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This publication was developed in the IOMC context. The contents do not necessarily reflect the views or stated policies of individual IOMC Participating Organisations.

The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The Participating Organisations are FAO, ILO, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD. UNDP is an observer. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

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FOREWORD

This document presents a second validation report of the harpacticoid copepod (*Amphiascus tenuiremis*) development and reproduction test.

In 2002, Sweden submitted a project proposal to the Working Group of the National Coordinators of the Test Guidelines Programme (WNT) to develop a new Test Guideline on the development and reproduction of copepods, an aquatic arthropod species. In 2007, A first validation report of the full life-cycle test with the harpacticoid copepods *Nitocra Spinipes* and *Amphiascus Tenuiremis* and the calanoid copepod *Acartia tonsa* (phase 1) was published in the Series on Testing and Assessment as No. 79. Further validation work was needed to ensure the relevance and reliability of the test method, and the species *Amphiascus tenuiremis* was selected for further experimental work.

In June 2009, a meeting of the OECD invertebrate testing expert group reviewed the outcome of the validation, acknowledged the challenges in applying this test method and made a number of recommendations for further work on this test method. These recommendations are included in the present report. The report was endorsed by the WNT at its meeting held on 12-14 April 2011. The Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology agreed to its declassification on 22 June 2011.

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology of the OECD.

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INTRODUCTION

1. This report covers progress as of May 2009 on the "second inter-laboratory calibration" of the draft OECD Copepod Test Guideline. The below chart shows the history of the Test Guideline's development, from its inception, as a co-project among Sweden, Denmark and the UK, up to the present time). The USA became involved in 2003 with the publishing of an American Society for Testing and Materials (ASTM) water quality standard test method for copepod full lifecycle testing (ASTM E2317-04). That method has been adapted here for this OECD copepod test guideline. In brief, the Test Guideline details how to culture, isolate and expose freshly hatched copepod larvae for full lifecycle exposures as individuals in microwells of 96-well microplates. It recommends the benthic harpacticoid copepod *Amphiascus tenuiremis*, but there are other copepod species that may be similarly suited for lifecycle testing using this approach (e.g., *Robertsonia propinquum*). There are now a dozen peer-reviewed journal articles using this method successfully to evaluate eight anthropogenic chemicals (see appended bibliography).

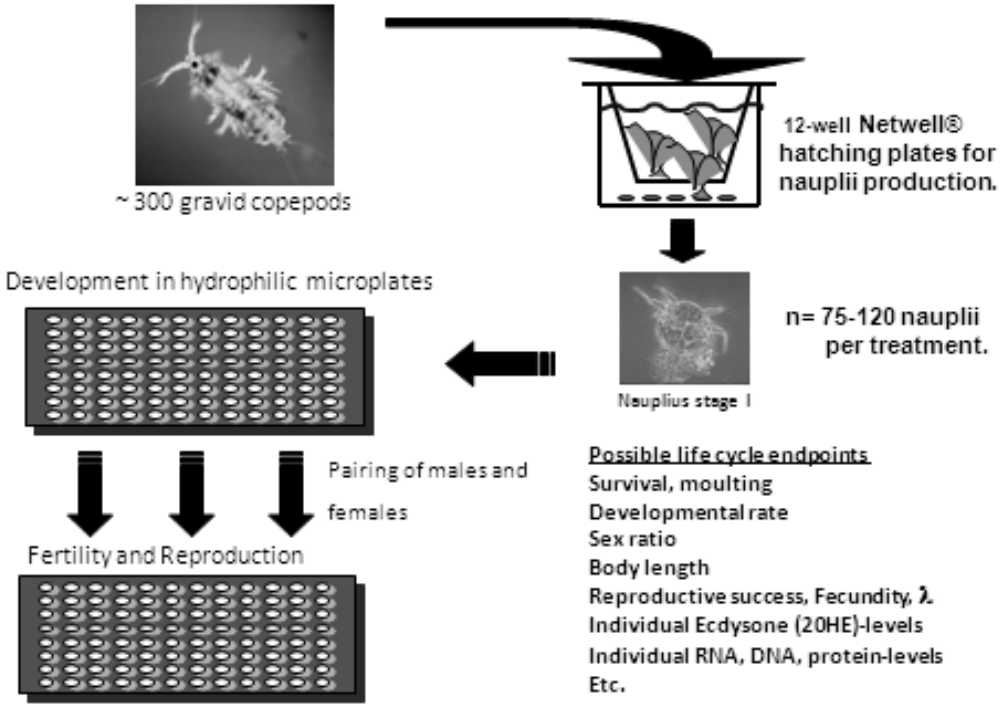
1. Background – Short history of the OECD copepod guideline

2002	Standard Project Submission Form for New Test Guideline (Sweden).
2003	**First draft** written as a co-project between UK, Denmark and Sweden. The harpacticoids *Nitocra spinipes* and *Tisbe battagliai* were included together with the calanoid *Acartia tonsa*.
2003	After circulation: Paris-meeting decided to **include *Amphiascus tenuiremis*** and change the experimental design **from cohort** exposure **to individual exposure** in microwells.
2004	**Revised drafts** sent out for comments (harpacticoid and calanoid parts separated). Reproduction added as new endpoint to the calanoid draft!
2005	Pre-validation including labs from Sweden, UK, Denmark (x2) and USA. Report finished in September and discussed in Paris. It was decided to continue with both drafts and use 3,5-DCP as a single reference substance. Minor changes to the drafts.
2006	Ring test including labs from Sweden (x3), USA (x2), UK (x1), Denmark (x2). Report finished in December.
2007	Decision to **proceed** with a second **intercalibration with *A. tenuiremis***.

2. Figure 1 below is a flow chart of the essence of this copepod-based test guideline. A revised draft guideline is forthcoming which will incorporate suggested improvements and eliminate some inconsistencies and ambiguities identified in the validation process for the most recent 2007 version. We direct the reader of this report to that draft OECD Copepod Test Guideline if there are questions regarding methods and materials used to produce the test data discussed and compared below for each participating laboratory. Four laboratories participated in this inter-laboratory calibration of the guideline: The University of South Carolina (hereafter called USC), the NOAA Center for Coastal Environmental Health and Biomolecular Research (NOAA), Stockholm University (Stockholm), and the SLU, Uppsala,

(Uppsala). Two reference chemicals (atrazine and lindane; analytical standard quality, > 98% purity) were tested individually over a range of 5 serially diluted concentrations plus controls for a 36 day (or less) "full lifecycle through two broods" exposure period. Although not part of the OECD copepod test guideline, life-table data produced by this method were used to estimate population instantaneous population growth rates (λ) and predict population growth and stage structure as a function of concentration as a demonstration for the reference chemical lindane (i.e., Table 18 and Figures 8 -16).

Figure 1. Essence of the copepod microplate test – Individualized exposures.



3. As of December 2008, all four labs (NOAA, USC, Stockholm and Uppsala) have completed lindane validation tests. NOAA, USC and Stockholm have also completed atrazine validation tests. Uppsala had planned to run a definitive atrazine test beginning March 9, 2009, but they encountered problems with algal culture contamination and paternity leave issues for their technician, and requested a further delay until June 2009. Fresh algal stocks were shipped to Uppsala by USC on May 15. After June 2009, the Uppsala atrazine test could not be carried out due to the persistent difficulties mentioned above with stock culture of marine algae. Copepods cannot be cultured in good health or at all if algal stocks cannot be established and maintained in good health. Uppsala has abandoned further efforts to validate the copepod Test Guideline.

4. In February 2008, all supplies and chemicals (atrazine and lindane) for the validation were ordered and received. Reference chemical concentrated stocks were prepared at the USC Chandler laboratory in Columbia, SC, using technical-grade chemicals (Atrazine = 98% purity; Lindane = 99.6%) in pesticide-grade acetone. Prior to shipping, chemical stocks were analyzed by Dr. Lee Ferguson (USC Chemistry Dept.) to verify nominal concentrations. Atrazine was measured in concentrate stocks using HPLC-UV (214 ± 38 ug/mL), and lindane using GC-ECD (47.3 ± 2.2 ug/mL). Limit of detection (LOD) for atrazine was 0.001 ug/mL. LOD for lindane was 0.0005 ug/mL. Supplies and stock reference chemicals were driven by courier to the NOAA lab in Charleston, SC. Supplies, pesticide-grade acetone and stock reference chemicals were sent to the laboratory of Dr. Magnus Breitholz in Stockholm via FedEx

International Priority. However, strict regulations on the importation and use of hazardous substances (i.e., the chemical stocks) in Sweden resulted in a significant delay in delivery (April 2008). Chemicals were returned to USC and had to be re-shipped to Sweden via a commercial shipping company that was able to gain Swedish Customs approval to deliver the concentrated pesticides in acetone. This initial obstacle set Sweden behind US lab test schedules by approximately two months. All participating laboratories used the same stocks of acetone and reference chemicals through all tests. All measured chemistries were performed by Dr. Lee Ferguson, Department of Chemistry, University of South Carolina.

5. *Amphiascus tenuiremis* for the validation study were cultured in a clean, sediment-based, flow-through seawater system. Sediment was collected from a pristine site located on Bread and Butter Creek in the NOAA national estuarine research reserve of North Inlet, Georgetown, South Carolina, and washed/processed per ASTM Guidelines (E2317-04). The water used was sterile-filtered 30 g/L Instant Ocean synthetic seawater with a dissolved oxygen (DO) level greater than 90% and a pH of 8.0. Preliminary rangefinders were used to set exposures at or below the expected 96 h LC_{30} for *A. tenuiremis*. USC served as the intercalibration source laboratory for copepod-culture seed stocks, a common lot of measured concentrated stock reference chemicals in acetone, a source of algal phytoplankton food stocks, and a supplier of all microplates, Hamilton® syringes, Costar Netwell® brood chambers, and silanized pipettes for copepod picking (see guideline). USC also hosted one technician from Stockholm for one week in early 2008 to learn copepod culturing and testing. NOAA participated in the first pre-validation test with 3,5 DCP in 2005 and had experience with the method prior to this broader intercalibration exercise. Uppsala received training from Dr. Magnus Breitholz and his technician in spring/summer 2008. Dr. Lee Ferguson (USC Chemistry Dept.) provided chemical analysis of all water samples from all participating laboratories for both reference chemicals.

1. Background – Second intercalibration

Two reference chemicals:

- Lindane (2.5, 5, 10, 15 and 20 µg/L)
- Atrazine (26, 43, 72, 120 and 200 µg/L)
- Acetone as carrier control for both substances
- Chemical analysis in USA by Dr. Lee Ferguson – Stored and shipped frozen.
  - *Fresh medium produced/sampled every 3 days*
  - *Used medium collected/analyzed from microplates on days 12, 24 and 36*

6. Beginning March 3rd, 2008, the NOAA laboratory began their atrazine validation. Subsequently, USC (April 4th), Stockholm (May 21st) and Uppsala (June 19th) started their atrazine validations. Bioassay experimental treatments (n=7 concentrations) consisted of a highest nominal concentration of 200 ug/L, a series of four dilutions decreasing by 60%, a solvent seawater control and a normal blank seawater control. Microplates were checked daily for temperature, mortality and

developmental life-stage (e.g., nauplius, copepodite and adult copepod). Every third day, each microwell was renewed (250 uL test media) and water quality criteria (temperature, salinity, pH and DO) was recorded. Every sixth day, test organisms were fed a 1:1:1 (by volume) algal mixture of a chrysophyte (*Isochrysis galbana*), a chlorophyte (*Dunaliella tertiolecta*) and a cryptophyte (*Rhodomonas salina*). This mixture provided each microwell with ~ 2 x 10^4 mixed cells / microwell per feeding. During testing, the microplates were placed in an opaque, covered humidity chamber and held in a temperature-regulated incubator at 25°C. A 12:12h light:dark cycle was also maintained throughout the duration of the test.

7. From March through December 2008, data and water samples were collected from each laboratory as they completed their atrazine and lindane bioassays. With the exception of Uppsala, all laboratories began testing lindane within a month of completing the atrazine bioassay. Testing periods for atrazine and lindane ranged from 30 – 36 days. The following are the results for lindane reference chemical exposures for all four participating laboratories. Atrazine results are reported for USC, NOAA, and Stockholm. Uppsala is conducting their atrazine validation in June 2009 with samples and data to follow.

8. For both reference chemicals, some laboratories experienced difficulties outside of the draft Test Guideline regarding consistent execution of reference chemical spikes using the agreed-upon Hamilton syringe delivery method. Some were unable to achieve spikes at or near nominal targets (see Tables 1 and 10 below), but generally all laboratories could achieve a serially ascending (descending) concentration range that was fairly evenly spaced. All results here are reported using nominal concentrations, but have identified in red those concentrations where mean measured concentrations exceeded nominal targets by more (or less) than 50%, and where toxicity responses are possibly affected.

9. Tables and figures in this report provide summary statistics (means and standard deviations) derived from spreadsheets and SAS® programming using data provided by each participating laboratory.

RESULTS:

ATRAZINE RESULTS

10. Chemical analyses: (**Table 1**) Based on measured chemistry, atrazine nominal spike concentrations exceeded ± 50% of nominal targets for NOAA time-zero seawater sample means at 26, 43 and 72 ug/L. Stockholm exceeded nominal targets by 50% or more at 43 and 120 ug/L. Across all labs, atrazine concentrations in pooled microwell water removed at 72 hours showed very little loss relative to initial measured atrazine concentrations. Atrazine was highly stable over each 72 hour spike:renewal time period for the 34-36 day bioassays. Hydrogel coating of microplate walls appears to prevent passive binding of atrazine to the test vessel.

Table 1: Atrazine measured chemistry (µg/L at 0 and 72 hrs; mean ± 1 SD)

LAB Atrazine [nominal]	USC 0 hours 72 hours	NOAA 0 hours 72 hours	Stockholm 0 hours 72 hours	Uppsala 0 hours 72 hours
Control	(ND) (ND)	(ND) (ND)	(ND) (ND)	-- --
Carrier	(ND) (ND)	(ND) (ND)	(ND) (ND)	-- --
26 µg/L	26.4 ± 4.0 31.1 ± 4.1	**45.5 ± 12.5 *** 31.5 ± 11.1	26.9 ± 6.6 36.1 ± 6.1	-- --
43 µg/L	53.0 ± 11.4 48.8 ± 5.0	**70.7 ± 22.6 *** 54.9 ± 4.5	**74.7 ± 38.9 *** 63.9 ± 21.5	-- --
72 µg/L	93.3 ± 35.1 94.1 ± 10.0	**109.8 ± 32.5 *** 93.6 ± 20.0	92.3 ± 26.9 88.6 ± 5.3	-- --
120 µg/L	140.3 ± 30.1 159.5 ± 34.2	163.6 ± 28.0 162.2 ± 14.9	**186.0 ± 82.3 *** 144.9 ± 33.4	-- --
200 µg/L	207.8 ± 25.2 208.5 ± 38.2	268.4 ± 48.1 210.4 ± 14.9	290.9 ± 88.6 221.0 ± 55.9	-- --

Note: (0 hour values in red are ≥ 50% above or below nominal targets)

Bioassay results:

Table 2: Percent total mortality (over 36d; mean ± 1 SD)

Lab / Atrazine	USC	NOAA	Stockholm	Uppsala
Control	11.1% ± 3.1 (ND)	13.3% ± 5.8 (ND)	**33.4% ± 9.7#*** (ND)	--
Carrier	7.3% ± 3.9 (ND)	18.3% ± 5.8 (ND)	11.9% ± 6.1 (ND)	--
26 µg/L	17.5% ±4.4 26.4 ± 4.0	10.0% ± 5.0 45.6 ± 12.5	10.3% ± 5.4 26.9 ± 6.6	--
43 µg/L	23.8% ± 3.3 53.0 ± 11.5	13.3% ± 2.9 70.7 ± 22.6	23.6% ± 7.9 74.7 ± 38.9	--
72 µg/L	23.3% ± 8.9 96.83 ± 29.1	6.7% ± 7.6 115.3 ± 22.9	32.9% ± 12.6 92.3 ± 26.9	--
120 µg/L	23.9% ± 9.5 140.3 ± 30.1	10.0% ± 5.0 163.6 ± 28.0	21.6% ± 5.9 186.00 ± 82.3	--
200 µg/L	14.8% ± 2.8 207.8 ± 25.3	5.2% ± 0.3 268.4 ± 48.1	28.7% ± 11.3 290.9 ± 88.6	--

Note:
- # Control mortality for Stockholm not typical due to non-random selection of individuals at start, and too few animals in stock cultures;
- red bold* means statistical significance

Total atrazine mortality divided among the life-stages is available in Annex 1 of the report.

Table 3: Sex ratio under exposure to atrazine (F:M)

Lab / Atrazine	USC	NOAA	Stockholm	Uppsala
Control	1.1 ± 0.4 (ND)	2.1 ± 0.4 (ND)	0.8 ± 0.2 (ND)	--
Carrier	0.5 ± 0.1 (ND)	1.4 ± 1.3 (ND)	1.3 ± 0.4 (ND)	--
26 µg/L	1.4 ± 0.5 26.4 ± 4.0	2.5 ± 0.9 45.6 ± 12.5	0.8 ± 0.4 26.9 ± 6.6	--
43 µg/L	1.4 ± 0.7 53.0 ± 11.5	1.7 ± 0.5 70.7 ± 22.6	1.1 ± 0.5 74.7 ± 38.9	--
72 µg/L	1.0 ± 0.3 96.8 ± 29.1	1.3 ± 0.4 115.3 ± 22.9	1.0 ± 0.5 92.3 ± 26.9	--
120 µg/L	0.5 ± 0.1 140.3 ± 30.1	1.1 ± 0.3 163.6 ± 28.0	1.1 ± 0.3 186.0 ± 82.3	--
200 µg/L	0.4 ± 0.0 207.8 ± 25.3	1.6 ± 1.2 268.4 ± 48.1	1.1 ± 0.6 290.9 ± 88.6	--

Table 4: Total Development Time -- Nauplius to Adult (80% pop.)

Lab / Atrazine	USC	NOAA	Stockholm	Uppsala
Control	19.6 (ND)	15.7 (ND)	19.8 (ND)	--
Carrier	21.3 (ND)	15.5 (ND)	18.8 (ND)	--
26 µg/L	19.4 26.4 ± 4.0	15.5 45.6 ± 12.5	17.5 26.9 ± 6.6	--
43 µg/L	20.0 53.0 ± 11.5	15.5 70.71 ± 22.57	17.1 74.7 ± 38.9	--
72 µg/L	20.7 96.8 ± 29.1	15.8 115.1 ± 22.9	18.3 92.3 ± 26.9	--
120 µg/L	20.7 140.3 ± 30.1	15.3 163.6 ± 28.0	17.7 186.0 ± 82.3	--
200 µg/L	18.1 207.8 ± 25.3	15.9 268.4 ± 48.1	17.3 290.9 ± 88.6	--

Table 5: Percent mating success through two broods

Lab Atrazine	USC	NOAA	Stockholm	Uppsala
Control	82.1% ± 16.0 (ND)	72.6% ± 8.4 (ND)	-- (ND)	--
Carrier	96.7% ± 5.6 (ND)	74.2% ± 3.9 (ND)	**44.2% ± 38.8** * (ND)	--
26 µg/L	73.1% ± 8.0 26.4 ± 4.0	73.0% ± 12.4 45.6 ± 12.5	56.7% ± 35.1 26.9 ± 6.6	--
43 µg/L	68.5% ± 17.6 53.0 ± 11.5	58.6% ± 20.0 70.7 ± 22.6	34.2% ± 31.7 74.7 ± 38.9	--
72 µg/L	90.0% ± 14.9 96.8 ± 29.1	55.6% ± 31.4 115.3 ± 22.9	36.7% ± 32.2 92.3 ± 26.9	--
120 µg/L	65.9% ± 13.5 140.3 ± 30.1	53.6% ± 5.1 163.6 ± 28.0	36.7% ± 32.2 186.0 ± 82.3	--
200 µg/L	74.1% ± 22.9 207.8 ± 25.3	57.1% ± 20.2 268.4 ± 48.1	35.6% ± 33.6 290.9 ± 88.6	--

Note: red bold* means statistical significance

Table 6: Mean viable offspring in two broods

Lab Atrazine	USC	NOAA	Stockholm	Uppsala
Control	11.6 ± 4.9 (ND)	11.8 ± 9.3 (ND)	-- (ND)	--
Carrier	12.6 ± 2.6 (ND)	12.7 ± 8.4 (ND)	9.9 ± 7.8 (ND)	--
26 µg/L	9.1 ± 5.6 26.4 ± 4.0	12.4 ± 8.6 45.6 ± 12.5	11.2 ± 8.7 26.9 ± 6.6	--
43 µg/L	8.0 ± 5.2 53.0 ± 11.5	6.8 ± 6.4 70.7 ± 22.6	10.2 ± 8.5 74.7 ± 38.9	--
72 µg/L	8.0 ± 6.3 96.8 ± 29.1	7.1 ± 5.9 115.3 ± 22.9	13.1 ± 6.8 92.3 ± 26.9	--
120 µg/L	**6.5 ± 4.8** * 140.3 ± 30.1	**6.8 ± 6.9** * 163.6 ± 28.0	9.8 ± 7.9 186.0 ± 82.3	--
200 µg/L	**5.5 ± 4.4** * 207.8 ± 25.3	**6.1 ± 6.2** * 268.4 ± 48.1	10.9 ± 8.0 290.9 ± 88.6	--

Note: red bold* means statistical significance

USC

11. USC survival rates over the atrazine lifecycle test (36 days) were 89.0% and 92.7% for the solvent control (carrier) and water controls respectively (**Table 2**), and fell within the copepod guideline survival rate criterion of ≥ 80%. USC found no concentration dependency or treatment-specific significant differences in mortality, development rate, sex ratios, or mating success at any test concentration or for any life-stage relative to controls (i.e., nauplius, copepodite, or adult; **see Annex 1** at end). However, the number of viable offspring produced through two clutches or broods at 120 and 200 ug/L atrazine were significantly depressed 52 to 57% relative to controls (see **Table 6**).

NOAA

12. NOAA control survival rates over the atrazine lifecycle test (35 days) were 86.7% and 81.7% for the carrier and non-solvent controls respectively (**Table 2**), and both rates fell within the guideline control survival rate criterion of ≥ 80%. NOAA found no concentration dependency or treatment-specific significant differences in mortality, development rate, sex ratios, or mating success at any atrazine test concentration for any life-stage (nauplius, copepodite, adult) relative to controls (**Tables 3-5**). However, the number of viable offspring produced through two clutches at 120 and 200 ug/L atrazine were both significantly depressed 46 to 52% relative to controls; and very similar to the USC response (**Table 6**).

STOCKHOLM

13. Stockholm control survival rates over the atrazine lifecycle test (34 days) were 66.6% and 88.1% for the carrier and non-solvent controls respectively (**Table 2**). The water-control survival rate was significantly below the OECD draft test guideline criterion of 80%, but the solvent control rate was similar to other laboratories and well within the guideline criterion. Stockholm attributed the normal-control result to non-random selection of control nauplii at experimental set up, and low stock culture densities prior to the beginning of the test. The Stockholm laboratory found no significant mortality differences relative to water control or solvent controls for any atrazine concentrations tested. No concentration dependency or significant differences were detected for development rate at any life-stage, nor did Stockholm see any treatment-related effects at any concentration for sex ratios, reproductive success or viable offspring production relative to controls. Solvent controls performed similarly to other laboratories for all endpoints except mating success. The guideline criterion for "percentage of control pairs able to produce two broods" is ≥ 70%. All labs achieved >70% mating pair success in controls except for Stockholm which achieved 44.2% success. Stockholm normal control matings could not be completed because that set of microplates was accidentally dropped prior to clutch extrusion. Solvent control matings that were successful by Stockholm produced two-brood clutch sizes of ~10 offspring which was close to the mean response seen in all atrazine treatments, and within the guideline two-brood clutch-size criterion of > 8 viable offspring in controls (**Table 6**).

LINDANE RESULTS

Chemical analyses: (**Table 7**)

14. Water control and solvent control seawater showed non detectable lindane levels in every sample for every lab (i.e., for time zero seawater and 72-h microplate pooled seawater). Lindane nominal spike mean concentrations exceeded 150% for every Uppsala time-zero seawater sample. However, beyond the 2.5 and 5.0 ug/L nominal treatments, this laboratory did achieve a declining measured concentration spacing of 58-60% as requested in the validation plan. USC exceeded nominal concentrations by 50% or more for the 15 ug/L nominal time-zero seawater target. Stockholm spikes were all within ± 50% of the nominal target concentrations. Across all labs, lindane concentrations in pooled microwell water removed at 72 hours usually showed higher loss rates than seen for atrazine, but concentrations were still reasonably stable for each 72 hour spike:renewal time period (**Table 7**; loss rates from 5 to 50%). Hydrogel coating of microplate walls appears to minimize passive binding of hydrophobic lindane to the test vessel.

Table 7: Lindane measured chemistry (µg/L at 0 and 72 hrs; mean ± 1 SD)

LAB Lindane [nominal]	USC 0 hours 72 hours	NOAA 0 hours 72 hours	Stockholm 0 hours 72 hours	Uppsala 0 hours 72 hours
Control	(ND) (ND)	(ND) (ND)	(ND) (ND)	(ND) (ND)
Carrier	(ND) (ND)	(ND) (ND)	(ND) (ND)	(ND) (ND)
2.5 µg/L	3.9 ± 1.4 3.7 ± 1.2	**5.1 ± 2.9 *** 3.1 ± 1.2	2.2 ± 0.8 1.5 ± 0.39	**7.0 ± 1.6 *** 16.3 ± 14.8
5 µg/L	7.6 ± 3.3 5.2 ± 0.70	7.5 ± 4.6 4.3 ± 2.2	4.7 ± 2.0 2.6 ± 0.50	**8.9 ± 4.2 *** 8.6 ± 0.9
10 µg/L	12.7 ± 4.2 **25.3 ± 10.4 ***	**15.9 ± 7.7 *** 7.3 ± 4.7	7.6 ± 3.4 4.3 ± 3.0	**16.7 ± 6.7 *** 53.8 ± 71.8
15 µg/L	**23.4 ± 7.6 *** 22.8 ± 6.8	**28.3 ± 15.1 *** 31.3 ± 28.3	11.6 ± 3.8 10.1 ± 2.3	**29.3 ± 9.4 *** 25.6 ± 6.2
20 µg/L	23.2 ± 5.1 22.4 ± 6.3	**36.7 ± 20.9 *** 24.5 ± 17.6	15.7 ± 7.3 11.5 ± 2.7	**53.2 ± 9.1 *** 51.1 ± 21.7

Note: (0 hour values in red are > 50% above or below nominal targets)

Bioassay results

Survival

Table 8: Total mortality (over 36 d; mean ± 1 SD)

Lab Lindane	USC	NOAA	Stockholm	Uppsala
Control	5.2% ± 2.6 (ND)	2.2% ± 1.9 (ND)	11.7% ± 12.6 (ND)	12.7% ± 6.2 (ND)
Carrier	10.2% ± 2.3 (ND)	6.7% ± 5.8 (ND)	13.9% ± 11.1 (ND)	2.0% ± 3.4 (ND)
2.5 µg/L	6.9% ± 5.3 3.9 ± 1.4	30.0% ± 8.8 5.1 ± 2.9	5.0% ± 5.0 2.2 ± 0.8	41.7% ± 36.3 7.0 ± 1.6
5 µg/L	10.3% ± 4.5 7.6 ± 3.3	**31.1% ± 16.8** * 7.5 ± 4.6	10.4% ± 5.0 4.7 ± 2.0	10.9% ± 5.8 8.9 ± 4.2
10 µg/L	**33.4% ± 1.7** * 12.7 ± 4.2	**81.1% ± 11.7** * 15.9 ± 7.7	22.2% ± 14.9 7.6 ± 3.4	**44.9% ± 18.9** * 16.7 ± 6.7
15 µg/L	19.2% ± 13.1 23.4 ± 7.6	**78.9% ± 3.9** * 28.3 ± 15.1	8.5% ± 10.3 11.6 ± 3.8	15.5% ± 12.5 29.3 ± 9.4
20 µg/L	**34.4% ± 7.8** * 23.2 ± 5.1	**57.8% ± 12.6** * 36.7 ± 20.9	**21.9% ± 11.3** * 15.7 ± 7.3	14.8% ± 7.4 53.2 ± 9.1

15. *USC* total control survival rates over the lindane lifecycle test were 94.8% and 89.8% respectively for the solvent control and water control (**Table 8**), and similar to the atrazine test control rates. These rates are within the copepod guideline survival rate criterion of ≥ 80%. USC saw strong concentration dependency and treatment-specific significant differences in mortality at 10, 15 and 20 ug/L. The naupliar larval stage was strongly sensitive to lindane at each of these concentrations (**Annex 2, Table 1**), but the copepodite stage (i.e., survivors from the preceding naupliar exposure window) was insensitive to lindane until 20 ug/L (**Annex 2, Table 2**). Adult *surviving* copepods were practically insensitive to all exposures over all laboratories (**Annex 2, Table 3**) for the mortality endpoint, except for 15 ug/L at NOAA. No USC mean mortalities exceeded 35% at any treatment level which is the guideline recommendation for treatment-related maximum mortalities.

16. *NOAA* total control survival rates over the lindane lifecycle test were 97.8% and 93.3% respectively for the water control and solvent control (**Table 8**), and significantly higher than the atrazine test control rates. All of these rates are within the copepod guideline survival rate criterion of ≥ 80%. NOAA saw strong and significant mortalities at all test concentrations which generally increased with concentration. Similar to USC, Stockholm and Uppsala, most mortality was manifest at the naupliar larval stage (**Annex 2, Table 1**). The 10, 15 and 20 ug/L treatments exceeded guideline recommendations of < 35% total mean mortality at any test concentration. The copepodite stage survivors were insensitive across all exposures (**Annex 2, Table 2**) for the mortality endpoint, but adults showed significant additional sensitivity at 5 and 15 ug/L which was not seen for other laboratories (**Annex 2, Table 3**).

Stockholm total control survival rates over the lindane lifecycle test were 88.3% and 86.1% respectively for the solvent control and water control (**Table 8**), and much improved over the atrazine-test normal

control rates. These rates are within the copepod guideline survival rate criterion of ≥ 80%. Stockholm mortality rates were variable across microplates within treatment, and showed no significant differences for any treatment. Mean mortality maximum was 17 nauplii at the highest 20 ug/L concentration (**Annex 2, Table 1**). Copepodites and adults were generally insensitive for the mortality endpoint -- similar to responses seen for the other laboratories **(Annex 2, Tables 1-3)**. No Stockholm mean mortalities exceeded 35% which is the test guideline recommendation for any treatment maximum mortality. Overall, Stockholm measured lindane concentrations were closer to the nominal targets than any other participating laboratory, but they were consistently lower by ~ 12 to 45%.

17. *Uppsala* total control survival rates over the lindane lifecycle test were 87.3% and 98.0% respectively for the water and solvent controls (**Table 8**). These rates are within the copepod test guideline survival rate criterion of ≥ 80%. Uppsala saw strong and significant mortalities at the 2.5 and 10 ug/L test concentrations, but concentration response was inconsistent for the mortality endpoint at Uppsala. Like USC, NOAA and Stockholm, observed mortality was almost completely manifest at the naupliar larval stage (**Annex 2, Table 1)** with almost zero copepodite and adult mortalities at any concentration (**Annex 2, Tables 1-3**). The 2.5 and 10 ug/L treatments exceeded the guideline recommendation of < 35% total mean mortality at any test concentration.

Development

Figure 1: Mean No. of Days from Nauplius to Copepodite (80% pop)

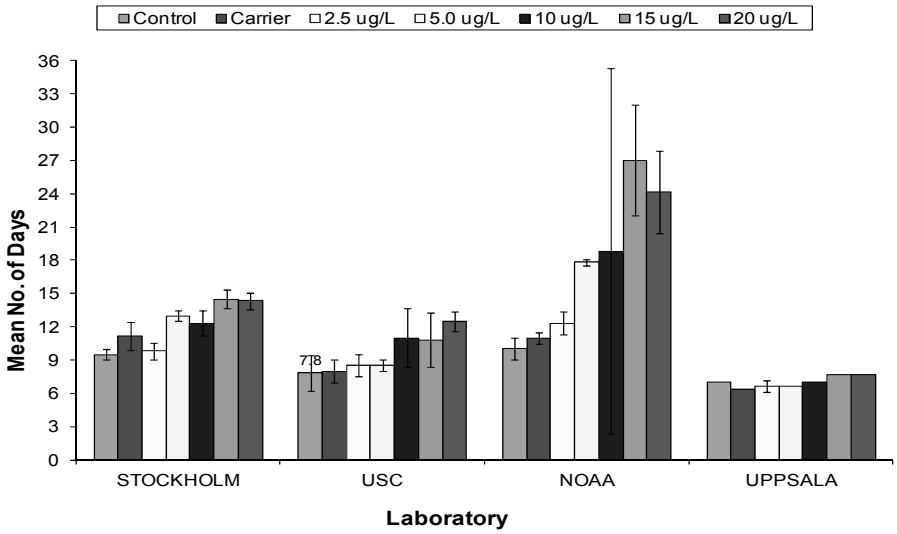
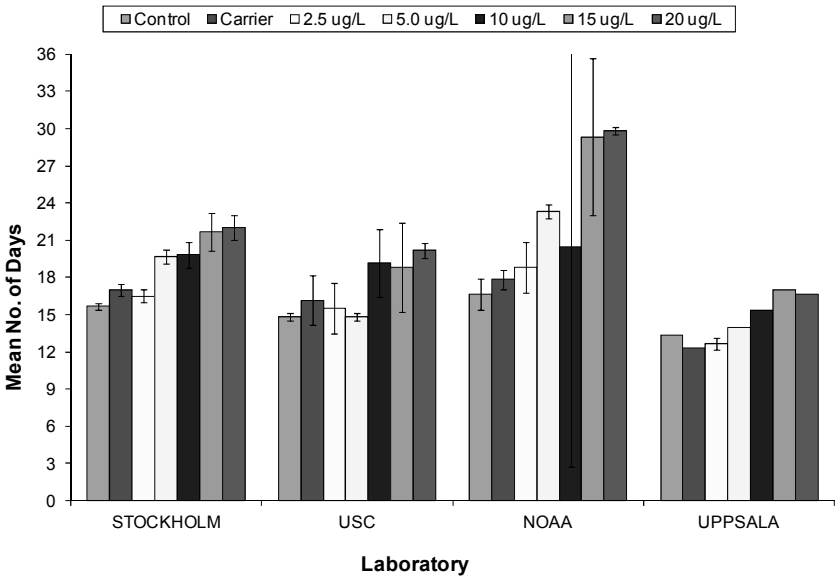


Figure 2: Mean No. of Days from Nauplius to Adult (80% pop)



USC. Unlike atrazine, the pesticide lindane had a strong concentration dependent affect on USC, NOAA and Stockholm copepod naupliar larval development time to adult (**Figure 2**). For USC, the normal and carrier-control development times for the nauplius-to-copepodite progression were almost identical at 7.8 and 8.0 days respectively (**Figure 1**). The USC copepodite-to-adult progression were similarly close at 14.8 and 16.2 days respectively. Lindane significantly delayed naupliar development at 10, 15 and 20 ug/L by 3 to 4.5 days relative to controls. Additional significant delays for the less acutely-sensitive copepodite stage were not seen by USC as all treatments and controls fell within the normal 5 to 7 day copepodite-to-adult time window.

18. NOAA control and carrier-control development rates for nauplii and copepodites were similar at 10 and 11 days, or 6.7 and 6.8 days, respectively. NOAA produced the most consistently high measured concentration of lindane among the four laboratories at the high end of the nominal range; and they saw correspondingly the strongest delays with concentration across the naupliar-to-copepodite time progressions (**Figure 2**). Significant naupliar delays were seen at 5, 15 and 20 ug/L that ranged from 7 to 16 days longer than that required for controls. 10 ug/L also elicited a strong mean delay of 8 additional days, but the response was highly variable across microplates possibly due to low sample sizes from high treatment mortalities. Additional significant copepodite delays were not seen by NOAA as all treatments and controls fell within the normal 5 to 7 day copepodite-to-adult time window.

19. Stockholm water control and solvent control development rates for nauplii and copepodites were similar to NOAA at 9.5 or 11.2 days (to copepodite), and 15.7 or 17.0 days (to adult), respectively. Stockholm produced the most consistently even spread of lindane concentrations of the four laboratories (Table 10). Correspondingly, they saw the least variable concentration-dependent increase in mean developmental delays across the naupliar-to-adult time window (**Figure 2**). Significant naupliar-to-copepodite delays of 2.7 to 4.1 days were seen at 5, 15 and 20 ug/L relative to controls. Additional significant copepodite delays were not seen as all treatments and controls fell within the normal 5 to 7 day copepodite-to-adult time window.

20. Uppsala water and solvent control development rates for nauplii and copepodites were the most rapid of all labs (**Figure 2**) at 7.0 or 6.3 days (to copepodite), and 13.3 or 12.3 days (to adult), respectively.

Uppsala saw almost zero differences in development time for the naupliar-to-copepodite progression with concentration (6.3 to 7.7 days total; < 1.5 day delay with concentration), but saw strong concentration dependency in copepodite-to-adult development rates at 15 and 20 ug/L (i.e., 3 to 3.3 days).

Sex ratios

Table 9: Sex ratios under Lindane exposure (F:M)

Lab Lindane	USC	NOAA	Stockholm	Uppsala
Control	1.1 ± 0.3 (ND)	1.3± 0.7 (ND)	0.8 ± 0.3 (ND)	1.1 ± 0.6 (ND)
Carrier	0.9 ± 0.1 (ND)	1.4 ± 0.3 (ND)	1.0 ± 0.2 (ND)	1.7 ± 0.7 (ND)
2.5 µg/L	1.1 ± 0.6 3.9 ± 1.4	2.5 ± 1.6 5.1 ± 2.9	1.1 ± 0.5 2.2 ± 0.8	0.8 ± 0.5 7.0 ± 1.6
5 µg/L	1.1 ± 0.1 7.6 ± 3.3	**3.7 ± 2.3** * 7.5 ± 4.6	0.9 ± 0.1 4.7 ± 2.0	1.7 ± 0.5 8.9 ± 4.2
10 µg/L	0.7 ± 0.3 12.7 ± 4.2	**5.5 ± 4.9** * 15.9 ± 7.7	1.0 ± 0.3 7.6 ± 3.4	1.1 ± 0.8 16.7 ± 6.7
15 µg/L	0.7 ± 0.6 23.4 ± 7.6	**15.0 ± 2.0** * 28.3 ± 15.1	1.1 ± 0.5 11.6 ± 3.8	0.9 ± 0.5 29.3 ± 9.4
20 µg/L	0.5 ± 0.2 23.2 ± 5.1	**4.8 ± 5.6** * 36.7 ± 20.9	0.9 ± 0.5 15.7 ± 7.3	1.0 ± 0.4 53.2 ± 9.1

21. *USC* water control and solvent control female-to-male ratios at adulthood were 1.1 and 0.9 respectively in the lindane test (**Table 9**). The guideline criterion for female-to-male adult ratio in controls is 0.6 to 1.8. One significant sex ratio shift to 0.5 ± 0.2 was observed by USC at 20 ug/L.

22. *NOAA* control and carrier-control female-to-male ratios at adulthood were 1.3 and 1.4 respectively in the lindane test (**Table 9**). The guideline criterion for female-to-male adult ratio in controls is 0.6 to 1.8. Strong and significant sex ratio shifts toward females were seen in the test populations at 10 through 20 ug/L. These shifts may have been caused by lindane acting on sex determination processes in larval and juvenile copepods, or possibly (likely) caused by differential male mortality from lindane since treatment mortality rates were high (81 to 58% at 10, 15 and 20 ug/L respectively) for NOAA. No other labs saw such strong sex ratio shifts with increasing concentration.

23. *Stockholm* water control and solvent control female-to-male ratios at adulthood were 0.8 and 1.0 respectively in the lindane test (**Table 9**). The guideline criterion for female-to-male adult ratio in controls is 0.6 to 1.8. No sex ratio shifts were seen by Stockholm at any test concentrations (range across treatments = 0.9 to 1.1; Table 15).

Uppsala. Similar to Stockholm and USC, Uppsala saw no significant sex ratio differences relative to normal (SR = 1.1) and carrier (SR = 1.7) controls (**Table 9**).

Mating success

Table 10: Mating pairs able to produce two broods

Lab / Lindane	USC	NOAA	Stockholm	Uppsala (all matings in one microplate)
Control	84.6% ± 15.4 (ND)	100% ± 0.0 (ND)	77.3% ± 19.8 (ND)	66.7 (ND)
Carrier	**50.0% ± 7.1** * (ND)	96.9% ± 5.8 (ND)	85.0% ± 6.6 (ND)	**50.0** * (ND)
2.5 µg/L	57.6% ± 19.5 3.9 ± 1.4	79.0% ± 12.5 5.1 ± 2.9	92.6% ± 7.3 2.2 ± 0.8	84.6 7.0 ± 1.6
5 µg/L	72.4% ± 11.7 7.6 ± 3.3	71.4% ± 14.4 7.5 ± 4.6	90.0% ± 6.4 4.7 ± 2.0	61.9 8.9 ± 4.2
10 µg/L	50.0% ± 38.2 12.7 ± 4.2	**25.0% ± 28.9** * 15.9 ± 7.7	**45.5% ± 32.8** * 7.6 ± 3.4	91.7 16.7 ± 6.7
15 µg/L	**10.0% ± 9.6** * 23.4 ± 7.6	**0% (n=1)** * 28.3 ± 15.1	69.6% ± 23.2 11.6 ± 3.8	75.0 29.3 ± 9.4
20 µg/L	**37.5% ± 50** * 23.2 ± 5.1	14.3% ± 19.2 36.0 ± 20.9	**27.3% ± 22.3** * 15.7 ± 7.3	66.7 53.2 ± 9.1

24. *USC* rates of control and carrier-control copepod mating pair success (i.e., ability of individually paired male:female couples to produce two viable broods of nauplii) were 84.6% and 50.0% respectively (**Table 10**). The test guideline criterion for minimal mating success is 70% . USC easily passed this criterion for water controls, but failed this criterion for the solvent control. Success rates were depressed at 10, 15 and 20 ug/L lindane, but were also variable across microplates within treatments. A significant and sharply depressed mating success rate of only 10% was seen at 15 ug/L.

25. *NOAA* rates of control and carrier-control copepod mating pair successe were 100% and 96.9% respectively (**Table 10**), and were the highest rates among all labs. The test guideline criterion for minimal mating success is 70% . NOAA success rates were sharply depressed at 5 through 20 ug/L lindane (Table 16), but were also variable across microplates within treatments. A significant and sharply depressed mating success rate of 0% was seen at 15 ug/L.

26. *Stockholm* rates of water control and solvent control copepod mating-pair success were 77.3% and 85.0% respectively (**Table 10**). The test guideline criterion for minimal mating success is 70%. Success rates were depressed at 10 and 20 ug/L lindane. A significant and sharply depressed mating success rate of only 27% occurred at 20 ug/L.

27. *Uppsala* rates of control and carrier-control copepod mating-pair success were 66.7% and 50.0% respectively (**Table 10**). The test guideline criterion for mating success of $\geq$ 70% in controls was not met by Uppsala. Success rates were not significantly different from controls for any lindane treatment in the Uppsala dataset.

Viable offspring production

Table 11: Mean viable offspring in two broods

Lab Lindane	USC	NOAA	Stockholm	Uppsala
Control	12.0 ± 3.3 (ND)	20.4 ± 4.6 (ND)	12.6 ± 7.3 (ND)	16.8±10.6 (ND)
Carrier	12.4 ± 3.9 (ND)	20.0 ± 6.0 (ND)	19.2 ± 6.2 (ND)	10.3 ± 7.3 (ND)
2.5 µg/L	11.2 ± 3.9 3.9 ± 1.4	15.5 ±7.7 5.1 ± 2.9	15.9 ± 7.0 2.2 ± 0.8	11.8 ± 8.0 7.0 ± 1.6
5 µg/L	13.0 ± 2.3 7.6 ± 3.3	11.7 ± 9.2 7.5 ± 4.6	15.4 ± 6.2 4.7 ± 2.0	**9.1 ± 7.4 *** 8.9 ± 4.2
10 µg/L	12.0 ± 2.9 12.7 ± 4.2	**4.0 ± 5.7 *** 15.9 ± 7.7	6.7 ± 6.8 7.6 ± 3.4	13.8 ± 6.2 16.7 ± 6.7
15 µg/L	**0.0 ± 0.0 *** 23.4 ± 7.6	**0 (n=1) *** 28.3 ± 15.1	8.8 ± 7.2 11.6 ± 3.8	13.6 ± 7.7 29.3 ± 9.4
20 µg/L	**0.0 ± 0.0 *** 23.2 ± 5.1	**4.3 ± 7.5 *** 36.0 ± 20.9	**3.7 ± 6.4 *** 15.7 ± 7.3	10.2 ± 7.4 53.2 ± 9.1

28. *USC* control and solvent control mean viable offspring production (i.e., number of live hatchlings through two broods) were almost identical at 12.0 or 12.3 offspring per mating pair respectively (**Table 11**). Offspring production remained stable for 2.5, 5 and 10 ug/L treatments, but was strongly depressed at 15 and 20 ug/L with zero offspring production. The test guideline criterion of ≥ 8 offspring per mating pair on average in controls was met by USC. **Figure 3** is a graph of the USC production response.

29. *NOAA* water control and solvent control mean viable offspring productions (i.e., number of live hatchlings through two broods) were also highest among all labs at 20.4 or 20.0 offspring per mating pair respectively (**Table 11**). Offspring production declined sharply at 5 ug/L to only 11.7 offspring, and then declined significantly to ≤ 4.0 offspring per mating pair at 10 to 20 ug/L. Only one mating pair could be constructed at 15 ug/L (offspring = 0) because of high lindane-induced mortality in earlier lifestages, and a dramatically skewed sex ratio that limited the number of mating pairs that could be constructed. The test guideline criterion of ≥ 8 offspring per mating pair on average in controls was met by NOAA. **Figure 4** is a graph of the NOAA production response.

30. *Stockholm* water control and solvent control mean viable offspring production was 12.6 or 19.2 nauplii respectively, and sharply depressed at 10, 15 and 20 ug/L (**Table 11**). Offspring production declined to only 3.7 offspring per pair on average at 20 ug/L which was statistically significant. The test guideline criterion of ≥ 8 offspring per mating pair on average in controls was met by Stockholm. **Figure 5** is a graph of the Stockholm production response.

31. *Uppsala* water control and solvent control mean viable offspring production was 16.8 or 10.3 nauplii per mating pair respectively (**Table 11**). No significant mating pair production rates were seen at any lindane concentration by Uppsala. The test guideline criterion of ≥ 8 offspring per mating pair on average in controls was met by Uppsala. **Figure 6** is a graph of the Uppsala production response.

Figure 3: Lindane -- Mean viable offspring in 2 broods - University of South Carolina, Columbia, SC, USA*

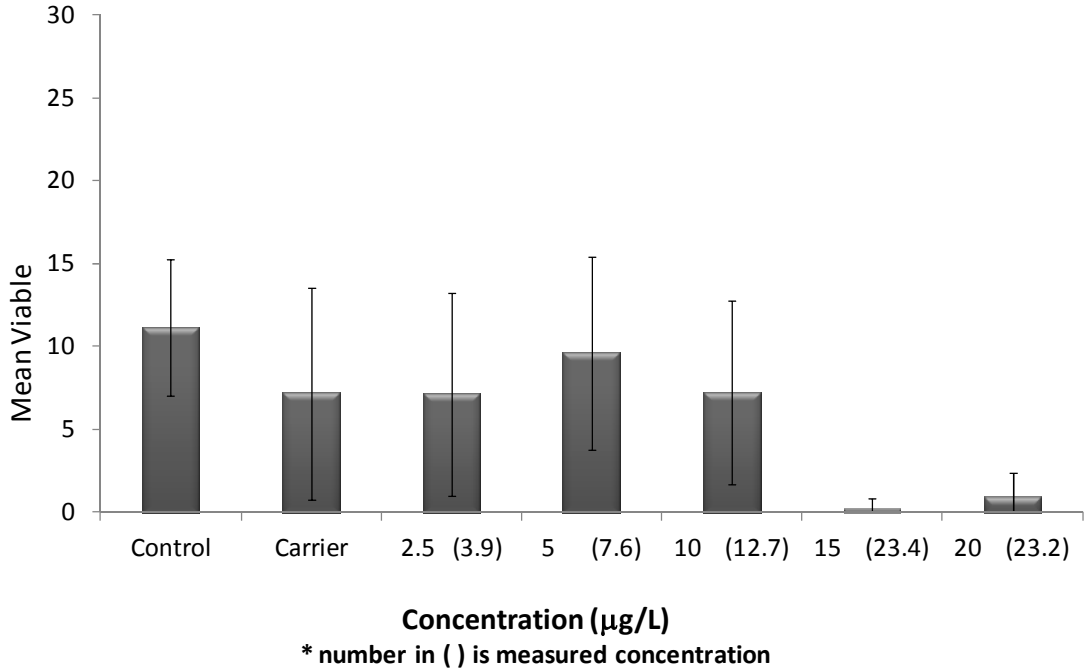

30
25
20
15
10
5
0
Mean Viable
Control
Carrier
2.5 (3.9)
5 (7.6)
10 (12.7)
15 (23.4)
20 (23.2)
Concentration (μg/L)
* number in ( ) is measured concentration

Figure 4: Lindane: Mean viable offspring in 2 Broods - NOAA, Charleston, SC, USA*

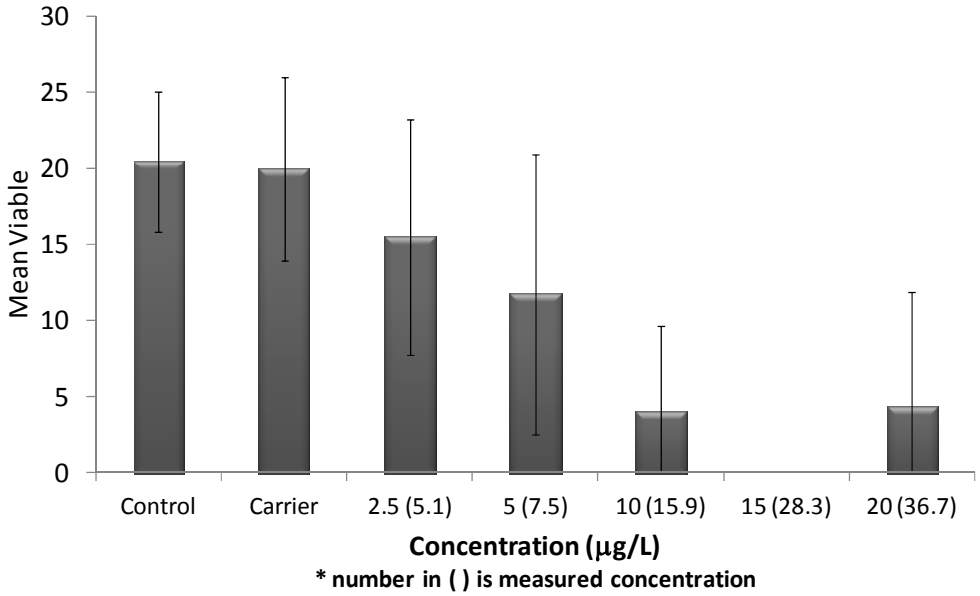


Figure 5: Lindane: Mean viable offspring in 2 Broods - Stockholm University, Stockholm, Sweden*

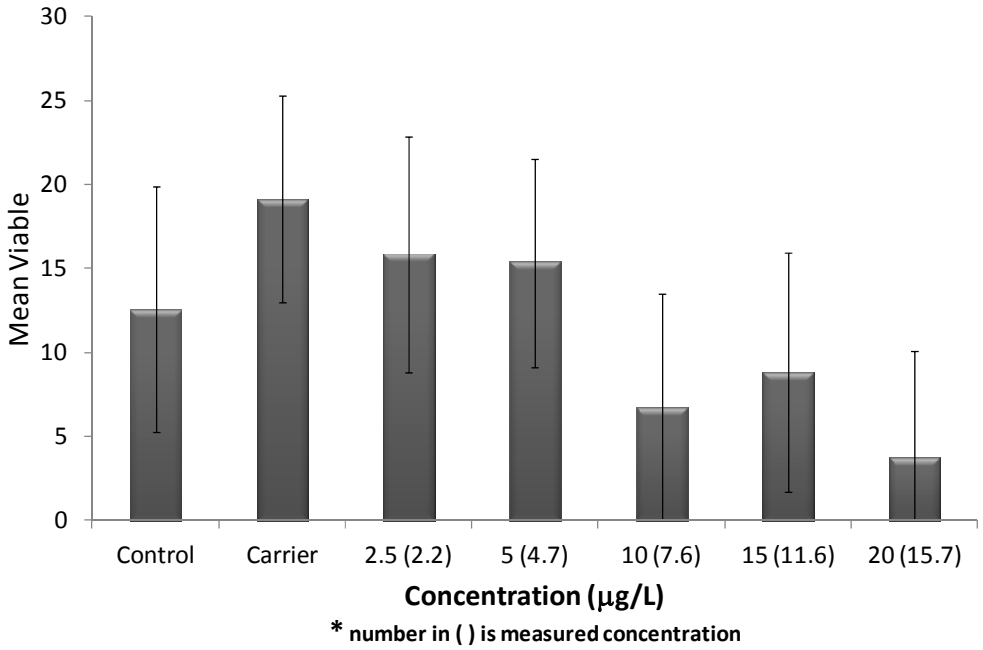
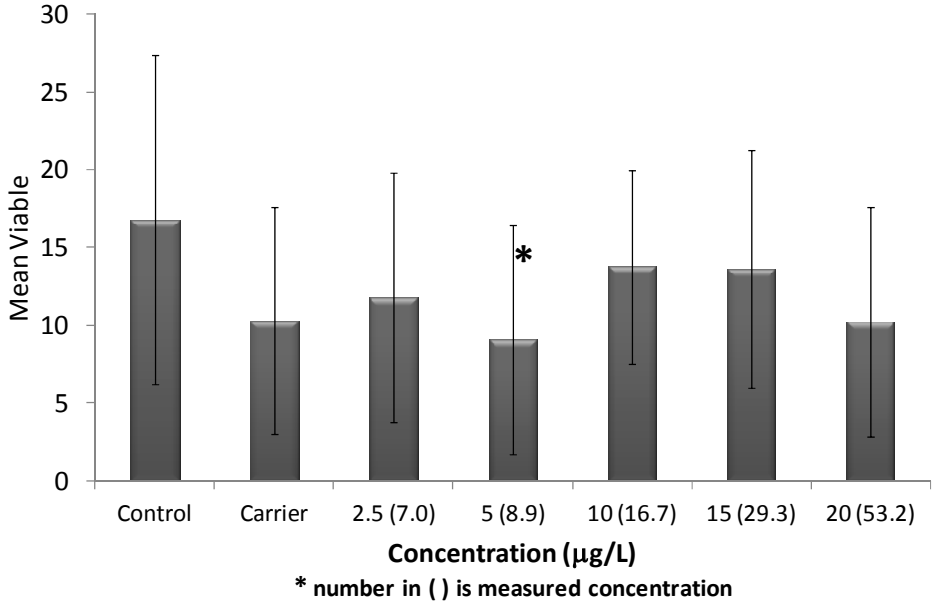


Figure 6: Lindane: Mean viable offspring in 2 Broods - SLU, Uppsala, Sweden*



Microplate Life-table Modeling: population growth rate

Table 12: Instantaneous Population Growth Rates λ^*

Lab Lindane	USC	NOAA	Stockholm	Uppsala
Control	1.43	1.57	1.23	1.21
Carrier	1.33	1.57	1.37	**1.13***
2.5 µg/L	1.26	1.51	1.35	1.09
10 µg/L	**1.19 ***	**1.01 ***	**1.09 ***	1.14
20 µg/L	**0.96 ***	**1.08 ***	**0.98 ***	1.11

***Normal control $\lambda \geq 1.2$**

32. USC measured lifetable parameters from the parental (F_0) microplate populations were modeled, by lindane treatment, using a matriarchal 5-stage Leslie matrix to produce estimates of λ (instantaneous rate of population increase; **Table 12**), projected life-stage abundances at the first filial generation (F_1), and projected total population sizes at the second filial generation (F_2) as a function of lindane exposure. The

five stages of the lifetable matrix are embryo:nauplius:copepodite: barren-female:gravid-female. All abundance estimates by the model exclude embryos and adult males from population counts in order to be able to capture sex ratio comparative effects on population growth. Parameters of the model were initial abundance of 90 naupliar-stage individuals, ceiling-type density dependence (with carrying capacity K = 20000 individuals), zero competition, and invoked stochasticity using microplate-measured standard deviations for each concentrations' stage-to-stage transition rates and for fecundity in the Leslie matrix. The recommended criterion for control copepod populations in microplate culture is $\lambda \geq 1.2$. USC water and solvent controls λ 's were 1.43 and 1.33 respectively, which implies these populations would be expected to experience a 43% or 33% increase in abundance at each successive generation. Lindane at 10 and 20 ug/L sharply reduced λ to 1.19 and 0.96 respectively (**Table 12**). A λ value of 1.0 means the population is exactly replacing itself over time; a λ value less than one means the population is in decline over time.

33. NOAA control and carrier-control λ 's (**Table 12**) were both high and identical at 1.57. This implies these populations would be expected to experience a large 57% increase in abundance at each successive generation. Recall that production rates for NOAA (i.e., fecundity) were high at 20.0 to 20.4 offspring through two broods, and λ is very sensitive to low (or high) mortalities and/or high fecundity, and/or high female production relative to males. NOAA lindane λ at 2.5 ug/L was close to control values at 1.51. However, λ's at 10 and 20 ug/L were dramatically lower at 1.01 and 1.08, implying these populations would be close to "replacement" values over time.

34. NOAA predicted total population abundance (**Figure 7** below) at the F2 (second filial, or third total) generation of exposure for each laboratory for the solvent control (SOLV CON) was high at 3578 individuals. Interestingly, at 2.5 ug/L the total predicted population size was only 46% of the control despite the identical λ value estimated from the Leslie matrix. Decreased fecundity and naupliar mortality rates produced a lower production estimate at 2.5 ug/L, but some of that negative effect was overcome by the higher proportion of females transitioning into the 2.5 ug/L population (see **Table 9**) relative to controls. More dramatically, at 10 and 20 ug/L the predicted population is either declining (only 46 individuals at 10 ug/L), or effectively just replacing itself at 20 ug/L just as λ alone would have predicted. **Annex 2, Figure 2** shows NOAA predicted life stage percentages in the population during the first filial generation (i.e., at the 6^{th} through 10^{th} time steps in the 3 generation model (15 time steps; 60 days). Stage structure is healthy and almost identical at 0, 2.5 and 10 ug/L -- similar to USC and Stockholm (**Annex 2, Figures 1-3**) -- but numbers are strongly depressed overall at 10 ug/L, and stage structure is predominated by barren females at 20 ug/L. High mortalities and low fecundities were the predominant drivers of low population predictions at these concentrations.

Figure 7: Leslie matrix projected population sizes (+ 1 SD) at the F2

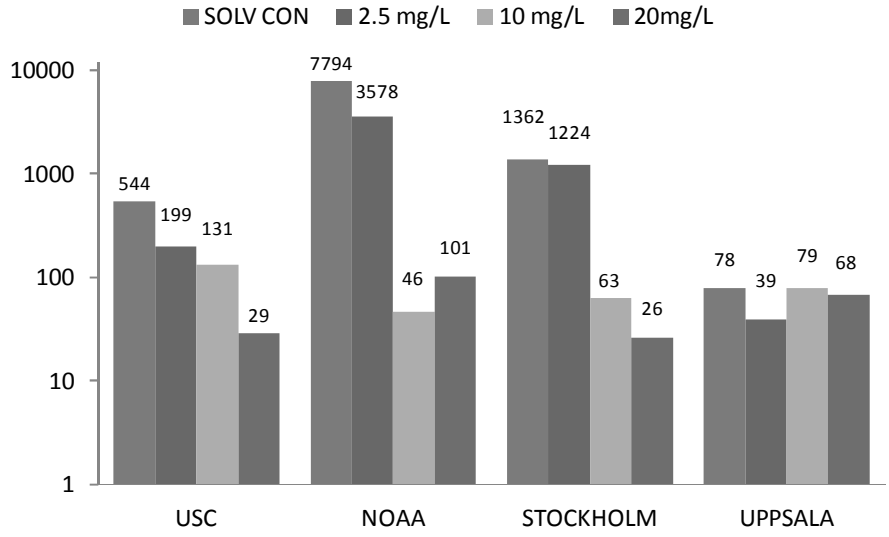


Figure 7 shows Leslie matrix predicted total population abundance at the F2 (second filial, or third total) generation of exposure for each laboratory for the solvent control (SOLV CON), and the 2.5, 10 and 20 ug/L microplate datasets. Error bars represent one standard deviation of the mean. USC lifetable data produced a smooth concentration responsive decline in population size from 544 individuals in controls to only 29 individuals at 20 ug/L. **Annex 2, Figure 1** shows USC predicted life stage percentages in the population during the first filial generation (i.e., at the 6th through 10th time steps in the 3 generation (15 time step; 60 day) of the model (note: white numbers are predicted stage abundances at the F1. Stage structure is almost identical at 0 and 2.5 ug/L, with typical within population distributions of highly abundant nauplii, copepodites, and normally fewer but sufficient barren and gravid females to support the population; but at 10 ug/L, copepodite and naupliar abundances decline sharply, and at 20 ug/L the population is composed completely of copepodites and barren females with zero gravids.

NOTE: Annex 2 Figures 5-8 show predicted F1 abundances by stage for each laboratory. This information is included as complementary to Annex 2, Figures 1-4 without discussion, because abundance differences and trends are discussed for the F2 in Figure 7.

35. Stockholm control and carrier-control λ 's (**Table 12**) were normal at 1.23 or 1.37 respectively. This implies that these populations would be expected to experience a 23 or 37% increase in abundance at each successive generation. Lindane λ at 2.5 ug/L was close to control values at 1.35. However, λ 's at 10 and 20 ug/L were similar to the NOAA lindane responses and were dramatically lower at 1.09 and 0.98. λ alone would predict that these populations would be close to "replacement" values over time, but that was not what the more integrative full model actually predicted:

36. Model predicted total population abundances (**Figure 7** above) at the F2 (second filial, or third total) generation for the solvent control (SOLV CON) and 2.5 ug/L treatment were high at 1362 or 1224 individuals respectively. Decreased fecundity and increased mortality at 10 and 20 ug/L produced sharply lower abundance estimates of only 63 or 26 individuals respectively at the F2. **Annex 2, Figure 3** shows the predicted life stage percentages (structure) in the Stockholm population during the first filial generation (i.e., at the 6^{th} through 10^{th} time steps) in the 3 generation model (i.e., 15 time steps total; 60 days). Stage structure is healthy and almost identical at 0, 2.5 and 10 ug/L -- similar to USC and NOAA (Figs. 9 and 10) -- but stage structure at 20 ug/L has a much higher proportion of barren females and almost zero gravids to support the population through time.

37. Uppsala's measured lifetable parameters from the parental (F_0) microplate populations produced estimates of λ for water controls and solvent controls of 1.21 and 1.13 respectively. The carrier fell below the recommended $\geq$ 1.2 criterion for λ when using this test guideline method. Lindane λ 's at 2.5, 10 and 20 ug/L were all close to the solvent control value of 1.13 (**Table 12**). Fecundity for Uppsala showed no concentration response, and, with the exception of 5 ug/L, fecundity was relatively flat at 10.2 to 13.8 offspring per mating pair across treatments and in the solvent control (**Table 11**). Uppsala mortalities were high at the 2.5 and 10 ug/L concentrations (42-45%), but much lower at 20 ug/L (only 15%). Mating pair success rates were sharply impaired at 20 ug/L and in the solvent control (only 50% and 67% respectively). Unlike the 2.5 and 10 ug/L treatments, the solvent control experienced only a 2% mortality rate; but with only a 50% mating success rate, and average fecundity, these microplate data yielded a model total abundance prediction for the control that was not significantly different from the lindane treatments. Therefore, not surprisingly and quite elegantly, the population model did what it should have done and predicted similar population abundances with high precision across 0, 2.5, 10 and 20 ug/L lindane (**Figure 7**). Correspondingly, population stage structures (**Annex 2, Figure 4**) were almost identical across treatments and control and uniformly showed sharp depressions in production of gravid females to support these populations.

SUMMARY PRELIMINARY CONCLUSIONS TO DATE (MAY 22, 2009):

- All participating laboratories experienced varying degrees of difficulty in producing consistently accurate and precise *measured* concentrations of atrazine and lindane. Measured chemistries of initial spike renewal seawater for some labs often exceeded 150%. Microplate pooled seawater was often much closer to the nominal targets at 72 h due to modest loss, bioaccumulation or degradation.

- All labs except for USC (the inventor of the method) experienced more frequent and more severe difficulties executing the guideline on the first validation run with atrazine than the second or later trials with lindane.

- Most labs were able to maintain physical parameters within guideline recommendations and requirements. Physical and copepod response data were not always recorded and reported consistently across labs.

- Atrazine mortality response was consistently low across labs, with toxicity manifest primarily in mating pair success rates and fecundity (offspring production) at exposures above 100 ug/L. Uppsala has not tested atrazine yet (planned for June 2009), so the atrazine dataset is not complete.

- For three of four participating laboratories, lindane produced consistent concentration-dependent delays in development rates, consistent naupliar-specific mortality, consistent mating-success depressions, and consistent reduction of fecundity (offspring production through 2 broods).

- Interlab comparisons of lindane lifetable data from the microplate exposures using the Leslie matrix model produced consistent patterns of response that could be explained integratively from the mortality, sex ratio, and fecundity measurements (and variance estimates) collected by each laboratory. One lab failed mating success criteria for controls, and therefore we could not discriminate a lindane effect on population growth using their data in the model.

- values alone were often predictable of Leslie matrix integrative projections of population abundance, but not always. Sex ratio shifts and mating success differences are often important indicators of reproductive and endocrine-based toxicity but they are not captured by values alone. **The OECD expert testing group should discuss the value of including population modeling in this guideline approach.** The method provides all of the information one needs to produce an individualized lifetable for each test population at each test concentration, which can then be easily modeled with off-the-shelf software (e.g., RAMAS Stage® or Metapop® programs).

- All laboratories demonstrated solid ability <u>most of the time</u> to produce control survival rates well within guideline criteria (80% or higher), control mating success above 70%, control clutch sizes above ten offspring through 2 broods, mean sex ratios for non solvent and solvent controls between 0.4 to 2 taking variability into account, and control lifetime development rates of 20 days or less at 25C. Uppsala conducted their lindane test at 21-22C rather than the guideline 25C, but still produced acceptable and the most rapid development rates to adult.

- All laboratories were able to culture *Amphiascus tenuiremis* in static bulk culture using seed stocks, sterile processed sediments, algal feed stocks, and 30S seawater shipped from USC, Columbia. All labs were able to harvest gravid copepods from culture, and get them to hatch healthy larval nauplii into Netwell® 12-well plates for harvest and delivery to the microplate test.

- Algal ration control seemed adequate for most labs given the relatively consistent control clutch sizes achieved across labs; but in some cases rations may have been set too high (e.g., the NOAA mean fecundity values of 20 offspring in controls is unusual and possibly tied to overfeeding.). **The OECD expert testing committee should discuss whether a minimum and maximum two brood range should be included in the guideline.** [We have tentatively suggested a criterion of $\geq$ 8 offspring but this needs group discussion.] The copepod guideline calls for an exact 2 L ration per microwell every 6th day over the 35 day bioassay. A departure to 3 L's can cause a

35% increase in total clutch size in controls. Good laboratory practice requires labs to maintain calibrated micropipettors, and a poorly performing micropipettor can deliver too much or too little food. As an example of expected reproductive output, consistency and food ration effects, below is a table of *Amphiascus tenuiremis* average offspring production through two broods for approximately 630 mating pairs scored by four USC technicians for 10 bioassays conducted over the past 5 years. The bottom row is data from four bioassays by two technicians that used a 3 □L food ration every sixth day (note, acetone carrier was not required in any of the 3 □L studies tabulated below):

CONTROL TYPE AND RATION	Mean Two Brood Size	STD. DEV	COEFFICIENT OF VARIATION
2 μL DIET			
NORMAL CONTROL (n=450 mating pairs)	11.13	3.14	28.2%
SOLVENT CONTROL (n=180 mating pairs)	11.63	3.78	32.5%
ALL CONTROLS (n=630 mating pairs)	**11.27**	**3.32**	**29.5%**
3 μL DIET			
NORMAL CONTROL (n=180 mating pairs)	17.65	3.62	20.5%

Most Frequently Reported Difficulties from Participants (not ranked):

- Lack of precision and accuracy in spiking pesticide: seawater solutions for each 72-h static renewal.

- Complaints about time and effort required on some weekends to score plates and/or perform water changes and feedings when the third or sixth static-renewal day falls on a Saturday/Sunday. (Note: fewer complaints with experience and facility with the method).

- Accidental aspiration of larval/juvenile copepods during water changes.

- Dessication of copepods during water changes (either by open exposure to atmosphere for extended periods or leaving copepods too long under warm incandescent illumination).

- Difficulty with male: female sex determinations for proper construction of mating pairs. (Note: Seemed to be an issue for labs in their first attempt at running the microplate test. Abilities improved with experience).

- Insufficient or inferior microscope optics for microplate reading, sex determination, and clutch size counting. Two labs reported difficulty seeing/counting the second clutch of new nauplii after the first clutch had hatched. (Note: Research quality dissection stereomicroscope with darkfield lighting and fiber optics, and a Hoffman DIC inverted compound microscope (4X, 10X and 20X) are highly recommended and make the assay much easier to perform. Optics are everything when working with harpacticoid copepods.)

- Lack of electronic particle counter for easy algal cell density determinations of food ration (i.e., recommended ~20,000 cells per microwell feeding).

CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER WORK

38. In June 2009, the Invertebrate Testing Expert Group met and reviewed the results and status of the validation of the copepod development and reproduction tests presented in this report. Several issues were addressed in the discussion: 1) the issue of proficiency and training in the application of the test method, 2) the absence of effects on most of the endpoints reported in the atrazine study, 3) the possible issue of sensitivity of the population of *Amphiascus tenuiremis* used in the study, 4) the influence of mortality (males more sensitive than females) on the sex ratio measured, 5) the expression of test results (NOEC/LOEC) based on nominal concentrations rather than measured concentrations, and finally 6) the manpower required in the study (50% time of a technician). Despite a recommendation from the group to collect the remaining data, laboratories with incomplete experiments were not able to finalize the recommended work.

39. The meeting encouraged the lead countries (Sweden and the United States) to further investigate the endpoints reliability through further experimental work. The following endpoints: mortality, fecundity (number of viable offspring in 2 broods), and development rate from nauplius to copepodite were considered to be important and reliable endpoints of the test method. The meeting also recommended performing the test method for controls alone to reassure that the test performance and the validity criteria set out in the revised draft TG can be met by the laboratories with no problem.

40. An effort led by the United States to carry out additional validation work is currently underway. The issues raised in this report in relation to consistent guideline execution, relative sensitivity and best selection of endpoints, and consistency of endpoint measurement across laboratories will hopefully be addressed in the validation study coordinated by the United States.

41. Additional recommendations for further work:

- There is a need for more information on the basic biology of this test species. It would be very useful to have more specific information on how different feeding levels affect the life history progression of this copepod.

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ANNEX 1

ADDITIONAL ATRAZINE RESULTS

Table 1: Percent Naupliar Mortality

Lab / Atrazine	USC	NOAA	Stockholm	Uppsala
Control	6.8% ± 3.9 (ND)	8.3% ± 10.4 (ND)	33.4% ± 9.7 (ND)	--
Carrier	6.3% ± 3.7 (ND)	18.3% ± 5.8 (ND)	11.9% ± 6.1 (ND)	--
26 µg/L	9.2% ± 5.8 26.4 ± 4.0	10.0% ± 5.0 45.6 ± 12.5	10.3% ± 5.4 26.9 ± 6.6	--
43 µg/L	18.2% ± 6.2 53.0 ± 11.5	13.3% ± 2.9 70.7 ± 22.6	21.7% ± 8.6 74.7 ± 38.9	--
72 µg/L	15.3% ± 11.0 96.8 ± 29.1	3.3% ± 5.8 115.3 ± 22.9	29.4% ± 16.2 92.3 ± 26.9	--
120 µg/L	23.0% ± 11.0 140.3 ± 30.1	6.7% ± 5.8 163.6 ± 28.0	18.1% ± 4.7 186.0 ± 82.3	--
200 µg/L	9.0% ± 8.1 207.8 ± 25.3	5.2% ± 0.32 268.4 ± 48.1	25.1% ± 13.4 290.9 ± 88.6	--

Table 2: Copepodite mortality

Lab / Atrazine	USC	NOAA	Stockholm	Uppsala
Control	2.8% ± 2.8 (ND)	5.1% ± 5.0 (ND)	0.0% ± 0.0 (ND)	--
Carrier	0.0% ± 0.0 (ND)	0.0% ± 0.0 (ND)	0.0% ± 0.0 (ND)	--
26 µg/L	6.4% ± 3.6 26.4 ± 4.0	0.0% ± 0.0 45.6 ± 12.5	0.0% ± 0.0 26.9 ± 6.6	--
43 µg/L	3.5% ± 0.1 53.0 ± 11.5	0.0% ± 0.0 70.7 ± 22.6	0.0% ± 0.0 74.7 ± 38.9	--
72 µg/L	7.3% ± 0.8 96.8 ± 29.1	1.9% ± 3.2 115.3 ± 22.9	0.0% ± 0.0 92.3 ± 26.9	--
120 µg/L	1.1% ± 1.9 140.3 ± 30.1	1.9% ± 3.2 163.6 ± 28.0	4.2% ± 7.2 186.0 ± 82.3	--
200 µg/L	3.4% ± 3.3 207.8 ± 25.3	0.0% ± 0.0 268.4 ± 48.1	2.6% ± 4.4 290.9 ± 88.6	--

Table 3: Adult stage mortality

Lab / Atrazine	USC	NOAA	Stockholm	Uppsala
Control	2.8% ± 2.8 (ND)	5.1% ± 5.0 (ND)	0.0% ± 0.0 (ND)	--
Carrier	0.0% ± 0.0 (ND)	0.0% ± 0.0 (ND)	0.0% ± 0.0 (ND)	--
26 µg/L	6.4% ± 3.6 26.4 ± 4.0	0.0% ± 0.0 45.6 ± 12.5	0.0% ± 0.0 26.9 ± 6.6	--
43 µg/L	3.5% ± 0.1 53.0 ± 11.5	0.0% ± 0.0 70.7 ± 22.6	0.0% ± 0.0 74.7 ± 38.9	--
72 µg/L	7.3% ± 0.8 96.8 ± 29.1	1.9% ± 3.2 115.3 ± 22.9	0.0% ± 0.0 92.3 ± 26.9	--
120 µg/L	1.1% ± 1.9 140.3 ± 30.1	1.9% ± 3.2 163.6 ± 28.0	4.2% ± 7.2 186.0 ± 82.3	--
200 µg/L	3.4% ± 3.3 207.8 ± 25.3	0.0% ± 0.0 268.4 ± 48.1	2.6% ± 4.4 290.9 ± 88.6	--

ANNEX 2

ADDITIONAL LINDANE RESULTS

Table 1. Naupliar mortality

Lab Lindane	USC	NOAA	Stockholm	Uppsala
Control	2.6% ± 0.4 (ND)	2.2% ± 1.9 (ND)	5.0% ± 5.0 (ND)	12.7% ± 6.2 (ND)
Carrier	3.4% ± 2.9 (ND)	3.3% ± 3.3 (ND)	8.8% ± 11.0 (ND)	2.0% ± 3.4 (ND)
2.5 µg/L	1.7% ± 2.9 3.9 ± 1.4	23.3% ± 8.8 5.1 ± 2.9	1.7% ± 2.9 2.2 ± 0.8	41.7% ± 36.3 7.0 ± 1.6
5 µg/L	5.1% ± 4.3 7.6 ± 3.3	31.1% ± 15.0 7.5 ± 4.6	8.5% ± 5.6 4.7 ± 2.0	10.9% ± 5.8 8.9 ± 4.2
10 µg/L	**28.8% ± 4.1** * 12.7 ± 4.2	**78.9% ±15.0** * 15.9 ± 7.7	13.8% ± 13.2 7.6 ± 3.4	**44.9% ± 18.9** * 16.7 ± 6.7
15 µg/L	**12.8% ± 10.1** * 23.4 ± 7.6	**74.4% ± 1.9** * 28.3 ± 15.1	8.5% ± 10.3 11.6 ± 3.8	15.5% ± 12.5 29.3 ± 9.4
20 µg/L	**26.9% ± 5.1** * 23.2 ± 5.1	**54.4% ± 10.2** * 36.7 ± 20.9	**16.7% ± 17.6** * 15.7 ± 7.3	10.8% ± 9.4 53.2 ± 9.1

Table 2. Copepodite mortality

Lab Lindane	USC	NOAA	Stockholm	Uppsala
Control	0.9% ± 1.5 (ND)	0.0% ± 0.0 (ND)	1.7% ± 3.0 (ND)	0.0% ± 0.0 (ND)
Carrier	0.0% ± 0.0 (ND)	0.0% ± 0.0 (ND)	1.9% ± 3.2 (ND)	0.0% ± 0.0 (ND)
2.5 µg/L	2.7% ± 2.7 3.9 ± 1.4	0.0% ± 0.0 5.1 ± 2.9	1.8% ± 3.0 2.2 ± 0.8	0.0% ± 0.0 7.0 ± 1.6
5 µg/L	3.6% ± 4.1 7.6 ± 3.3	1.6% ± 2.7 7.5 ± 4.6	2.1% ± 3.6 4.7 ± 2.0	0.0% ± 0.0 8.9 ± 4.2
10 µg/L	5.2% ± 1.7 12.7 ± 4.2	0.0% ± 0.0 15.9 ± 7.7	0.0% ± 0.0 7.6 ± 3.4	0.0% ± 0.0 16.7 ± 6.7
15 µg/L	6.6% ± 5.8 23.4 ± 7.6	0.0% ± 0.0 28.3 ± 15.1	0.0% ± 0.0 11.6 ± 3.8	0.0% ± 0.0 29.3 ± 9.4
20 µg/L	**14.4% ± 5.5** * 23.2 ± 5.1	2.6% ± 4.4 36.7 ± 20.9	0.0% ± 0.0 15.7 ± 7.3	2.2% ± 3.9 53.2 ± 9.1

Table 3. Adult-stage mortality

Lab Lindane	USC	NOAA	Stockholm	Uppsala
Control	1.8% ± 1.6 (ND)	0.0% ± 0.0 (ND)	5.6% ± 9.6 (ND)	0.0% ± 0.0 (ND)
Carrier	7.5% ± 1.8 (ND)	3.5% ± 3.4 (ND)	4.1% ± 3.7 (ND)	0.0% ± 0.0 (ND)
2.5 µg/L	3.0% ± 0.3 3.9 ± 1.4	8.7% ± 4.5 5.1 ± 2.9	1.7% ± 2.9 2.2 ± 0.8	0.0% ± 0.0 7.0 ± 1.6
5 µg/L	2.1% ± 1.9 7.6 ± 3.3	13.9% ± 5.5 7.5 ± 4.6	0.0% ± 0.0 4.7 ± 2.0	0.0% ± 0.0 8.9 ± 4.2
10 µg/L	1.3% ± 2.3 12.7 ± 4.2	6.7% ±11.6 15.9 ± 7.7	10.3% ± 7.4 7.6 ± 3.4	0.0% ± 0.0 16.7 ± 6.7
15 µg/L	1.9% ± 3.2 23.4 ± 7.6	**28.1% ± 19.2** * 28.3 ± 15.1	0.0% ± 0.0 11.6 ± 3.8	0.0% ± 0.0 29.3 ± 9.4
20 µg/L	0.0% ± 0.0 23.2 ± 5.1	6.1% ± 10.5 36.7 ± 20.9	5.3% ± 9.1 15.7 ± 7.3	2.1% ± 3.6 53.2 ± 9.1

Figure 1. USC Leslie matrix F1 stage structure (stage abundance in white)

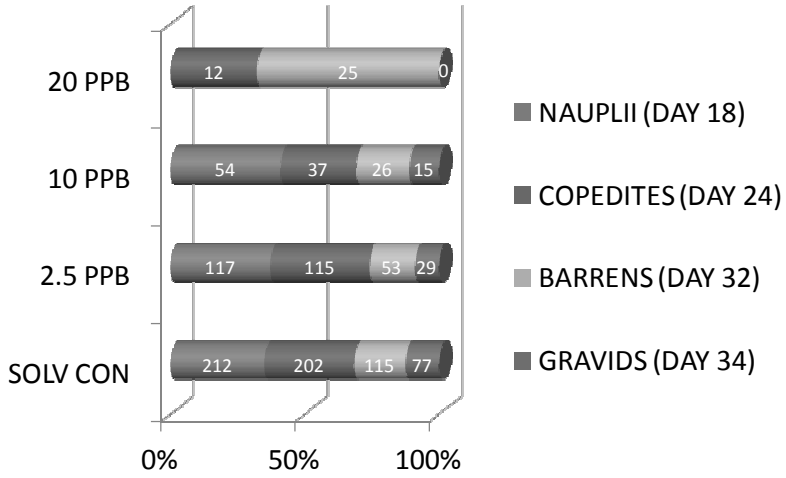

Figure 2. NOAA Leslie matrix F1 stage structure (stage abundance in white)

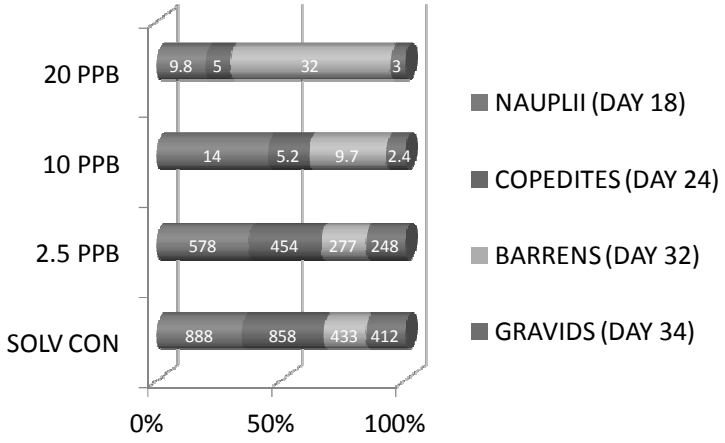

Figure 3. STOCKHOLM Leslie matrix F1 stage structure (stage abundance in white)

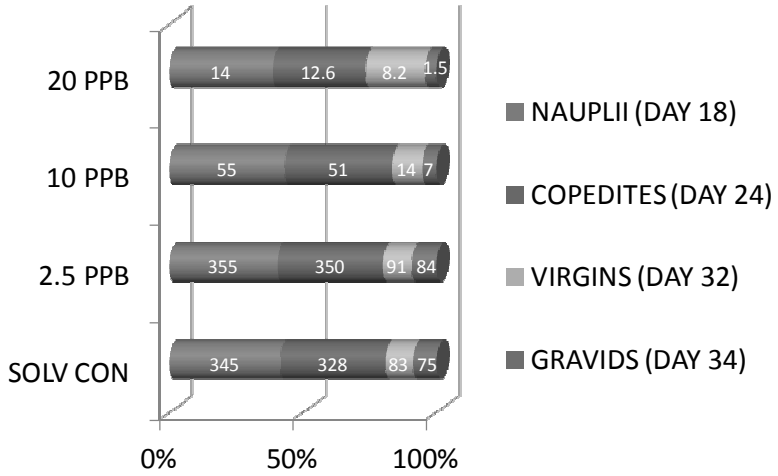

Figure 4. UPPSALA Leslie matrix F1 stage structure (stage abundance in white)

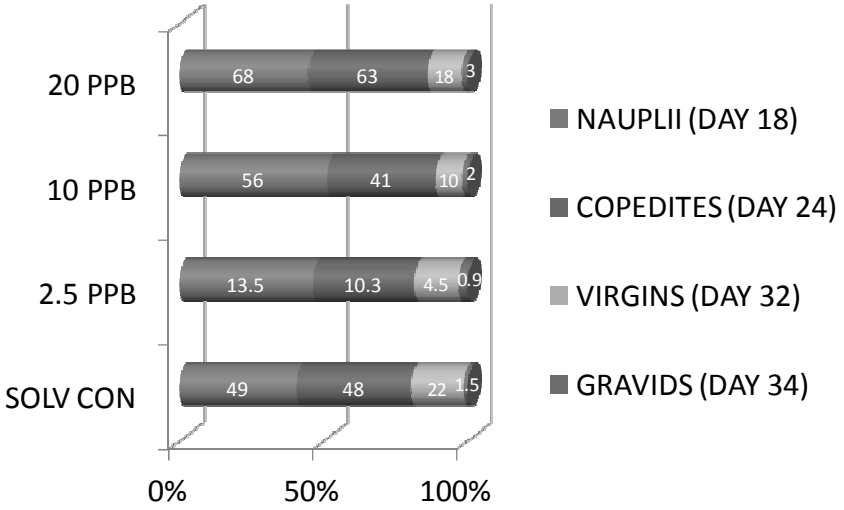

Figure 5. USC Leslie matrix F1 stage abundances

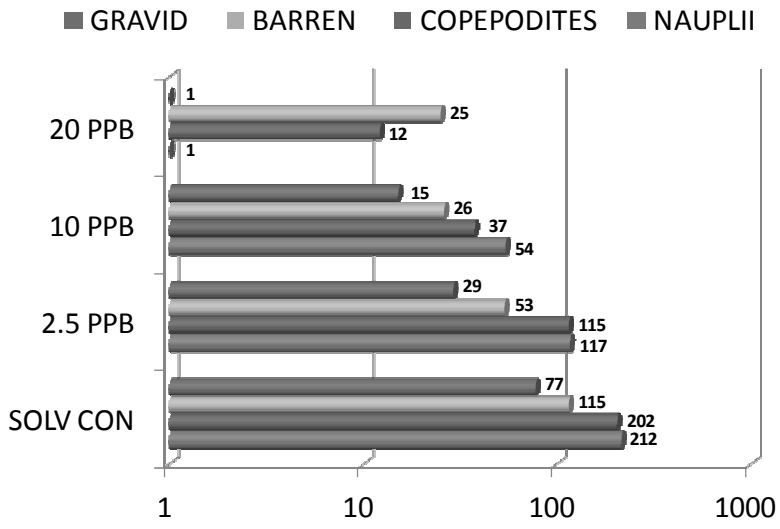

Figure 6. NOAA Leslie matrix F1 stage abundances

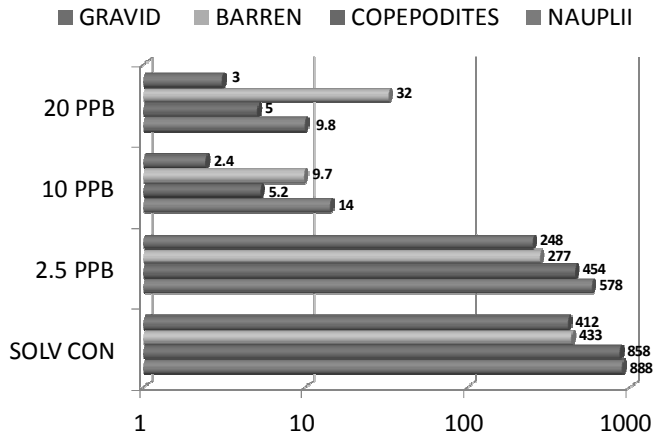

Figure 7. STOCKHOLM Leslie matrix F1 stage abundance

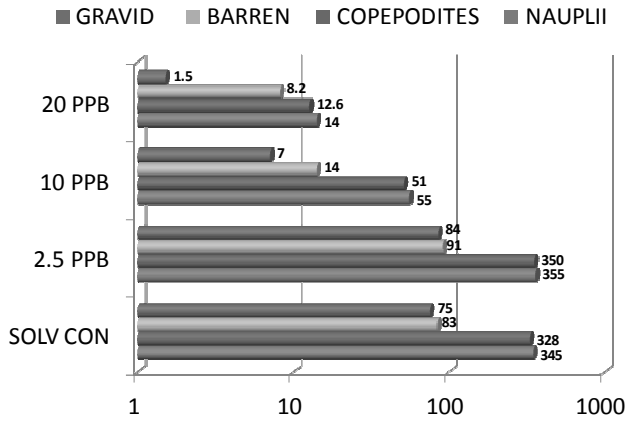

Figure 8. UPPSALA Leslie matrix F1 stage abundance