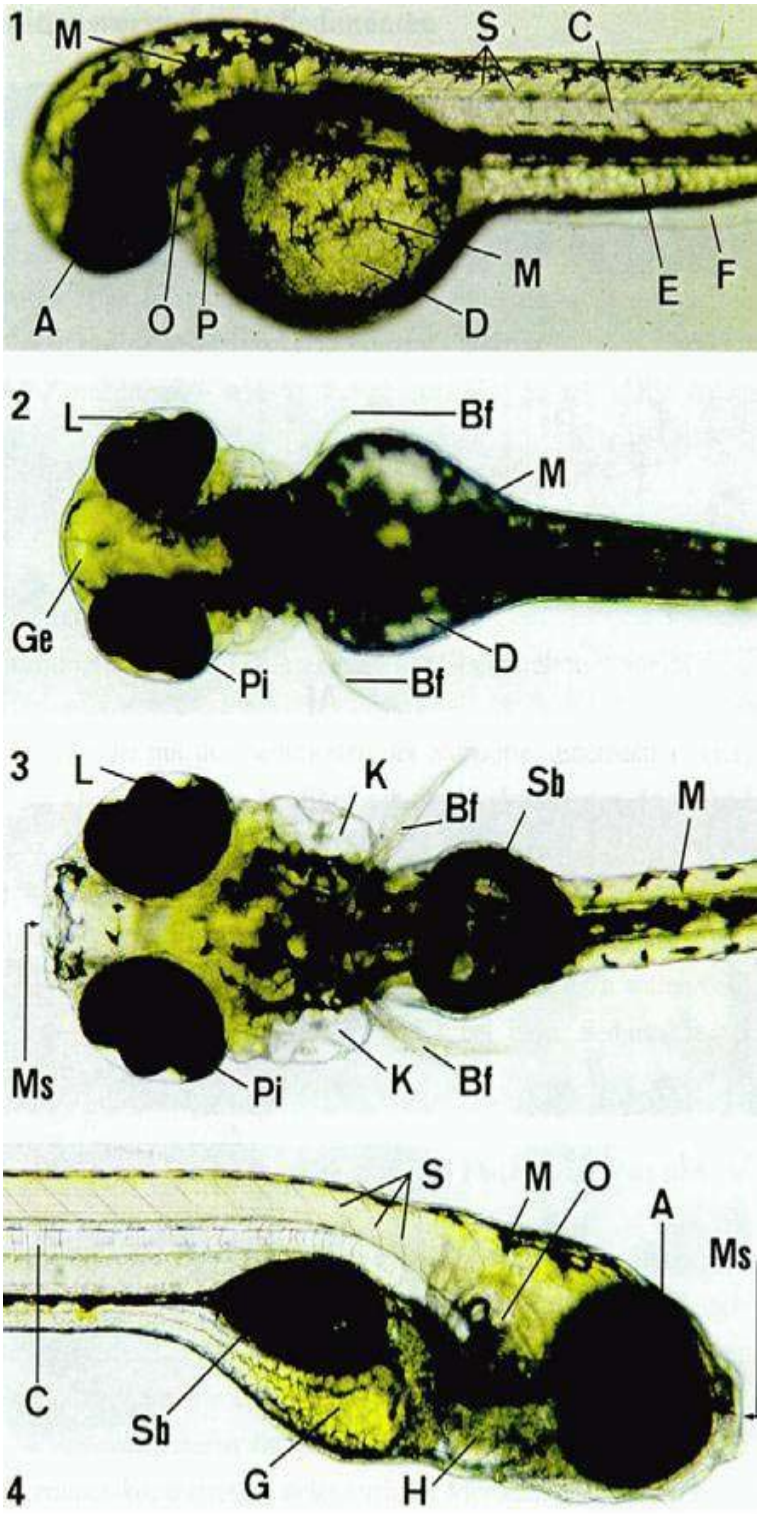


Fig. A1f: **Normal development of zebrafish (*Danio rerio*) embryos** IV: (1) 48 h; (2) 72 h; (3) 144 h; (4) 144 h. A – eye bud; Bf – pectoral fin; C – chorda; D – yolk sac; E – gut; F – fin; G – gastrointestinal tract, Ge – brain; H – heart; K – gills; L – eye lens; M – melanophores; Ms – mouth slit; O – ear; P – pericardium; Pi – ocular pigment layer; S – somites (muscle segments); Sb – swimming bladder (from Braunbeck & Lammer 2005).

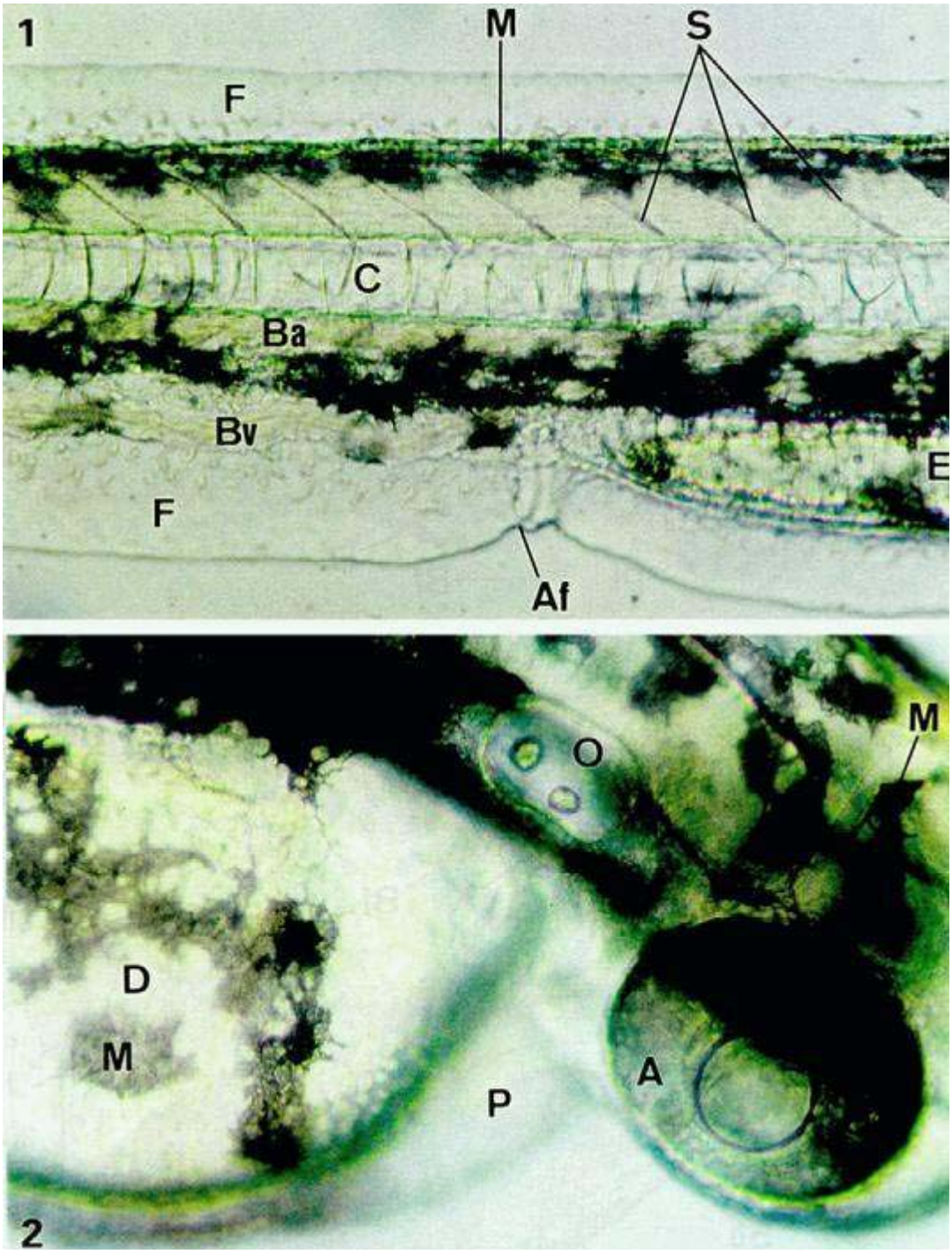


Fig. A1g: **Normal development of zebrafish (*Danio rerio*) embryos** V (following dechorionation): (1) 48 h, anal region; (2) 48 h, ear region. A – eye bud; Af – anus; Ba – dorsal aorta; Bv – central ventral axial vein; C – chorda; D – yolk sac; E – peritoneum; F – fin; M – melanophores; P – pericardium; O – ear; S – somites (muscle segments; from Braunbeck & Lammer 2005).

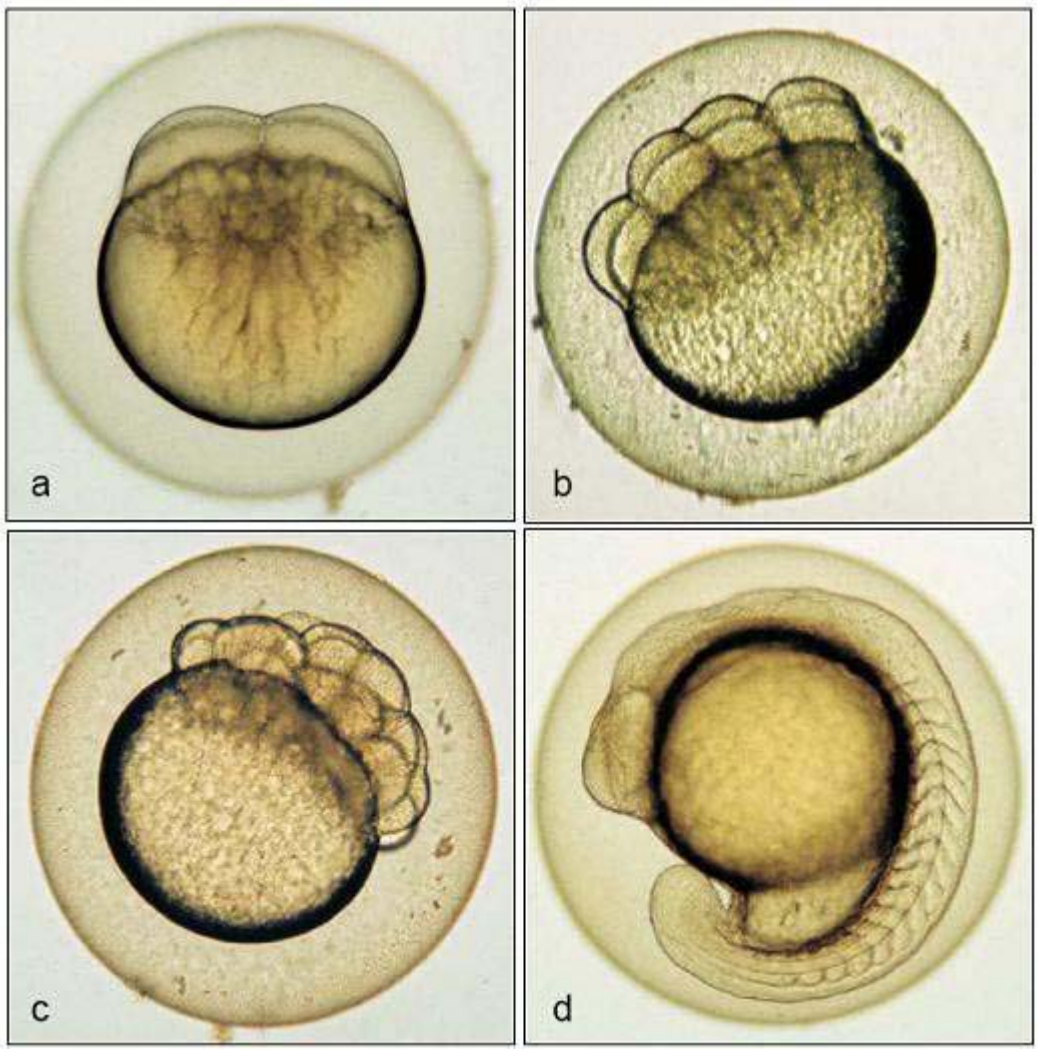


Fig. A1h: **Selected stages of zebrafish (*Danio rerio*) development**: (a) 4-cell stage (approx. 1 h); (b) 16-cell stage (approx. 1.3 h); (c) 64-cell stage (approx. 1.8 h); (d) detachment of tail (approx. 17.5 h; from Braunbeck & Lammer 2005).

# ANNEX 2

## Atlas of lethal endpoints for the Zebrafish Embryo Toxicity Test

The following apical endpoints indicate acute toxicity and, consequently, death of the embryos: coagulation of the embryo, non-detachment of the tail, non-formation of somites and non-detection of the heartbeat. The following micrographs have been selected to illustrate these endpoints.

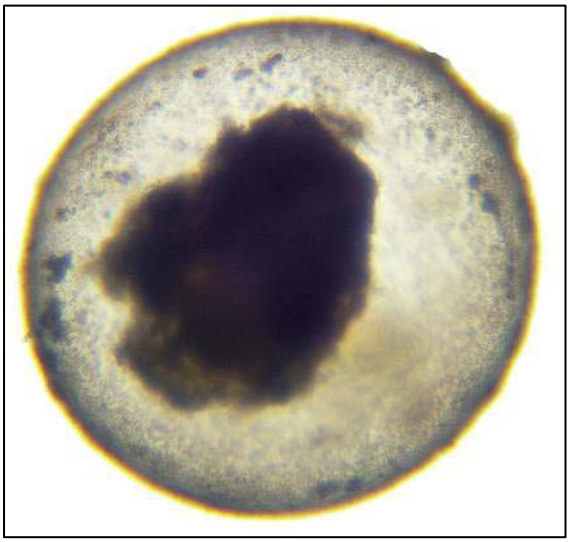

Fig. A2a: **Coagulation of the embryo**: Under bright field illumination, coagulated zebrafish embryo show a variety of intransparent inclusions.

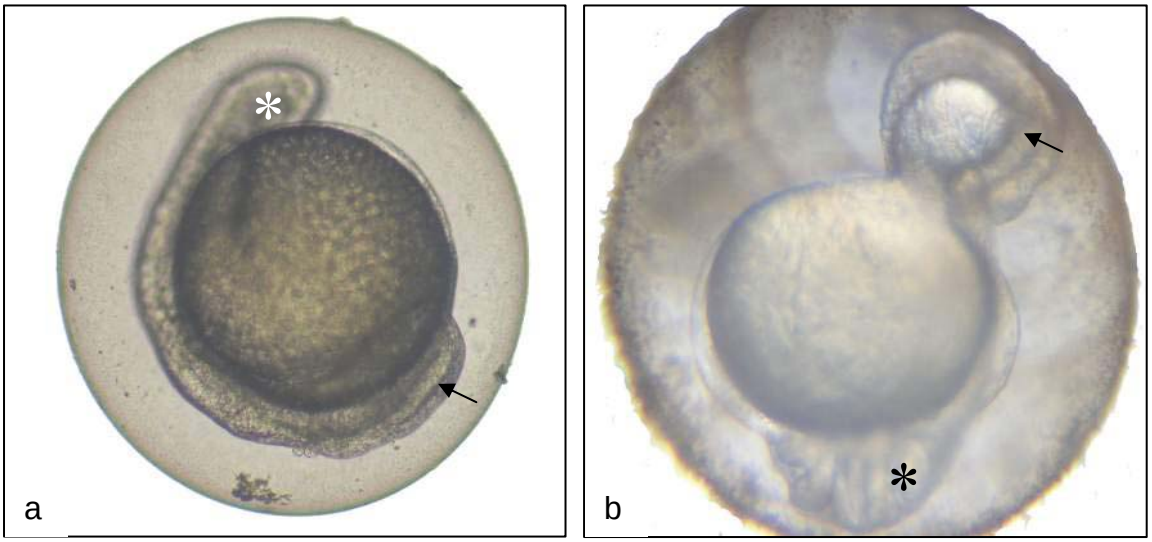


Fig. A2b: **Non-detachment of tail** bud in lateral view (a: →; 96 h old zebrafish embryo) and frontal rear view (b: →; 96 h old zebrafish embryo). Note also the lack of the eye bud (*).

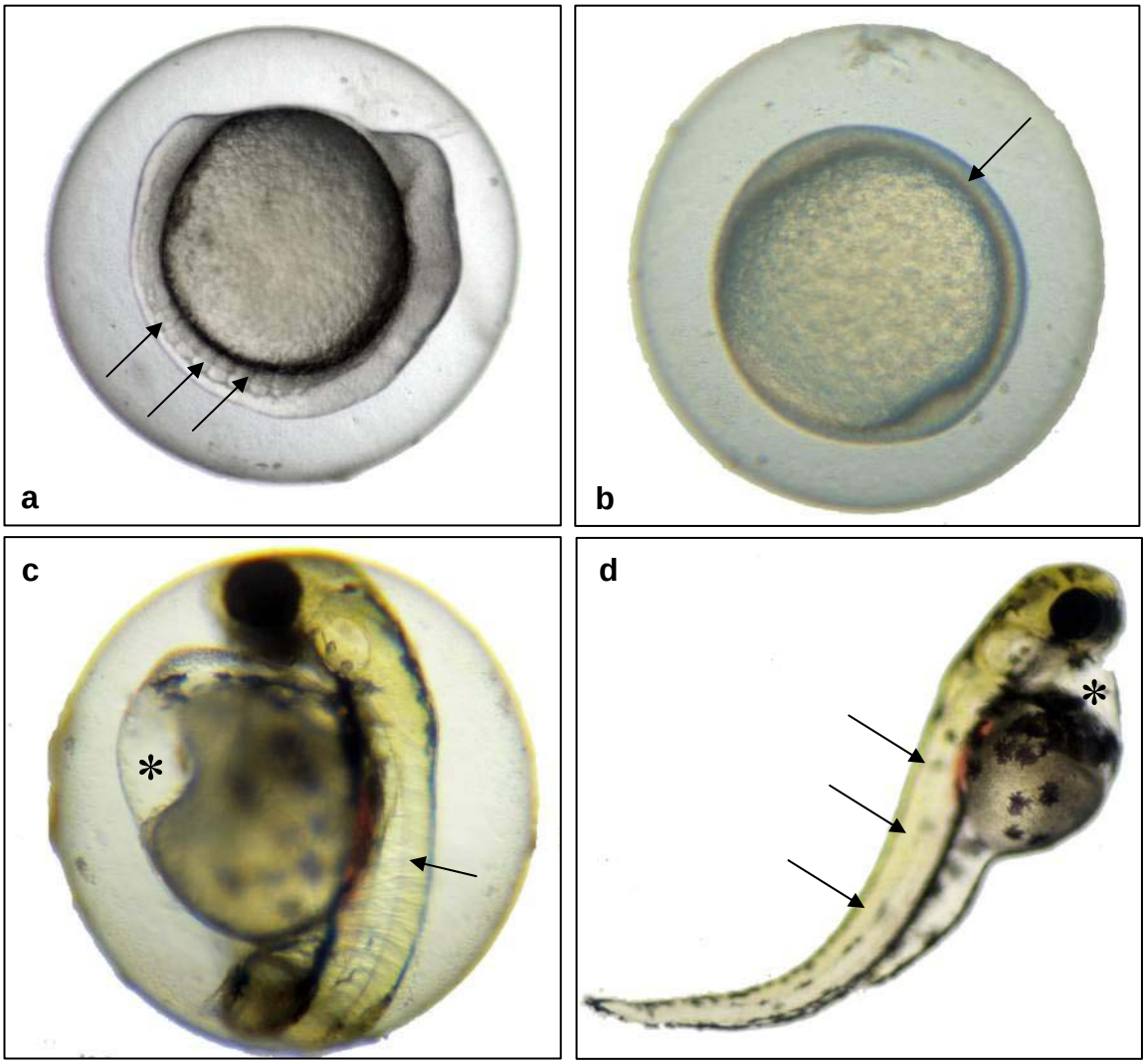


Fig. A2c: **Non-formation of somites**: Although retarded in development by approx. 10 h, the 24 h old zebrafish embryo in (a) shows well-developed somites (a: →), whereas the embryo in the right micrograph does not show any sign of somite formation (b: →). Although showing a pronounced yolk sac edema (*), the 48 h old zebrafish embryo in (c) shows distinct formation of somites (→), whereas the 96 h (!) old zebrafish embryo depicted in (d) does not show any sign of somite formation (→). Note also the spinal curvature (scoliosis) and the pericardial edema in the embryo shown in Fig. (d), see also figure A2d.

.

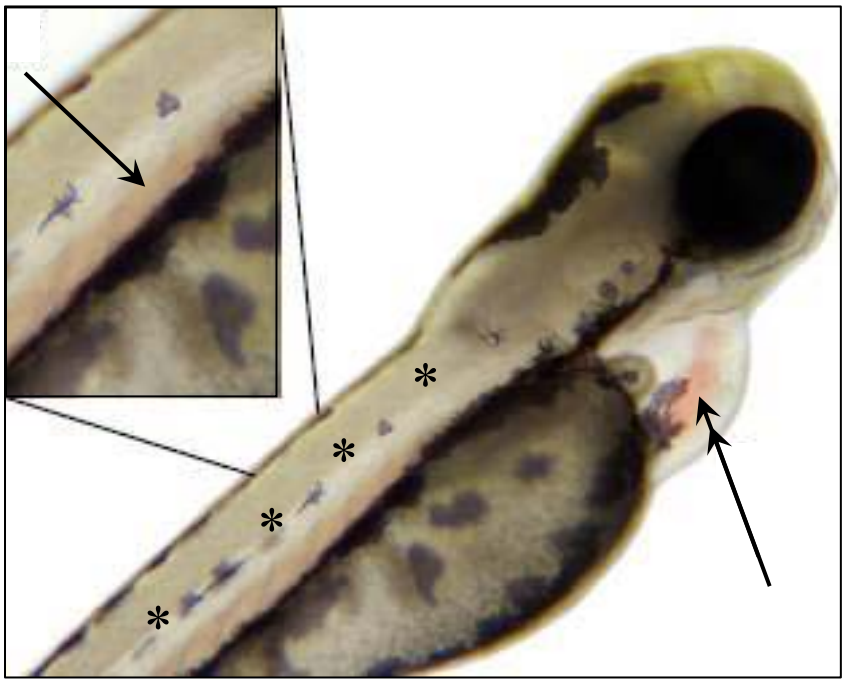

Fig. A2d: **Lack of heart beat** is, by definition, difficult to illustrate in a micrograph. Lack of heart beat is indicated by non-convulsion of the heart (double arrow). Immobility of blood cells in, e.g., the aorta abdominalis (→ in insert) is not an indicator for lack of heart beat. Note also the lack of somite formation in this embryo (*, homogenous rather than segmental appearance of muscular tissues). The observation time to record an absence of heart beat should be at least of 1 min with a minimum magnification of 80×.

# ANNEX 3

## Egg production in spawning groups as performed at University of Heidelberg

The day before a test, males and females in a ratio of 2:1 are placed in spawning tanks (Fig. 1) immediately before the onset of darkness. Since spawning groups of zebrafish may occasionally fail to spawn, the parallel use of at least three spawning tanks is strongly recommended. Artificial plants serve as breeding stimulant and substrate. Mating, spawning and fertilization take place within 30 min after the onset of light in the morning.

Since zebrafish is known to feed upon its own offspring, the bottom of the spawning tanks should be covered with a grid of stainless steel (mesh size approx. 2 mm), thus allowing the eggs to be sampled without interference by the adults. The egg trays should be replaced under the spawning tanks at the latest possible time (less recommended) or on the next day before the light is turned on. In the authors´ laboratory, for collection of eggs, the bottom of the 3 L spawning tanks are replaced by a stainless steel grid with a mesh size of 1.25 mm in order to prevent predation of eggs. The spawning tanks are placed on rectangular full-glass dishes of similar dimensions (egg trays; Fig. 1).

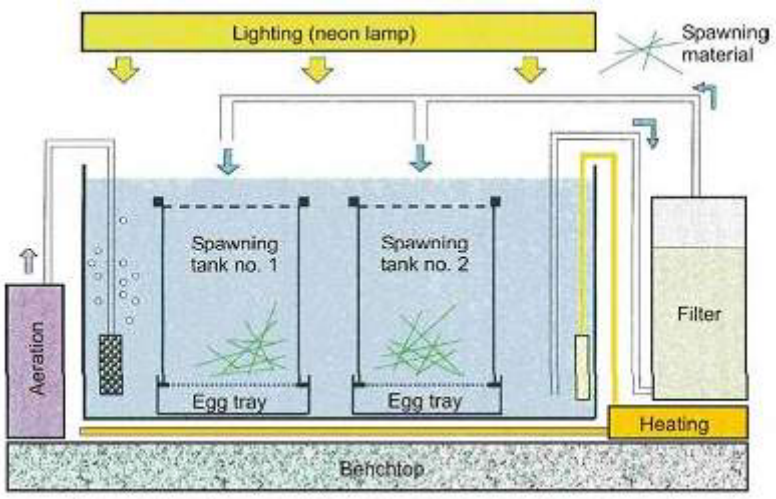


Fig. 1: Tank setup used for breeding zebrafish (*Danio rerio*). Up to 10 tanks, the bottoms of which are replaced by a stainless steel grid, were placed on top of spawning dishes of similar dimensions. All spawning tanks were immersed into one bigger tank equipped with fully conditioned aquarium water. To collect the eggs after spawning, the egg trays can easily be removed from the breeding facility.

As a spawning stimulus, artificial plants made of green plastic or glass should be fixed to the grid covering the egg trays (Fig. 1). About 30 - 60 minutes after spawning, the egg trays can be carefully removed.

For selection of fertilized eggs see 6.3.2.1.

# Zebrafish Embryo Toxicity Test

## Evaluation of transferability, intra- and interlaboratory reproducibility

Phase 2a

Trial Plan for Training of New Laboratories

*TP_ZFET_OECD_2a V01*
8th October 2010

| | | | |
|---|---|---|---|
| **Title** | **Zebrafish Embryo Toxicity Test: Evaluation of transferability, intra- and interlaboratory reproducibility – Phase 2a: Training of new laboratories** | | |
| **Sponsor:** | **-** | | |
| **Identification:** | *TP_ZFET_OECD_2a* | | |
| **Start Date:** | October 2010 | **End Date:** | November 2010 |

| | | |
|---|---|---|
| Trial Coordinator | Name | Marlies Halder |
| | Date | August 2010 |
| | Signature | |
| Reviewer 1 | Name | Participating laboratories |
| | Date | September 2010 |
| | Signature | |
| Reviewer 2 | Name | Validation Management Group |
| | Date | October 2010 |
| | Signature | |

**Table of Contents**

1. Introduction....4
2. Purpose of the study....5
3. Validation management group....5
4. Participating laboratories....6
5. Standard operation procedure....6
6. Time schedule and design of the study....7
7. Study performance....8
7.1. General considerations....8
7.2. Pre-saturation of glass vessels used for selection of fertilised eggs and 24-well plates 8
7.3. Daily semi-static renewal of test solutions/controls....8
7.4. Measurements of test conditions....9
8. Test chemical....9
8.1. Information on 3,4-dichloroaniline....9
8.2. Preparation of 3,4-dichloroaniline stock solution (100mg/l)....9
8.3. Test concentrations....9
9. Controls....10
10. Sampling and storage of stock solutions....10
10.1. Sampling....10
10.2. Labeling....10
10.3. Sample storage:....10
11. Reporting of results....11
12. Statistical analysis....11
13. Archiving....11
14. Quality assurance statement....11
15. References....12
ANNEX 1: Layout of 24-well plates for Phase 2a....13
ANNEX 2: Laboratory contact details....14
ANNEX 3: 3,4-DCA material safety data sheet....15

# Zebrafish Embryo Toxicity Test
# Evaluation of transferability, intra- and interlaboratory reproducibility

## Phase 2a: Training of new laboratories

## 1. Introduction

The acute fish toxicity test is a mandatory component in the environmental safety assessment of industrial chemicals, agrochemicals, pharmaceuticals, feed stuff etc. In the European Union, Council Directive 86/609/EEC on the protection of laboratory animals (EC, 1986) and, in particular, the legislation on chemicals (REACH; EC 2007) demand that tests on vertebrate animals are reduced, refined or replaced whenever possible.

One of the most promising alternative approaches to the LC50 96h fish toxicity test (OECD 203 [OECD, 1992]; C.1 [EC, 2008]) is based on the use of fish embryos.

In Germany, the Fish Egg Toxicity test (DIN 2001) was validated and replaced the 48 h acute fish test for routine whole effluent testing in 2005. Recently, a modified international version of the fish egg toxicity test was published (ISO 2007).

Extensive efforts have been undertaken to adapt the method to also meet chemical testing requirements (Nagel 2002, Braunbeck *et al.,* 2005, Lammer *et al.,* 2009). In fall 2005, the German Federal Environment Agency submitted the draft guideline “Fish embryo toxicity (FET) test” to the OECD Test Guideline program together with a Draft Detailed Review Paper (Braunbeck *et al.,* 2005). Based on the comments received from the national coordinators, the OECD decided to establish the *ad hoc Expert Group on the Fish Embryo Toxicity Test*. During several teleconference and face-to-face meetings, the submitted documents were reviewed taking into consideration the scientific basis, reproducibility and predictive capacity of the FET. A thorough re-evaluation of existing data demonstrates that the zebrafish fish embryo test correlates well with acute fish toxicity tests (Lammer *et al.* 2009).

The ad hoc Expert Group on the Fish Embryo Toxicity Test noted that most data are available for the zebrafish embryo toxicity test, however, data providing sufficient evidence for the reproducibility of the method are lacking.

## 2. Purpose of the study

The zebrafish embryo toxicity test (ZFET) is designed to determine the lethal effects of chemicals on embryonic stages of fish and constitutes an alternative test method to the acute toxicity tests with juvenile and adult fish, i.e. the OECD Test Guideline 203 (OECD 1992).

Following the advice of the OECD ad hoc Expert Group on Fish Embryo Tests, OECD decided to perform a ring trial in a restricted number of laboratories. The purpose is to evaluate:

- the transferability,
- the intralaboratory reproducibility, and
- the interlaboratory reproducibility of the ZFET.

The study is steered by a validation management group.

The study is divided into two phases, where Phase 1 constitutes the transferability of the ZFET from the Lead laboratory to the other laboratories (Phase 1a – Transferability/Training) and consequent the testing of six substances (Phase 1b). Based on the outcome of Phases 1a and 1b, the standard operation procedure (SOP) might undergo revisions. In Phase 2, 13 substances will be tested.

As agreed upon by the validation management group and the OECD ad hoc Expert Group on Fish Embryo Tests, new laboratories joining the study for Phase 2 would need to undergo training. This training step is based on the trial plan used for Phase 1a (TP_ZFET_OECD_1a_V01.7).

## 3. Validation management group

The validation management group (VMG) will steer the study and is responsible for the overall study design. Specific roles and responsibilities are listed below:

| **Name** | **Affiliation/contact** | **Role** |
|---|---|---|
| Marlies Halder<br>François Busquet | JRC/IHCP/IVM-ECVAM<br>marlies.halder@jrc.ec.europa.eu<br>francois.busquet@jrc.ec.europa.eu | Coordination/reporting |
| Patric Amcoff | OECD<br>patric.amcoff@oecd.org | OECD TG Program |
| Thomas Braunbeck | University of Heidelberg<br>braunbeck@zoo.uni-heidelberg.de | Lead laboratory, SOP |
| Scott Belanger | Procter & Gamble<br>belanger.se@pg.com | Chemical analysis, participating laboratory |

| | | |
|---|---|---|
| Greg Carr | Procter & Gamble<br>carr.gj@pg.com | data analysis |
| Adam Lillicrap | NIVA<br>Adam.Lillicrap@niva.no | Independent adviser |
| Susanne Walter-Rohde | Umweltbundesamt (UBA)<br>Susanne.Walter-Rohde@uba.de | OECD lead country |

## 4. Participating laboratories

| **Name** | **responsible/contact** | **Role** |
|---|---|---|
| BASF | Sabine Zok<br>sabine.zok@basf.com | Participating laboratory |
| Instituto Politecnico Nacional | Fernando Martinez Jeronimo<br>fjeroni@ipn.mx | Participating laboratory |
| Merck KGaA | Nicole Huebler<br>nicole.huebler@merck.de | Participating laboratory |
| UBA | Christian Polleichter<br>christian.polleichtner@uba.de | Participating laboratory |

Full contact details and alternate person to be contacted are given in Annex 2.

## 5. Standard operation procedure

The use of the SOP_ZFET_OECD_V02.9 is mandatory. Any deviation from the SOP must be reported in the reporting template.

## 6. Time schedule and design of the study

The study design covers training / transferability aspects of the method and allows to intervening at any stage.

| Step | Action | Responsible |
|---|---|---|
| 1 | Distribution of 3,4-dichloroaniline<br>– labs confirm receipt | ECVAM for UBA, Mexico; Braunbeck for Merck, BASF |
| 2 | Distribution of final Phase 2a trial plan, SOP, reporting templates via e-mail<br>– labs confirm receipt | ECVAM |
| 3 | a) Preparation of 3,4-DCA stock solution (see 8.2) and storage of samples (see 10)<br>b) Testing of 3,4-DCA (5 concentrations) in 1 run;<br>c) Submission of data to ECVAM | Participating labs |
| 4 | Analysis of data | ECVAM |
| 5 | Discussion of data | VMG |
| 6 | a) Use stock solution prepared under step 2 for the below 3 independent runs provided that it is not older than 2 months, otherwise prepare a new stock solution (see 8.2)<br>b) Testing of 3,4-DCA (5 concentrations) in 3 independent runs *("independent" means that the experiments are performed with different batches of zebrafish eggs, on different days and with newly prepared test concentrations)*<br>c) Submission of data to ECVAM | Participating labs |
| 7 | Analysis of data | ECVAM |
| 9 | Discussion of data & decision on progression to Phase 2b | VMG |

## 7. Study performance

### *7.1. General considerations*

The zebrafish embryo toxicity test is performed as described in the SOP_ZFET_OECD_V02.9.
The materials and equipment described in the SOP have to be used. The test substance and controls are described in chapters 8 – 9.
Any deviation from the trial plan or the SOP must be reported.
For the training of new laboratories, 3,4-dichloroaniline (3,4-DCA) is used as test chemical and tested in five concentrations as described in chapter 8. 3,4-DCA induces acute toxicity to zebrafish embryos and is used as positive control during the other phases of the overall study.

*Note: For the training, it will not be necessary to run a positive and a solvent control. However, a negative control is mandatory.*

For all experiments, the plate layout shown in Annex 1 has to be used.

All experiments have to be recorded using the reporting template (*RT_ZFET_OECD_2a_V01.0_laboratory code_run*), which will be distributed by François Busquet to the participating laboratories.

### *7.2. Pre-saturation of glass vessels used for selection of fertilised eggs and 24-well plates*

The 24-well plates and glass vessels must be pre-saturated with the respective concentrations of test substances and controls **24 hrs** before the day of the test. They are filled with the required quantity of freshly prepared test concentrations (freshly = prepared on the same day) and respective controls, e.g. glass vessels, at least 50 ml and 24-well plates, at least 2 ml/well (see also SOP_ZFET_OECD_V02.9; see *Note* in 6.3.2).

### *7.3. Daily semi-static renewal of test solutions/controls*

*Note: Analysis of the test concentrations at P&G showed a loss of more than 30% over the course of the test. The VMG therefore agreed that daily renewal of the test solutions/controls is mandatory.*

Daily semi-static renewal of test solutions/controls should be performed according to SOP_ZFET_OECD_V02.9 section 6.5.

### *7.4. Measurements of test conditions*

Measurements of test conditions should be performed according to SOP_ZFET_OECD_V02.9 section 6.6.

## 8. Test chemical

### *8.1. Information on 3,4-dichloroaniline*

| | |
|---|---|
| **Name** | 3,4-dichloraniline |
| **CAS** | 95-76-1 |
| **Supplier** | Sigma-Aldrich<br>(Fluka Pestanal® analytical standard) |
| **Purchase number** | 35827 |
| **Lot number** | SZE6080X |
| **Colour** | Dark brown |
| **Form** | Solid |
| **Purity (%)** | 99.9 |
| **Storage** | room temperature |
| **Molecular weight (g/mol)** | 162.02 |

The 3,4-dichloroaniline material safety data sheet is attached in Annex 3.

### *8.2. Preparation of 3,4-dichloroaniline stock solution (100mg/l)*

a) Dissolve 50 mg 3,4-DCA in 500 ml dilution water

b) Stir in a closed, light-proof vessel for 24 h at room temperature

c) Adjust pH to the pH of the dilution water (within the range of ± 0.5). For adjustment of the pH, the use of HCl and NaOH is recommended.

d) Stock solution can be kept dark in refrigerator (1-8°C) for up to 2 months

e) Before use of the stock solution, stir at room temperature for at least 30 min to ensure a uniform concentration of the substance.

### *8.3. Test concentrations*

a) The following concentrations of 3,4-DCA have to be tested in Phase 2a:
**0.5, 1.0, 2.0, 4.0, 8.0 mg/l.**

b) Test concentrations are freshly prepared (= on the same day) with dilution water (see 5.3 of SOP_ZFET_OECD_V02.9). The temperature of the dilution water should be 26±1˚C.

## 9. Controls

- Negative control
  dilution water as described in SOP_ZFET_OECD_V02.9

- Positive control
  not applicable in Phase 2a

- Solvent control
  not applicable in Phase 2a

## 10. Sampling and storage of stock solutions

Two samples per stock solution should be stored.

### *10.1. Sampling*

- Use appropriate sampling containers, e.g. amber borosilicate glass, VWR catalogue 80076-572 or similar (e.g., Wheaton #W224604), with screw caps (solid-top lined with PTFE faced 14B white styrene-butadiene rubber).
- Minimum volume 10 mL, maximum volume 20 mL
- Pre-rinse any sample container with an initial sample
- Fill container completely and cap
- Wrap cap with Parafilm or equivalent
- Wrap entire sample in aluminum foil

### *10.2. Labeling*

- Samples should be clearly and legibly labeled with the following information at a minimum:
  - Researcher name
  - Laboratory name
  - Material name and CAS nº
  - Nominal concentration of sample
  - Date sample was taken
  - Type of sample (i.e., stock solution)
  - Study code (i.e., ZFET_OECD_2a)

### *10.3. Sample storage:*

- Store the samples of each new stock solution in the refrigerator (1-8°C) until further notice from the study coordinators.

## 11. Reporting of results

The results (also of failed experiments) should be reported using the reporting template. A brief report summarising observations, deviations from SOP, comments etc should be added to the “remarks” sheet in the reporting template. The reporting templates are returned to François Busquet (e-mail: francois.busquet@jrc.ec.europa.eu).

## 12. Statistical analysis

The statistical data analysis will be carried out by ECVAM as described for Phase 1.

## 13. Archiving

Reporting templates either filled in electronically, printed and signed, or handwritten, that are produced during the study are defined as raw data and should be archived by the participating laboratories.

## 14. Quality assurance statement

The participating laboratories should document their quality assurance system.

## 15. References

Braunbeck, T., Böttcher, M., Hollert, H., Kosmehl, T., Lammer, E., Leist, E., Rudolf, M. & Seitz, N. (2005) Towards an alternative for the acute fish LC50 test in chemical assessment: The fish embryo toxicity test goes multi-species – an update. ALTEX 22: 87-102.

DIN (2001) German standard methods for the examination of water, waste water and sludge – Subanimal testing (group T) – Part 6: Toxicity to fish. Determination of the non-acute-poisonous effect of waste water to fish eggs by dilution limits (T 6). DIN 38415-6; German Standardization Organization.

European Commission (1986). Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. Official Journal of the European Communities L358, 1-29.

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European Commission, 2008. Regulation (EC) No 440/2008. Laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). C.1. Acute Toxicity For Fish. Official Journal of European Union. L142, 446-455.

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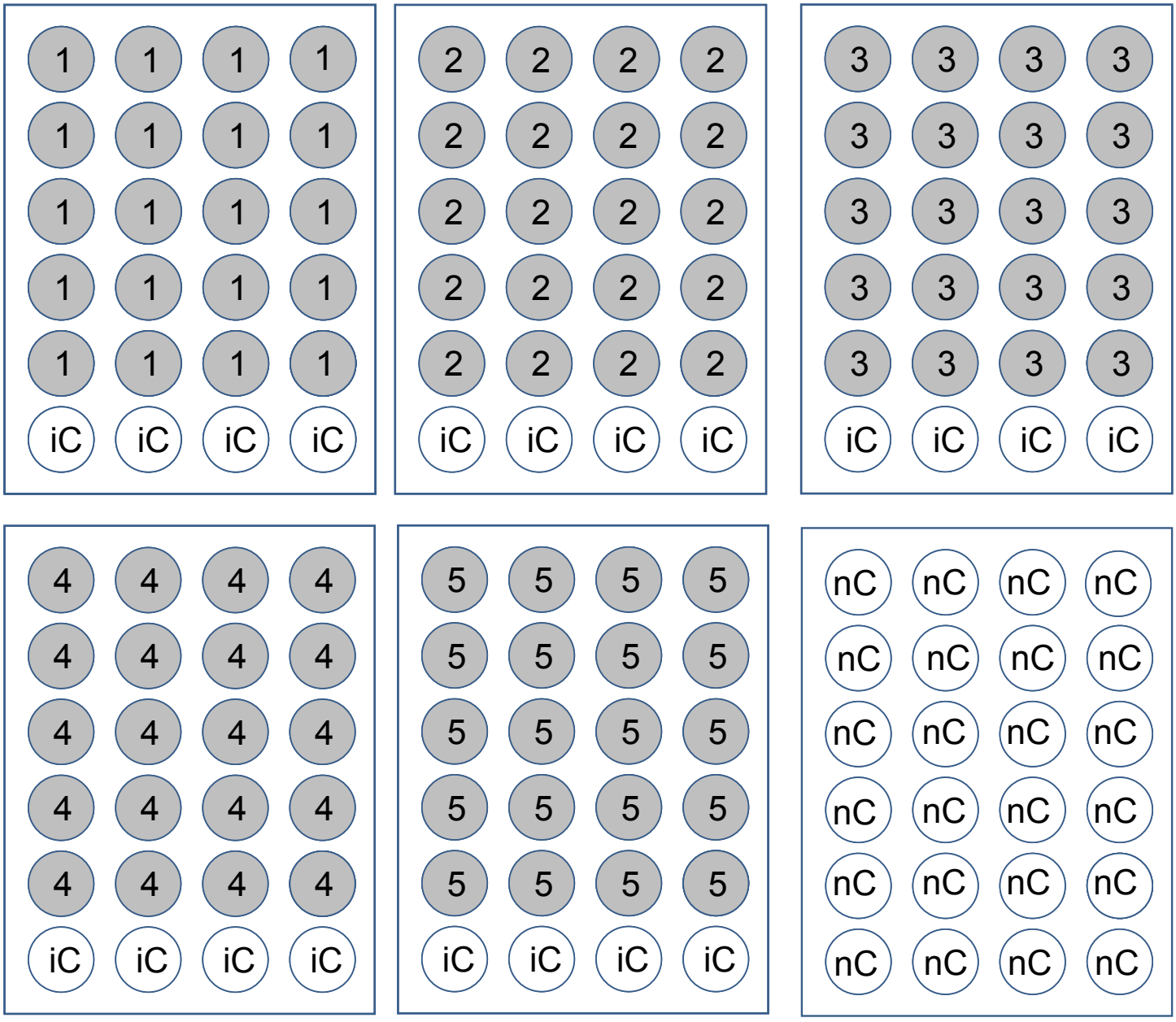
Nagel, R. (2002) DarT: The embryo test with the zebrafish Danio rerio – a general model in ecotoxicology and toxicology. ALTEX 19: 38-48.

OECD (1992) Test Guideline 203. OECD Guideline for Testing of Chemicals. Fish, Acute Toxicity Test. Available: [http://www.oecd.org/document/22/0,2340,en_2649_34377_1916054_1_1_1_1,00.html].

# ANNEX 1: Layout of 24-well plates for Phase 2a




1 to 5 = five concentrations of 3,4-DCA
nC = negative controls
iC = internal controls

# ANNEX 2: Laboratory contact details

| Laboratory contact person 1 | Laboratory contact person 2 |
|---|---|
| **Fernando Martínez-Jerónimo**<br>Escuela Nacional de Ciencias Biológicas, I.P.N.<br>Prol. Carpio esq. Plan de Ayala S/N<br>Col. Santo Tomás,<br>México, D. F. 11340<br>MÉXICO<br>+52(55)57296000 ext. 62424<br>fjeroni@ipn.mx<br>ferjeronimo@hotmail.com | **Roberto Carlos Valerio García**<br>Escuela Nacional de Ciencias Biológicas, I.P.N.<br>Prol. Carpio esq. Plan de Ayala S/N<br>Col. Santo Tomás,<br>México, D. F. 11340<br>MÉXICO<br>+52(55)57296000 ext. 62424<br>ferjeronimo@hotmail.com |
| **Dr. Nicole Hübler**<br>Merck KGaA<br>Frankfurter Str. 250<br>64291 Darmstadt<br>GERMANY<br>+49(0)6151 72 2326<br>nicole.huebler@merck.de | |
| **Sabine Zok, PhD**<br>BASF - The Chemical Company<br>BASF SE, GV/TC - Z570,<br>67056 Ludwigshafen,<br>GERMANY<br>+49 621 60-55944<br>sabine.zok@basf.com | |
| **Christian Polleichtner**<br>Umweltbundesamt<br>FG IV 2.4<br>Schichauweg 58<br>12307 Berlin<br>GERMANY<br>+49-(0)30-8903-4245<br>Christian.Polleichtner@uba.de | |

# ANNEX 3: 3,4-DCA material safety data sheet

# SAFETY DATA SHEET

according to Regulation (EC) No. 1907/2006
Version 4.0 Revision Date 12.03.2010
Print Date 27.09.2010
GENERIC EU MSDS - NO COUNTRY SPECIFIC DATA - NO OEL DATA

## 1. IDENTIFICATION OF THE SUBSTANCE/MIXTURE AND OF THE COMPANY/UNDERTAKING

| | | |
|---|---|---|
| Product name | : | 3,4-Dichloroaniline |
| Product Number | : | 35827 |
| Brand | : | Fluka |
| Company | : | Sigma-Aldrich S.r.l.<br>Via Gallarate 154<br>I-20151 MILANO |
| Telephone | : | +390233417310 |
| Fax | : | +390238010737 |
| Emergency Phone # | : | +39 02-6610-1029 (Centro Antiveleni Niguarda Ca' Granda - Milano) |
| E-mail address | : | eurtechserv@sial.com |

## 2. HAZARDS IDENTIFICATION

### Classification of the substance or mixture

According to Regulation (EC) No1272/2008
Serious eye damage (Category 1)
Skin sensitization (Category 1)
Acute aquatic toxicity (Category 1)
Chronic aquatic toxicity (Category 1)
Acute toxicity, Inhalation (Category 3)
Acute toxicity, Dermal (Category 3)
Acute toxicity, Oral (Category 3)

According to European Directive 67/548/EEC as amended.
Toxic by inhalation, in contact with skin and if swallowed. Risk of serious damage to eyes. May cause sensitization by skin contact. Very toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment.

### Label elements

Pictogram

Signal word Danger

Hazard statement(s)

| | |
|---|---|
| H301 | Toxic if swallowed. |
| H311 | Toxic in contact with skin. |
| H317 | May cause an allergic skin reaction. |
| H318 | Causes serious eye damage. |
| H331 | Toxic if inhaled. |
| H410 | Very toxic to aquatic life with long lasting effects. |

Precautionary statement(s)

| | |
|---|---|
| P261 | Avoid breathing dust/fume/gas/mist/vapours/spray. |
| P273 | Avoid release to the environment. |
| P280 | Wear protective gloves/eye protection/face protection. |
| P301 + P310 | IF SWALLOWED: Immediately call a POISON CENTER or doctor/physician. |

| | |
|---|---|
| P305 + P351 + P338 | IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. |
| P311 | Call a POISON CENTER or doctor/physician. |

Hazard symbol(s)

| | |
|---|---|
| T | Toxic |
| N | Dangerous for the environment |

R-phrase(s)

| | |
|---|---|
| R23/24/25 | Toxic by inhalation, in contact with skin and if swallowed. |
| R41 | Risk of serious damage to eyes. |
| R43 | May cause sensitization by skin contact. |
| R50/53 | Very toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment. |

S-phrase(s)

| | |
|---|---|
| S26 | In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. |
| S36/37/39 | Wear suitable protective clothing, gloves and eye/face protection. |
| S45 | In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible). |
| S60 | This material and its container must be disposed of as hazardous waste. |
| S61 | Avoid release to the environment. Refer to special instructions/ Safety data sheets. |

**Other hazards** - none

## 3. COMPOSITION/INFORMATION ON INGREDIENTS

Formula : C6H5Cl2N
Molecular Weight : 162,02 g/mol

| CAS-No. | EC-No. | Index-No. | Classification | Concentration |
|---|---|---|---|---|
| **3,4-Dichloroaniline** | | | | |
| 95-76-1 | 202-448-4 | 612-202-00-1 | Eye Dam. 1; Skin Sens. 1; Aquatic Acute 1; Aquatic Chronic 1; Acute Tox. 3; H301, H311, H317, H318, H331, H410<br>T, N, R23/24/25 - R41 - R43 - R50/53 | - |

For the full text of the H-Statements mentioned in this Section, see Section 16.

## 4. FIRST AID MEASURES

**General advice**
Consult a physician. Show this safety data sheet to the doctor in attendance.

**If inhaled**
If breathed in, move person into fresh air. If not breathing give artificial respiration Consult a physician.

**In case of skin contact**
Wash off with soap and plenty of water. Take victim immediately to hospital. Consult a physician.

**In case of eye contact**
Rinse thoroughly with plenty of water for at least 15 minutes and consult a physician.

**If swallowed**
Never give anything by mouth to an unconscious person. Rinse mouth with water. Consult a physician.

## 5. FIRE-FIGHTING MEASURES

**Suitable extinguishing media**
Use water spray, alcohol-resistant foam, dry chemical or carbon dioxide.

**Special protective equipment for fire-fighters**
Wear self contained breathing apparatus for fire fighting if necessary.

## 6. ACCIDENTAL RELEASE MEASURES

**Personal precautions**
Wear respiratory protection. Avoid dust formation. Avoid breathing dust. Ensure adequate ventilation. Evacuate personnel to safe areas.

**Environmental precautions**
Prevent further leakage or spillage if safe to do so. Do not let product enter drains. Discharge into the environment must be avoided.

**Methods and materials for containment and cleaning up**
Pick up and arrange disposal without creating dust. Keep in suitable, closed containers for disposal.

## 7. HANDLING AND STORAGE

**Precautions for safe handling**
Avoid contact with skin and eyes. Avoid formation of dust and aerosols.
Provide appropriate exhaust ventilation at places where dust is formed. Normal measures for preventive fire protection.

**Conditions for safe storage**
Store in cool place. Keep container tightly closed in a dry and well-ventilated place.

## 8. EXPOSURE CONTROLS/PERSONAL PROTECTION

**Personal protective equipment**

**Respiratory protection**
Where risk assessment shows air-purifying respirators are appropriate use a full-face particle respirator type N99 (US) or type P2 (EN 143) respirator cartridges as a backup to engineering controls. If the respirator is the sole means of protection, use a full-face supplied air respirator. Use respirators and components tested and approved under appropriate government standards such as NIOSH (US) or CEN (EU).

**Hand protection**
The selected protective gloves have to satisfy the specifications of EU Directive 89/686/EEC and the standard EN 374 derived from it.

Handle with gloves.

**Eye protection**
Face shield and safety glasses

**Skin and body protection**
Choose body protection according to the amount and concentration of the dangerous substance at the work place.

**Hygiene measures**
Avoid contact with skin, eyes and clothing. Wash hands before breaks and immediately after handling the product.

## 9. PHYSICAL AND CHEMICAL PROPERTIES

**Appearance**

| | |
|---|---|
| Form | solid |
| Colour | dark brown |

**Safety data**

| | |
|---|---|
| pH | no data available |
| Melting point | 70 °C |
| Boiling point | 272 °C at 1.013 hPa |
| Flash point | 135,00 °C - closed cup |
| Ignition temperature | 265 °C |
| Lower explosion limit | 2,8 %(V) |
| Upper explosion limit | 7,2 %(V) |
| Water solubility | no data available |
| Relative vapour density | 6,49 |

## 10. STABILITY AND REACTIVITY

**Chemical stability**
Stable under recommended storage conditions.

**Conditions to avoid**
no data available

**Materials to avoid**
Acid anhydrides, Oxidizing agents

**Hazardous decomposition products**
Hazardous decomposition products formed under fire conditions. - Hydrogen chloride gas

## 11. TOXICOLOGICAL INFORMATION

**Acute toxicity**
LD50 Oral - rat - 545 mg/kg

**Skin corrosion/irritation**
Skin - rabbit - Severe skin irritation

**Serious eye damage/eye irritation**
Eyes - rabbit - Severe eye irritation

**Respiratory or skin sensitization**
May cause allergic skin reaction.

Causes sensitization.

**Germ cell mutagenicity**

Genotoxicity in vitro - Human - lymphocyte
Sister chromatid exchange

**Carcinogenicity**

IARC: No component of this product present at levels greater than or equal to 0.1% is identified as probable, possible or confirmed human carcinogen by IARC.

**Reproductive toxicity**
no data available

**Specific target organ toxicity - single exposure**
no data available

**Specific target organ toxicity - repeated exposure**
no data available

**Aspiration hazard**
no data available

**Potential health effects**

| | |
|---|---|
| **Inhalation** | Toxic if inhaled. May cause respiratory tract irritation. |
| **Ingestion** | Toxic if swallowed. |
| **Skin** | Toxic if absorbed through skin. May cause skin irritation. |
| **Eyes** | Causes serious eye irritation. |

**Additional Information**
RTECS: BX2625000

## 12. ECOLOGICAL INFORMATION

**Toxicity**

| | |
|---|---|
| Toxicity to fish | LC50 - Pimephales promelas (fathead minnow) - 7 - 10 mg/l - 96,0 h |
| Toxicity to daphnia and other aquatic invertebrates. | EC50 - Daphnia magna (Water flea) - 0,05 - 2,20 mg/l - 48 h |
| Toxicity to algae | EC50 - Pseudokirchneriella subcapitata (green algae) - 4,9 mg/l - 72 h |
| | Growth inhibition LOEC - Algae - 1 - 10 mg/l - 28 d |

**Persistence and degradability**

| | |
|---|---|
| Biodegradability | Result: - Not readily biodegradable. |

**Bioaccumulative potential**

| | |
|---|---|
| Bioaccumulation | Poecilia reticulata (guppy) - 48 h<br>Bioconcentration factor (BCF): 96 |

**Mobility in soil**
no data available

**PBT and vPvB assessment**
no data available

**Other adverse effects**
Very toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment.

## 13. DISPOSAL CONSIDERATIONS

**Product**
Observe all federal, state, and local environmental regulations. Contact a licensed professional waste disposal service to dispose of this material. Dissolve or mix the material with a combustible solvent and burn in a chemical incinerator equipped with an afterburner and scrubber.

**Contaminated packaging**
Dispose of as unused product.

## 14. TRANSPORT INFORMATION

**ADR/RID**
UN-Number: 3442 Class: 6.1 Packing group: II
Proper shipping name: DICHLOROANILINES, SOLID

**IMDG**
UN-Number: 3442 Class: 6.1 Packing group: II EMS-No: F-A, S-A
Proper shipping name: DICHLOROANILINES, SOLID
Marine pollutant: Marine pollutant

**IATA**
UN-Number: 3442 Class: 6.1 Packing group: II

Proper shipping name: Dichloroanilines, solid

## 15. REGULATORY INFORMATION

This safety datasheet complies with the requirements of Regulation (EC) No. 1907/2006.

## 16. OTHER INFORMATION

### Text of H-code(s) and R-phrase(s) mentioned in Section 3

| | |
|---|---|
| Acute Tox. | Acute toxicity |
| Aquatic Acute | Acute aquatic toxicity |
| Aquatic Chronic | Chronic aquatic toxicity |
| Eye Dam. | Serious eye damage |
| H301 | Toxic if swallowed. |
| H311 | Toxic in contact with skin. |
| H317 | May cause an allergic skin reaction. |
| H318 | Causes serious eye damage. |
| H331 | Toxic if inhaled. |
| H410 | Very toxic to aquatic life with long lasting effects. |
| Skin Sens. | Skin sensitization |
| N | Dangerous for the environment |
| T | Toxic |
| R23/24/25 | Toxic by inhalation, in contact with skin and if swallowed. |
| R41 | Risk of serious damage to eyes. |
| R43 | May cause sensitization by skin contact. |
| R50/53 | Very toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment. |

### Further information


The above information is believed to be correct but does not purport to be all inclusive and shall be used only as a guide. The information in this document is based on the present state of our knowledge and is applicable to the product with regard to appropriate safety precautions. It does not represent any guarantee of the properties of the product. Sigma-Aldrich Co., shall not be held liable for any damage resulting from handling or from contact with the above product. See reverse side of invoice or packing slip for additional terms and conditions of sale.

# Zebrafish Embryo Toxicity Test

## Evaluation of transferability, intra- and interlaboratory reproducibility

Phase 2b

Trial Plan for the testing of 13 chemicals

*TP_ZFET_OECD_2b V01.1*

17th January 2011

| | | | |
|---|---|---|---|
| **Title** | **Zebrafish Embryo Toxicity Test: Evaluation of transferability, intra- and interlaboratory reproducibility – Phase 2b: Trial plan for the testing of 13 chemicals** | | |
| **Sponsor:** | **-** | | |
| **Identification:** | *TP_ZFET_OECD_2b* | | |
| **Start Date:** | January 2011 | **End Date:** | July 2011 |

| | | |
|---|---|---|
| Trial Coordinator | Name | Marlies Halder |
| | Date | November 2010 |
| | Signature | |
| Reviewer 1 | Name | Validation Management Group |
| | Date | December 2010 |
| | Signature | |
| Reviewer 2 | Name | Participating laboratories |
| | Date | December 2010 |
| | Signature | |

PAGES OF CHANGES

| Date of change | Version number | Changed pages/sections | Summary of the change(s) | Changed by/sign |
|---|---|---|---|---|
| 13 January | V.1.1 | 17 | DNOC Table on concentration – correction of g/L to mg/L | M. Halder |
| | | | | |
| | | | | |

**Table of Contents**

1. Introduction ..... 5
2. Purpose of the study ..... 6
3. Validation management group ..... 7
4. Participating laboratories ..... 8
5. Standard operation procedure ..... 8
6. Time schedule and design of the study ..... 9
7. Study performance ..... 10
7.1. General considerations ..... 10
7.2. Chemicals tested per laboratory ..... 10
7.3. Pre-saturation of glass vessels and 24-well plates ..... 12
7.4. Daily semi-static renewal ..... 12
7.5. Measurements of test conditions ..... 12
7.6. Analytical measurements of stock solutions and test concentrations ..... 12
8. Test chemicals ..... 13
8.1. Carbamazepine ..... 13
8.2. Copper(II) sulfate pentahydrate ..... 14
8.3. 4,6-Dinitro-o-cresol (DNOC) ..... 16
8.4. 2,4-Dinitrophenol (2,4-DNP) ..... 17
8.5. Dimethyl sulfoxide (DMSO) ..... 18
8.6. Luviquat HM 552 ..... 19
8.7. Malathion ..... 21
8.8. Merquat 100 ..... 22
8.9. Methylmercury (II) chloride ..... 23
8.10. 1-Octanol ..... 25
8.11. Prochloraz ..... 26
8.12. Tetradecyl sulfate sodium salt (TSSS) ..... 27
8.13. Triethylene glycol (TEG) ..... 29
9. Controls ..... 30
9.1. Internal control ..... 30
9.2. Negative control ..... 30
9.3. Positive control ..... 30
10. Reporting of results ..... 31
11. Statistical analysis ..... 31
12. Archiving ..... 31
13. Quality assurance statement ..... 31
14. References ..... 32
ANNEX 1: Layout of 24-well plates for Phase 2b ..... 33
ANNEX 2: Statistical analysis ..... 34
ANNEX 3: Details for sampling the stock solutions ..... 36
ANNEX 4: Laboratory contact details ..... 37

# Zebrafish Embryo Toxicity Test
# Evaluation of transferability, intra- and interlaboratory reproducibility

## Phase 2b: Testing of 13 chemicals

## 1. Introduction

The acute fish toxicity test is a mandatory component in the environmental safety assessment of industrial chemicals, agrochemicals, pharmaceuticals, feed stuff etc. In the European Union, the former Council Directive 86/609/EEC on the protection of laboratory animals (EC, 1986), the recently published Directive 2010/63/EU on the protection of laboratory animals (EC, 2010), in particular, the legislation on chemicals (REACH; EC 2007) demand that tests on vertebrate animals are reduced, refined or replaced whenever possible.

One of the most promising alternative approaches to the LC50 96h fish toxicity test (OECD 203 [OECD, 1992]; C.1 [EC, 2008]) is based on the use of fish embryos.

In Germany, the Fish Egg Toxicity test (DIN 2001) was validated and replaced the 48 h acute fish test for routine whole effluent testing in 2005. Recently, a modified international version of the fish egg toxicity test was published (ISO 2007).

Extensive efforts have been undertaken to adapt the method to also meet chemical testing requirements (Nagel 2002, Braunbeck *et al.,* 2005, Lammer *et al.,* 2009). In fall 2005, the German Federal Environment Agency submitted the draft guideline “Fish embryo toxicity (FET) test” to the OECD Test Guideline program together with a Draft Detailed Review Paper (Braunbeck *et al.,* 2005). Based on the comments received from the national coordinators, the OECD decided to establish the *ad hoc Expert Group on the Fish Embryo Toxicity Test*. During several teleconferences and face-to-face meetings, the submitted documents were reviewed taking into consideration the scientific basis, reproducibility and predictive capacity of the FET. A thorough re-evaluation of existing data demonstrates that the zebrafish fish embryo test correlates well with acute fish toxicity tests (Lammer *et al.* 2009).

The ad hoc Expert Group on the Fish Embryo Toxicity Test noted that most data are available for the zebrafish embryo toxicity test, however, data providing sufficient evidence for the reproducibility of the method are lacking.

## 2. Purpose of the study

The zebrafish embryo toxicity test (ZFET) is designed to determine the lethal effects of chemicals on embryonic stages of fish and constitutes an alternative test method to the acute toxicity tests with juvenile and adult fish, i.e. the OECD Test Guideline 203 (OECD, 1992).

Following the advice of the OECD ad hoc Expert Group on Fish Embryo Tests, OECD decided to perform a ring trial in a restricted number of laboratories. The purpose is to evaluate:

- the transferability,
- the intralaboratory reproducibility, and
- the interlaboratory reproducibility of the ZFET.

The study is steered by a validation management group.

The study is divided into two phases, where Phase 1 constitutes the transferability of the ZFET from the Lead laboratory to the other laboratories (Phase 1a – Transferability/Training) and consequent the testing of six chemicals (Phase 1b). Based on the outcome of Phases 1a and 1b, the standard operation procedure (SOP) might undergo revisions. In Phase 2, 13 chemicals will be tested.

As agreed upon by the validation management group and the OECD ad hoc Expert Group on Fish Embryo Tests, new laboratories joining the study for Phase 2 would need to undergo training. This training step is based on the trial plan used for Phase 1b (TP_ZFET_OECD_1b_V01.8).

## 3. Validation management group

The validation management group (VMG) will steer the study and is responsible for the overall study design. Specific roles and responsibilities are listed below:

| **Name** | **Affiliation/contact** | **Role** |
|---|---|---|
| Marlies Halder<br>François Busquet | JRC/IHCP/IVM-ECVAM<br>marlies.halder@jrc.ec.europa.eu<br>francois.busquet@jrc.ec.europa.eu | Coordination/reporting |
| Patric Amcoff | OECD<br>patric.amcoff@oecd.org | OECD TG Program |
| Thomas Braunbeck | University of Heidelberg<br>braunbeck@uni-hd.de | Lead laboratory, SOP |
| Scott Belanger | Procter & Gamble<br>belanger.se@pg.com | Chemical analysis, participating laboratory |
| Greg Carr | Procter & Gamble<br>carr.gj@pg.com | Data Analysis |
| Adam Lillicrap | NIVA<br>Adam.Lillicrap@niva.no | Independent adviser |
| Susanne Walter-Rohde | Umweltbundesamt (UBA)<br>Susanne.Walter-Rohde@uba.de | OECD lead country |

## 4. Participating laboratories

| Name | responsible/contact | Role |
|---|---|---|
| University of Heidelberg | Thomas Braunbeck<br>braunbeck@uni-hd.de | Lead laboratory |
| BASF | Edward Salinas<br>edward.salinas@basf.com | Participating laboratory |
| Instituto Politecnico | Fernando Martinez-Jeronimo<br>fjeroni@ipn.mx | Participating laboratory |
| Ipo-Pszczyna | Przemys•aw Fochtman<br>fochtman@ipo-pszczyna.pl | Participating laboratory |
| IVM | Juliette Legler<br>juliette.legler@ivm.vu.nl | Participating laboratory |
| Merck KGaA | Nicole Huebler<br>nicole.huebler@merck.de | Participating laboratory |
| Procter & Gamble | Scott Belanger<br>belanger.se@pg.com | Participating laboratory |
| RIVM | Leo van der Ven<br>Leo.van.der.ven@rivm.nl | Participating laboratory |
| UBA | Carola Kussatz<br>carola.kussatz@uba.de | Participating laboratory |

Full contact details and alternate person to be contacted are given in Annex 4.

## 5. Standard operation procedure

The use of the SOP_ZFET_OECD_V02.10 is mandatory. Any deviation from the SOP must be reported in the reporting template.

## 6. Time schedule and design of the study

The study design covers the testing of 13 chemicals by the participating laboratories.

*Table 1: Study design*

| Step | Action | Responsible |
|---|---|---|
| 0 | Distribution of draft documents for Phase 2b, i.e. trial plan, SOP, reporting templates via e-mail<br>– labs should carefully read the documents and contact ECVAM if explanations are required | ECVAM |
| 1 | Distribution of final documents for Phase 2b, i.e. trial plan, SOP, reporting templates via e-mail<br>– labs confirm receipt | ECVAM |
| 2 | Distribution of the 13 test chemicals, MSDS and CoA as indicated in Table 2<br>– labs confirm receipt | ECVAM |
| 3 | a) A new stock solution of 3,4-DCA must be prepared (see section 9.3)<br>b) Preparation of stock solutions of test chemicals as described in section 8. *Note: A new stock solution must be prepared for each run*<br>c) Sampling and storage of stock solutions as described in section 8 and Annex 3<br>d) Testing of test chemicals (5 concentrations) in 3 independent runs with the appropriate controls (see sections 8-9). *Note: "independent" means that the experiments are performed with different batches of zebrafish eggs, on different days and with newly prepared test concentrations*<br>e) Submission of data to ECVAM | Participating labs |

| | | |
|---|---|---|
| 4 | P&G and at Ipo-Pszczyna will carry out analytical measurements of stock solutions and test concentrations for a number of chemicals as agreed | P&G and Ipo-Pszczyna |
| 5 | Analysis of data | Greg Carr |
| 6 | Discussion of results | VMG |

## 7. Study performance

### 7.1. General considerations

The zebrafish embryo toxicity test is performed as described in the SOP_ZFET_OECD_V02.10.

The materials and equipment described in the SOP have to be used. The test chemicals and controls are described in section 8 – 9.

Any deviation from the trial plan or the SOP must be reported in the reporting template.

For all experiments, the plate layout shown in Annex 1 has to be used.

All experiments have to be recorded using the reporting template (*RT_ZFET_OECD_2b_V01.0_Laboratory code_Chemical_Run*), which will be distributed by ECVAM to the participating laboratories.

### 7.2. Chemicals tested per laboratory

*Note:* Since not all of the 9 laboratories have the capacity to test all of the 13 chemicals in 3 independent runs, the VMG decided to distribute the chemicals amongst the 9 laboratories as given in Table 2. This distribution ensures that each chemical is tested in at least 3 laboratories.

*Table 2: Chemicals to be tested by the participating laboratories*

| | RIVM | IVM | IPO-PSZCZYNA | HEIDELBERG | P&G | MERCK | UBA | BASF | INSTITUTO POLITECNICO |
|---|---|---|---|---|---|---|---|---|---|
| Carbamazepine | | X | X | X | | | | X | X |
| Copper(II) sulphate pentahydrate | | X | | X | X | X | | | |
| Dimethyl sulfoxide | | | | X | | X | | X | X |
| 4,6-Dinitro-o-cresol | | | X | X | | X | | X | X |
| 2,4-Dinitrophenol | | X | | X | | | X | X | X |
| Luviquat HM 552 | | X | | X | | X | X | | |
| Malathion | X | | | X | | | | X | X |
| Merquat 100 | | X | | X | | | | X | X |
| Methylmercury (II) chloride | | X | | X | | X | | X | |
| 1-Octanol | X | | | X | X | X | | | |
| Prochloraz | | | X | X | | X | X | | |
| Tetradecyl sulfate sodium salt | X | | | X | X | X | | | |
| Triethylene glycol | X | | X | X | | | X | | |

### 7.3. Pre-saturation of glass vessels and 24-well plates

The 24-well plates and glass vessels used for selection of fertilised eggs must be pre-saturated with the respective concentrations of test chemicals and controls at least **24 hrs** before the day of the test. They are filled with the required quantity of freshly prepared test concentrations (freshly = prepared on the same day) and respective controls, e.g. glass vessels, at least 50 mL and 24-well plates, at least 2 mL/well (see also *Note* in 6.3.2 of SOP_ZFET_OECD_V02.10).

### 7.4. Daily semi-static renewal

Daily semi-static renewal of test concentrations and controls should be performed according to SOP_ZFET_OECD_V02.10 section 6.5.

In case that all 20 embryos of a test concentration are coagulated for more than 24 h, these coagulated embryos and the medium can be removed. This should be reported in the "remarks section" of reporting template as well as the observation ("C") until the end of the exposure period.

**Note: the internal negative controls should be kept, observed and daily renewal continued until the end of the test.**

### 7.5. Measurements of test conditions

Measurements of test conditions should be performed according to SOP_ZFET_OECD_V02.10 section 6.6.

In case the plate with the highest concentration was removed before the end of the exposure, measurements of test conditions will be carried out using the plate with the second highest test concentration.

### 7.6. Analytical measurements of stock solutions and test concentrations

- For each laboratory, stock solutions should be aliquoted and stored for analysis as described in section 8 and Annex 3.

- Two laboratories will perform analytical measurements of stock solutions and test concentrations:
  - P&G: 1-octanol, Tetradecyl sulphate sodium salt and copper (II) sulphate pentahydrate
  - Ipo-Pszczyna: Carbamazepine and Prochloraz (to be confirmed)

## 8. Test chemicals

***Note:*** ECVAM will distribute the 13 chemicals to the laboratories with the Material Safety Data Sheets (MSDS) and the certificate of analysis (CoA).

Laboratories will be informed by ECVAM on the date of sending, the tracking number, and the number of samples. On receipt, laboratories must control the status of the samples and report back to ECVAM (e-mail to François Busquet).

### 8.1. Carbamazepine

#### 8.1.1. Information on Carbamazepine

| | |
|---|---|
| **Product name** | Carbamazepine |
| **CAS number** | 298-46-4 |
| **Supplier** | SIGMA |
| **Purchase number** | C4024 |
| **Lot number** | 119K1317V |
| **Lot number quality control date release** | December 2009 |
| **Recommended retest period** | November 2015 |
| **Colour** | White |
| **Form** | Powder |
| **Purity (%)** | 100 |
| **Storage** | In the fridge: 2 - 8 °C |
| **Molecular weight (g/mol)** | 236.27 |

#### 8.1.2. Preparation of Carbamazepine stock solution (210 mg/L)

***Note 1:*** The long term stability of this compound is not known under storage conditions; therefore, a new stock solution must be prepared for each run.

***Note 2:*** The Carbamazepine stock solution is stable only if stored **>26°C** (e.g. in the incubator).

- Dissolve 210 mg Carbamazepine in 1L of dilution water.
- Stir in a closed, light proof vessel for at least 1-2h at 40°C and the next 6-8h at 80-90°C with appropriate stirring (1250rpm) to ensure Carbamazepine to be completely dissolved.
- Adjust pH to the pH of the dilution water (± 0.5) using the acid/base solution indicated in the SOP.
- The stock solution must be kept **>26°C** during a single run (e.g. in the incubator).

- Before use of the stock solution, stir for 30 min to ensure uniform concentration of the chemical.

- Of each stock solution prepared two samples should be stored at -20°C (see Annex 3 for details of sampling).

#### 8.1.3. Carbamazepine test concentrations

- The following concentrations of Carbamazepine will be tested in Phase 2b: **54.7, 76.5, 107.1, 150 and 210 mg/L**.

- Test concentrations are prepared fresh (on the same day) with dilution water (see section 5.3 of SOP_ZFET_OECD_V02.10). The temperature of the dilution water should be 26 ± 1°C.

- Prepare test concentrations as given below:

| **Test concentrations (mg/L)** | Volume of carbamazepine to be added (ml) | Volume of dilution water to be added (ml) |
|---|---|---|
| **54.7** | 26.048 | 73.952 |
| **76.5** | 36.429 | 63.571 |
| **107.1** | 51 | 49 |
| **150** | 71.429 | 28.571 |
| **210** | 100 | 0 |

### 8.2. Copper(II) sulfate pentahydrate

#### 8.2.1. Information on copper(II) sulfate pentahydrate

| | |
|---|---|
| **Product name** | Copper(II) sulfate pentahydrate |
| **CAS number** | 7758-99-8 |
| **Supplier** | SIGMA |
| **Purchase number** | 209198 |
| **Lot number** | mkbd0338 |
| **Lot number quality control release date** | July 2010 |
| **Recommended retest period** | June 2012 |
| **Colour** | Blue |
| **Form** | Crystals |
| **Purity (%)** | 99.1 |
| **Storage** | At room temperature<br>Air sensitive and hygroscopic |
| **Molecular weight (g/mol)** | 249.68 |

### 8.2.2. Preparation of copper(II) sulfate pentahydrate stock solution (5 mg/L)

***Note 1:*** This compound is an inorganic salt and known to be stable under diverse storage conditions; therefore, a single preparation of a stock solution will be sufficient.

- Dissolve 10 mg copper(II) sulfate pentahydrate ($CuSO_4*5H_20$) in 2L of dilution water. The final stock solution is thus 5 mg/L.
- Stir in a closed, light proof vessel for a minimum of 1 hr at room temperature to ensure the $CuSO_4*5H_20$ is completely dissolved.
- Store the stock solution in the dark at room temperature.
- Before use of the stock solution, stir at room temperature for 30 minutes to ensure uniform concentration of the stock solution

***Note 2:*** Throughout this section, mass of test chemical refers to the entire Formula Weight of $CuSO_4*5H_20$ (249.68 g/mol). When reporting use of test material, identification of test concentrations and subsequent test results, the mass of compound will be for $CuSO_4*5H_20$. However, it is also known that the salt will dissociate completely and the actual toxicity will be a function of the concentration of the copper ion.

### 8.2.3. Copper(II) sulfate pentahydrate test concentrations

The following concentrations of copper(II) sulfate pentahydrate will be tested in Phase 2b: **0.15, 0.30, 0.60, 1.2, and 2.4 mg $CuSO_4*5H_20$/L**

- Test concentrations are prepared fresh (on the same day) with dilution water (see section 5.3 of SOP_ZFET_OECD_V02.10). The temperature of the dilution water should be 26 ± 1°C.
- Prepare test concentrations as given below:

| **Test concentrations (mg/L)** | Volume of $CuSO_4*5H_20$ to be added (ml) | Volume of dilution water to be added (ml) |
|---|---|---|
| **0.15** | 3 | 97 |
| **0.30** | 6 | 94 |
| **0.60** | 12 | 88 |
| **1.2** | 24 | 76 |
| **2.4** | 48 | 52 |

## 8.3. 4,6-Dinitro-o-cresol (DNOC)

### 8.3.1. Information on DNOC

| | |
|---|---|
| **Product name** | DNOC |
| **CAS number** | 534-52-1 |
| **Supplier** | SIGMA |
| **Purchase number** | 45464 |
| **Lot number** | SZE6159X |
| **Expiry date** | June 2013 |
| **Colour** | Dark yellow |
| **Form** | Sheets |
| **Purity (%)** | 99.9 |
| **Storage** | At room temperature |
| **Molecular weight (g/mol)** | 198.13 |

### 8.3.2. Preparation of DNOC stock solution (20 mg/L)

***Note:*** The long term stability of this compound is not known under storage conditions; therefore, a new stock solution must be prepared for each run.

- Dissolve 20 mg DNOC in 1L of dilution water.
- Stir in a closed, light proof vessel for at least 30 min to several hours to ensure the DNOC to be completely dissolved.
- Adjust pH to the pH of the dilution water (± 0.5) using the acid/base solution indicated in the SOP.
- The stock solution should be kept stored refrigerated in the dark at 1-8°C during a single run.
- Before use of the stock solution, stir for 30 min to ensure uniform concentration of the chemical.
- Of each stock solution prepared two samples should be stored at -20°C (see Annex 3 for details of sampling).

### 8.3.3. DNOC test concentrations

- The following test concentrations of DNOC will be tested in Phase 2b:
  **0.18, 0.32, 0.58, 1.05 and 1.89 mg/L**.
- Test concentrations are prepared fresh (on the same day) with dilution water (see section 5.3 of SOP_ZFET_OECD_V02.10). The temperature of the dilution water should be 26 ± 1°C.

- Prepare test concentrations as given below:

| **Test concentrations (mg/L)** | Volume of DNOC to be added (mL) | Volume of dilution water to be added (mL) |
|---|---|---|
| **0.18** | 0.9 | 99.1 |
| **0.32** | 1.6 | 98.4 |
| **0.58** | 2.9 | 97.1 |
| **1.05** | 5.25 | 94.75 |
| **1.89** | 9.45 | 90.55 |

### 8.4. 2,4-Dinitrophenol (2,4-DNP)

#### 8.4.1. Information on 2,4-DNP

| | |
|---|---|
| **Product name** | 2,4-Dinitrophenol |
| **CAS number** | 51-28-5 |
| **Supplier** | SIGMA |
| **Purchase number** | 34334 |
| **Lot number** | sze9167x |
| **Expiry date** | June 2013 |
| **Colour** | Not given |
| **Form** | Solid |
| **Purity (%)** | 99.9 |
| **Storage** | Room temperature<br>Light sensitive. Heat sensitive. |
| **Molecular weight (g/mol)** | 184.11 |

#### 8.4.2. Preparation of 2,4-DNP stock solution (400 mg/L)

***Note 1:*** The long term stability of this compound is not known under storage conditions; therefore, a new stock solution must be prepared for each run.

***Note 2:*** This test chemical must be tested in the **dark**, e.g. either in an incubator without light cycle or place a box over the plates.

- Dissolve 40 mg 2,4-DNP in 100 mL of dilution water.
- Stir in a closed, **light proof** vessel for 30-60 minutes at room temperature to ensure that the 2,4-DNP is completely dissolved.
- Adjust pH to 7.7 using the acid/base solution indicated in the SOP (± 0.5).
- The stock solution can be kept refrigerated in the dark (1-8°C) during a single run.
- Before use of the stock solution, stir at room temperature for 30 min to ensure uniform concentration of the chemical.

- Of each stock solution prepared two samples should be stored at -20°C (see Annex 3 for details of sampling).

#### 8.4.3. 2,4-DNP test concentrations

- The following concentrations of 2,4-DNP will be tested in Phase 2b:
  **0.625, 1.25, 2.5, 5.0, and 10.0 mg/L**.

- Test concentrations are prepared fresh (on the same day) with dilution water (see section 5.3 of SOP_ZFET_OECD_V02.10). The temperature of the dilution water should be 26±1°C.

- Prepare test concentrations as given below:

| **Test concentration (mg/L)** | Volume of 2,4-DNP stock solution to be added (ml) | Volume of dilution water to be added (ml) |
|---|---|---|
| **0.625** | 0.156 | 99.844 |
| **1.25** | 0.313 | 99.687 |
| **2.5** | 0.625 | 99.375 |
| **5** | 1.25 | 98.75 |
| **10** | 2.5 | 97.5 |

### 8.5. Dimethyl sulfoxide (DMSO)

#### 8.5.1. Information on DMSO

| | |
|---|---|
| **Product name** | DMSO |
| **CAS number** | 67-68-5 |
| **Supplier** | Gruessing GmbH |
| **Purchase number** | 10282 (The product number is incorrect on the MSDS given by the supplier) |
| **Lot number** | 215 |
| **Colour** | Colourless |
| **Form** | Liquid, clear |
| **Purity (%)** | 99.1 |
| **Storage** | Room temperature<br>Hygroscopic |
| **Molecular weight (g/mol)** | 78.13 |

#### 8.5.2. Preparation of DMSO stock solution

- Use pure DMSO for preparing the respective test concentrations.

#### 8.5.3. DMSO test concentrations

- The following concentrations of DMSO will be tested in Phase 2b:
  **10, 17, 28.9, 49.13 and 83.521 g/L**.

- Test concentrations are prepared fresh (on the same day) with dilution water (see section 5.3 of SOP_ZFET_OECD_V02.10). The temperature of the dilution water should be 26 ± 1°C.

- The chemical is a liquid, hence calculation is based on the density (1.1 g/cm$^3$).

- Prepare test concentrations as given below:

| **Test concentrations (g/L)** | Volume of DMSO to be added (mL) | Volume of dilution water to be added (mL) |
|---|---|---|
| **10** | 0.909 | 99.091 |
| **17** | 1.545 | 98.455 |
| **28.9** | 2.627 | 97.373 |
| **49.13** | 4.466 | 95.534 |
| **83.521** | 7.593 | 92.407 |

### 8.6. Luviquat HM 552

#### 8.6.1. Information on Luviquat HM 552

| | |
|---|---|
| **Product name** | Luviquat HM 552 |
| **CAS number** | 95144-24-4 |
| **Supplier** | SIGMA |
| **Purchase number** | 59059 |
| **Lot number** | 1322472 |
| **Date of quality control release** | February 2007 |
| **Colour** | Very faintly greenish yellow |
| **Form** | Clear, viscous liquid |
| **Purity (%)** | 19.3% active ingredient in H2O |
| **Storage** | Room temperature |
| **Molecular weight (g/mol)** | 255.74 |

#### 8.6.2. Preparation of Luviquat HM 552 stock solution (500 mg/L)

***Note:*** The long term stability of this compound is not known under storage conditions; therefore, a new stock solution must be prepared for each run.

- Since Luviquat HM 552 is extremely viscous it is recommended to weigh the chemical.

- Luviquat HM 552 is 19.3 % in water (=193 g/L). Moreover, the chemical is a liquid; hence calculation is based on the density (1.05 g/cm$^3$).

- Due to the high viscosity of Luviquat HM 522, preparation of the stock solution must be made by weighing, not by adding a certain volume. Place an empty 100 ml screw cap bottle on a precision scale, weigh 272 mg Luviquat HM 552 (equivalent to 259 µL) and add 99.74 mL of dilution water.

- The resulting final concentration of the stock solution is 500 mg/L with a total volume of 100 mL.

- Stir in a closed, light proof vessel for 30 minutes at room temperature to ensure that the Luviquat HM 552 is completely dissolved.

- Adjust pH to the pH of the dilution water (± 0.5) using the acid/base solution indicated in the SOP.

- The stock solution can be kept refrigerated in the dark (1-8°C) during a single run.

- Before use of the stock solution, stir at room temperature for 30 min to ensure uniform concentration of the chemical.

- Of each stock solution prepared two samples should be stored at -20°C (see Annex 3 for details of sampling).

### 8.6.3. Luviquat HM 552 test concentrations

- The following concentrations of Luviquat HM 552 will be tested in Phase 2b: **0.125, 0.25, 0.5, 1 and 2 mg/L**.

- Test concentrations are prepared fresh (on the same day) with dilution water (see section 5.3 of SOP_ZFET_OECD_V02.10). The temperature of the dilution water should be 26±1°C.

- Prepare test concentrations as given below:

| **Test concentrations (mg/L)** | Volume of Luviquat HM552 to be added (µL) | Volume of dilution water to be added (mL) |
|---|---|---|
| **0.125** | 25 | 99.975 |
| **0.25** | 50 | 99.95 |
| **0.5** | 100 | 99.9 |
| **1** | 200 | 99.8 |
| **2** | 400 | 99.6 |

## 8.7. Malathion

### 8.7.1. Information on Malathion

| | |
|---|---|
| **Product name** | Malathion |
| **CAS number** | 121-75-5 |
| **Supplier** | SIGMA |
| **Purchase number** | PS86 |
| **Lot number** | 447-115b |
| **Lot number expiration date** | August 2012 |
| **Colour** | Colourless |
| **Form** | Liquid |
| **Purity (%)** | 98.7 |
| **Storage** | Room temperature |
| **Molecular weight (g/mol)** | 330.4 |

### 8.7.2. Preparation of Malathion stock solution (50 mg/L)

***Note:*** The long term stability of this compound is not known under storage conditions; therefore, a new stock solution must be prepared for each run.

- The chemical is a liquid; hence calculation is based on the density (1.23 g/cm$^3$).
- Add 41.2 µL of Malathion in 1L of dilution water which corresponds to 50.65 mg (purity was taken in consideration).
- Stir in a closed, light proof vessel for 30 minutes at room temperature to ensure the malathion is completely dissolved.
- Adjust pH to the pH of the dilution water (± 0.5) using the acid/base solution indicated in the SOP.
- Before use of the stock solution, stir at room temperature for 30 min to ensure uniform concentration of the chemical.
- Of each stock solution prepared two samples should be stored at -20°C (see Annex 3 for details of sampling).

### 8.7.3. Malathion test concentrations

- The following concentrations of Malathion will be tested in Phase 2b:
  **0.5, 1.0, 2.0, 4.0, and 8.0 mg/L**.
- Test concentrations are prepared fresh (on the same day) with dilution water (see section 5.3 of SOP_ZFET_OECD_V02.10). The temperature of the dilution water should be 26 ± 1°C.

- Prepare test concentrations as given below:

| **Test concentrations (mg/L)** | Volume of Malathion to be added (ml) | Volume of dilution water to be added (ml) |
|---|---|---|
| **0.5** | 1 | 99 |
| **1** | 2 | 98 |
| **2** | 4 | 96 |
| **4** | 8 | 92 |
| **8** | 16 | 84 |

### 8.8. Merquat 100

#### 8.8.1. Information on Merquat 100

| | |
|---|---|
| **Product name** | Merquat 100 |
| **CAS number** | 26062-79-3 |
| **Supplier** | SIGMA |
| **Purchase number** | 409022 |
| **Lot number** | mkbf2018v |
| **Date of quality control release** | August 2010 |
| **Colour** | Colourless |
| **Form** | Viscous liquid |
| **Purity (%)** | 20.8% active ingredient in H2O |
| **Storage** | Room temperature |
| **Molecular weight (g/mol)** | Average Mw 200,000-350,000 (medium molecular weight) |

#### 8.8.2. Preparation of Merquat 100 stock solution (500 mg/L)

***Note:*** The long term stability of this compound is not known under storage conditions; therefore, a new stock solution must be prepared for each run.

- Merquat from Sigma Aldrich is 20.8 % in water (=208 g/L). Add 0.24 mL from the 20 % solution to 99.76 mL artificial water.

- Stir in a closed, light proof vessel to ensure the Merquat 100 is completely dissolved.

- Adjust pH to the pH of the dilution water (± 0.5) using the acid/base solution indicated in the SOP.

- The stock solution can be kept refrigerated in the dark (1-8°C) during a single run.

- Before use of the stock solution, stir at room temperature for 30 min to ensure uniform concentration of the chemical.

- Of each stock solution prepared two samples should be stored at -20°C (see Annex 3 for details of sampling).

#### 8.8.3. Merquat 100 test concentrations

- The following concentrations of Merquat 100 will be tested in Phase 2b: **0.1, 0.2, 0.4, 0.8 and 1.6 mg/L**.

- Test concentrations are prepared fresh (on the same day) with dilution water (see section 5.3 of SOP_ZFET_OECD_V02.10). The temperature of the dilution water should be 26 ± 1°C.

- Prepare test concentrations as given below:

| **Test concentrations (mg/L)** | Volume of Merquat 100 to be added (µL) | Volume of dilution water to be added (mL) |
|---|---|---|
| **0.1** | 20 | 99.98 |
| **0.2** | 40 | 99.96 |
| **0.4** | 80 | 99.92 |
| **0.8** | 160 | 99.84 |
| **1.6** | 320 | 99.68 |

### 8.9. Methylmercury (II) chloride

#### 8.9.1. Information on Methylmercury (II) chloride

| | |
|---|---|
| **Product name** | Methylmercury (II) chloride |
| **CAS number** | 298-46-4 |
| **Supplier** | SIGMA |
| **Purchase number** | 33368 |
| **Lot number** | SZBA172X |
| **Lot number expiry date** | June 2015 |
| **Colour** | Not given |
| **Form** | Solid |
| **Purity (%)** | 99.9 |
| **Storage** | Room temperature |
| **Molecular weight (g/mol)** | 251.08 |

#### 8.9.2. Preparation of Methylmercury (II) chloride stock solution (20 mg/L)

***Note:*** The long term stability of this compound is not known under storage conditions; therefore, a new stock solution must be prepared for each run.

- Dissolve 20 mg Methylmercury (II) chloride in 1L of dilution water.

- Stir in a closed, light proof vessel for at least 30 min to ensure the Methylmercury (II) chloride to be completely dissolved.

- Adjust pH to the pH of the dilution water (± 0.5) using the acid/base solution indicated in the SOP.

- The stock solution can be kept refrigerated in the dark (1-8°C) during a single run.

- Before use of the stock solution, stir for 30 min to ensure uniform concentration of the chemical.

- Of each stock solution prepared two samples should be stored at -20°C (see Annex 3 for details of sampling).

### 8.9.3. Methylmercury (II) chloride test concentrations

- The following concentrations of Methylmercury (II) chloride will be tested in Phase 2b: **6.25, 12.5, 25, 50 and 100 µg/L**.

- Test concentrations are prepared fresh (on the same day) with dilution water (see section 5.3 of SOP_ZFET_OECD_V02.10). The temperature of the dilution water should be 26 ± 1°C.

- Prepare test concentrations as given below:

| **Test concentrations (µg/L)** | Volume of Methylmercury (II) chloride to be added (µL) | Volume of dilution water to be added (mL) |
|---|---|---|
| **6.25** | 31.3 | 99.969 |
| **12.5** | 62.5 | 99.937 |
| **25** | 125 | 99.875 |
| **50** | 250 | 99.75 |
| **100** | 500 | 99.5 |

## 8.10. 1-Octanol

### 8.10.1. Information on 1-Octanol

| | |
|---|---|
| **Product name** | 1-Octanol |
| **CAS number** | 111-87-5 |
| **Supplier** | SIGMA |
| **Purchase number** | 293245 |
| **Lot number** | STBB5181 |
| **Lot number quality control release** | September 2010 |
| **Colour** | Colourless |
| **Form** | Liquid |
| **Purity (%)** | 99.7 |
| **Storage** | Room temperature |
| **Molecular weight (g/mol)** | 130.23 |

### 8.10.2. Preparation of 1-Octanol stock solution (150 mg/L)

***Note:*** The long term stability of this compound is not known under storage conditions; therefore, a new stock solution must be prepared for each run.

- The chemical is a liquid, hence calculation via density (0.827 g/cm$^3$) is necessary. Dissolve 90.7 µL 1-Octanol in 500 mL dilution water.
- Stir in a closed, light proof vessel for at least 30 min to ensure the 1-Octanol to be completely dissolved.
- No pH adjustment is necessary.
- The stock solution can be kept refrigerated in the dark (1-8°C) during a single run.
- Before use of the stock solution, stir for 30 min to ensure uniform concentration of the chemical.
- Of each stock solution prepared two samples should be stored at -20°C (see Annex 3 for details of sampling).

### 8.10.3. 1-Octanol test concentrations

- The following concentrations of 1-Octanol will be tested in Phase 2b:
  **2.5, 5, 10, 20 and 40 mg/L**.
- Test concentrations are prepared fresh (on the same day) with dilution water (see section 5.3 of SOP_ZFET_OECD_V02.10). The temperature of the dilution water should be 26±1°C.

- Prepare test concentrations as given below:

| **Test concentrations (mg/L)** | Volume of 1-Octanol to be added (mL) | Volume of dilution water to be added (mL) |
|---|---|---|
| **2.5** | 1.67 | 98.33 |
| **5** | 3.33 | 96.67 |
| **10** | 6.67 | 93.33 |
| **20** | 13.33 | 86.67 |
| **40** | 26.67 | 73.33 |

## 8.11. Prochloraz

### 8.11.1. Information on Prochloraz

| | |
|---|---|
| **Product name** | Prochloraz |
| **CAS number** | 67747-09-5 |
| **Supplier** | SIGMA |
| **Purchase number** | 45631 |
| **Lot number** | SZE6220X |
| **Lot number expiry date** | August 2012 |
| **Colour** | Colourless |
| **Form** | liquid |
| **Purity (%)** | 99.1 |
| **Storage** | Room temperature |
| **Molecular weight (g/mol)** | 376.67 |

### 8.11.2. Preparation of Prochloraz stock solution (20 mg/L)

***Note 1:*** The long term stability of this compound is not known under storage conditions; therefore, a new stock solution must be prepared for each run.

***Note 2:*** The Prochloraz stock solution precipitates if stored in the fridge. Keep the stock solution at room temperature.

- Dissolve 20.2 mg Prochloraz (purity was taken in consideration) in 1L of dilution water.

- Stir at least at 1250 rpm overnight at room temperature to ensure the Prochloraz is completely dissolved. Heating (max up to 35°C) while stirring may accelerate the dissolution of Prochloraz.

- Adjust pH to the pH of the dilution water (± 0.5) using the acid/base solution indicated in the SOP.

- The stock solution must be kept at room temperature in a closed container.

- Before use of the stock solution, stir for 30 min to ensure uniform concentration of the chemical.

- Of each stock solution prepared two samples should be stored at -20°C (see Annex 3 for details of sampling).

#### 8.11.3. Prochloraz test concentrations

- The following concentrations of Prochloraz will be tested in Phase 2b:
  **0.5, 1.0, 2.0, 4.0, and 8.0 mg/L**.

- Test concentrations are prepared fresh (on the same day) with dilution water (see section 5.3 of SOP_ZFET_OECD_V02.10). The temperature of the dilution water should be 26±1°C.

- Prepare test concentrations as given below:

| **Test concentrations (mg/L)** | Volume of Prochloraz to be added (mL) | Volume of dilution water to be added (mL) |
|---|---|---|
| **0.5** | 2.5 | 97.5 |
| **1** | 5 | 95 |
| **2** | 10 | 90 |
| **4** | 20 | 80 |
| **8** | 40 | 60 |

### 8.12. Tetradecyl sulfate sodium salt (TSSS)

#### 8.12.1. Information on TSSS

| | |
|---|---|
| **Product name** | Tetradecyl sulfate sodium salt |
| **CAS number** | 1191-50-0 |
| **Supplier** | SIGMA |
| **Purchase number** | 293938 |
| **Lot number** | 06008LC |
| **Lot number quality control release** | September 2004 |
| **Colour** | White crystalline powder |
| **Form** | flakes |
| **Purity (%)** | 97.5 |
| **Carbon content (%)** | 53.24 |
| **Storage** | Room temperature<br>Hygroscopic |
| **Molecular weight (g/mol)** | 316.43 |

### 8.12.2. Preparation of TSSS stock solution (5 mg/L)

***Note 1:*** The long term stability of this compound is not known under storage conditions; therefore, a new stock solution must be prepared for each run. The volumes used are required due to low solubility of TSSS and a need to measure enough mass to be accurate. The measured solubility of tetradecyl sulfate is 5.3 mg/L (note the sodium counter-ion is dissociated). .

- Dissolve 10 mg TSSS in 2L of dilution water. The final stock solution is thus 5 mg/L.
- Heat the solution in a closed, light proof vessel at 60$^{o}$C for a minimum of 1 h.
- Remove from heat and stir to ensure the TSSS is completely dissolved.
- Let stand in the dark at room temperature overnight to return to volume.
- Before use of the stock solution, stir at room temperature for 30 min to ensure uniform concentration of the stock solution

***Note 2:*** throughout this section, mass of test chemical refers to the entire Formula Weight of tetradecyl sulfate sodium salt (Na-$C_{14}SO_4$) (316.43 g/mol). When reporting use of test material, identification of test concentrations and subsequent test results, the mass of compound will be for $C_{14}SO_4^{\cdot(-1)}$. It is known that the salt will dissociate completely and the actual toxicity will be a function of the concentration of the anion.

### 8.12.3. TSSS test concentrations

The following concentrations of TSSS will be tested in Phase 2b:
**0.156, 0.3125, 0.625, 1.25, and 2.5 mg/L**

- Test concentrations are prepared fresh (on the same day) with dilution water (see section 5.3 of SOP_ZFET_OECD_V02.10). The temperature of the dilution water should be 26 ± 1°C.
- Prepare test concentrations as given below:

| **Test concentrations (mg/L)** | Volume of TSSS Stock to be added (ml) | Volume of dilution water to be added (ml) |
|---|---|---|
| **0.156** | 3.125 | 96.875 |
| **0.3125** | 6.25 | 93.75 |
| **0.625** | 12.5 | 87.5 |
| **1.25** | 25 | 75 |
| **2.5** | 50 | 50 |

## 8.13. Triethylene glycol (TEG)

### 8.13.1. Information on TEG

| | |
|---|---|
| **Product name** | Triethylene glycol |
| **CAS number** | 112-27-6 |
| **Supplier** | SIGMA |
| **Purchase number** | T59455 |
| **Lot number** | STBB7542 |
| **Lot number quality control release date** | May 2010 |
| **Colour** | Colourless |
| **Form** | Clear, viscous liquid |
| **Purity (%)** | 99.8 (GC) |
| **Storage** | Room temperature<br>Hygroscopic |
| **Molecular weight (g/mol)** | 150.17 |

### 8.13.2. Preparation of TEG stock solution

- Use pure TEG for preparing the respective concentrations.

### 8.13.3. TEG test concentrations

- The following concentrations of TEG will be tested in Phase 2b:
  **20, 30, 45, 67.5 and 101.25 g/L**.

- No pH adjustment is necessary.

- Test concentrations are prepared fresh (on the same day) with dilution water (see section 5.3 of SOP_ZFET_OECD_V02.10). The temperature of the dilution water should be 26 ± 1°C.

- The chemical is a liquid; hence calculation is based on the density (1.124 g/cm$^3$).

- Prepare test concentrations as given below:

| **Test concentrations (g/L)** | Volume of TEG to be added (mL) | Volume of dilution water to be added (mL) |
|---|---|---|
| **20** | 1.779 | 98.221 |
| **30** | 2.669 | 97.331 |
| **45** | 4.004 | 95.996 |
| **67.5** | 6.006 | 93.994 |
| **101.25** | 9.008 | 90.992 |

# 9. Controls

## 9.1. Internal control

Dilution water (described in SOP_ZFET_OECD_V02.10) is used for internal controls (see Annex 1 for the layout of 24-well plates).

## 9.2. Negative control

Dilution water (described in SOP_ZFET_OECD_V02.10) is used for negative controls (see Annex 1 for the layout of 24-well plates).

## 9.3. Positive control

3,4-Dichloraniline (3,4-DCA) is used as positive control (see Annex 1 for the layout of 24-well plates).

### 9.3.1. Information on 3,4-Dichloraniline (3,4-DCA)

| | |
|---|---|
| **Product name** | 3,4-DCA |
| **CAS number** | 95-76-1 |
| **Supplier** | SIGMA |
| **Purchase number** | 35827 |
| **Lot number** | SZE6080X |
| **Expiry date** | March 2013 |
| **Colour** | Dark brown |
| **Form** | Solid |
| **Purity (%)** | 99.9 |
| **Storage** | room temperature |
| **Molecular weight (g/mol)** | 162.02 |

### 9.3.2. Preparation of 3,4-DCA stock solution (100 mg/L)

- Dissolve 50 mg 3,4-DCA in 500 mL dilution water.
- Stir in a closed, light-proof vessel for 24 h at room temperature.
- Adjust pH to the pH of the dilution water (±0.5) using the acid/base solution indicated in the SOP.
- Stock solution can be kept dark in fridge (1-8°C) for up to 2 months maximum.
- Before use of the stock solution, stir at room temperature for at least 30 min to ensure a uniform concentration of the chemical.

### 9.3.3. Concentration of positive control

A concentration of ***4.0 mg/L*** of 3,4-DCA is used as a positive control.

The 3,4-DCA solution is freshly prepared (= on the same day) with dilution water (see 5.3 of SOP_ZFET_OECD_V02.10). The temperature of the dilution water should be 26 ± 1•C.

## 10. Reporting of results

The results (also of failed experiments) should be reported using the reporting template provided by ECVAM to each laboratory. The results are made available according to the deadlines given in section 6. A brief report summarising observations, deviations from SOP, comments etc should be added to the “remarks” sheet in the reporting template. The reporting templates are returned to:

- François Busquet (e-mail: francois.busquet@jrc.ec.europa.eu).

## 11. Statistical analysis

An outline of the statistical data analysis is given in Annex 2.

## 12. Archiving

Reporting templates either filled in electronically, printed and signed, or handwritten, that are produced during the study are defined as raw data and should be archived by the participating laboratories.

## 13. Quality assurance statement

The participating laboratories should document their quality assurance system.

## 14. References

Braunbeck, T., Böttcher, M., Hollert, H., Kosmehl, T., Lammer, E., Leist, E., Rudolf, M. & Seitz, N. (2005) Towards an alternative for the acute fish LC50 test in chemical assessment: The fish embryo toxicity test goes multi-species – an update. ALTEX 22: 87-102.

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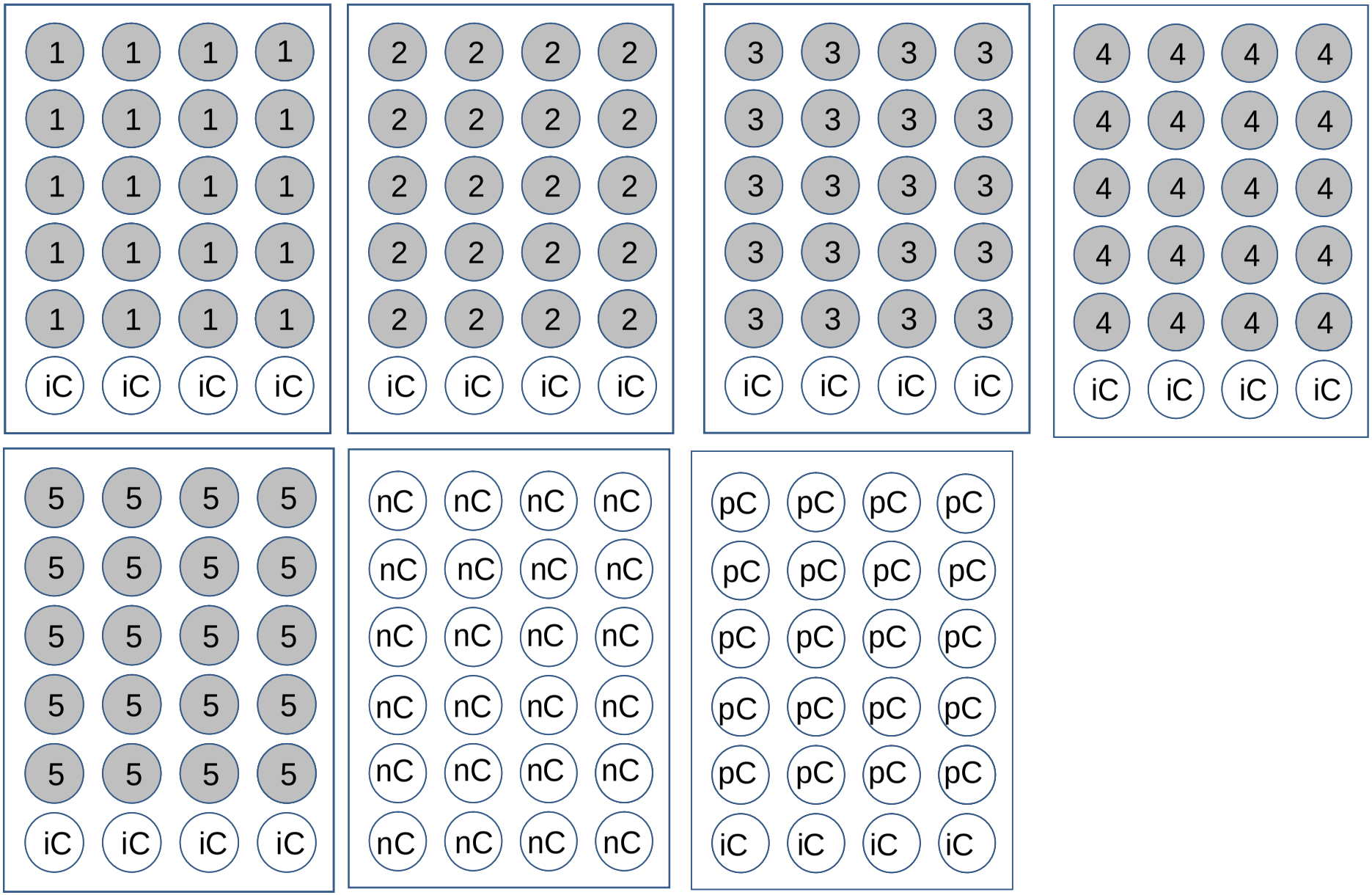
Lammer, E., Carr, G.J., Wendler, K., Rawlings, J.M., Belanger, S.E., Braunbeck, T. (2009) Is the fish embryo toxicity test (FET) with the zebrafish (Danio rerio) a potential alternative for the fish acute toxicity test? Comparative Biochemistry and physiology, Part C, Toxicol Pharmacol -149 (2), 196-209.

Nagel, R. (2002) DarT: The embryo test with the zebrafish Danio rerio – a general model in ecotoxicology and toxicology. ALTEX 19: 38-48.

OECD (1992) Test Guideline 203. OECD Guideline for Testing of Chemicals. Fish, Acute Toxicity Test. Available: [http://www.oecd.org/document/22/0,2340,en_2649_34377_1916054_1_1_1_1,00.html].

**ANNEX 1: Layout of 24-well plates for Phase 2b**




1 to 5 = five concentrations of the test chemical
nC = negative controls (dilution water) pC = positive control (3,4-DCA at 4 mg/l) iC = internal controls (dilution water

## ANNEX 2: Statistical analysis

***Responsible – Greg Carr***

As a basis, the following data analyses steps will be performed. Any deviations should be justified and explained in the report of the statistical data analysis. The analyses are not necessarily limited to the given steps.

***1. Quality checks***

1.1. Is the information complete?
1.2. Are acceptance criteria met?
1.3. Are reported results consistent? (i.e. Is an embryo reported as dead at 24 h still reported as dead at 96 h?)

***2. Descriptive statistics***

2.1. Summarise quality checks
2.2. Summarise results of chemical and control in tables and figures
2.3. Count failed (e.g. acceptance criteria not met (see 7.1 in SOP) or following the judgement of the operator) and summarise in tables
2.4. Summarise remarks

***3. Inferential statistics***

3.1. Choose appropriate model for estimating the LC50 including (robust) confidence intervals by following recommendations of the OECD Guidance Document No. 54 on current approaches in the statistical analysis of ecotoxicity data will be considered. As target one model should be chosen which showed an acceptable fit and robustness for all results (exceptions are possible but only for experiments where the chosen model obviously does not show an acceptable fit or the maximization process fails).
3.2. Quality criteria for fitting a model:
- Do the assumptions of the model reflect the biological context?
- Inspection of residuals
- Transformations of the variables are indicated (e.g. log dose)?
- Convergence of maximization process

3.3. Estimate LC50 and confidence intervals per experiment
- Summarise model fits, quality criteria and confidence intervals
- Summarise dose-response curves in figures

3.4. Test of effect on internal controls caused by the increasing test concentrations using Cochran-Armitage trend test in a stratified manner (strata: laboratory).
3.5. Fisher test internal control vs. external control plate in a stratified manner (strata: laboratory).

***4. Intralaboratory variability***

4.1. Calculate coefficient of variation (CV) based upon LC50 estimates per lab. Will be performed on a log scale if necessary.
4.2. Fitting of a global random effects model on all LC50 estimates, on log scale (if necessary), which simultaneously estimates the components of variability due to within-lab replication, and between lab.

***5. Interlaboratory variability***

5.1. Calculate CV based upon the LC50 estimate per lab. Will be performed on a log scale if necessary.

5.2. ANOVA and Post-hoc with laboratory (independent variable) vs. LC50 (depended variable).

5.3. See 4.2

***6. Report of statistical data analysis***

The outcome will be summarised in a report to the validation management group.

***7. Quality assurance of data analysis and reporting***

An independent statistician (e.g. of IHCP) will review the data analysis and the report.

## ANNEX 3: Details for sampling the stock solutions

Two samples per stock solution per laboratory are requested. Sample 1 will be the primary sample for analysis and Sample 2 will serve as a back-up in reserve in the case of spillage or other laboratory issue.

1. Labelling sample containers:

    - Samples should be clearly and legibly labelled with the following information at a minimum:
        - Researcher name
        - Laboratory name
        - Material name and CAS number
        - Nominal concentration of sample
        - Date sample was taken
        - Type of sample (i.e., stock solution)
        - Sample code (consisting of two letter location indicator, date on DDMMYY format followed by -1 or -2 as further described below)
            - An example code from Procter & Gamble's Aquatic Toxicology Laboratory may look like "PG030509-1" for a sample taken by Procter & Gamble on 3 May 2009, sample 1).

2. Sample containers:

    - Use amber borosilicate glass, VWR catalogue 80076-572 or similar (e.g., Wheaton #W224604), with screw caps (solid-top lined with PTFE faced 14B white styrene-butadiene rubber).
    - Minimum volume 10 mL, maximum volume 20 mL
    - Pre-rinse any sample container with an initial sample
    - Fill container and cap
    - Wrap cap with Parafilm or equivalent
    - Wrap entire sample in aluminum foil

## ANNEX 4: Laboratory contact details

| Laboratory contact person 1 | Laboratory contact person 2 |
|---|---|
| Scott Belanger, PhD<br>The Procter & Gamble Company<br>11810 East Miami River Road<br>Cincinnati, OH 45252<br>USA<br>Tel: +1 513-627-1928<br>Fax: +1 513-277-8156<br>belanger.se@pg.com | Jane Rawlings<br>The Procter & Gamble Company<br>11810 East Miami River Road<br>Cincinnati, OH 45252<br>USA<br>Tel: +1 513-627-1183<br>Fax: +1 513-386-1472<br>rawlings.jm@pg.com |
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| | |
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| | |
|---|---|
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# Zebrafish Embryo Toxicity Test

## Evaluation of transferability, intra- and interlaboratory reproducibility

Phase 2b

**Amendment to**
Trial Plan for the testing of 13 chemicals

*TP_ZFET_OECD_2b V01.1*

23 February 2011

**Amendment to**
**Chapter 8.1.3.Carbamazepine test concentrations of**
**Trial Plan TP_ZFET_OECD_2b_V01.1**

### 8.1.3 Carbamazepine test concentrations

- The following concentrations of Carbamazepine will be tested in Phase 2b: **54.7, 76.5, 107.1, 150 and 210 mg/L**.

- Test concentrations are prepared fresh (on the same day) with dilution water (see section 5.3 of SOP_ZFET_OECD_V02.10). The temperature of the dilution water should be 26 ± 1°C.

- Prepare test concentrations as given below:

*100 ml (for presaturation of test vessels and 24-well plates)*

| **Test concentrations (mg/L)** | Volume of carbamazepine to be added (ml) | Volume of dilution water to be added (ml) |
|---|---|---|
| **54.7** | 26.048 | 73.952 |
| **76.5** | 36.429 | 63.571 |
| **107.1** | 51 | 49 |
| **150** | 71.429 | 28.571 |
| **210** | 100 | 0 |

*50 ml (for daily renewal)*

| **Test concentrations (mg/L)** | Volume of carbamazepine to be added (ml) | Volume of dilution water to be added (ml) |
|---|---|---|
| **54.7** | 13.024 | 36.976 |
| **76.5** | 18.214 | 31.786 |
| **107.1** | 25.5 | 24.5 |
| **150** | 35.714 | 14.286 |
| **210** | 50 | 0 |

# Annexes VIII - X

**Annex VIII: Evaluation of Time-Dependent Changes in LC50s during the Zebrafish Fish EmbryoTest Using Data Gathered from Phase 1 and 2 of the OECD Validation of the Zebrafish FET.....3**

**Annex IX: Evaluation of Hatching during the Zebrafish Fish Embryo Test Using Data Gathered from Phase 2 of the OECD Validation of the Zebrafish FET .....13**

**Annex X: Impact of Group Size on the Estimation of LC50 in the Zebrafish Fish Embryo Test .....19**

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# ANNEX VIII: Evaluation of Time-Dependent Changes in $LC_{50}$s during the Zebrafish Fish EmbryoTest Using Data Gathered from Phase 1 and 2 of the OECD Validation of the Zebrafish FET

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# Annex VIII

**Overview**

The present document details determinations of $LC_{50}$s at 24, 48, 72 and 96 h to assess the time-dependent changes in toxicity using the ZFET. Based on these determinations it may be possible to develop recommendations to perform the ZFET at durations shorter than 96 h for certain groups of chemicals.

Some observations were targeted at differences in $LC_{50}$ determinations with respect to time. Selected chemicals, e.g. Merquat 100 and Luviquat HM 552, possessed very large differences with respect to 48 and 96 h $LC_{50}$s. Presumably, this is due to the chorion acting as a barrier for some high molecular weight compounds. Outright toxicity was only consistently observed at 96 h (i.e., post-hatch) for these polymers. Other chemicals possessed $LC_{50}$s that were only slightly lower at 96 h versus 48 h; however, $LC_{50}$s remained in the same order of magnitude. Further statistical analysis was suggested to provide evidence whether the exposure time could be restricted to 72h without losing information on the toxicity of the chemicals.

**Patterns of Toxicity Across Chemicals**

For reference, Table 1 provides geometric mean $LC_{50}$s based on individual lab mean results as detailed in OECD (2011a). Information at 48 and 96 h only is presented for Phase 1 chemicals and the full analyses are given for those in Phase 2 and for 3,4-dichloraniline (DCA), which was tested by all participating laboratories.

For the purposes of plotting trends, at times $LC_{50}$s could not be generated due to lack of toxicity. In these cases, for this analysis only, the highest exposure concentration in the exposure series was inserted for comparative purposes. Notes will be made throughout where this may have a bearing on interpretations for a given chemical.

*Group 1: Chemicals whose toxicity is observed primarily early in exposure.*

This group of chemicals had $LC_{50}$s that were roughly equivalent across all time periods (Fig. 1). Most of the observed mortalities were seen in the first 24 h, and the observed effect was therefore primarily egg coagulation. This group of chemicals spanned a wide range of toxicity, chemical functional groups, and modes of action, thus it would appear that no generalization could be made about the relationship between the rapidity and stability of toxicity and other toxicologically relevant parameters. Prochloraz is considered a member of this group, but it should be noted that 2 of 4 laboratories did not generate LC50s until the final time point of 96 h. This is due, at least in part, to the relatively low solubility of the compound and the proximate distance to its acute toxicity (i.e., toxicity occurs very close to the limit of solubility).

In Table 2, the ratio of 96 h $LC_{50}$ to previous timepoints is given. The group as a whole has ratios of 96 h:24, 48, or 72 h that exceeds 0.9. Chemicals in Phase 1 that are likely to be members of this group include ethanol, sodium chloride and trimethylphenol based on the individual 96:48 h $LC_{50}$ ratios.

*Group 2: Chemicals whose toxicity continues to steadily progress throughout the exposure*

This group of chemicals had $LC_{50}$s that displayed a downward trend (lower $LC_{50}$s through time) with each exposure period slightly to somewhat lower than the previous (Figure 2, Table 2). Carbamazepine, malathion, DMSO, and DCA are members of this group. As with Group 1, the members of Group 2 have have a wide array of modes of action, represents a number of different chemical categories, but are all moderate to less toxic ($LC_{50}$s of 2 mg/L or higher). Two members of this group (malathion and carabamazepine) did not have measurable toxicity at 24 h and mixed results across labs at 48 h. By 72 h, however, all labs had converged and had measurable responses to the chemical. Chemicals in Phase 1 that are likely to be members of this group possibly include methyl hepten-one.

*Group 3: Chemicals whose toxicity rapidly changes after 24 h*

This group is a more extreme version of Group 2, but differs in that larger differences between 24 and 48 h can be observed (Figure 3, Table 2). Similar to Group 2, however, is an on-going lowering of the $LC_{50}$ through time for the entire period of exposure. This group contains the chemicals methylmercury (an organometal) and two uncouplers of phosphorylation (dintrophenol and dintro-*o*-cresol). This group is widely spaced in its potencies. Dibutyl maleate and triclosan from Phase 1 likely fit into this category given their 96:48 h ratios.

*Group 4: Chemicals whose toxicity is mostly expressed following hatch at 72 h*

This group of chemicals, originally envisioned by the validation management group, was assessed for the expressed purpose to reveal instances where toxicity may be delayed after hatch because the chemicals could not cross the chorion. The cationic polymers Merquat and Luviquat appear to fit this description. Some small amount of toxicity before 72 h was observed in some laboratories, but the majority of the response was seen only following hatch (Table 2, Fig. 4). It is possible that prochloraz would also fit this description, but is much less clear cut given the difficulties testing this compound.

**Overall Patterns of Toxicity Across All Chemicals Tested**

When looking at the overall patterns of toxicity on a temporal basis, and across all compounds, it is remarkable that the changes observed across time are indeed relatively low in overall variability (Figure 5). The differences cited in the groupings above are revealed only by very close inspection of the studies themselves and one compound at a time. Taken as a whole, the geometric mean average ratios of the 96:72 h, 96:48 h, and 96:24 h for all compounds is 0.86, 0.83, and 0.71. In other words, less than a 30% change in toxicity is generally observed from 24 proceeding to 96 h. However, for any one compound, this change could be much greater.

**Recommendations and Additional Observations**

(1) Four relatively distinct temporal patterns of toxicity were identified in the chemicals evaluated during the OECD validation of the Zebrafish Embryo Test (ZFET). These were: (Group 1) chemicals whose toxicity was observed primarily early in exposure, (Group 2) chemicals whose toxicity continues to steadily progress throughout the exposure, (Group 3) chemicals whose toxicity rapidly changes after 24 h, and (Group 4) chemicals whose toxicity is mostly expressed following hatch at 72 h. These remain relatively arbitrary groupings and indicate trends, and should not be over-interpreted.

(2) There is no clear pattern of chemical category, functional use, mode of action or potency that is associated with any grouping of chemicals using temporal patterns of $LC_{50}$s as a guide.

(3) Some chemicals possess properties that would result in erroneous assessments of overall potential to be toxic to fish if the ZFET would be terminated before hatch. Only 1 chemical category, the cationic polymers, may be typified as consistent members of this group. Other members may eventually be indicated by physical-chemical properties such as possessing low solubility and high hydrophobicity (high log $K_{ow}$), or whose potency is very close to the limits of solubility.

**Literature**

OECD. 2011a. Validation Report (Phase 1) for the Zebrafish Embryo Toxicity Test, Part I. Series on Testing and Assessment No. 157. Organization for Economic Cooperation and Development, Paris, France. 25 August 2011, 123 pg.

OECD. 2011b. Validation Report (Phase 1) for the Zebrafish Embryo Toxicity Test, Part 2. Series on Testing and Assessment No. 157. Organization for Economic Cooperation and Development, Paris, France. 25 August 2011. 185 pg.

Table 1. Geometric Mean LC50s for each compound across time from the different phases of the ZFET validation study. A indicates not assessed as a time point in this exercise. CNC indicates that a mean $LC_{50}$ could not be calculated.

| | 24 h Mean $LC_{50}$ (mg/L) | 48 h Mean $LC_{50}$ (mg/L) | 72 h Mean $LC_{50}$ (mg/L) | 96 h Mean $LC_{50}$ (mg/L) |
|---|---|---|---|---|
| Transferability | | | | |
| DCA | 3.63 | 3.17 | 2.97 | 2.72 |
| | | | | |
| Phase 1 | | | | |
| Triclosan | A | 0.417 | A | 0.304 |
| Dibutyl maleate | A | 1.38 | A | 0.694 |
| Trimethylphenol | A | 13.2 | A | 13.1 |
| Methyl hepten-one | A | 279 | A | 243 |
| Sodium chloride | A | 5340 | A | 5140 |
| Ethanol | A | 13200 | A | 12000 |
| | | | | |
| Phase 2 | | | | |
| Methylmercury | 0.0571 | 0.0421 | 0.0330 | 0.0280 |
| Copper | 0.309 | 0.308 | 0.308 | 0.291 |
| Tetradecyl sulfate | 0.335 | 0.337 | 0.339 | 0.339 |
| Merquat | CNC | CNC | 1.21 | 0.48 |
| Dintro-o-cresol | 1.04 | 0.72 | 0.59 | 0.57 |
| Luviquat | CNC | CNC | CNC | 0.86 |
| Dintrophenol | 6.5 | 4.13 | 3.43 | 3.00 |
| Malathion | CNC | CNC | 5.01 | 4.56 |
| Prochloraz | CNC | CNC | CNC | 5.60 |
| Octanol | 20.1 | 20.7 | 20.7 | 20.7 |
| Carbamazepine | CNC | 177 | 174 | 154 |
| DMSO | 45100 | 40200 | 35300 | 34100 |
| Triethylene glycol | 74500 | 71300 | 60400 | 54800 |

Table 2. Grouping of chemicals based on patterns of toxicity observed in the ZFET. A indicates not assessed as a timepoint in this exercise. CNC indicates ratio could not be calculated.

| | Chemical | Ratio of LC50 at 96:24 h | Ratio of LC50 at 96:48 h | Ratio of LC50 at 96:72 h |
|---|---|---|---|---|
| Group 1 | Copper | 0.942 | 0.945 | 0.945 |
| | Tetradecyl sulfate | 1.012 | 1.006 | 1.000 |
| | Prochloraz | CNC | CNC | CNC |
| | Octanol | 1.030 | 1.000 | 1.000 |
| | Ethanol | A | 0.909 | A |
| | Trimethylphenol | A | 0.992 | A |
| | Sodium chloride | A | 0.963 | A |
| Group 2 | Carbamazepine | CNC | 0.870 | 0.885 |
| | Malathion | CNC | CNC | 0.910 |
| | DMSO | 0.756 | 0.848 | 0.966 |
| | DCA | 0.750 | 0.857 | 0.914 |
| | Methyl hepten-one | A | 0.871 | A |
| Group 3 | Methylmercury | 0.490 | 0.665 | 0.848 |
| | Dintrophenol | 0.462 | 0.726 | 0.875 |
| | Dintro-o-cresol | 0.545 | 0.784 | 0.961 |
| | Dibutyl maleate | A | 0.503 | A |
| | Triclosan | A | 0.729 | A |
| Group 4 | Merquat | CNC | CNC | 0.397 |
| | Luviquat | CNC | CNC | CNC |

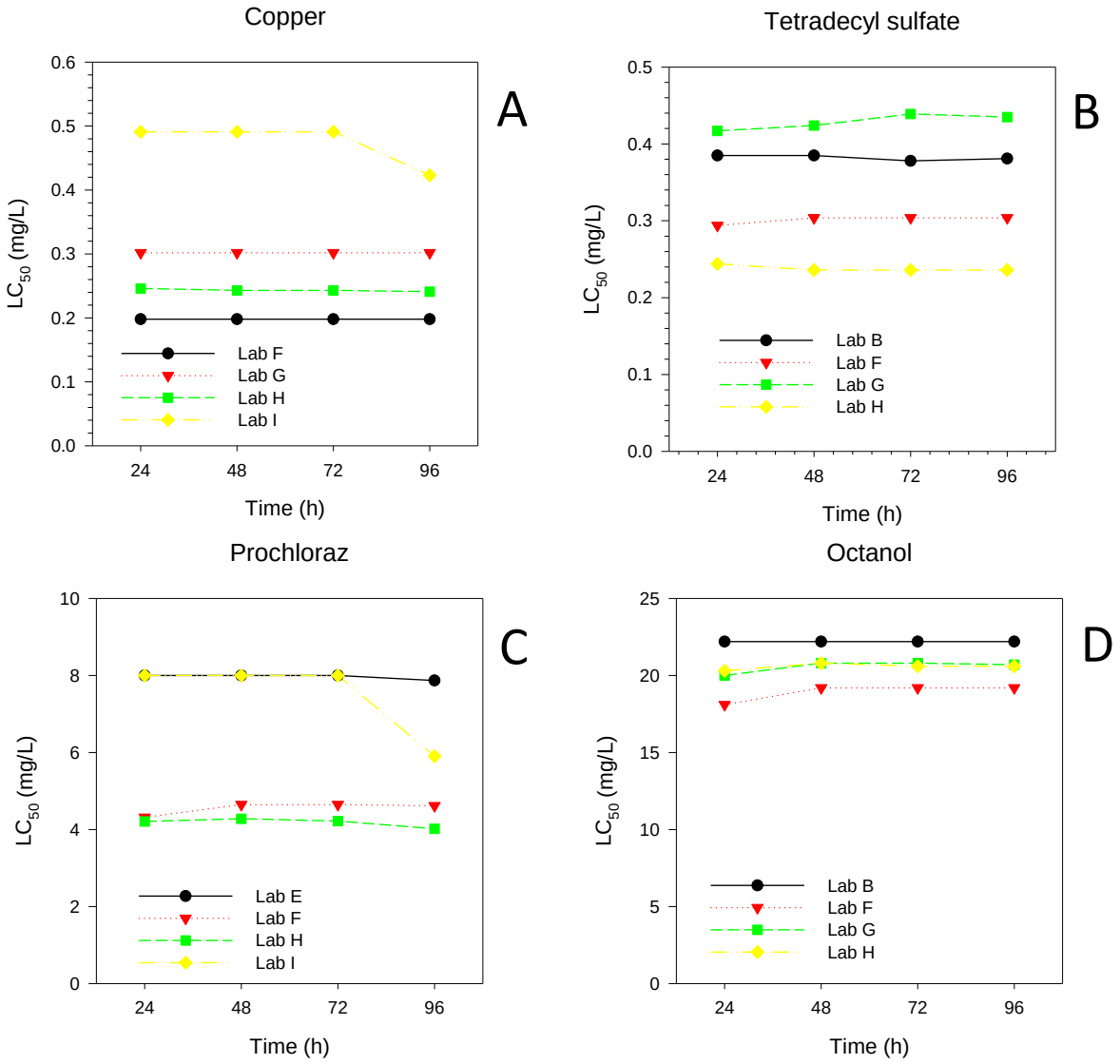


Figure 1: Chemicals whose toxicity is primarily observed in the first 24 h of exposure.

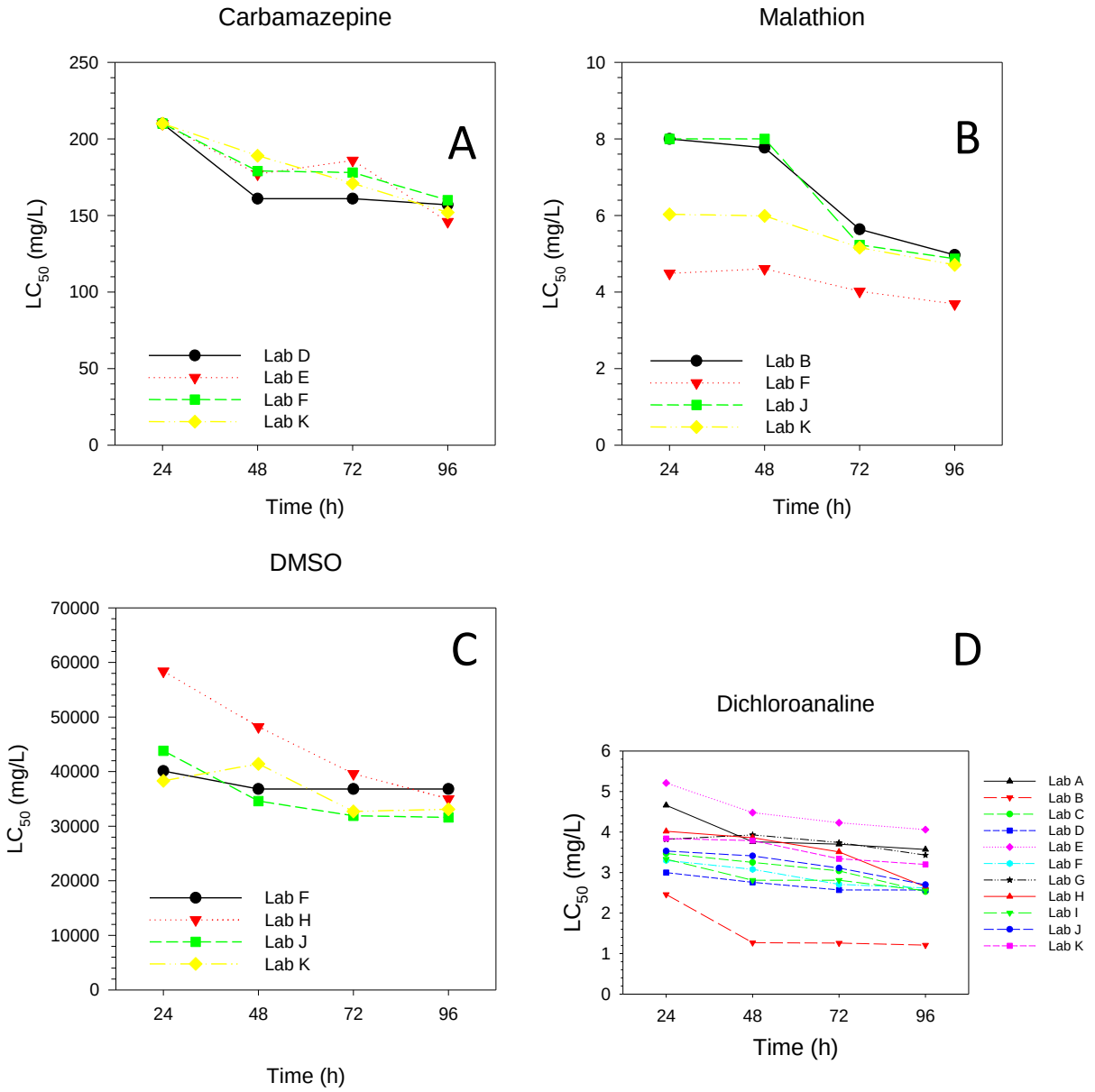


Figure 2. Chemicals whose measured toxicity continues to change at an on-going rate throughout the observed period of 96 h exposure.

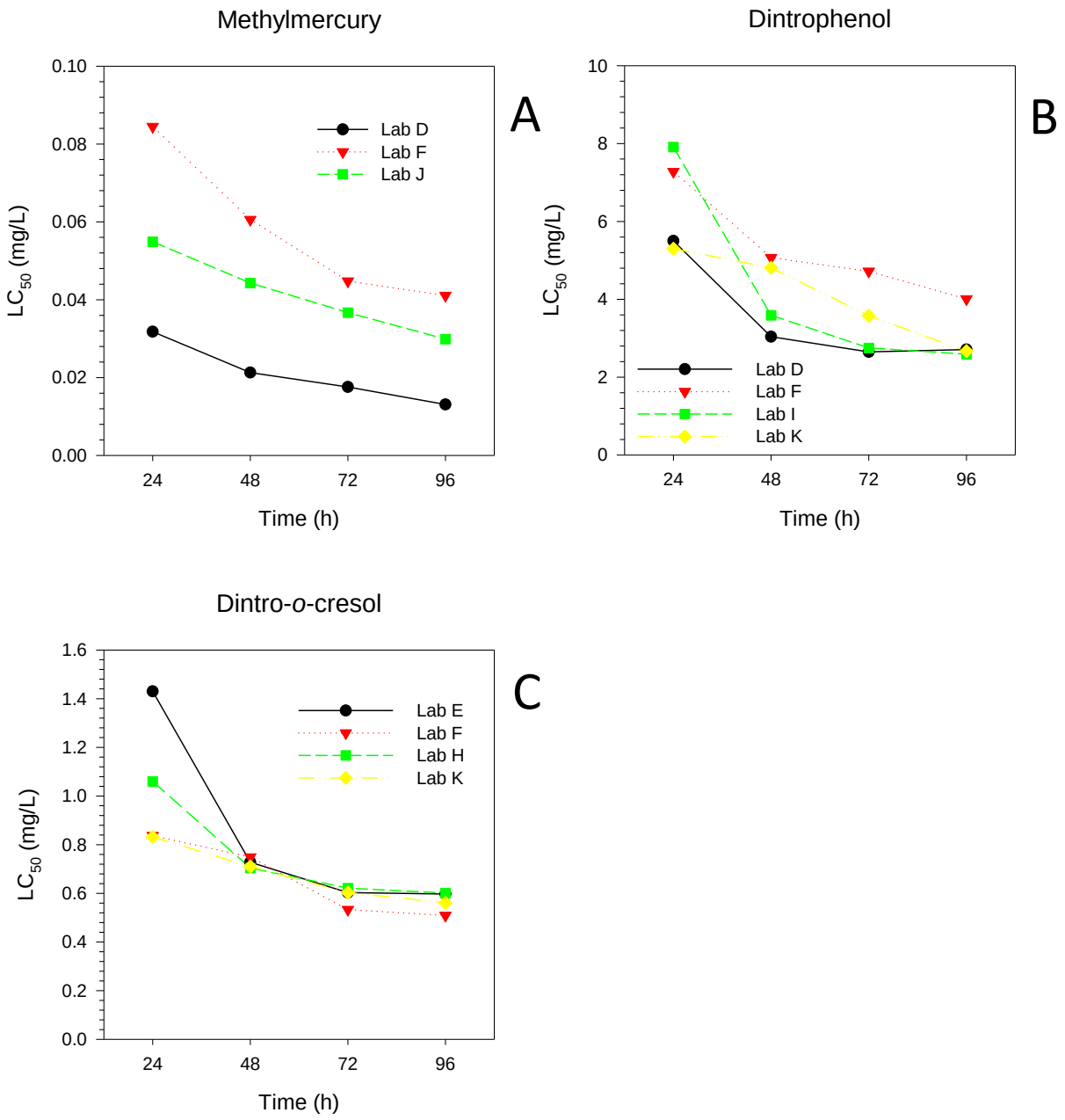


Figure 3. Chemicals whose toxicity rapidly changes after 24 h

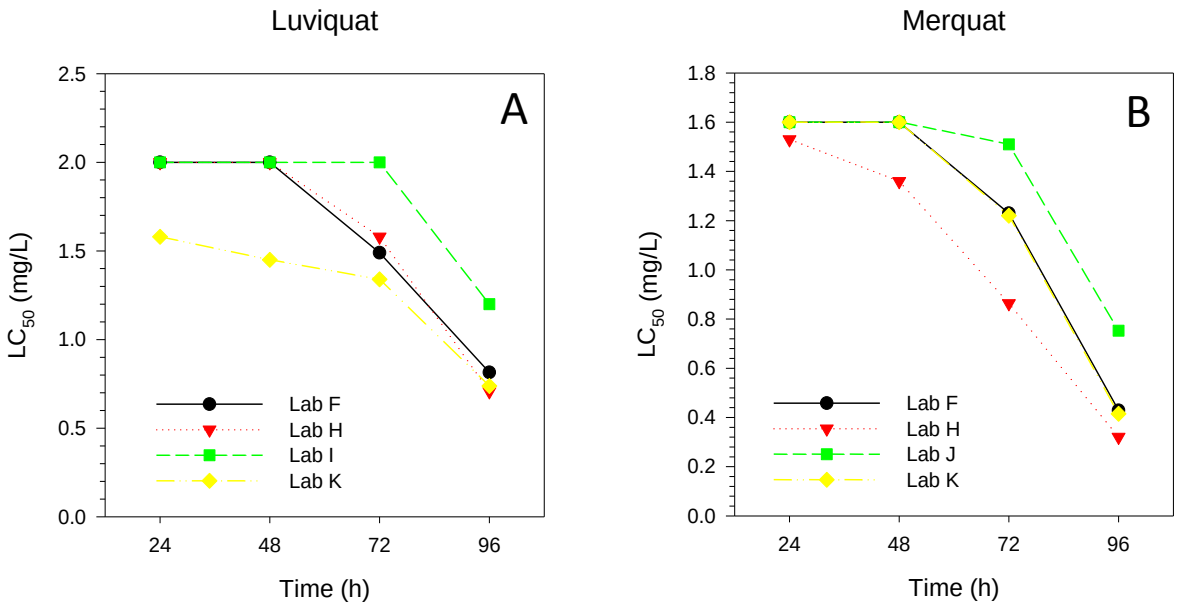


Figure 4. Chemicals whose toxicity is mostly expressed following hatch at 72 hr.

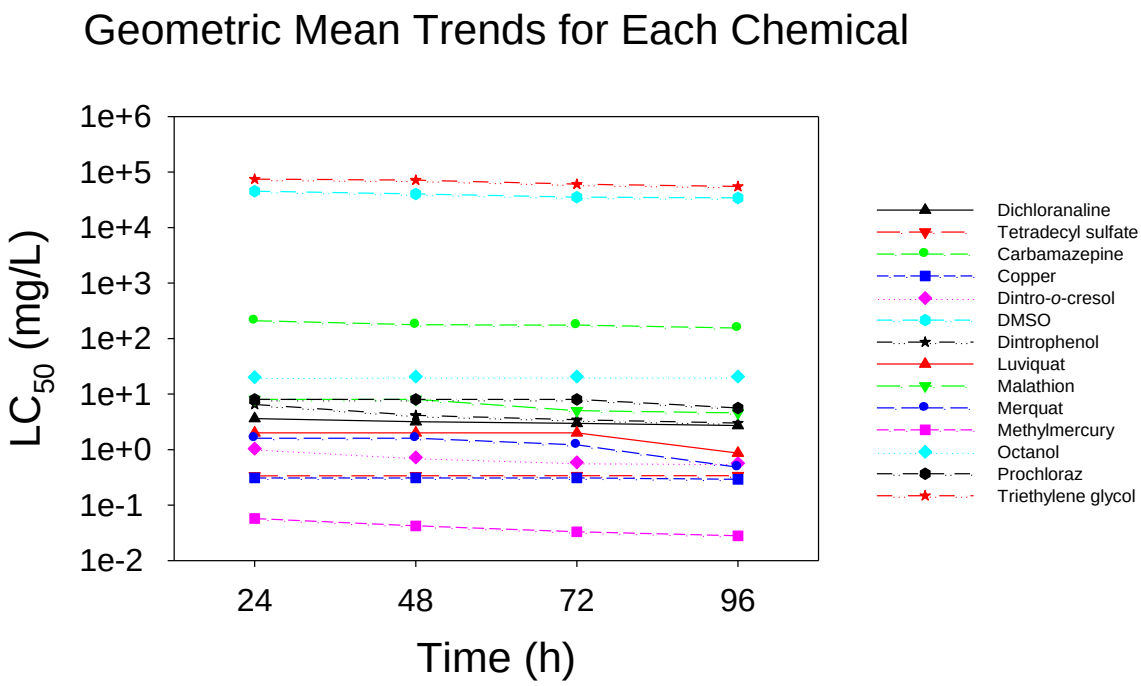


Figure 5. Overall time-course of the changes in $LC_{50}$s from 24 to 96 h for all compounds evaluated in Phase 2 of the OECD ZFET Validation Study.

# Annex IX: Evaluation of Hatching during the Zebrafish Fish Embryo Test Using Data Gathered from Phase 2 of the OECD Validation of the Zebrafish FET

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# Annex IX

**Overview**

Lethal effects of chemicals in the Fish Embryo Test are identified by observations of embryo coagulation, lack of heartbeat, lack of somite development and lack of detachment of the tail. Each one of these endpoints leads to death. Observation of developmental endpoints is also possible to consider and have long been documented for purposes other than establishing acute toxicity (Schulte and Nagel 1994; Nagel and Isberner 1998; Nagel 2000). A common endpoint used in longer-term fish toxicity tests is hatch rate (most often expressed as percent hatch). Delay in hatch is considered by some as a highly relevant sub-lethal effect from chemical exposure. In addition, hatchability in the negative control is an important criterion to judge quality of the brood stock as high levels of hatch indicate health of the parent generation. In the conduct of FET assays, hatching is also quite important as post-hatch exposure to the still developing eleutheroembryo may eliminate the issue of the chorion acting as a barrier to chemical exposure. It is clear from the time course analyses in Annex VIII that for most chemicals, the chorion does not act as a barrier to chemical penetration and uptake. Recent research in the laboratory of Th. Braunbeck (University of Heidelberg, personal communication) suggests penetration of chemicals through the chorion, just like passage across biological membranes in general, is a complex interaction of molecular size, hydrophobicity, and electro-static charge interactions.

During Phase 1 and Phase 2 of the OECD ZFET validation study, additional information on hatching was gathered by all participating laboratories during each test. Numbers hatched in the negative control through time are of particular interest as this provides an indication of the likelihood of embryos being exposed both with and without the chorion present. Hatching rate is also often used as a quality criterion in chronic fish tests. This report provides description of hatching rates and success during Phase 2 of the ZFET validation study and further compares whether inter-laboratory differences for hatching may have affected $LC_{50}$ determinations. The presumption here is that if negative controls did not hatch or hatching rates across different tests were largely different, then exposure to chemicals by embryos may have been reduced and $LC_{50}$s underestimated as a result.

**Patterns of Hatching in Negative Controls**

Overall, approximately <1%, 71%, and 97% of embryos hatched by 48, 72, and 96 h, respectively. Based on experience with contract laboratory facilities, the rate of hatch observed in Phase 2 of the ZFET validation study is within the normal range for experienced laboratories. Figure 1 depicts the percent of negative control hatch for all runs in Phase 2 along with the median, lower 75$^{th}$ and lower 90$^{th}$ percentiles. By test termination, only 3 runs of 153 did not achieve 80% or greater hatch. The median hatch rate was 80% by 72 h and at the 75$^{th}$ percentile over half of embryos were hatched at this time point.

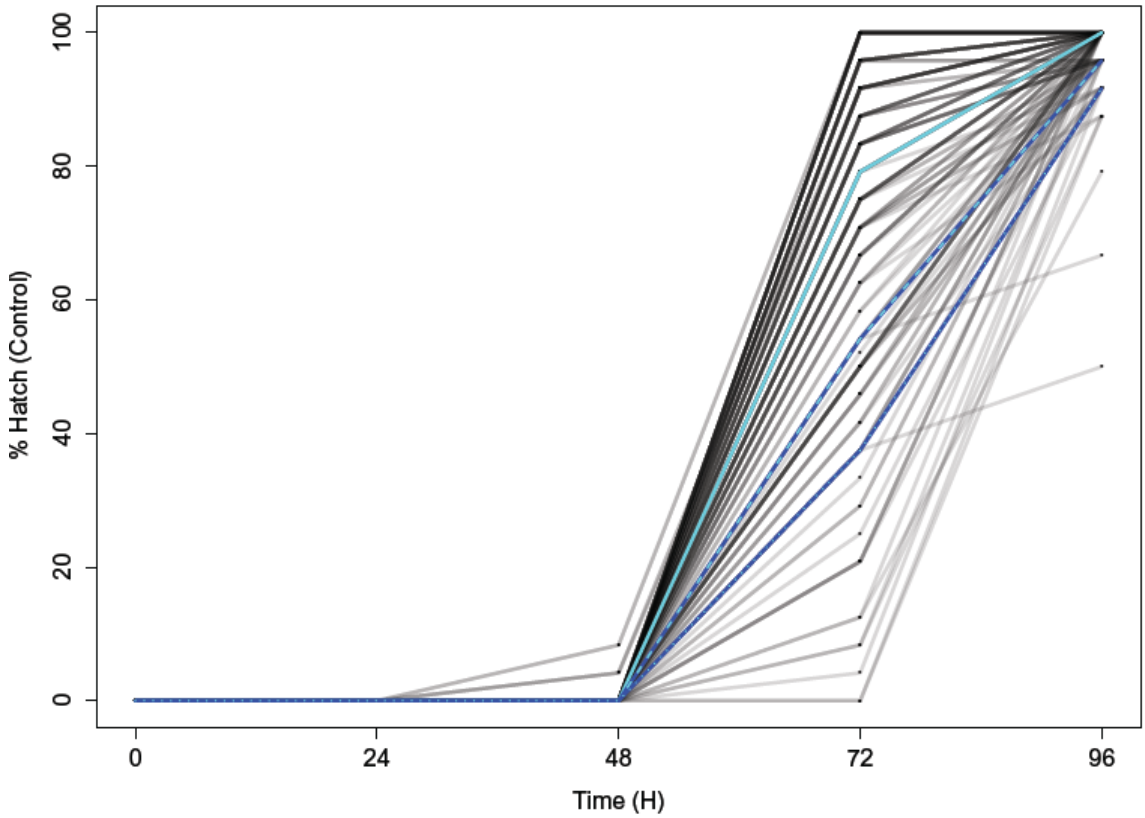


Figure 1. Percent hatch at each time point in controls. The light solid blue, medium dashed blue, and short dashed blue lines are the median, 75$^{th}$ percentile and 90$^{th}$ percentile (n=153 tests).

**Effect of Hatch on 96 h LC50s**

If hatch was significantly delayed for some reason, it could be hypothesized that the 96 h $LC_{50}$ would be higher assuming that the penetration of the chorion by the chemical was an important phenomenon. One way to assess this would be to evaluate the 96 h $LC_{50}$ in light of the amount of embryos that had hatched at 72 h. One would expect that if the hypothesis were true, a downward trend in the 96h $LC_{50}$ with hatch rate. As seen in Figure 2, this appears to not be the case. There could be several reasons for this, among them that toxicity of many chemicals is not affected by the chorion at all. Indeed, as given in Annex IX, most chemicals express toxicity long before hatch identified as Groups 1 through 3.

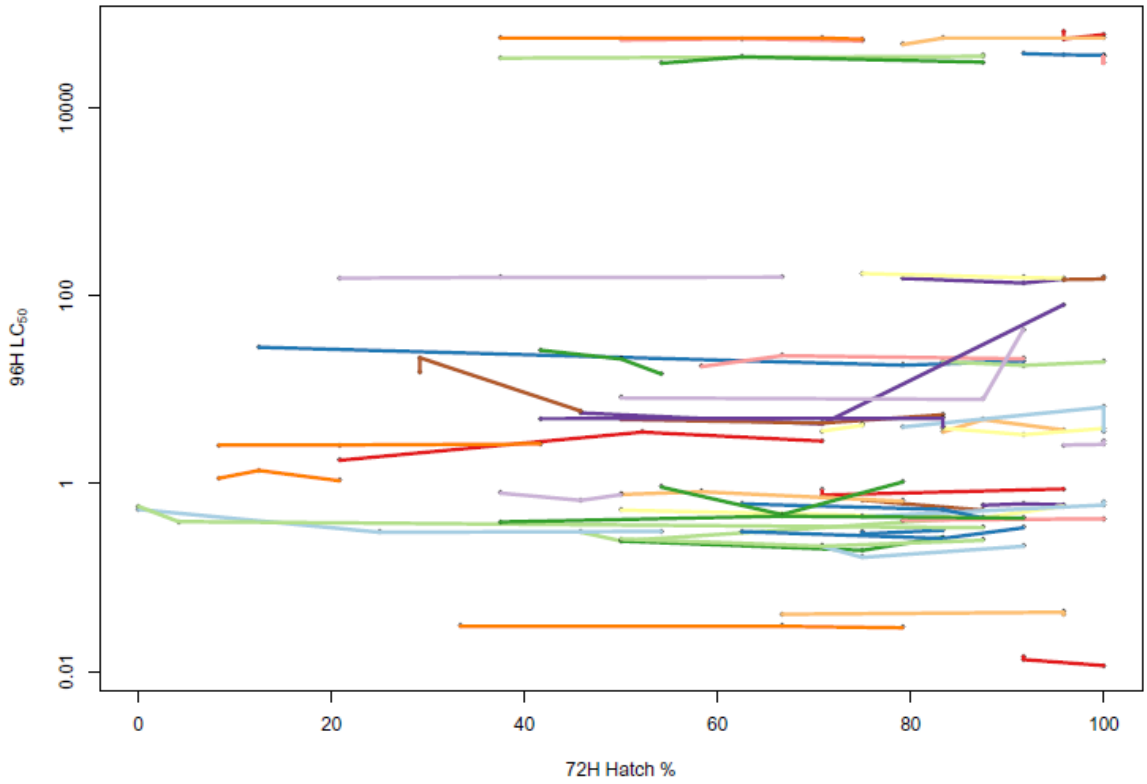


Figure 2. Assessment of the relationship of the final 96 hr $LC_{50}$ as a function of the number of embryos hatched at 72 h.

**Conclusions and Recommendations**

(4) Negative control hatch rates for zebrafish are high and quite consistent. Over 80% of negative control zebrafish hatch by 72 h and the 90$^{th}$ percentile exceeds 90% hatch by 96 h.
(5) Only 3 out of 153 runs did not achieve 80% hatch at 96 h. These occurred in laboratories B, D and I.
(6) The 96 h $LC_{50}$ appears to be unrelated to the percent of embryos hatched at 72 h.

**Literature**

Nagel R, Isberner K. 1998. Testing of Chemicals with fish-a critical evaluation of tests with special regard to zebrafish. In, Braunbeck T, Hinton DE, Streit B, eds, *Fish Ecotoxicology*, Birkhauser Verlag, Basel, Switzerland, pp 337-352.

Nagel R. 2002. DarT: The embryo test with the Zebrafish Danio rerio - a general model in ecotoxicology and toxicology. *ALTEX* 19:38-48.

Schulte C, Nagel R. 1994. Testing acute toxicity in the embryo of zebrafish, Brachydoanio rerio, as an alternative to the acute fish test: preliminary results. *ATLA* 22:12-19.

# ANNEX X: Impact of Group Size on the Estimation of $LC_{50}$ in the Zebrafish Fish Embryo Test

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# Annex X

**Summary**

In the OECD validation study of the ZFET method, 20 zebrafish embryos per group were used in a series of five concentrations. A statistical computer simulation study was used to quantify the effect of group test size on the estimation of the $LC_{50}$ and its confidence interval. Under conditions where there is the potential for conducting a test for which the true (but unknown) $LC_{50}$ is not well centered in the concentration range chosen, and when the response trend is relatively flat, the simulations demonstrate that reducing the group size also diminishes the likelihood of deriving a sound $LC_{50}$ with reasonably useful 95% confidence intervals. The advantages quantified in the simulation results, and the non-protected nature of the life stage being tested, leads to the recommendation that 20 per group will optimize the value of the tests in practice. In practical terms, this also has the benefit of reducing the likelihood that if fewer embryos are used/group, that multiple (non-random) exposure concentrations would be employed on the same exposure plate (based on the standard 24-well plate).

# Annex X

**Introduction**

The statistical analysis of ZFET experiments in the validation study is done by a logistic regression model of the probability of mortality as a function of the log concentration. It has two parameters, $LC_{50}$ and $\beta$, where

$$\Pr\left(Dead|x\right) = \frac{1}{1+\exp\left(-b\left(x - LC_{50}\right)\right)}$$

The concentrations, $x$, and the corresponding $LC_{50}$ are assumed to be on the log10 scale. This model has a smooth, sigmoidal shape that has a long history of use in toxicity testing for methods that count a number of events of interest (here, mortalities) among individuals, where independent sets of individuals are tested at a variety of exposure levels. In practice, the logistic model used here is indistinguishable from the analogous probit model.

The one improvement to the standard analysis implemented in the validation study statistical analysis is the use of profile likelihood confidence intervals (PLL) instead of conventional "Wald-style" intervals that are calculated in the typical +/-2*SE manner (Venzon and Moolgavkar, 1988). This report will quantify the improvement due to the use of PLL over the standard Wald-type method (STD).

Statistical computer simulations (also called Monte Carlo methods, see for example http://en.wikipedia.org/wiki/Monte_Carlo_method), are used here for the ZFET method to quantify important properties of statistical estimates obtained from experiments—specifically, the quality of the estimates of the $LC_{50}$ and its confidence interval. As for any statistical analysis method, the number of individuals tested must affect the statistical properties of estimates, but so too does the positioning of the tested concentrations relative to the true underlying model, and the steepness of the concentration-response trend. In practice, the true underlying model is unknown. By using statistical simulations, we can define representative true models for the data, and then investigate how well the estimation works, taking into account the natural variability that will be present in the data.

**Cases covered in simulations**

This report describes simulated experiments that always test the same concentrations: 1, 2, 4, 8, 16. The actual value of concentrations is arbitrary. They could be any set of five equally spaced values with multiplicative spacing. The general observations made will still hold true. All figures cited in the text of this report can be found at the end this document, following the text.

# Annex X

A number of factors will influence the quality of results from ZFET experiments. Those covered in this report are:

1. The number of individuals exposed at each concentration (hereafter referred to as the group size): simulations evaluated 7, 10, 15, 20/group. Group sizes 7, 10, and 20 are of greatest practical interest to the ZFET method, as three sets of 7, two sets of 10, or a single set of 20, fit nicely with the 24-well plates in common use.
2. The location of the $LC_{50}$ relative to the concentrations tested. The simulations covered the full range from all of the concentrations being above the true $LC_{50}$ (so every group expects at least 50% mortality), through the case that all of the tested concentrations are below the $LC_{50}$ (so every group expects less than 50% mortality)
3. The slope of the concentration-response curve. The slope is related to the practical issue of spacing concentrations. A very shallow slope represents cases for which the concentrations are too close together, and a very steep slope represents concentrations too far apart. Too close together (shallow slope) is more problematic than too far, as it may not even be possible to bracket the $LC_{50}$ among tested concentrations when too close together. A total of six slopes were simulated ($\beta$ = 2, 3, 5, 7, 15, 20), but only 3, 7, and 15 are summarized in this report. Each of the main simulation results figures (5, 8, 11 – 14) contains three panels, one for each slope value.

Figure 1 illustrates the three basic shapes of response trend summarized in this report. These all have an $LC_{50}$ equal to 4 (the vertical reference line), exactly in the center of the concentrations tested. In the simulations, the $LC_{50}$ was shifted through the concentration range, at each of the tick values at the bottom of the figure region. For each combination of $LC_{50}$ and slope, a set of simulated experiments was run for each of the group sizes.

**Example of simulated experiment**

Consider one hypothetical estimation problem for which the true model has $LC_{50}$ equal to 3, the slope parameter $\beta$ is 5, and the concentrations tested are 1, 2, 4, 8, 16. The model is shown in Figure 2, with points on the curve showing the true response probabilities for each concentration (as fractions, they are 0.084, 0.293, 0.651, 0.894, 0.974). The vertical reference line shows the $LC_{50}$.

Experimental data can only approximate these true probabilities, subject to the natural sampling variability that will be a function of the group size. If 10 individuals are tested at each concentration with these probabilities, a single experimental result derived from this model, by simulation, is shown in Figure 3. The confidence interval is shown as a horizontal bar crossing the fitted model at 0.5. Some groups have response rates above the true rate, some below, but the model successfully estimates the $LC_{50}$, at least in terms that the confidence interval covers the true value of 3. A second simulated experiment from this same case is shown in Figure 4.

The Monte Carlo evaluation of any given case (the underlying model, concentrations tested, and group sizes) requires a high number of independent simulated experiments in order to accurately quantify how well the experimental setup (model, concentrations, group sizes) will estimate the $LC_{50}$. The full simulation results repeated the process numerous times (100,000 simulated experiments/case) in order to achieve very precise estimates of the performance of the statistical methods. For any single experimental setup the simulations quantify three attributes: (1) the long-run percentage of times the confidence interval will contain the true value; (2) the average bias in the estimates of the $LC_{50}$; and (3) the percentage of problematic results, such as no significant trend in responses or the failure of the model to cross 50% within the range of tested concentrations. For the readers not well versed in the use of computer simulations, its major advantage is that the true values of the parameters are really known, versus actual experimental data for which they are not, giving an ability to answer a question such as "Is the true LC50 inside of the calculated confidence interval?"

In simulated experiments, for which we know the true parameter values, a 95% confidence interval should encompass the true $LC_{50}$ value 95% of the time, as a long-run average. The confidence interval is nominally called 95%, but that is based on a statistical approximation that might require group sizes larger than those used in practice. Computer simulations provide an understanding of the performance of such "asymptotic" confidence intervals under practical use conditions.

Various summary figures are presented to assimilate the information as a function of (1) the slope and (2) location of $LC_{50}$ relative to test concentrations. These main figures have three parts A, B, and C corresponding to three response trend slopes.

**Results**

Evaluation of trend

Prior to attempting to fit a regression model to experimental data, it is reasonable to check whether there is evidence of an increasing trend in the response rates. For a shallow slope ($\beta$=3), Figure 5A shows that using 15 or 20/group would assure that a trend in responses would be detected with near certainty, as long as the true $LC_{50}$ lies somewhere between 2 and 8. Even for the larger group sizes, if the $LC_{50}$ is not in the middle half of tested concentrations (outside of 2 – 8 interval), a trend might not be evident. If the $LC_{50}$ is below 2, the practical setting of concern is one in which the responses are all relatively high, even at the lowest concentration. At the other end of the $LC_{50}$ range, the phenomenon is

reversed, with the responses all relatively low because the $LC_{50}$ is high in the range of concentrations tested.

For example, Figure 6 shows a case for which the true model (in black) has $LC_{50}$ =16 and $\beta$=3. It is a slowly rising curve. The Figure 5 indicates that about 6-7% of cases when group size is 10 will not show statistical evidence of a trend. An example of such data is superimposed on the model in Figure 6, in red. Using 7/group is even more problematic, because even if test groups are perfectly centered on the $LC_{50}$ =4, there is about a 3% chance that the data will not show a trend. An example of such a result is given in Figure 7.

Figure 5A shows that, when the slope is shallow ($\beta$=3), experiments using N=7/group clearly carry a risk that the results could be inconclusive, no matter where the $LC_{50}$ lies inside the range of tested concentrations. The more off-center the concentrations are relative to the $LC_{50}$, the more likely a trend will not be present in the data. 7/group is less problematic if the response trend is steeper (Figure 5B and 5C). In this case, any of the group sizes should reliably show a statistically significant response trend, as long as the true $LC_{50}$ is in the middle half of the concentration range tested. If the $LC_{50}$ is actually between the outer two concentrations, the smaller size studies will still sometimes fail to demonstrate a significant trend, and will likely also not have percent values that span the 50% response rate that is needed to estimate the $LC_{50}$.

Model/Data Failures

Even if positive trend in response is significant, it is possible that the fitted model will fail to cross the 50% level across the range of concentrations tested. In this case, the estimation of the $LC_{50}$ is suspect, being an extrapolation outside of the range of tested concentrations. The combination of these two attributes of study results gives a more comprehensive picture of the likelihood of success from a study under given conditions, and is summarized in Figure 8. For shallow slopes (Figure 8A), even 20/group can lead to failed studies when the true $LC_{50}$ is in the outer reaches of the concentrations tested. Down to 7/group, the rate of study failures can be fairly high, even when the $LC_{50}$ lies in the middle half of concentration range. In the following figures, study failure percentage rates are the gap between the lines and 100%.

An example of 7/group data for which the data exhibit trend, but model crosses 50% outside of the range tested is given in Figure 9. A true underlying model for which this is possible is when $\beta$=3 and $LC_{50}$ =1.70.

In the validation study, problems were more often associated with low toxicity throughout the range tested, such as in Figure 10, for which trend is significant, but the model is always below 50%.

Larger group sizes improve the chances that the highest concentration would exceed 50%, both in actual data value and model prediction.

Bias

The simulations clearly demonstrate a tendency for estimates of the $LC_{50}$ to be too high when the true $LC_{50}$ lies within the span of the two lowest concentrations, and a tendency for the estimates to be too low when the true $LC_{50}$ lies between the two highest concentrations (Figure 11). If the average predicted $LC_{50}$ from models fit were identical to the true $LC_{50}$, the lines in the figures would track on the line of equality. There is some departure from equality, especially in the shallow response trend case (A), and very little otherwise. This fact could be taken into account when setting exposure levels for the main test, and for interpreting results when the estimated $LC_{50}$ lies near the extremes of the concentrations tested.

This assessment for bias was conducted only on simulated experiments that were acceptable by having evidence of trend and predictions that cross the 50% response level, as in Figure 8.

Confidence Interval Coverage

In this summary of confidence interval coverage, again only data that fit our definition of success are summarized ($p<0.05$ for evidence of a trend in responses, and the model predictions cross 50% within the 1-16 range of concentrations assumed.).

In Figure 12, the coverage probabilities of 95% confidence intervals are summarized, for the PLL method adopted for the validation analyses. If the confidence interval calculation were perfect, the lines shown in these figures would be perfectly flat at 95% across the range of true $LC_{50}$'s. Obviously that is not the case. In shallow response trend cases (A) a pattern exists in which the CI widths tend to become wider than necessary (well over 95% coverage) when the true $LC_{50}$ is not well centered on the tested concentrations, but the PLL method does achieve nearly 95% true coverage for all group sizes. For steeper slopes ($\beta$=7, 15), the coverages become more sensitive to the group size used. For $\beta$=7, N=15 or 20/group performs reasonably well, but the lower sizes have widths that are too narrow on average. At N=7, even in the middle half of the concentration range, the coverage values are sometimes near 90%,

rather than 95%. Given this fact, it might make some sense to implement a procedure that makes the intervals wider, if small sizes like N=7/group are used in real experiments.

Still, the PLL CI method is far superior to the STD method. The STD method of calculating CIs has much different properties (Figure 13). In the first panel for ($\beta$=3) the STD method fails in a larger region near the concentration limits, but otherwise works well. But, beyond the shallow slope case, the STD method fails to cover the true $LC_{50}$ at anything near 95% ($\beta$=7 or $\beta$=15). For very steep response trend ($\beta$=15), the STD method simply does not work.

Both Figures 12 and 13 are presented on the same scales, making it easy to contrast the two procedures, and conclude that PLL is superior.

CI width

Even if confidence intervals cover the true $LC_{50}$ at the correct percentage, that does not imply that the widths from different group sizes are the same. Larger group sizes will yield more narrow CIs. Figure 14 shows the percentage of times the width of the PLL confidence interval on $LC_{50}$ can be expected to be larger than a 2X ratio of the upper endpoint to the lower. This 2X value was chosen because it is the exposure spacing. For a shallow slope ($\beta$=3), the confidence intervals can be expected to be wider than the concentration spacing, even if 20/group is used, and no matter where the true $LC_{50}$ lies in the concentration range. At an intermediate slope ($\beta$=7), 20/group will almost always give a smaller CI, as long as the $LC_{50}$ lies in the middle half of exposures. 7/group will more than 50% of the time yield wider CIs, even if the $LC_{50}$ is in the middle of exposure concentrations. In steep response models ($\beta$=15) N=7/group will still produce wider CIs, even if the $LC_{50}$ is in the middle.

**Discussion**

These simulations provide detailed information on the performance of the statistical methods used for ZFET experiments. There is very clear evidence that as long as the concentration spacing is meaningful (ie, not so wide as to provide no useful information), and the concentrations capture the true (but unknown in practice) $LC_{50}$ within the middle half of concentration range, the method will almost always provide an estimate of the $LC_{50}$, and a confidence interval that is usually contained within the range of concentrations tested. However, for smaller group sizes (especially 7/group) and shallow response trend, the confidence intervals can be relatively wide, and this should be taken into account prior to the study execution. The ultimate decision on a study size recommendation would partly depend on how often the $LC_{50}$ is judged to lie between the outer concentrations, along with the frequency of shallow response trend. Higher group sizes provide insurance against the potential complication of the need to

rerun an experiment, and they will also deliver less biased estimates, and tighter confidence intervals within which the true $LC_{50}$ probably lies.

There is a quantifiable advantage to larger group sizes. N=20/group will clearly produce more robust experimental results, and fits well with the practical in-lab considerations, such as the 24-well plate in common use.

**References**

Venzon, D.J. and Moolgavkar, S.H. (1988), "A Method for Computing Profile-Likelihood-Based Confidence Intervals," *Applied Statistics*, 37, 87-94.

Figure 1: Three basic shapes of response trend summarized in this report. For each curve shown, the $LC_{50}$ is 4. All other $LC_{50}$ values studied (vertical ticks at bottom) are a simple horizontal shift of the curve and vertical line to the value of interest.

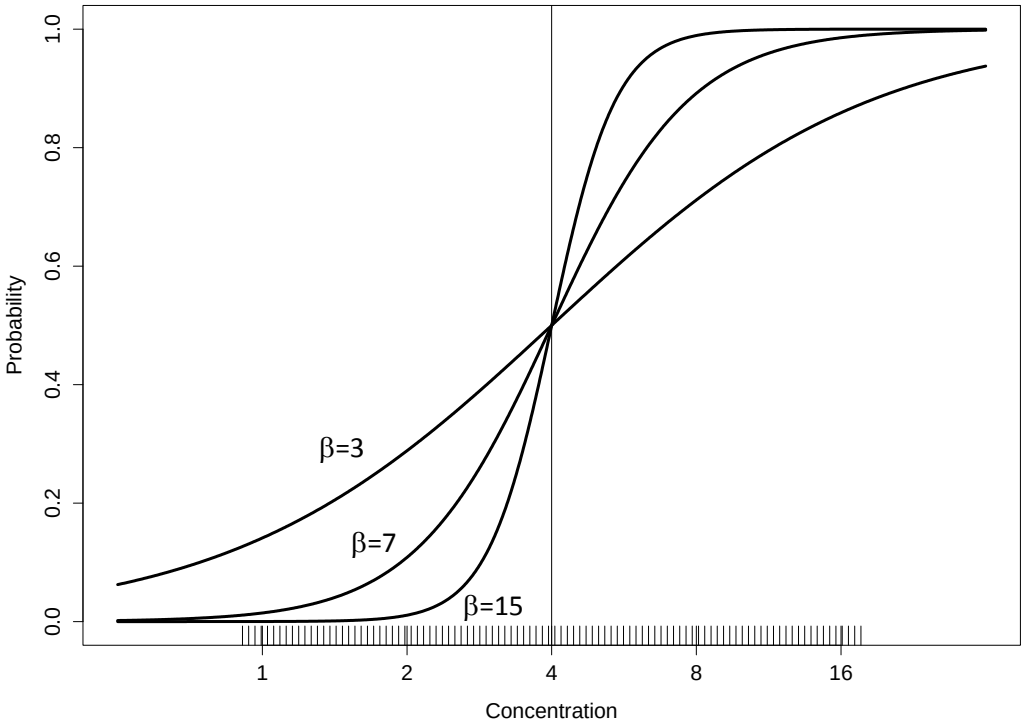


Figure 2: Model with true $LC_{50}$ = 3 and slope parameter $\beta$ = 5.

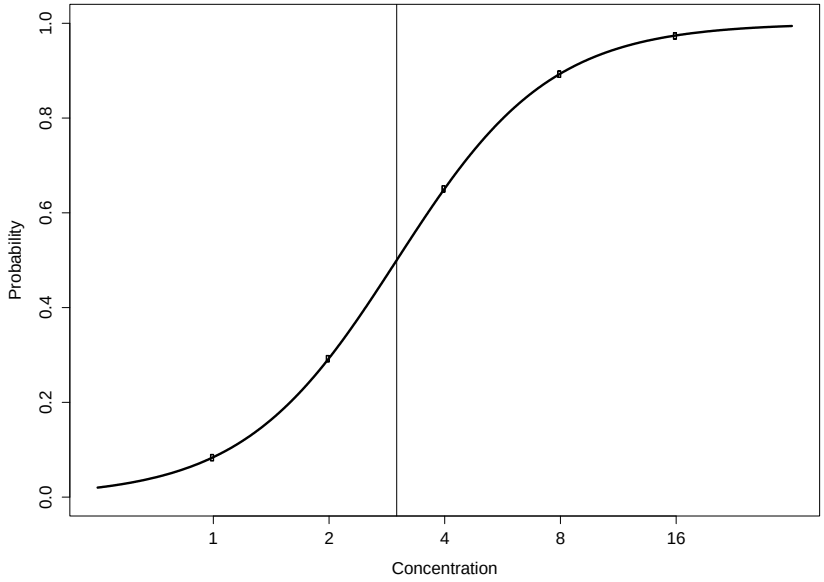

Figure 3: A single simulated experiment using 10/group and fitted model plus 95% CI (blue), from true model with $LC_{50}$ = 3 and the slope parameter $\beta$ = 5 (black).

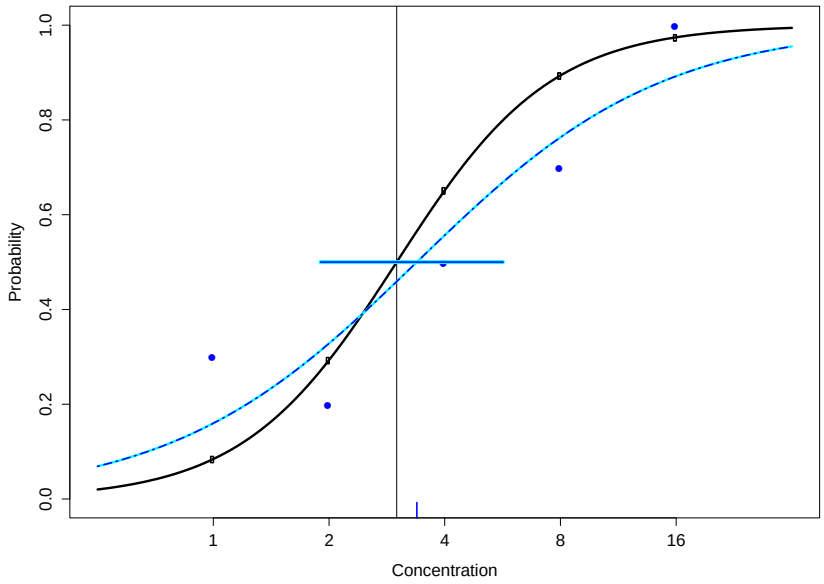


Figure 4: A single simulated experiment using 10/group and fitted model plus 95% CI (blue), from true model with $LC_{50}$ = 3 and the slope parameter $\beta$ = 5 (black).

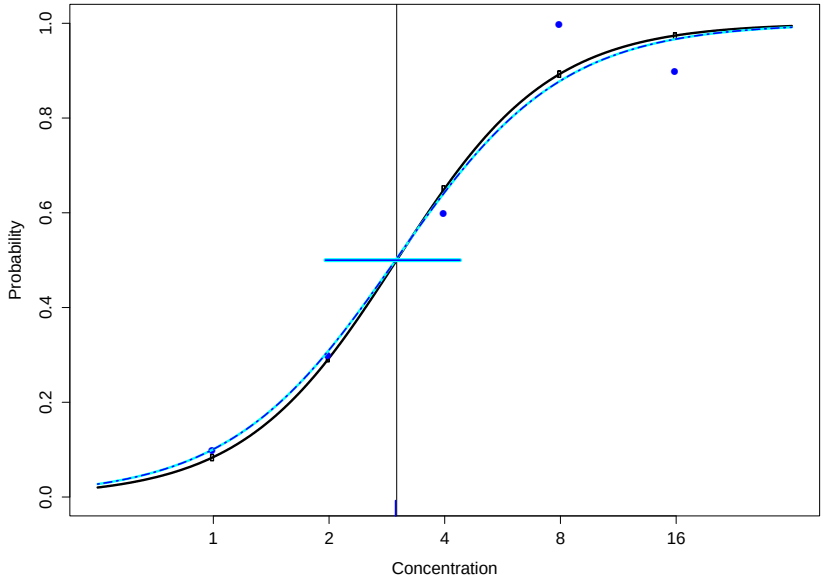

Figure 5: The effect of group size on evidence of a response trend (statistical power to detect the trend) for three response trend slopes.

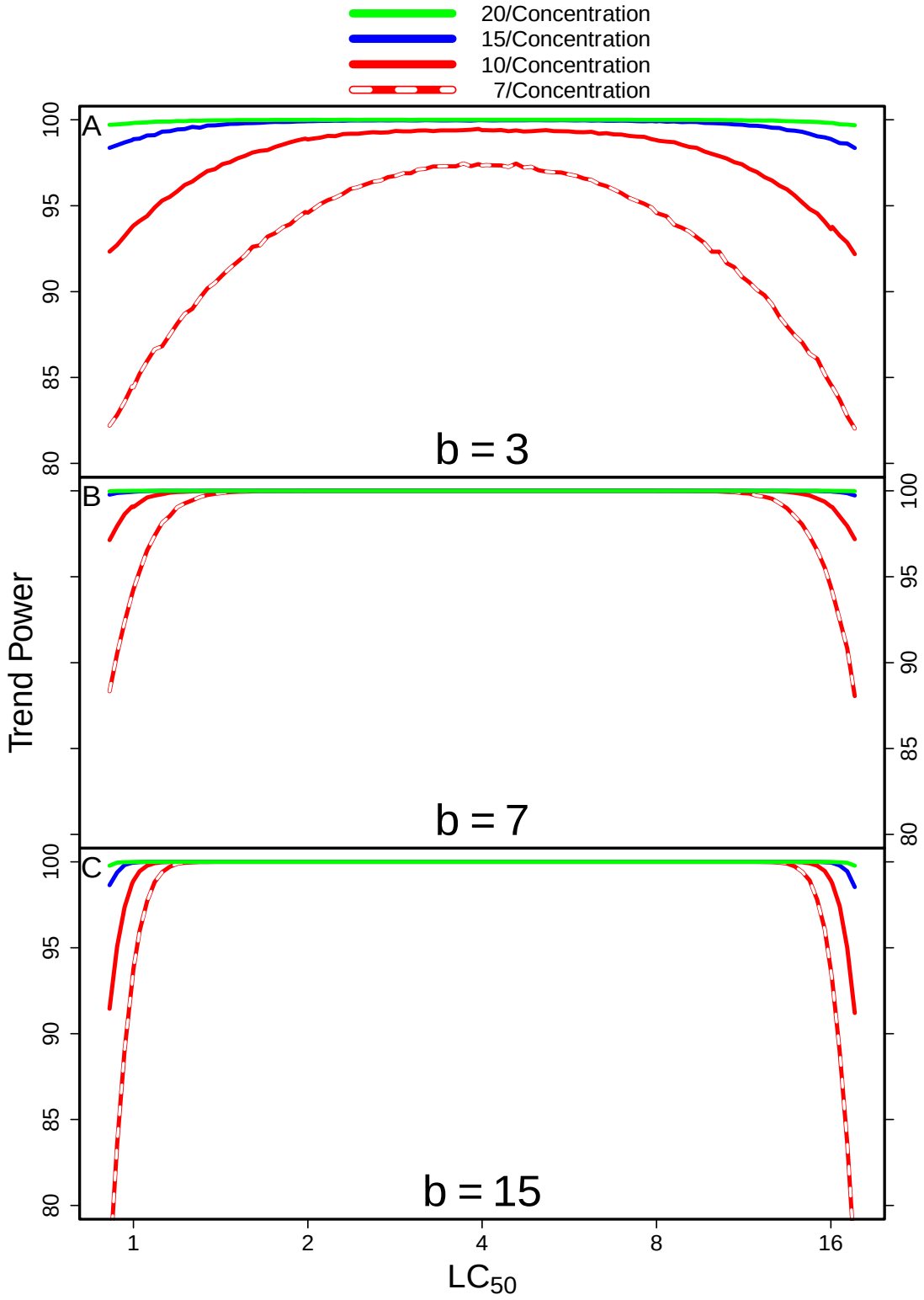

Figure 6: A single simulated experiment using 10/group (red) from true model with $LC_{50}$ = 16 and the slope parameter $\beta$ = 3 (black). The response trend in the data is not statistically significant (p>0.05)

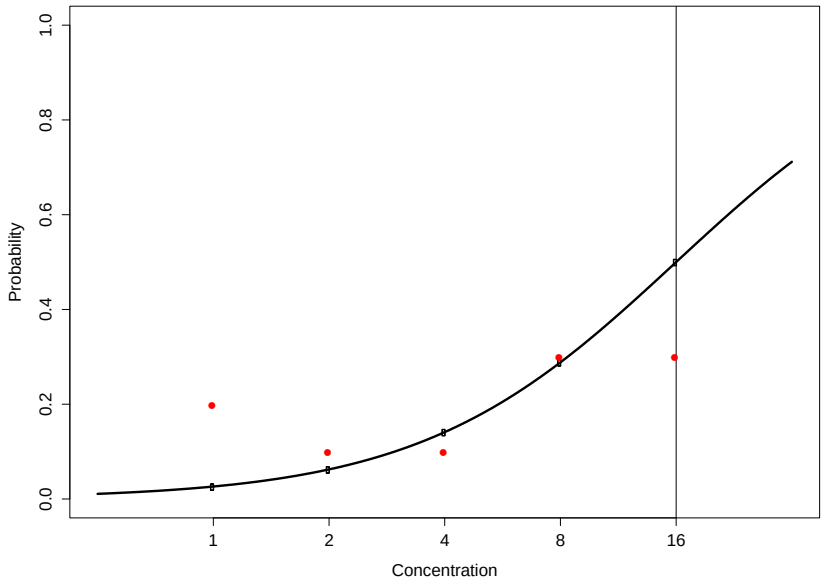


Figure 7: A single simulated experiment using 7/group (red) from true model with $LC_{50}$ = 4 and the slope parameter $\beta$ = 3 (black). The response trend in the data is not statistically significant (p>0.05)

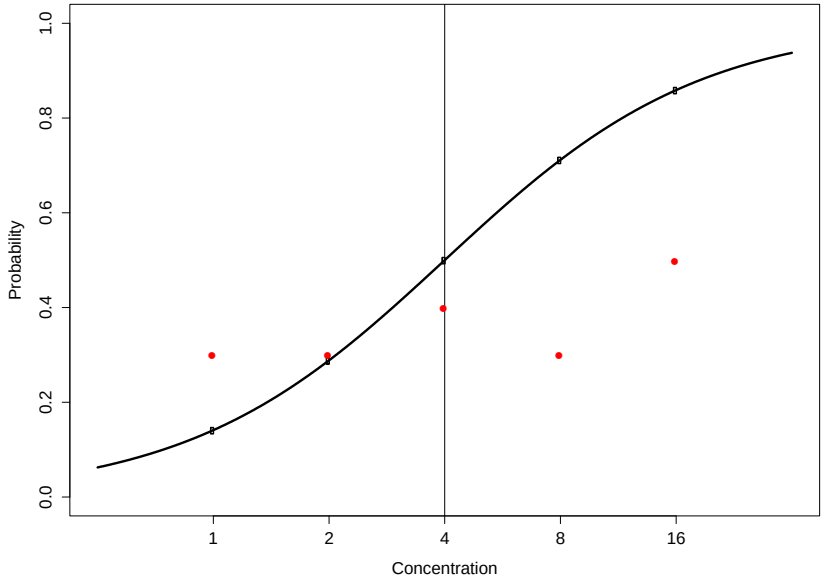

Figure 8: The effect of group size on the likelihood that a good experimental result will be obtained (both significant trend and prediction that crosses 50%) under three response trend slopes.

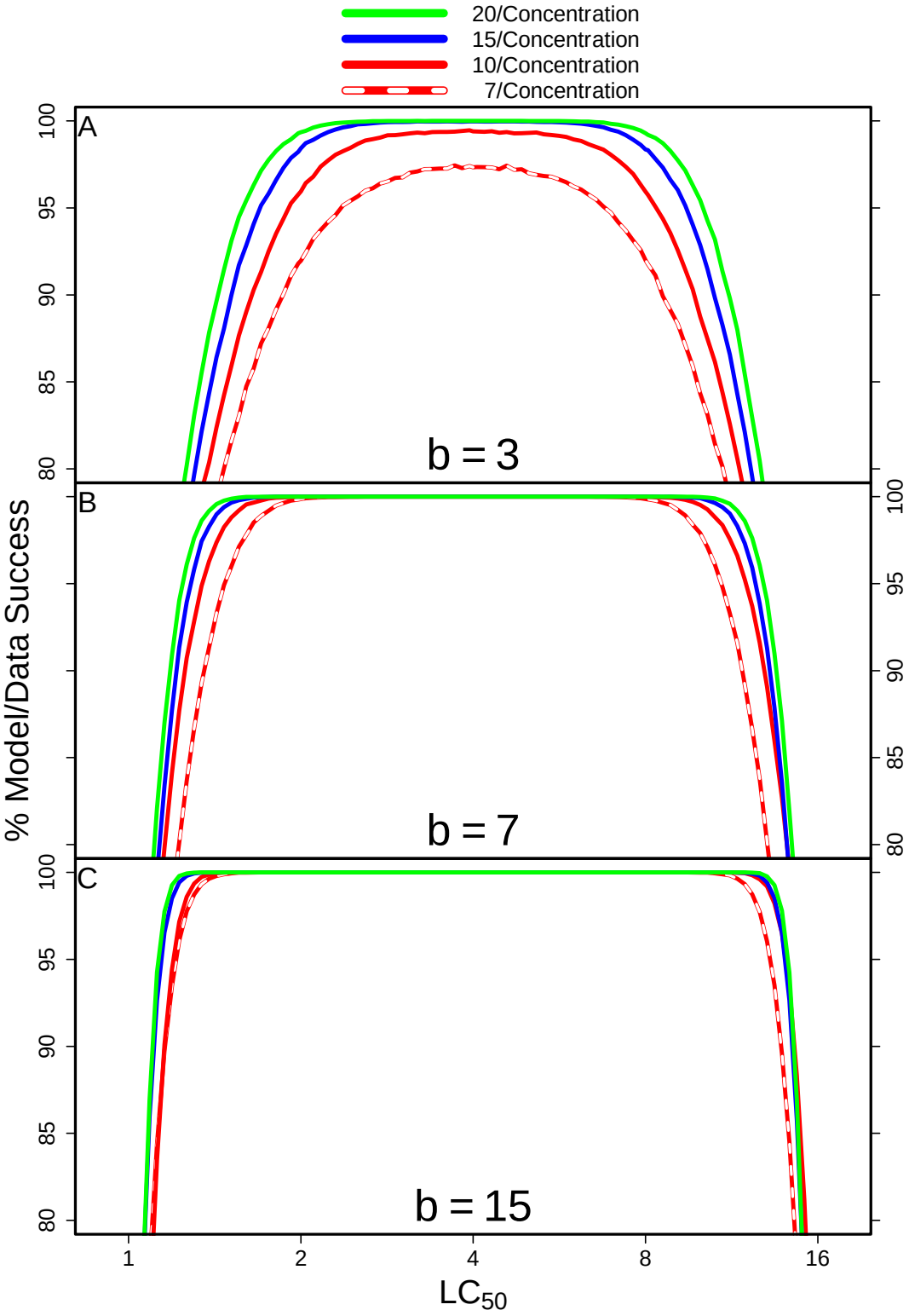

Figure 9: A single simulated experiment using 7/group and the fitted model plus 95% CI (blue), from a true model with $LC_{50}$ = 1.7 and the slope parameter $\beta$ = 3 (black). Data show a trend, but the $LC_{50}$ is estimated to lie below all of the tested concentrations.

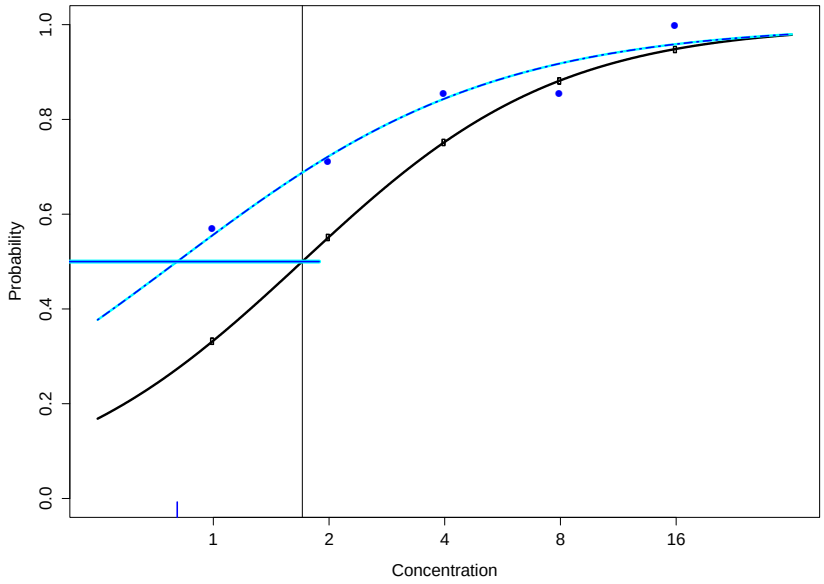


Figure 10: A single simulated experiment using 7/group and the fitted model plus 95% CI (blue), from a true model with $LC_{50}$ = 12.3 and the slope parameter $\beta$ = 3 (black). Data show a trend, but the $LC_{50}$ is estimated to lie above all of the tested concentrations.

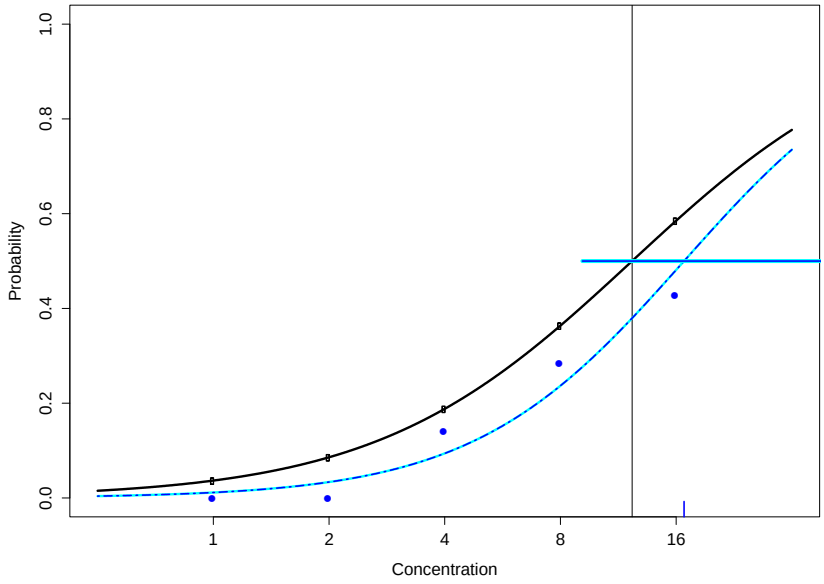

Figure 11: The effect of group size on estimation of the $LC_{50}$ . When colored lines line on top of the line of equality, there is no bias present.

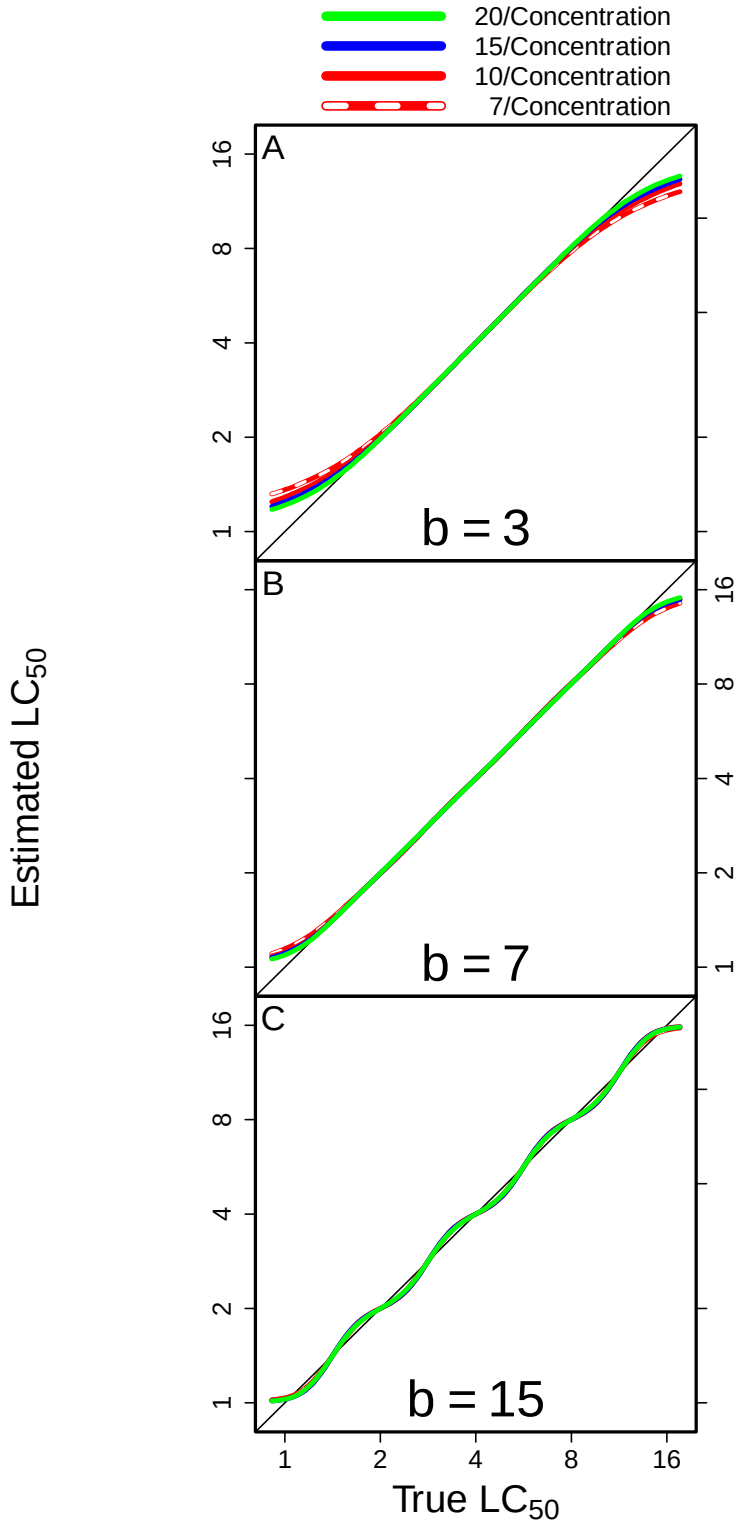

Figure 12: The effect of group size on the rate at which 95% PLL confidence intervals contain the true $LC_{50}$ . The horizontal reference lines are at 94, 95, and 96% to show the target and a window within which the result would be considered very good.

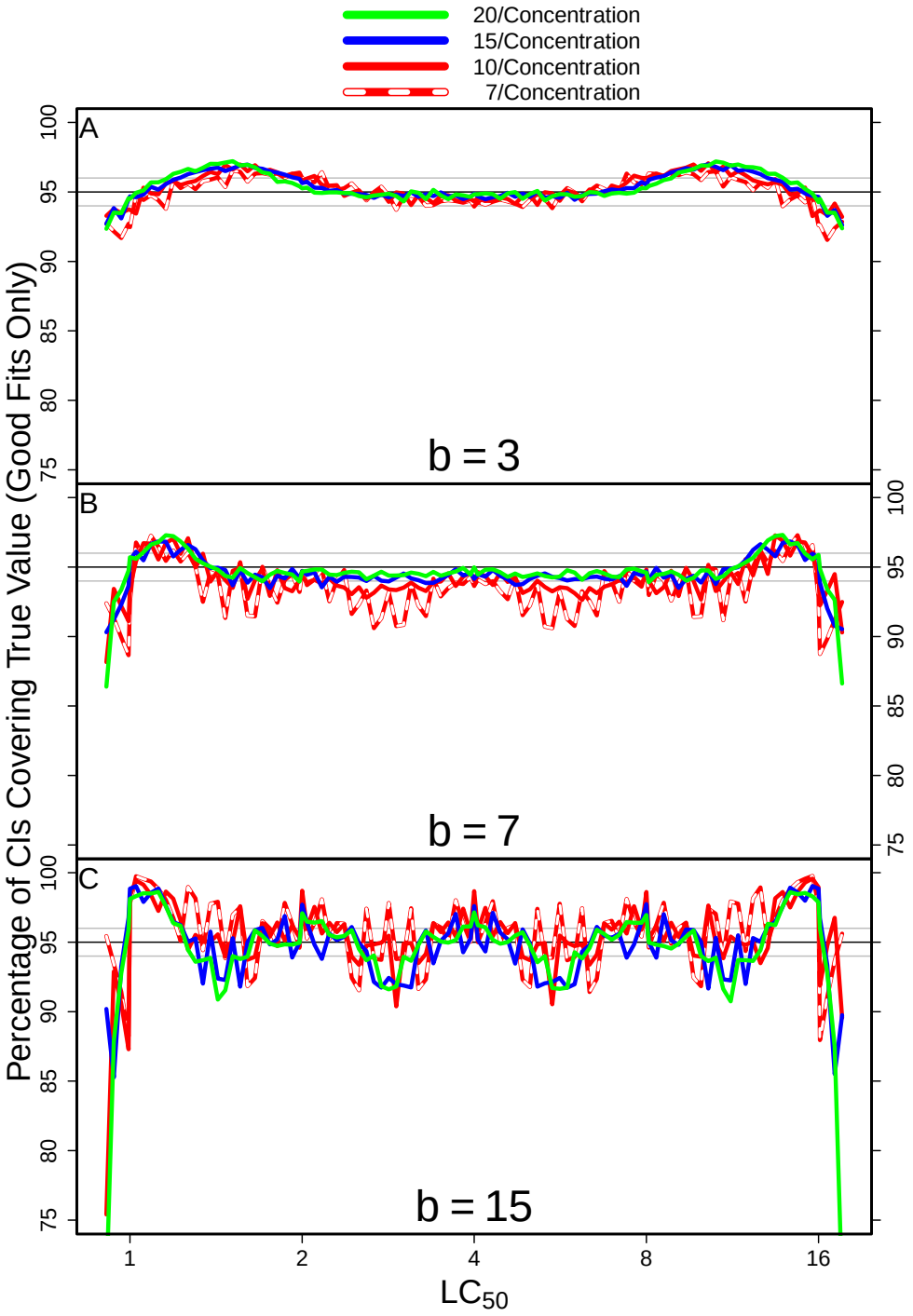

Figure 13: The effect of group size on the rate at which 95% PLL confidence intervals contain the true $LC_{50}$ . The horizontal reference lines are at 94, 95, and 96% to show the target and a window within which the result would be considered very good.

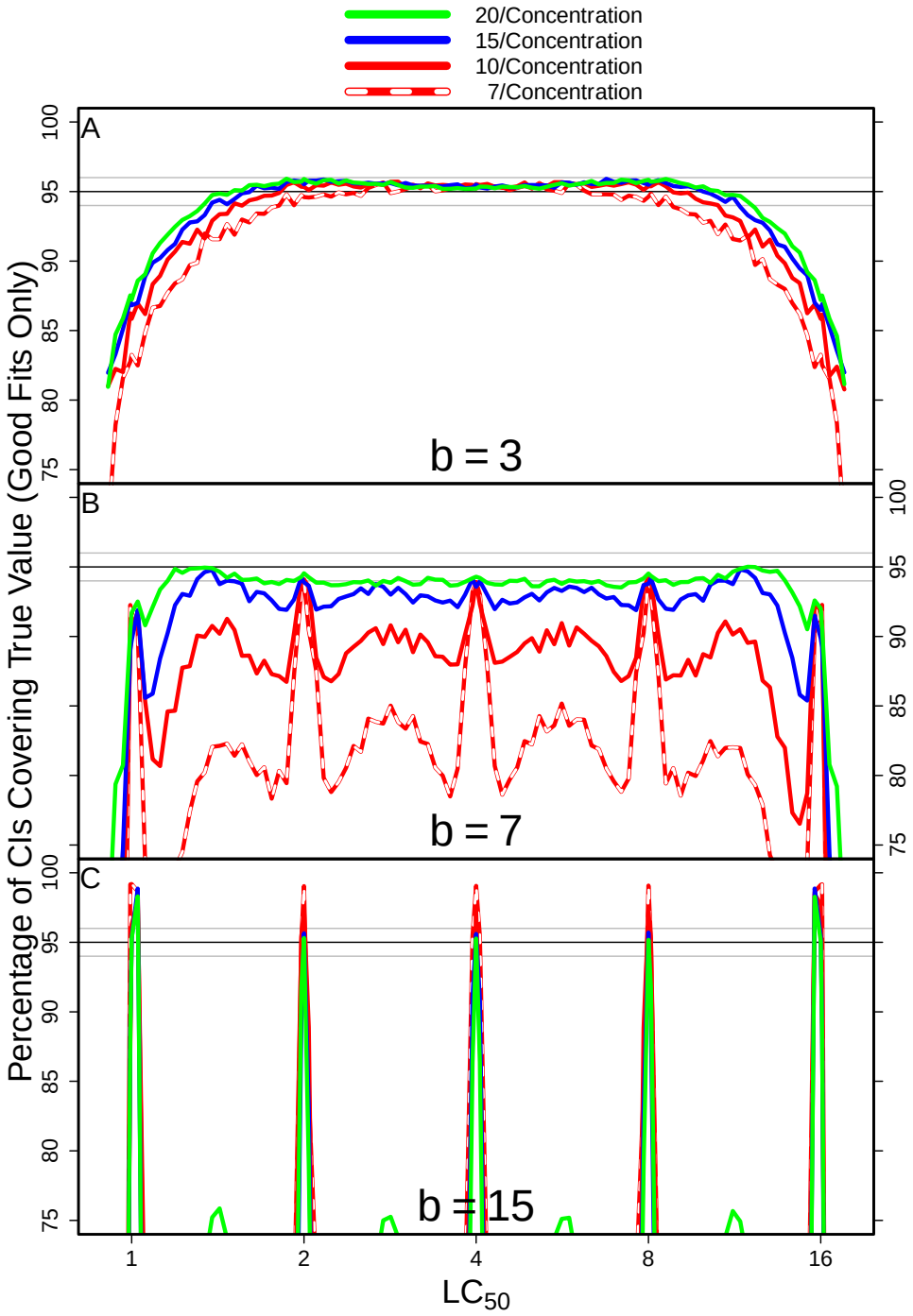

Figure 14: The effect of group size on the rate at which the width of 95% PLL confidence intervals are less than the 2X concentration spacing.