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NEW GUIDANCE DOCUMENT ON HARPACTICOID COPEPOD DEVELOPMENT AND REPRODUCTION TEST WITH AMPHIASCUS

Series on Testing and Assessment

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IOMC

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

Environment Directorate
ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT
Paris 2014

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FOREWORD

A Project proposal for a Copepod Test Guideline (HCDRT) was submitted by Sweden to the OECD Working Group of the National Coordinators of the Test Guidelines Programme (WNT) in 2002. Unlike the Daphnia reproduction test, the proposed method allows for assessing toxicity of chemicals on sexual reproduction of a crustacean species. Validation of this test method was conducted between 2006 and 2012. Several reports were published in the Series on Testing and Assessment (Nos. 79 and 158). There is currently limited experience in the ecotoxicological laboratory community for reproducible and successful completion of this test with the proposed organisms. Previous problems in handling copepods and defining maturation and gender identification were highlighted; these are clearly areas requiring experience and proficiency demonstration. Experts recognised that technical proficiency of the laboratory in acclimating, rearing and using the organisms is critical to the success of the test.

In October 2013, the OECD Validation Management Group for Ecotoxicity Testing (VMG-eco) recommended developing, at this stage, a Guidance Document to ensure the test method is available to potential users who can progressively get the experience needed. Such a guidance document is not covered by the Mutual Acceptance of Data like OECD Test Guidelines. The document was approved by the WNT at its 26th meeting in April 2014. The Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology agreed to its declassification on 7th July, 2014.

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

TABLE OF CONTENTS

NEW GUIDANCE DOCUMENT ON HARPACTICOID COPEPOD DEVELOPMENT AND REPRODUCTION TEST WITH *AMPHIASCUS TENUIREMIS*

INTRODUCTION

1. Since 1995, revision and development of OECD Test Guidelines in aquatic ecotoxicity testing have assigned high priority to the development of acute and chronic tests with species of marine Crustacea (1). Specific recommendations for developing subchronic/chronic test methods with both warm and cold-water marine and brackish water species have been made (2). This Guidance Document describes test procedures for measuring the (sub)chronic effects of chemicals on an estuarine/marine harpacticoid copepod species under semistatic conditions; the biological endpoints include survival and development rates of the early life-stages and multiple aspects of its reproduction (*e.g.*, sexual maturation, mating success and numbers of viable offspring produced (fecundity). These procedures are applicable to most chemicals, either individually, or in formulations, commercial products, or known mixtures, that can be solubilized and measured accurately at the necessary concentration in seawater. Test chemicals that have a high oxygen demand, are highly volatile, or are rapidly biologically or chemically transformed in aqueous solutions, might not be suited for use in this test.

2. Copepods occur widely in marine, brackish and freshwater ecosystems. They represent important prey items for the larvae of many fish and larger invertebrates (3), and they are increasingly used as a live food source in aquaculture (4). A number of copepod species (predominantly marine and brackish water species) have been used for many years to evaluate the acute and chronic toxicity of single substances and complex mixtures (5, 6). For assessment of marine contaminants in temperate ecosystems in this test protocol, *Amphiascus tenuiremis* is used as test organisms. Several inter-laboratory studies have been performed to standardize the methodology and results are available in reports (7) (8). Other species have also proven to be valuable test organisms *Nitocra spinipes*, *Acartia tonsa* and *Tisbe battagliai* (9-15).

3. The biological attributes of harpacticoid copepods (i.e., laboratory culturable, rapid lifecycles, sexual dimorphism) facilitate the measurement of developmental, reproductive and life-table parameters, which provide sensitive and ecologically relevant endpoints that can be used to determine the potential (sub)chronic toxicity of many water-borne contaminants on development, reproduction and population growth.

4. Test chemical represent what is tested. Definitions used are given in Annex 1.

PRINCIPLE OF TEST

5. Newly hatched larvae (termed F_0 generation nauplii), aged less than 24 hours at the start of the test, are exposed individually (60-120 nauplii allocated over three replicate microplates per treatment) in microwell (300-µL total volume) test chambers to seawater (*i.e.*, control) and to the test chemical added to seawater at least in five test concentrations. The test duration is usually up to 36 days at 25ºC, which is sufficient time for the control animals to reach adulthood, be paired, mate and produce at least two clutches of offspring (termed the F_1 generation). The duration of the test may be increased in order to investigate longer-term effects on the survival and reproductive success of the F_0 and/or F_1 and subsequent F_N generations.

6. When the copepods reach adulthood, which normally takes 15-18 days for the present species at 25 ºC, the gender of each copepod is determined by light microscopy. Individual adult male: female mating pairs are then constructed and allowed to mate for 7-14 days in new isolated microwell test chambers containing seawater and the test chemical added to seawater at the same range of concentrations as during maturation/ development. The mating pairs are followed until the females have been fertilised and have released two clutches of offspring. The two-clutch reproductive output (*i.e.*, the number of viable hatched F_N nauplii) of the copepods exposed to the test chemical is compared to that of the control(s) in order to determine the reproductive no observed effect concentration (NOEC). In addition, and as far as possible, the data may be analysed using a regression model in order to estimate the concentration that would cause an X% reduction in reproductive output, *i.e.,* EC_x (*e.g.*, EC_{50}, EC_{20} or EC_{10})**.** The mean development rates to the copepodite and adult stages, respectively, sex and survival of the parent animals, and time to production of first and second clutches should also be reported. Other chemical-related effects on reproduction such as reproductive success, failed fertilization, depressed clutch sizes, infertile or unhatched eggs, altered time intervals between successive clutches and possibly depressed intrinsic or instantaneous rates of population increase (r_m or λ), may also be recorded or calculated depending on the needs of the study.

DATA ON THE TEST CHEMICAL

7. The water solubility, the vapour pressure, pK_a, and K_{ow} of the test chemical should be known, and a reliable analytical method for the quantification of the chemical in the test solutions with known and reported accuracy and limit of quantification should be available. Useful information for establishing the optimal test conditions includes the test chemical structural formula, purity of the chemical, stability in light, stability under the conditions of the test (abiotic and biotic degradation) and dissipation/fugacity. Loss of test chemical (volatilization, adsorption, accumulation, degradation, etc.) during the exposure period should be considered and evaluated before starting the test. Further guidance for testing chemicals with properties that make them difficult to test with the described methods is provided in (16). Also personal safety precautions when working with the test chemical should be taken. Waste containing the test chemical should be treated in a way that does not cause human or environmental harm or concerns.

8. Results of an acute toxicity test (see (17)), preferably performed with the same species (*Amphiascus tenuiremis*), should be available. The results will be needed for selecting an appropriate range of test concentrations in the definitive chronic tests (See paragraphs 32 to 34).

REFERENCE SUSBTANCES

9. Any of the reference substances cadmium chloride ($CdCl_2$), fipronil or sodium dodecyl sulfate (SDS) may be tested periodically as a means of assuring that the test protocol and test conditions are reliable, and that the sensitivity of copepod test stocks is consistent over time and between stocks. Example calculated *Amphiascus tenuiremis* 96-h LC_{50}'s (with 95% confidence intervals) for these reference substances at 30 ‰ salinity are:

Fipronil:	7.3 µg/L (95% C.I.: 5.7-9.3)
Cd (as CdCl2):	224 µg/L (95% C.I.: 213 – 235)
SDS:	12.0 mg/L (95% C.I.: 10.8 – 13.4)

10. For regular sensitivity checks of stock culture populations it is recommended that sodium dodecyl sulfate be used as a reference toxicant. The highest acceptable value for SDS toxicity to the

University of South Carolina stock population of *A. tenuiremis* is a running average 96-hour $LC_{50} \leq 18$ mg-SDS/L-seawater at 30 ‰ salinity.

VALIDITY OF TEST

11. For a test to be valid, the following validity criteria apply to the control:

- the average survival of the parent F_0 generation in the control(s) should be at least 70% over the duration of the chemical exposure. No individual control replicate microplate should exceed 40% mortality;
- sex ratio at maturity for F_0-controls (♀♀ : ♂♂) should on average be between 35-65%male:%female or %female:%male in the test population;
- at least 75% of control mating pairs are able to produce offspring by the end of the 36 day test;
- the average number of viable offspring through two clutches in the controls is at least eight individuals
- the average number of days to extrusion of the first control egg clutch at a temperature of 25 °C should be less than or equal to 25 days in this 36 day test.

Test physico-chemical acceptance criteria

12. The following physico-chemical criteria should be met during the test to ensure copepod response data quality:

- the dissolved oxygen concentration of excess stock solution or seawater removed and pooled from microplate wells is at least 60% of the air saturation value (ASV) over the exposure period;
- daily temperature variation is less than ± 4°C over the entire test with a 36-day mean temperature of 25 ± 2.0 °C; seawater pH must not vary more than 0.5 units from the average control pH with a 36 day mean pH of 8.0 ± 0.4;
- conductivity/salinity must not vary more than ± 10% from the control start value (*e.g.,* 30‰ ± 3 ‰).

DESCRIPTION OF THE METHOD

Apparatus

13. Microwell test chambers and other test vessels and apparatus which will come into contact with the test solutions should, if possible, be made entirely of glass or other materials chemically inert to the test chemical. Microwell plates are typically made of polystyrene plastic. Polystyrene is not well suited to testing with hydrophobic organic chemicals because of non-specific plastic: organic binding, which can reduce chemical bioavailability to copepods. Therefore, microplates with microwells pre-coated with glass or polyacrylamide gel (*i.e.*, hydrogel microplates) may be better suited for copepod testing of hydrophobic chemicals than microplates with naked polystyrene wells. Hydrogel microplates show low binding of hydrophobic organic chemicals, but they must be pre-hydrated for one hour with distilled water or test chemical seawater solution and then emptied before adding copepods and test media. Prehydration

saturates the polyacrylamide coating with water to create a hydrophilic low-binding microwell surface. Testing of metals or other non-hydrophobic chemicals may be conducted in microplates with naked polystyrene wells if preliminary chemical binding studies show minimal test chemical affinity for polystyrene.

14. In addition, some or all of the following equipment will be required:

- oxygen meter (with microelectrode or other suitable equipment for measuring dissolved oxygen in low volume (*e.g.*, ≤ 10-mL) samples);
- pH meter;
- conductivity meter, osmometer or salinity refractometer;
- adequate apparatus for the control of the test lighting regime;
- stereo compound microscope, preferably inverted with dark-field, Nomarski or Hoffman DIC illumination;
- dissection stereomicroscope, preferably with dark-field illumination and fiber optic episcopic illumination;
- climate-controlled environmental chamber(s) or a temperature regulated environmental room;
- 0.3 m^3 glass or chemically inert plastic rectangular containers with sealable lids to create humidity chambers for the placement of microplates (to minimize evaporative water loss);
- various plastic or stainless-steel mesh sieves (0.05 mm to 0.5 mm) to culture, capture and sort copepods;
- Pasteur pipettes, preferably silanized to prevent copepods from adhering to pipette walls;
- adjustable single and multi-channel volumetric pipettors (1-10 µL, 100-500 µL) for algal cell food additions;
- 250 µL Hamilton glass volumetric syringes with 23 gauge bevelled stainless-steel needles for test solution manipulation;
- hemacytometer cell counting chamber or electronic particle counter/analyser (*e.g.*, a Coulter principle impedance-based system) for algal cell count verifications.

Test organism

15. The species to be used in the test is *Amphiascus tenuiremis*, which has widespread amphi-Atlantic distribution and occurs commonly in harpacticoid copepod field surveys. However, the taxonomic identification of this species may be difficult and therefore great care should be taken to correctly identify species used to establish laboratory cultures if collected in the field. It is recommended that test animals be obtained from already established laboratory cultures.

16. Test animals should be derived from a healthy stock (*i.e.*, showing no signs of stress such as high mortality, poor fecundity, etc.). The stock animals are maintained in culture conditions (light, temperature,

seawater medium and feeding regimen) similar to those to be used in the test (a detailed culturing method is contained in Annex 2).

Test medium

17. Natural or synthetic seawater may be used as the test medium. If natural seawater is used, it should be collected from a location free from known sources of pollution, filtered at < 0.2 µm to remove indigenous organisms, and chemically analyzed for the presence of environmental pollutants (e.g., metals, PAHs, pesticides). If synthetic seawater is used, it should be prepared by dissolving reagents of known analytical grade, or a commercially available formulation, in distilled or de-ionised water. However, for this species, there is insufficient information on the use of multiple synthetic seawater formulations to allow that a particular brand or type be recommended. The suitability of synthetic seawater for culture and testing should be evaluated in trials. Any seawater in which the copepods show suitable long-term survival with normal development and fecundity may be used as the test medium.

18. *A. tenuiremis* can be used at salinities between 18-40‰, but is normally cultured at a salinity of 25-30‰. The use of a lower salinity, which may be more appropriate for tests concerning estuarine brackish water situations, should be justified in the test report. For testing at salinities greater than 3 ‰ above or below the normal stock culturing salinity (usually 25-30 ‰), it is recommended that stock cultures be acclimatized in 2 ‰ daily increments up or down to the salinity target and then held at that target for at least 25 days (i.e., one full generation) to achieve population salinity acclimation. For testing, dilution seawater should be within $\pm$ 2 ‰ of the salinity target, have a dissolved oxygen concentration above 65% of the air saturation value, and a pH of 8.0 ± 0.4 before being used to prepare the test solutions. It is recommended that all dilution water be gently aerated for 24 hours and then filtered at 0.2 µm immediately prior to test solution preparation in order to minimize bacterial contamination.

19. The dilution water used for testing should be from the same source as water that has been found to support culture of the organisms for at least two generations. However, if the medium to be used in the test is different from that used for routine culture, it is a good practice to include an adequate pre-test acclimation period (*i.e.*, sufficient time to culture the copepods through at least two generations) in order to avoid stress related artifacts.

20. The dilution water should be of constant quality and samples should, if possible**,** be taken at regular intervals for analysis. Measurement of heavy metals (*e.g.*, Cu, Pb, Zn, Hg, Cd, Ni), major anions and cations (*e.g.*, Ca, Mg, Na, K, Cl, SO_4), pesticides (*e.g.*, total organophosphorus and total organochlorine pesticides), total organic carbon and suspended solids should be made, for example, every three months where a dilution water is known to be relatively constant in quality. If water quality has been demonstrated to be constant over at least one year, determinations can be less frequent and intervals extended (*e.g.*, every six months).

Test solutions

21. The test solutions of the chosen test chemical concentrations are usually prepared by dilution of a concentrated stock chemical solution. Stock solutions preferably should be prepared by dissolving the test chemical directly in test medium. For chemicals with low water solubility, separate concentrated stock solutions in an appropriate minimally-toxic carrier solvent should be prepared for dilution in seawater to the desired test chemical concentrations. The preferred options for preparing test solutions are physical methods, such as stirring and sonication (18). Saturation columns (solubility columns) can be used for achieving a suitable concentrated stock solution of poorly water-soluble test chemicals.

22. The use of organic solvents may be required in some cases in order to produce a suitably concentrated stock solution for testing. Further, solvents may be essential for handling some special test chemicals; for example, for preparing stock solutions of hydrolytically unstable or highly viscous chemicals. Every effort should be made to avoid the use of solvents, but, if unavoidable, a solvent control with adequate replication must be run concurrently with a dilution water control and the various test concentrations at adequate replication. Only solvents or dispersants that have been investigated to have no significant or only minimal effects on the response variable should be used in the test. Examples of suitable solvents (e.g. acetone, ethanol, methanol, dimethylformamide and triethylene glycol) and dispersants (e.g. Cremophor RH40, methylcellulose 0.01% and HCO-40) are given in (15). Where a solvent or dispersant is used, its final concentration should be $\leq$ 0.1 ml/l (15) and it should be the same concentration in all test vessels except the dilution water control. Every effort should be made to keep the required solvent concentration to a minimum. Care should be taken when using readily biodegradable solvent compounds (*e.g.*, DMSO, acetone) as these can cause problems with bacterial build-up and possibly lower dissolved oxygen in the test vessels.

23. Test solutions should be renewed at 72-h intervals or less (see paragraphs 36 and 37). The frequency of medium renewal will depend on the stability of the test chemical. There must be evidence that the concentration of the test chemical being tested has been satisfactorily maintained. Microwell water may be collected from each treatment concentration, pooled, and analyzed for chemical concentration.

PROCEDURE

Culture of copepods

24. The life stages, culture and husbandry procedures for *Amphiascus tenuiremis* are described in Annex 2.

Conditions of exposure

Duration

25. The overall test duration for the complete life-cycle method is up to 36 days (mainly depending on temperature, food quality/quantity, and any developmental-rate changes induced by the test chemical of interest. At 25 ^{o}C, 36 days is usually sufficient time for nauplii to develop to the adult stage, be mated, extrude two clutches of eggs and hatch offspring (broods) (i.e., the F_1 generation). Daily observation of each surviving mating pair is normally ended after extrusion/hatch (and counting) of the second clutch of eggs, or on Day 36, whichever comes first. Longer-term observation of F_1 naupliar survival and development to adult will extend the total bioassay time by an additional 15-18 days. Multigenerational testing to the F_n is possible using F_1 surviving nauplii and repeating the guideline through "n" lifecycles. Extended observation beyond the F_1 is not required under this test guideline**.** At 25°C, two generations of testing through the second F_2 brood would require 45-70 days depending on the degree of any developmental rate delays induced by the test material(s).

Loading

26. Because of their small size, copepods are exposed in small volumes (*e.g.*, <40 ml total) of test solution at each test solution renewal. However, larger volumes are usually prepared in order to meet minimum mass: volume requirements for physical-chemical analysis (*e.g.*, pH, DO, and determination of the test chemical concentration). For this microplate-based test, 100-mL of test solution per concentration per water renewal (see paragraph 32) should provide adequate volume for microplate-based bioassay and chemical analysis of most test chemicals.

Test animals

27. Newly hatched synchronous nauplii (*i.e.*, within 24 hours old) are obtained by isolating 400-600 ovigerous females from a mass culture at least three weeks old and allowing them to hatch offspring over a 24 h period into a Netwell 12-well culture plate (Annex 2, Figure 3.a.). It is recommended that no more than 20-40 nauplii be assayed per microplate so that most end and edge microwells can be avoided and left empty. Edge microwells are the most exposed to temperature and dessication extremes because of their marginal locations in the microplate (Annex 2, Figure 3.b). If, for example, it is desired that 40 individually isolated nauplii are to be tested in a microplate, they should be dispensed individually into ten microwells per row over four rows (see Figure 3.b) of each of three replicate test vessels (microplates) per concentration and control(s). This will yield a total initial test population per treatment or control of 120 individuals dispersed over triplicate microplates. If a test population of > 120 individuals per treatment or control is desired, then a fourth microplate should be added per concentration. The recommended minimum test population is 20 nauplii per microplate per test concentration; i.e., 60 nauplii/concentration.

28. Treatments should be dispersed randomly among separate replicate microplates, as should all subsequent handling, loading, observation and feeding. Failure to do this may result in bias that could be construed as being a chemical-dependent effect. In particular, if experimental units are handled in treatment or concentration order, then some time-related effect, such as operator fatigue or other error, could lead to greater or lesser effects at the higher or lower concentrations. Only one test chemical concentration should be tested within each microplate to minimize opportunities for microwell cross contamination. Furthermore, if it is known or suspected a priori that test results may be affected by an environmental condition of the test, such as position in the laboratory or environmental chamber, then consideration should be given to blocking the test, or using a Latin-square or similar experimental design for physical dispersion of treatment and control microplates in the test system.

Feeding

29. Copepod feeding should be done at every other microwell water renewal, *i.e.*, at every 6^{th} day of the up to 36-day test. Deviations from this schedule should be reported. When using small volumes of test solution in semi-static tests, it is important to consider the volume of food fed (particularly for weekly or more frequent feeding), and the potential for diluting exposure concentrations by addition of food. Food quality and quantity are critically important for optimal development and reproduction of copepods. Individual copepods in test chambers during maturation/development and reproduction are fed 2 µl/microwell of a 1:1:1 volume mixed-algal peak growth phase cell suspension (i.e., 50-mL peak algal culture harvested, centrifuged for 12 minutes to pellet at 4700 RCF, supernatant removed, and pellet re-constituted in ~ 2-mL fresh 30 ‰ seawater to 10^7 cells/mL final density checked by Coulter Countrer or equivalent). A chlorophyte (*e.g.*, *Dunaliella tertiolecta*), a chrysophyte (*e.g.*, *Isochrysis galbana*) and a cryptophyte (*e.g.*, *Rhodomonas salina*) are recommended as food, and delivered as 2 µl per above every six days during both maturation/development and reproductive periods. Therefore the cell density added to each test microwell at each feeding should target as nearly as possible 2×10^4 cells/microwell/feeding. This cell density will support copepod maturation and reproduction without fouling the microwell environment. Centrifuged cell density in reconstituted seawater media should be measured by Coulter Counter, hemacytometer, or equivalent particle counting technology. 10^7 cells/mL is an easy and useful target concentration for delivery to microwells via 2 µL aliquots from micropipettor or Hamilton syringe. Alternatively, on the day of feeding, cells may be mixed into test chemical solutions at the required 10^7 cells/mL density and delivered concurrently with test media at the time of test solution renewal. It is important that healthy algal stocks are maintained during the 36-day test, and that all treatments and controls receive the same rations and sources/types of algal cells. High variance in feeding regime across treatments will disproportionately affect development rates, clutch sizes, and possibly survival in these

sensitive organisms. Specific details of stock culture algal feeding regimes used for *A. tenuiremis* are contained in Annex 2.

Light and temperature

30. During testing, microplates are covered and placed in a temperature-regulated incubator or room set at a desired controlled temperature (normally 25 ± 2 ºC), with a 12-h light and 12-h dark photoperiod. 12 hours light should be at an intensity not exceeding 15-20 $\mu E \cdot m^{-2} \cdot s^{-1}$ measured at the water surface of the vessel. For light-measuring instruments calibrated in lux, an equivalent range of 1000 – 1500 lux for cool white light corresponds near to the recommended light intensity 15-20 $\mu E \cdot m^{-2} \cdot s^{-1}$.

Aeration

31. The test vessels are not aerated during the test; however the dilution seawater should be aerated for 24 h before addition of the test chemical, then filtered at 0.2 μm, and then mixed with test chemical stocks at the appropriate volumes and concentrations before addition to microplate wells.

Test concentrations

32. Preliminary knowledge of the toxicity of the test chemical (*e.g.*, from an acute test and/or from range-finding studies) should help in selecting appropriate test concentrations. As guidance, the highest concentration in the chronic test should be set between one tenth to one fifth of the 96 hr nauplius or copepodite LC_{50} to avoid strong negative effects on lifetime survival. This bioassay is a full lifecycle test and care has to be taken to determine an appropriate dose range that will elicit effects of interest without reducing the test population to a number insufficient to provide at least ten or more adult mating pairs. Concentrations that produce more than 35% mortality over the lifecycle should be reduced and re-tested if insufficient adults are produced to provide ten or more mating pairs. Normally, there should be at least five test concentrations, each comprising 20-40 separate microwells in each of three replicate microplates *per* test chemical concentration (60-120 microwells/concentration). Test concentrations should be arranged in arithmetic series with each successively lower concentration being 60% of the preceding higher concentration. For some test chemicals a geometric series with a concentration separation factor of 3.2 or less may be more appropriate depending on the slope of the dose response curve and the goals of the study. Justification should be provided if fewer than five concentrations are used. In the seawater test medium, chemicals should not be tested above their solubility limits or > 10 mg/L, whichever is less.

33. When planning the test it should be taken into consideration whether the goal is to achieve a NOEC/LOEC (by use of ANOVA-type techniques) or an EC_x value (by use of statistical regression techniques). With an ANOVA approach, higher replication at the expense of fewer test concentrations often yields a more statistically robust experimental outcome. With regression techniques, more test concentrations at a finer scale of separation but with fewer microplate replicates will often yield a more robust prediction of an EC_x.

34. In setting the range of test concentrations, the following should be borne in mind:

(i) If the aim is to obtain the NOEC/LOEC, the selected lowest test concentration is low enough so that the biological end point (*e.g.*, fecundity) of interest at that concentration is not significantly lower than that of the control. If this is not the case, the test will have to be repeated with a reduced lowest concentration.

(ii) If the aim is to obtain the NOEC/LOEC, the selected highest test concentration is high enough to cause a statistically significant effect when compared to the control for the

biological end point (*e.g.*, fecundity) of interest. If this is not the case, the test will have to be repeated with an increased highest concentration unless this concentration occurs at the functional limit of solubility or 10 mg/L, whichever is lower.

(iii) If an EC_x for effects on reproduction is estimated, it is advisable that sufficient concentrations be used to define an EC_x with the appropriate level of confidence. If the EC_{50} for effects on reproduction is estimated, it is recommended that the highest test concentration be greater than this EC_{50}. Otherwise, although it will still be possible to estimate an EC_{50}, the confidence interval for the EC_{50} will be unacceptably large and it may not be possible to satisfactorily assess the adequacy of the fitted model.

(iv) The range of test concentrations should preferably not include any concentrations that have a statistically and biologically significant effect on survival since the main objective of the test is to measure sublethal effects (*e.g.*, on development and reproduction). The occurrence of mortality significantly higher than the control(s) in the test may require more complex statistical analysis depending on the size of the difference.

Controls

35. One test-medium control series and, if relevant, one carrier control series containing the solvent carrier used to promote test chemical solubility in seawater should be run. When used, the solvent control concentration should be set at the highest solvent concentration used in any microwell test chamber containing the test chemical. This is determined most often as that solvent concentration required for solubilizing the highest test chemical concentration in seawater but not exceeding 0.1 mL-solvent/L-seawater. The number of replicates in the control and carrier control should be at least the same number as for any test chemical concentration; typically 60-120 microwells spread uniformly over three microplates.

Test medium renewal

36. The frequency of medium renewal in each test microwell will depend on the stability of the test chemical, but it should occur at least every third day of the test. If, from preliminary stability tests, the test chemical concentration is not stable (*i.e.*, outside the range 70 – 130% of nominal or falling below 50% of the measured initial concentration) over the maximum renewal period (*i.e.*, 2-3 days), consideration should be given to more frequent medium renewal. Potential evaporation losses of media from the test chambers should be monitored daily and, if needed, be corrected by more frequent medium renewal. When using microwell test chambers (300 µL total volume; 250 µL working volume), which are normally held in temperature-regulated incubators or rooms (see paragraph 30), care should be taken so that evaporation losses do not exceed 10% on a daily basis. To prevent such a scenario, microplates may be placed in a 0.2-0.3 m^3 humidity box on a simple elevated platform over a 1-cm deep water-layer or over water-saturated sponges inside the temperature regulated incubator or room.

37. When the medium is renewed, a fixed volume (~ 90% of the total test medium volume) should be aspirated from each microwell chamber under dissection stereomicroscopic observation and cool fiber optic or tangential lighting using a Hamilton gas syringe or micropipette of suitable needle or capillarity bore diameter (*e.g.*, 23 gauge or less, beveled edge). The copepod will remain in a small drop in the bottom of the microplate. Most test media can be removed this way without drawing out the copepod. Care should be taken that copepods are not aspirated into the syringe. If copepods are aspirated they may experience stress or egg sac damage; thus, such aspirated copepods should be disqualified from the test.

Observations

38. Copepod lifecycle endpoints are monitored daily in each microplate using a stereoscopic inverted compound microscope with phase-contrast or better quality illumination (*e.g.,* Hoffman differential interference contrast illumination) for the naupliar life stages. A 4X objective lens with 10X ocular lenses will allow 100% field of view coverage of a single microwell through the bottom of the microplate. For sex determinations and egg sac counts, a 10X objective may be more helpful. Phase-contrast, Nomarski or Hoffman DIC illumination makes microplate scoring, sex determinations and hatched naupliar counting especially efficient at these magnifications. For initial microplate loading, water renewals, and later scoring of the copepodite-to-adult life stages, a research quality dissection stereomicroscope with dark- and light-field illumination is required. Test observations for each treatment must include life stage-specific mean mortalities, mean development rates from nauplius to the copepodite, and copepodite to adult stages, respectively, fertilization success (*i.e.*, mean number of mating pairs able to produce two successive clutches), total mean viable offspring production per mating pair (*i.e.* naupliar production through two clutches averaged over the number of mating pairs created and tested), time to production of first clutch, and time interval between successive clutches. Observations may also be made for any abnormal behaviour (relative to the controls), including uncoordinated swimming, loss of equilibrium, change in pigmentation, inability to molt, and atypical quiescence. Sex ratio may be determined by observations of adult-stage male and female secondary sexual characteristics, which in harpacticoid copepods normally can be distinguished in the 5th copepodite stage at the earliest (see Annex 2, Figure 2.A-D) for micrographs of how to determine differences between adult males and females). Numbers of aborted egg sacs and necrotic/infertile eggs may also be counted if desired.

39. Regular observations of F_0-generation nauplii and copepodites as well as counting of F_1-generation hatched nauplii should be facilitated by the use of light microscopy. An inverted stereomicroscope designed for microplate scanning is most suitable for this purpose (4-20X magnification; see paragraph 38). General sorting of gravid females for initial naupliar production and harvesting is conducted under an upright dissection stereomicroscope with darkfield illumination and fiber optic episcopic illumination.

40. The results of the observations made during the test should be recorded daily on data sheets, examples of which are provided in Annex 3 with notes on their use.

Development and Mating of Adults

41. The number of days it takes for development of each individually isolated copepod, from a first stage nauplius to a juvenile copepodite to a reproductively mature adult, is observed and recorded daily. It is not necessary nor recommended that investigators try to record each of the five naupliar stages or six copepodite stage progressions through time (see Annex 2, Figure 1). Within a given treatment or control set of microplates, the development rate to adult will usually be consistent across copepods within 1-2 days. On the first test solution renewal/feeding day that 50% or more of the copepods can be sexed visually (usually after 15-18 days at 25 ºC), mature virgin males and females should be gently pipetted from their respective treatment or control microplates and placed separately by sex into 25- or 60-mm glass Petri dishes filled ~50% with the appropriate same-concentration test chemical solution. If the test chemical does not have binding affinity for plastic (polystyrene) then plastic Petri dishes may be used for the same treatment same-sex adult collections.

42. These collections of virgin males or virgin females provide the mating stock to be used for construction of individual mating pairs in the bioassay. For greatest ease, the removal and mating of sexually dimorphic males and females should be conducted on days when test solution renewal and feeding is scheduled to occur. This normally occurs on Days 18 to 24 of the copepod-mating time window. If

development to adulthood is delayed by the test chemical(s), then sexual maturity and subsequent matings may be delayed in those treatments until the Day 24 or even Day 30 feeding schedule. Matings are constructed as follows: Adults are pipetted haphazardly from Petri dishes and paired randomly with an opposite sex individual (*i.e.*, one male:female pair per microwell) into a new or unused microwell in the same treatment microplate from which it came. If the previously unused microwell is hydrogel coated then it must be filled with test chemical seawater solution and pre-hydrated for one hour prior to use as described above (14). The pre-hydration solution should be aspirated away prior to loading of mating pairs to the microwell. The test solution transferred with the copepod is the same concentration as used for rearing. After male:female pairing is complete, the microwell is filled as needed with the appropriate test solution concentration plus 2 µl of algal food as described above. This pairing method is performed for as many male:female pairs as possible for each treatment and control replicate microplate. With practice it also may become straightforward for the operator to create mating pairs by simple direct transfer of an adult male or female from a rearing microwell to a mating microwell without use of the Petri dish adult pooling method. If direct transfers are conducted, it is important to aspirate away $\geq$ 90% of transfer water using a Hamilton syringe as described above, and then replacing the liquid with fresh test solution (250 µL).

43. If survival is 100% in a treatment or control group up to the point of mating, then the yield of mating pairs will be approximately 50% of the starting naupliar n-size if sex ratios are balanced. If sex ratios are skewed, or mortality rates have produced insufficient numbers of males and/or females to produce 10 or more mating pairs within a given microplate within a given treatment, then adults should be mated *across* microplates within treatments. However, this cross-microplate mating must be noted in any report of results, and an *a priori* assumption of no significant microplate to microplate variability within the given treatment(s) must be made by the investigator.

Offspring production

44. After pairing of male:female mating pairs, observations are made daily for the presence of females carrying their first and subsequent egg sacs. In controls, two fertile egg sacs per female (> 4 eggs/clutch normally) usually are extruded simultaneously from the abdomen in 1-2 days after mating. Offspring (nauplii) will normally take two days to hatch from the egg sacs at 25 ^{o}C. The sacs are carried by the female up until a few hours prior to hatching when they are dropped to the bottom of the microwell to hatch. The mean number of viable offspring produced through two broods in this test should be $\geq$ 8 offspring per mating pair in the controls. In the case of uneven sex ratios in a treatment or control, spare unmated males or females may be retained and monitored for survival as individuals to the end of the test exposure period with renewal of test media continued as described above. Once naupliar offspring are present in the mating-pair microwells, water:food renewals, when required, should only be conducted after nauplii are counted, and they should be conducted under dissection stereomicroscopy (250-300X magnification) with Hamilton syringe to avoid copepod aspiration. Total *egg* number per mating pair can be counted by direct observation of all eggs in each egg sac if desired. Numbers of successfully hatching nauplii (*i.e.*, called total viable clutch, or brood, or fecundity) should be recorded for the first and second successive clutches of eggs. Hatching number can be obtained by direct counting numbers of hatched nauplii crawling on the bottom of each microwell using direct microscopic observation. Once the second clutch is hatched (usually 3-5 days after the first), then it is counted, and the bioassay is complete for a given mating pair. Clutch-one nauplii will be visibly larger than clutch-two nauplii at hatching, which facilitates clutch-two counting. Any two clutch production of viable offspring by a mating pair in the 36-day test period should be recorded as a "successful mating" event in the datasheet. Percent mating success should be calculated as the ratio of successful mating pairs versus unsuccessful pairs (*i.e.*, those pairs unable to produce two viable clutches of eggs in $\leq$ 36 days) for each group of mating pairs in each microplate within each treatment and control. The average percent mating success (and standard deviation) should be calculated from these data for each treatment and control and reported. The observed

two-clutch brood size (mean fecundity) should be calculated as the average number (plus standard deviation) of hatched offspring through two broods per mating pair per treatment or control in ≤ 36 days. Depending on the needs and goals of a study, investigator may choose to extend the bioassay to a multigenerational test by harvesting and using F_1 to F_N offspring and repeating the lifecycle exposure with a fresh set of microplates and over another ≤ 36 days. At the end of the exposure period, all remaining copepods (*i.e.*, including unfertilised females and males) are either discarded, frozen or fixed in glutaraldehyde (4%) for possible determination of sex-specific toxicant whole-body burdens, egg mass toxicant burdens, egg quality, imposex, developmental defects, DNA integrity, etc.

Mortality

45. Survival of larvae (nauplii), juveniles (copepodites) and adults is checked daily during maturation/ development/ mating up to and including the last day of the test. Daily mortality counts of the F_1 offspring after hatching are difficult and not required or recommended in this guideline.

Other endpoints

46. Other endpoints that can be measured or calculated include time from appearance of first egg sacs to release of first and second clutch, number of necrotic and infertile eggs per clutch, and morphological abnormalities in nauplii, copepodites or adults. This species is sexually dimorphic so indicators of intersexuality may be examined using light microscopy (see Annex 2 for further information on easily-observed secondary sexual characteristics in the present species). Population growth rate (*e.g.*, the intrinsic (or instantaneous) rate of natural increase [r_m *or* λ]) may be calculated from the resulting lifetable survival, sex-ratio and fecundity data produced by this bioassay (see Annex 4, Figures 1 and 2 for further information on growth rate calculations).

Analytical determination and measurements

Physical-chemical parameters

47. During the test, dissolved oxygen, pH, salinity and temperature should be measured in the controls and in all test concentrations each time test medium is renewed. If a temperature microprobe is available, temperature may be measured in a blank microplate filled with seawater and placed in the test chamber with the test microplates. If not available, then a 100 mL Griffin beaker filled with seawater should be placed in the test chamber near the test microplates and temperature measured via direct immersion thermometry. Seawater measurements of dissolved oxygen, pH, salinity and temperature should be made on all fresh renewal test solutions, and where possible on the 72-hour old seawater removed and pooled from each microplate treatment or control. As a minimum, these measurements should be made in the control(s) and in the highest test concentration.

Concentration of the test chemical

48. During the test, the concentrations of test chemical are determined at regular intervals, if possible, for all stock renewal test solutions and if possible in some of 72-hour old removed/pooled test solution, e.g. lower and higher test concentrations. Used microwell seawater can be collected and pooled to yield 1-mL for chemical analysis from every 4-5 microwells within a given treatment. As a minimum, the controls (water and solvent if used), the highest and lowest test chemical concentrations, and the positive controls (if used) should be analysed when freshly prepared at the start of the test and immediately prior to each test solution renewal in the microplate. For tests where the concentration of the test chemical is not expected to remain within ± 20% of the initial measured concentration after 72 h or less, it is necessary to analyse ALL control(s) and test concentrations when freshly prepared and at test medium renewal. If the test chemical is known or expected to degrade quickly, then preliminary blank microplate loadings without

copepods should be conducted with concentrations monitored/measured over time to determine how frequently seawater renewals should be made to maintain exposure integrity.

49. In the case of rapid test chemical breakdown, test results should be expressed in terms of time-weighted mean concentrations (see guidance for calculation in OECD TG 211 Annex 6). Note that care should be taken when testing very lipophilic (*i.e.*, log K_{ow} > 5) and hence poorly water-soluble chemicals in the present test system (14). Use of radiolabelled chemicals can give crucial information on chemical partitioning in the test system, which may facilitate the verification of nominal concentrations. However, if the major fractions (*i.e.*, >75%) of the radiolabelled chemicals are found to be adsorbed to particulate material (*e.g.*, the food items) and/or walls of the test chambers, an alternative test system or methodology should be used, where the effect levels are reported relative to contaminant levels in, *e.g.,* food.

50. It is recommended that bioassay results be reported based on mean measured test chemical concentrations. If there is evidence that the measured concentrations have been satisfactorily maintained throughout the test within ± 20 percent of the nominal concentrations , then results can be reported based on nominal or mean measured initial test chemical concentrations (i.e., measured concentrations in stock solutions averaged across renewals).

DATA AND REPORTING

Treatment of results

51. Statistical procedures should be chosen to best match the test data. The following methods are examples and compilations of suitable statistical procedures (19, 20). If desired, acute mortality data for selected time periods (*e.g.*, ≤ 96 hours) can be analysed based on the methods of Stephan (21) in order to calculate median lethal (LC_{50}) concentrations of the test chemical.

52. Statistically significant effects on survival of adults may be calculated using a contingency table procedure, Cochran-Armitage Test (19), which should be reported as a separate endpoint. Those concentrations where mortality is ≥ 35% are not included in the statistical analysis of reproduction data unless ten or more mating pairs were able to be constructed and survived to the end of the test.

53. Larval/juvenile development rates, stage-specific mortality, and percent mating success of the F_0 may all be analysed statistically for significant differences relative to controls. Reproductive endpoints may be analysed as single end-of-test measurements or relative to different time intervals during the exposure. The mean number of F_1 offspring produced from fertilised females (F_0) in each replicate is calculated. Generally, the reproduction data is not adjusted for mortality but are presented on a *per female or per mating-pair* basis. The mean number of offspring produced by each control and treatment mating-pair is calculated through two broods or up to 36 days – whichever arrives first. If a female dies after producing young, the total number of offspring produced up to the time of death may be used. However, those mating pairs that die during the exposure (*i.e.*, before giving birth to any young) are not included in the fertility or fecundity endpoint calculations and subsequent statistical analyses. One-way nested analysis of variance (ANOVA) is used to evaluate effects of the test chemical on the reproduction (and other chronic endpoints) of the copepods, and a multiple comparison procedure (*e.g.*, William's or Dunnett's parametric test (19)(20);or Jonckheere-Terpstra non-parametric test (22)) is used to define the treatment level resulting in e.g., significantly different reproduction from the control (p = 0.05). For ANOVA, individual microwell mating pairs are nested within their particular microplate, which is then replicated within a given treatment. Therefore microwells within a microplate are not strictly independent of one another, and a nested model is best even if data are not evenly balanced across microplates across treatments.

54. Analysis of variance is a parametric procedure and is based on the assumptions that the observations are independent and normally distributed, and that the variance of the observations is homogeneous across all toxicant concentrations and the control. These assumptions should be validated prior to ANOVA using Shapiro-Wilk's test for normality, and Levene's test for variance homogeneity across treatments or microplates within treatments. Non-normally distributed data may be subjected to normalizing transformations such as log transforms, arc-sine square root transforms, etc., provided both treatment and control datasets are subjected to the same transformation. If normality cannot be achieved with data transformation, then the Jonckheere-Terpstra step-down non-parametric ANOVA approach may be preferable if the test endpoint data exhibit a monotonic response to increasing concentration. For specific comparisons to the control, Dunn's non-parametric test is preferable. For the statistical guidance on analysis of test results see also the OECD Guidance document No. 54 (19).

55. If a statistically significant difference in survival, development rate, reproductive success or fecundity is detected between the control and solvent control, only the solvent control is used as the basis for the calculation and reporting of "comparison to control" results. If no significant differences exist between control and solvent control data, and replication levels have provided good statistical power to detect differences if they are real, then these control datasets may be pooled for greater statistical power in comparisons of control response with test chemical response.

Test report

56. The test report includes the following information:

- reference to this Guidance Document.

Test chemical:

- physical nature and relevant physicochemical properties;
- chemical identification data (name, structural formula, CAS number, etc.) including purity;
- analytical method for quantification of the test chemical where appropriate;
- supplier or source of the test chemical.

Test species:

- the species of copepod used, supplier or source (if known), and the stock culturing conditions used.

Test conditions:

- test procedure used (*e.g.*, microplate based semi-static renewal)
- photoperiod and light intensity;
- test design (*e.g.*, test concentrations used, number of replicates, number of copepods per replicate, number of mating pairs constructed per treatment);
- method of preparation of stock solutions and frequency of renewal (the solubilising agent and its concentration must be given for each treatment, when used);

- the nominal and measured test chemical concentrations in stock media at each seawater renewal; the means of measured test concentrations and their standard deviations in the test vessels when measured and the method by which these were attained; and evidence that the measurements reflect actual concentrations of the test chemical(s) in true solution;

- dilution water characteristics (including pH, salinity, temperature, dissolved oxygen concentration, total organic carbon, suspended solids, ammonia, and any other measurements made);

- detailed information on feeding (*e.g.*, type of food(s), source, amount given, frequency of feeding, and analyses for relevant unintended contaminants if available (*e.g.*, metals, PCBs, PAHs and organochlorine pesticides).

Results:

- results from any preliminary studies on the chemical stability of the test chemical;

- the nominal test concentrations and the methods/results of all analyses used to determine the concentrations of the test chemical in the test vessels; the recovery efficiency of the analytical method and the limit of detection should also be reported;

- water quality of test media introduced to test vessels (*i.e.*, pH, temperature, salinity and dissolved oxygen concentration;

- a full record of all the biological effects which were observed or measured and the statistical techniques used to analyse the data;

- the Lowest Observed Effect Concentration (LOEC) and No Observed Effect Concentration (NOEC) for the biological end point(s) employed and how they were calculated/determined;

- EC_x and 95% confidence intervals and a graph of the fitted model used for its calculation; the slope of the concentration-response curve and its standard error, where appropriate, for the test chemical(s), and statistical methods used for its determination;

- other observed effects;

- an explanation of any and all deviations from the Test Guideline recommendations.

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ANNEX 1

DEFINITIONS

Nauplii = Larval copepods

Copepodites = Juvenile copepods

LOEC
The lowest observed effect concentration is the lowest concentration within the experimental range at which a statistically significant effect is observed when compared with the control, within the stated exposure period of the test.

NOEC
The no observed effect concentration is the tested concentration just below the LOEC.

EC_x
EC_x is the calculated effective concentration from which an effect of x% is expected. EC_x is a function of exposure time as well as exposure concentration.

Confidence intervals
A x% confidence interval is a high and low range of values within which the measured or calculated value of an endpoint is likely to be present with a probability of x%.

Offspring production or fecundity
Number of offspring produced per time unit per copepod mating pair

Reproductive output
Number of eggs (or offspring) produced per time unit.

Fertility or mating success
Number or percentage of mating pairs able to produce viable offspring, usually through two successive clutches or broods.

Instantaneous rate of population increase (r_i or λ)
Uses initial and final number of animals over x generations to measure the ability of a population to increase arithmetically, geometrically, or exponentially over time.

Intrinsic rate of population increase (r_m)
Uses life-table endpoints (*e.g.*, life-stage survivorship and fecundity) to measure the ability of a population to increase arithmetically, geometrically or exponentially in a limited or unlimited resource environment.

ANNEX 2

BIOLOGY AND CULTIVATION OF *AMPHIASCUS TENUIREMIS*

The organism

A. tenuiremis c.f. Mielke is an easily-cultured, diosaccid harpacticoid copepod that is amphi-Atlantic in distribution ranging from the North Sea/Baltic intertidal to the southern Gulf of Mexico (1). It is an epifaunal to deep burrowing (0 to 15 mm) species that ingests sediments for diatoms and bacteria, and produces epibenthic burrowing larvae with no swimming ability. Sexes are dimorphic with females reaching 0.4 mm in length and males 0.25 to 0.30 mm. Males are also more narrowly streamlined in shape than females and have a swollen geniculate segment on their first antenna to clasp the female in mating. Figure 1 below is a composite micrograph of each of the major life stages of this copepod.

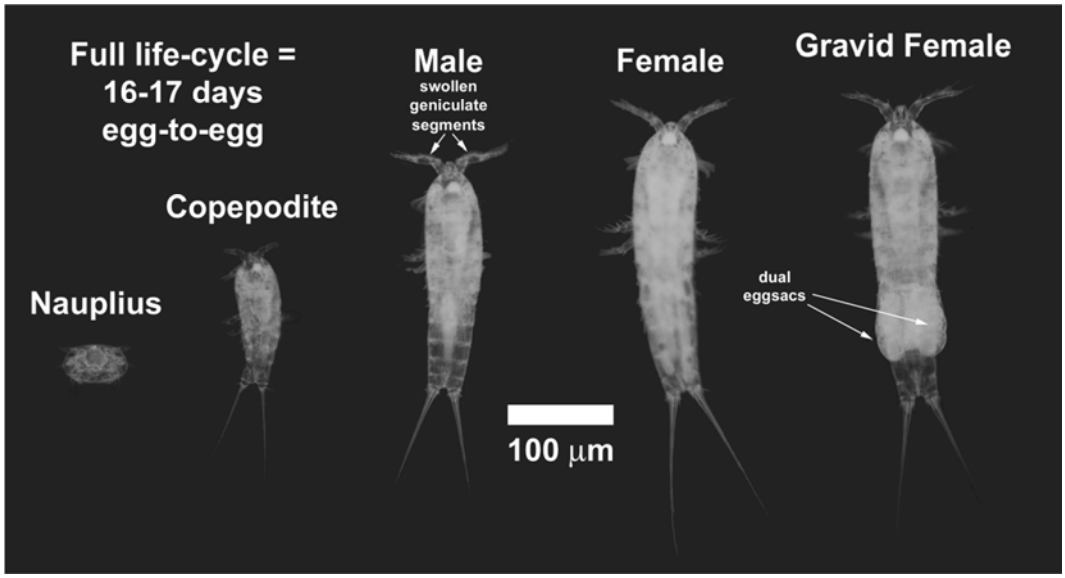

Figure 1. Photomicrographs of *Amphiascus tenuiremis* major life stages.

A. tenuiremis has a usual generation time (egg to egg) of 16-17 days at 25°C in microplate culture, or 14-15 days in 25°C sediment culture. At 21°C in sediments, the generation time is 20-21 days egg to egg. Development rates depend on food quality as well as temperature. Females are sexually mature after the fifth copepodite stage (that is, in the twelfth life stage). They then mate and produce their first clutch in two to three days. The fifth and last pair of legs are added at the 5th copepodite molt to adulthood. The presence of the fifth leg(s) is another morphometric indicator of complete metamorphosis to adult. Sperm transfer is via a membranous spermatophore sac attached externally by the male to the female genital pore. Isolated virgin females can be fertilized successfully up to 14 days post adulthood, and females will not extrude viable eggs unless paired with a reproductively-functional mature

male. Females will produce eight to nine clutches over their median lifetime of 49 ± 2 days. Nauplii hatch within two to three days of egg extrusion and normally reach the copepodite stage in six to eight days at 25 ºC. Figure 2 below is a composite comparison micrograph of male:female sexual dimorphism in the adult (final) molt stage. Figure 2.A. shows the swollen male geniculate antennal segment (circled in red); 2.B.1. shows an adult male viewed from below in a hydrogel-coated microplate (geniculate segment circled in red); 2.B.2.(inset) shows a third-stage copepodite juvenile viewed from below in a hydrogel-coated microplate; 2.C. shows a full view of an adult male and its more narrow, streamlined body morphology (geniculate antennal segments circled in red); 2.D. shows an adult female with normal non-swollen female antennae and the broader, more robust female body shape.

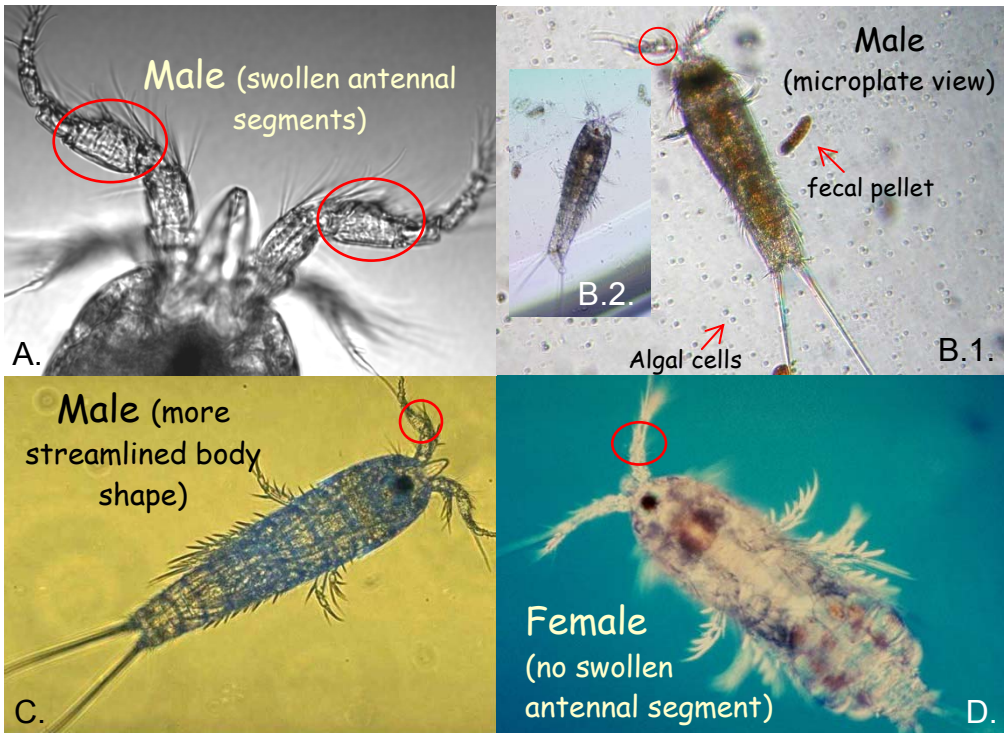

Figure 2: comparison micrograph of male:female sexual dimorphism in the adult (final) molt stage.

Culturing

General

Successful harpacticoid copepod culture is most dependent on meeting three important conditions: (a) a toxicant-free muddy sediment substrate consisting of clay, silt, and fine sand particles less than 0.100 mm in size (median grain diameter approximately 0.004 to 0.02 mm); (b) a highly-filtered carbon-polished seawater source saturated with oxygen and relatively free of ammonia (less than 30 µg-NH_4/L; pH = 8.0 to 8.3); (c) a varied diet of at least three easily cultured phytoplankton species (for example, a chlorophyte, *Dunaliella tertiolecta*, a chrysophyte, *Isochrysis galbana*, a cryptophyte, *Rhodomonas salina*, and/or a diatom, *Phaeodactylum tricornutum*). All marine algae are cultured in nutrient-enriched seawater and can be purchased from the University of Texas Culture Collection (http://web.biosci.utexas.edu/utex) or similar algal culture collections around the world. Copepods can be purchased from the laboratory of Dr. G. T. Chandler at the University of South Carolina (tchandler@sc.edu), or collected from the field and placed into

sediment culture. If collected from the field, it is important that an experienced copepodologist verify the species as *Amphiascus tenuiremis*.

All copepods in a test should be from the same brood stock. Before a test is initiated, at least two previous generations should have been raised in sediments from birth using the same food, seawater, and approximate temperature as that to be used in the life-cycle test. This will not only acclimate the copepods, but will also demonstrate the acceptability of the food, seawater, and culturing procedures before the test is begun. Copepods may be grown continuously as static renewal sediment cultures provided the culture water is changed twice weekly (80% renewal) and gently but continuously aerated.

Preparation of Clean Stock Sediments

Stock sediments for harpacticoid culture are prepared (after Chandler 1986 [2]) as follows: Collect toxicant-free muddy sediments from a pristine estuarine habitat and pass them through 0.5-mm and 0.1-mm sieves into deionized water (~1 kg sediment per 5L water). Let sediments settle for 1 hour, remove the supernatant, and then re-suspend in fresh de-ionised water. Repeat this wash at least three times after settling, and then centrifuge (3 min at 4000 RCF in 500-mL centrifuge bottles) to remove 50 to 60 % of the water. Homogenize "dry" sediment pellets in a solvent-cleaned electric blender for 3-5 minutes, and then aliquot 600-mL of the homogenate into 1-L Griffin beakers, cover loosely with aluminium foil, and autoclave for 15 min at 125°C and 30 psi. Store autoclaved sediment stocks sealed at 4ºC until use. Prepare culture sediment substrate by taking 100 g of the autoclaved stock, blending it for 3 min at high speed with 300-mL 5-µm-filtered seawater, and then pouring the blended stock through a 0.063-mm sieve into 3.5 L of filtered seawater in a 4-L Griffin beaker. Allow sediments to settle for 30 min, and then aspirate away the supernatant water. Re-suspend the remaining settled sediments again in 3.5 L filtered seawater and allow to re-sediment for 24 h at 4ºC. After 24 h, aspirate away all supernatant water, leaving approximately 300 mL wet sediment slurry. The sediment to water ratio for copepod cultures should be approximately 1:100 for static renewal cultures, or up to 1:20 for flow-through cultures. Wet sediment media has a consistent water-to-solids percentage of 87:13 to 88:12 using this technique. Sediment organic carbon content (based on C:H:N analysis) should range from 3 to 4 % in sediments after passing through these procedures. Final organic carbon content in wet sediment culture media will vary depending on the organic carbon content of the raw source sediments. A muddy source sediment of 4-5% organic carbon will usually yield a 3-4% organic carbon final sediment medium after passing through these preparation steps.

Inoculation, culture and feeding of copepods

Wet sediments (see above) should be poured slowly into ~ 1-L glass or bisphenol-A free plastic dishes pre-filled with 500 mL seawater and allowed to settle for 1 h. Sediments are then inoculated with a copepod starter population. Typically greater than 100 gravid copepods are required to start a viable culture. Flush each copepod culture dish slowly (approximately 3 to 5 mL/min) but continuously with polished natural or synthetic seawater from a recirculating (3) or ambient 0.005-mm filtered seawater system, or replace 80% of the culture seawater twice weekly with fresh aerated seawater. If static-renewal culture is employed, install a continuous gentle bubble stream at the edge of the culture vessel to maintain oxygen saturation above 70%. Feed cultures a 1:1:1 mixed algal cell suspension at a level not more than they will consume (for example, approximately $2.5 \cdot 10^8$ centrifuged live cells for a mature copepod culture) twice per week. Culture algal cells to exponential growth using standard f/2 enriched seawater media (4) and centrifuge for 12 min at 4700 RCF. Remove the supernatant, re-suspend algae in ~1-mL clean sediment slurry (see above sediment preparation) and 50-mL filtered seawater, microwave for 15 s (at 800 W) to diminish swimming abilities, cool to 4ºC, and pipette 10-50 mL into each culture depending on consumption rates. One hundred gravid copepods will often yield a peak-density culture (> 10,000 copepods) in 2 to 3 months if food, sediment, and water quality are high.

Harvesting stock cultures

A. tenuiremis should be handled as little as possible. When handling is necessary, it should be done gently, carefully, and quickly so that the copepods are not unnecessarily stressed, and gravid females will not drop their externally carried egg sacs. When collecting gravid *A. tenuiremis* females, monoculture stock sediments should be sieved gently over a 125 µm stainless steel sieve, with the retained sediment fraction and copepods washed into a sterile, plastic petri dish (10 cm diameter) using seawater at the same salinity as used for the life-cycle test. High-quality borosilicate glass Pasteur pipettes 146 mm in length should be used for collection and transfer of gravid female *A. tenuiremis* to 50-mL glass crystallizing dishes using a research-grade dissection stereomicroscope and cool fiber-optic illumination. Copepods should be introduced into seawater solutions beneath the air to water interface. Copepods that touch dry surfaces or are dropped or visibly injured should be discarded.

Harvesting Young for Testing

Less than 24-h old young (nauplii) are obtained by first collecting adult and gravid females from a mature stock culture, as stated above, and then apportioning ~ 100 gravid females equally among each well of a six-well, polystyrene NetWell plate filled individually with 15 mL of 0.2-µm filtered seawater. Within each well, a 73-75-µm nylon mesh basket (Corning Costar NetWells) is suspended to retain females approximately 3 mm above the well bottom. The basket allows newly hatched non-swimming nauplii to fall through the mesh and away from adult females (see Fig. 2A below). At 24 hours, the mesh basket and post-hatch females are removed, and nauplii are rinsed from all wells into 60- or 100-mm Petri dishes. These dishes are placed on a research quality dissection stereomicroscope with dark-field illumination, and nauplii then pipetted and transferred individually to 96-well microplate microwells using silanized fine-tipped (0.1 mm diameter) borosilicate glass Pasteur pipets. Pipettes may be made "fine-tipped" by pulling the narrow end to a smaller diameter over a simple Bunsen burner. After all wells are successfully loaded with a single nauplius, the overlying transfer seawater is removed under a stereomicroscope using an analytical grade 250-µL glass Hamilton syringe (23 gauge; beveled needle) so that only approximately 5-10 µL of seawater remains. This standardizes the starting volume in each well and allows for minimal dilution of the test solutions which are immediately added back into each well. Once the overlying water is removed, 250 µL of control(s) or test solution is added, then partially aspirated under microscopic viewing so as not to suction away the nauplius, and then added again to the appropriate wells using an appropriate volumetric micropipettor or microsyringe (250-µL). After test solutions have been added, each well should receive 2 µL of a fresh, 10^7 cells·mL^{-1} 1:1:1 mixed algal cell suspension as described previously. The algal species *Rhodomonas*, *Isochrysis* and *Dunaliella* work well in this method guideline. Silicious diatoms can be useful for stock culturing, but they frequently foul microwells when used repeatedly over time.

Quality Assurance

To increase the chances of an acceptable test, the test should not begin with nauplii that were from injured or dying (that is, immobile) adult females. All nauplii should be handled and checked for viability using cool light microscopy. All 24-h old nauplii must be alive before beginning the test. *Amphiascus tenuiremis* nauplii are non-swimming so their survivability can be checked under a stereomicroscope by gently tapping on the petri dish holding the representative nauplii and checking for appendage movement or gut peristalsis. Representative copepods from the brood stock should be analyzed for the test material of interest before beginning a test if there is a possibility that the test material might be present in the culturing environment (*e.g.*, metals).

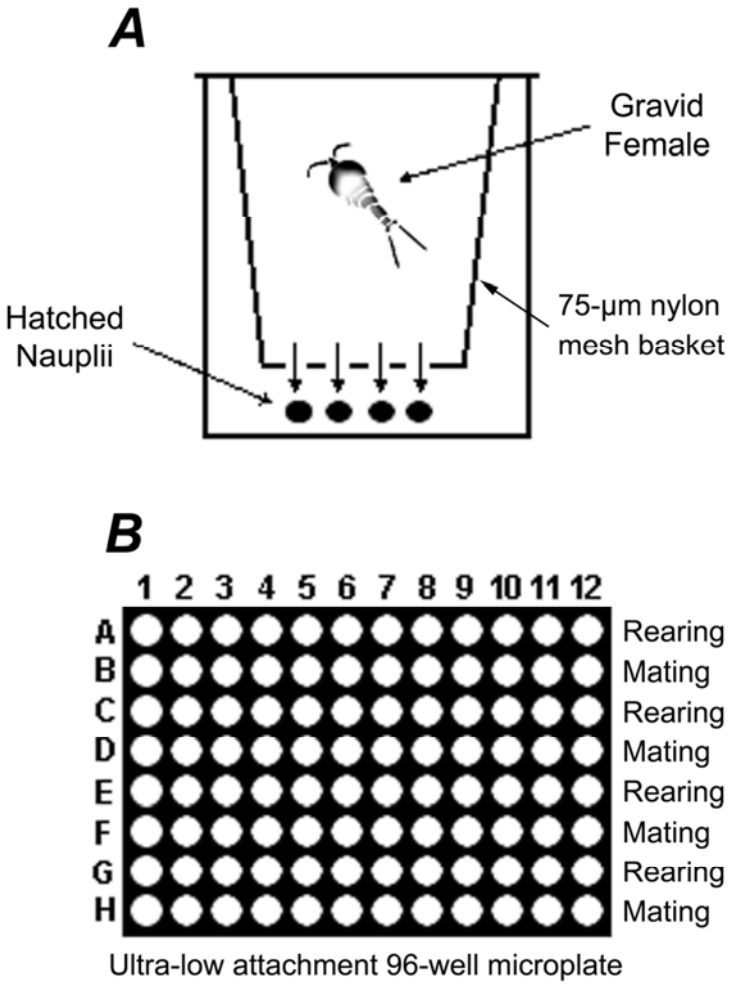

Figure 3. Schematic of (A) naupliar Netwell collection apparatus, and (B) microplate test layout and design.

Literature

(1) Lang, K. 1948. *Monographie der Harpacticiden*. Hakan Ohlsson, Lund, Sweden.

(2) Chandler, G.T. 1986. High density culture of meiobenthic harpacticoid copepods within a muddy sediment substrate. Canadian Journal of Fisheries and *Aquatic Sciences* 43, 53-59.

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ANNEX 3

EXAMPLE DATA SHEETS FOR RECORDING MAJOR MORTALITY, DEVELOPMENTAL AND REPRODUCTIVE ENDPOINTS
(assumes the use of 96-well microplates)

Daily log sheet template ***(Note: death events and day of metamorphosis to copepodite or adult male or adult female are recorded daily as observed in the microplate microwell row:column positions)***

Treatment: **Date:** **Time:** **Technician:**

1

Microplate I	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

2

Microplate II	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

3

Microplate III	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Mating log-sheet template ***(note: the day of death of any male or female in any mating pair is recorded)***

Test Material: Start date: Start time: Technician:

Treatment Concentration:								Replicate: One	
	♀	♂	**Cell #**	**Day Mated**	**Day of 1st Extrusion**	**# Naups on 1st Extrusion**	**Day of 2nd Extrusion**	**# Naups on 2nd Extrusion**	**Days b/t Extrusions**
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									

Treatment Concentration:								Replicate: Two	
	♀	♂	**Cell #**	**Day Mated**	**Day of 1rst Extrusion**	**# Naups on 1st Extrusion**	**2nd Day of Extrusion**	**# Naups on 2nd Extrusion**	**Days b/t Extrusions**
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									

Treatment Concentration:								Replicate: Three	
	♀	♂	**Cell #**	**Day Mated**	**Day of 1rst Extrusion**	**# Naups on 1st Extrusion**	**2nd Day of Extrusion**	**# Naups on 2nd Extrusion**	**Days b/t Extrusions**
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									

ANNEX 4

CALCULATIONS

Population Growth Rate

There are numerous mathematical techniques for calculating population growth rate from individual life-table endpoints. The life-stage transition rates produced by collection of individual survival, development and reproduction data from microplates can be entered directly into commercially available life-table modeling software to yield robust predictions of instantaneous population growth rates (λ) under exposure to a test chemical concentration. The Leslie matrix is available in reference number 1 and the following references 2-5 describe how to use the Leslie matrix. Figures (1-2) describe how to use Leslie (Lefkovich) matrices for this purpose. The Leslie matrix is powerful and relevant for predicting population response and future age:stage structure using life stage data from individually isolated/reared copepods over one or multiple life cycles under exposure to chemical stress:

(1) Leslie, P.H. 1945. On the use of matrices in certain population mathematics. *Biometrika 33*, 183-212.

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(6) Chandler, G.T., Cary, T.L., Bejarano, A.C., Pender, J., Ferry, J.L. 2004. Population consequences of fipronil and degradates to copepods at field concentrations: an integration of lifecycle testing with Leslie-matrix population modeling. Environmental Science and Technology 38:6407-6414.

(7) Akçakaya, H.R., Stark, J.D., Bridges, T.S. 2008. Demographic Toxicity: Methods in ecological risk assessment. Oxford Univ. Press, 288 pp.

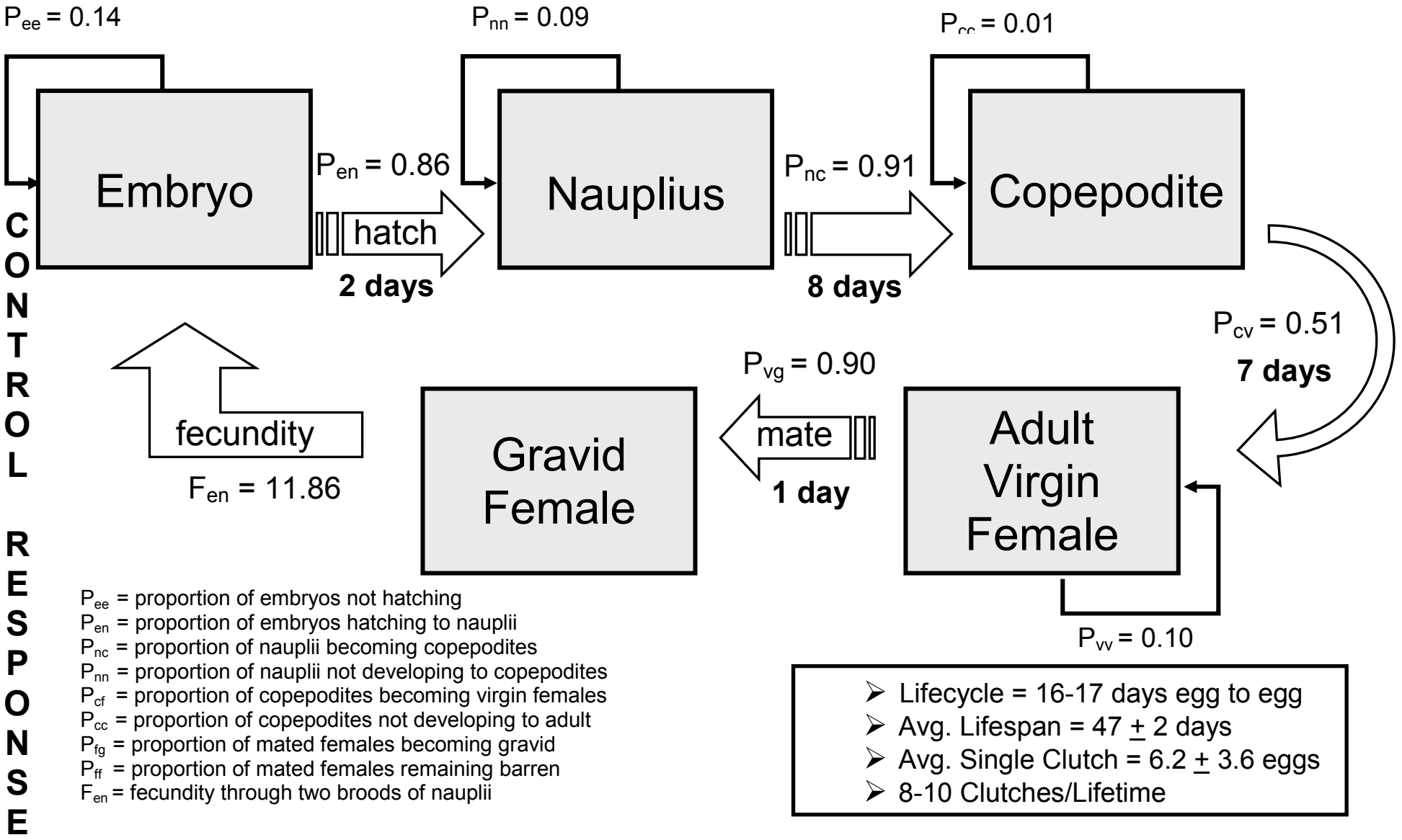
P_{ee} = 0.14
P_{nn} = 0.09
P_{cc} = 0.01
Embryo
P_{en} = 0.86
hatch
2 days
Nauplius
P_{nc} = 0.91
8 days
Copepodite
P_{cv} = 0.51
7 days
P_{vg} = 0.90
mate
1 day
Adult Virgin Female
Gravid Female
fecundity
F_{en} = 11.86
P_{vv} = 0.10
CONTROL RESPONSE
P_{ee} = proportion of embryos not hatching
P_{en} = proportion of embryos hatching to nauplii
P_{nc} = proportion of nauplii becoming copepodites
P_{nn} = proportion of nauplii not developing to copepodites
P_{cf} = proportion of copepodites becoming virgin females
P_{cc} = proportion of copepodites not developing to adult
P_{fg} = proportion of mated females becoming gravid
P_{ff} = proportion of mated females remaining barren
F_{en} = fecundity through two broods of nauplii
Lifecycle = 16-17 days egg to egg
Avg. Lifespan = 47 ± 2 days
Avg. Single Clutch = 6.2 ± 3.6 eggs
8-10 Clutches/Lifetime

Figure 1. Example lifecycle graph of *Amphiascus tenuiremis* life-stage development times showing the observed proportions of each life stage progressing (or failing to progress) to the next life stage through 21-24 days (i.e., the time normally required for controls to produce two hatched broods of nauplii) at 25ºC in control microplates. All proportions are exclusive of mortality (i.e., dead individuals were not scored as developmental failures) and can be inserted into the Leslie matrix to estimate population growth, age and stage structure over n-generations.

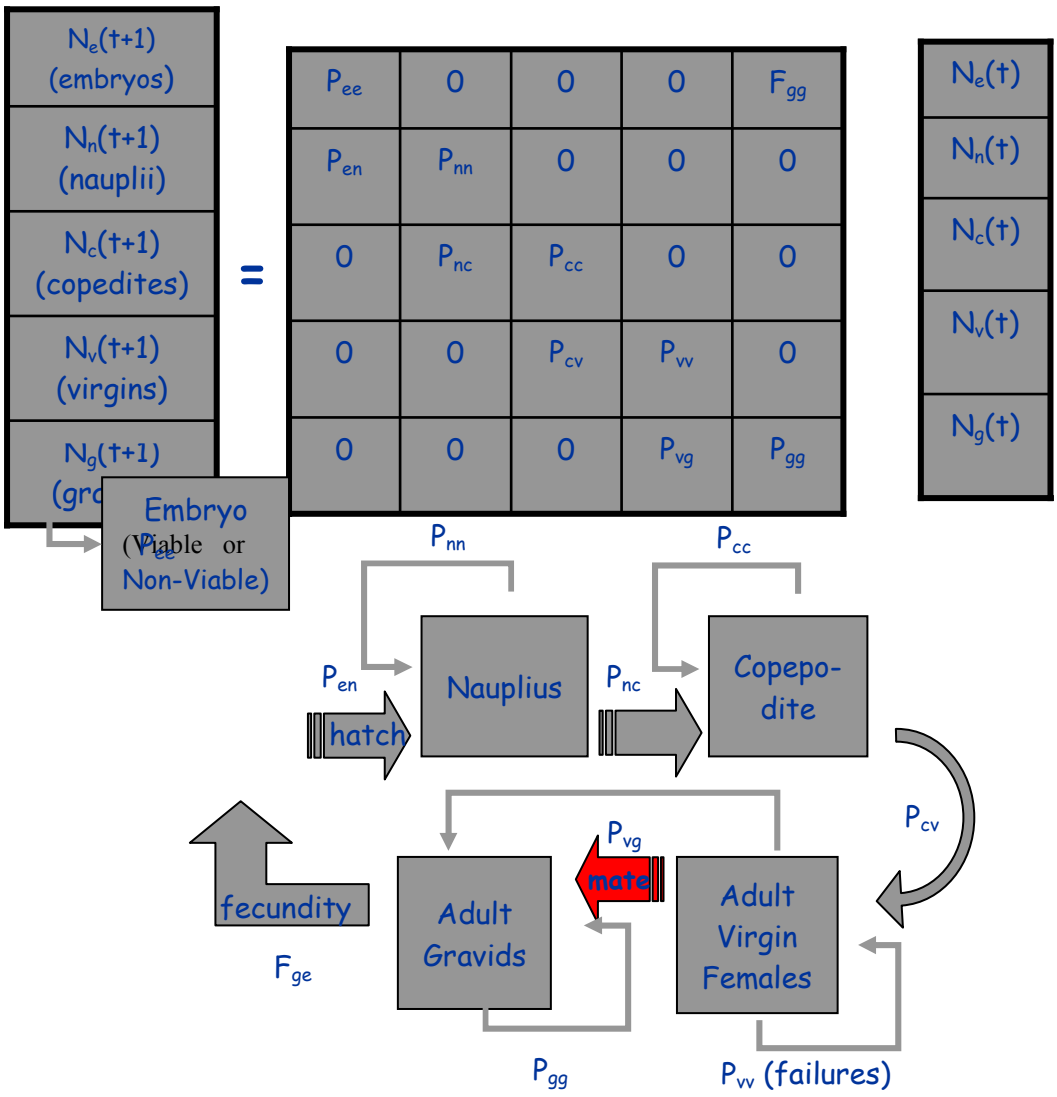

Figure 2. Example matriarchal stage-structured Leslie (Lefkovich) matrix population growth model for copepod development and reproduction in water-borne chemicals. Model yields population growth rate and N generation projections per control and toxicant concentration which are then compared.

P's = proportion from one life-stage surviving and developing to the next life stage, with/without exposure, through the empirical microplate life-cycle test.