Adopted
29 July 2016

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Potamopyrgus antipodarum Reproduction Test

INTRODUCTION

1. The need for testing the reproductive toxicity of some chemicals in aquatic molluscs has been highlighted in the OECD Detailed Review Paper on mollusc toxicity testing (1). In particular, the absence of toxicity test guidance for such an ecologically and economically important group was highlighted. This guideline is therefore designed to assess potential effects of prolonged exposure to chemicals on reproduction and survival of parthenogenetic lineages of the freshwater mudsnail *Potamopyrgus antipodarum*, known as the freshwater or New Zealand mudsnail. Typical habitats of *P. antipodarum* are running freshwaters from small creeks to streams, lakes and estuaries. Mudsnails are able to survive and reproduce in brackish water with salinity up to 15‰. In their ancestral distribution area, the populations have an almost balanced ratio of males to females with a sympatric coexistence of biparental and parthenogenetic populations. In other parts of the world, populations consist almost entirely of female snails reproducing parthenogenetically. In Europe, male snails are found only very rarely and did not occur in a long-term laboratory culture until now. The embryos are released through the female aperture when the egg shell tears open. This kind of reproduction is called ovovivipary (see also Annex 2).

2. The measured parameters in this test are mortality and the assessment of the reproduction as the total number of embryos in the brood pouch per female after 28 days exposure without distinction of developmental stages.

3. Before use of the test guideline on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.

PRINCIPLE OF THE TEST

4. The primary objective of the test is to assess the effect of chemicals on reproductive output of *P. antipodarum* by evaluating embryo numbers in the brood pouch (without distinction of developmental stages) at the end of 28 days exposure. To this end, adult female *P. antipodarum* are exposed to a concentration range of the test chemical. The test chemical is dispersed into the reconstituted dilution

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water, added to test beakers, and adult snails are subsequently introduced into the test beakers. When testing "difficult chemicals" (i.e. volatile, unstable, readily biodegradable and adsorbing chemicals) the test can be conducted under flow-through conditions as an alternative to the semi-static design with fixed renewal periods of the medium (see paragraph 29). *P. antipodarum* survival over the 28 days exposure period and reproduction at the end of the test after 28 days are examined.

5. The toxic effect of the test chemical on embryo numbers is expressed as EC_X by fitting an appropriate regression model in order to estimate the concentration that would cause x % reduction in embryo numbers or alternatively as the No Observed Effect Concentration and Lowest Observed Effect Concentration (NOEC/LOEC) value (2). The test concentrations should bracket the lowest of the used effect concentrations (e.g. EC_{10}) which means that this value is calculated by interpolation and not extrapolation.

INFORMATION ON THE TEST CHEMICAL

6. Information on the test chemical which may be useful in establishing the test conditions includes the structural formula, purity of the test chemical, stability in light, stability under the conditions of the test, pK_a, P_{ow} and results of a test for ready biodegradability (3, 4).

7. The water solubility and the vapour pressure of the test chemical should be known and a reliable analytical method for the quantification of the test chemical in the test solutions with reported recovery efficiency and limit of quantification (LOQ) should be available.

REFERENCE SUBSTANCES

8. Reference substances should be tested periodically, as needed, to ensure consistency and sensitivity of test organism and experimental test conditions of the method. Toxicants successfully used in international validation studies are cadmium chloride, tributyltin chloride and prochloraz with EC_{50} ranges of 5 to 20 µg Cd/L, 35 to 190 ng TBT-Sn/L and 100 to 800 µg prochloraz/L, for reproductive output as number of embryos (see chapter "Reproduction" below), respectively (5).

VALIDITY OF THE TEST

9. For a test to be valid the following conditions should be fulfilled:

- the mean mortality (accounting for all control replicates) should not exceed 20% at the end of the test;
- the mean number of embryos in the controls should be at least 5 embryos per female at the end of the test;
- the dissolved oxygen content should be at least 60% of the air saturation value in both control and exposure groups throughout the test; and
- water mean temperature should be 16 ± 1.5°C throughout the test in both control and exposure groups.

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DESCRIPTION OF THE METHOD

Apparatus

10. Test vessels and other apparatus which will come into contact with the test solutions should be made entirely of glass or other chemically inert material. Any plastic material used during the test should ensure no leaching of plasticizers or other compounds. Additionally, the following equipment will be required:

- 500 mL glass beakers which can be covered with perforated lids, gauze, or any device that prevent snails from escaping but allows air inflow;
- air pumps, air tubes, Pasteur pipettes;
- adjustable valves for airflow control;
- volumetric flasks and other laboratory glassware for the preparation of solutions;
- glass pipettes;
- oxygen meter;
- pH meter;
- conductivity meter;
- test kits or other adequate equipment for ammonium, nitrite, nitrate measurements in water, total organic carbon and water hardness;
- stereomicroscope equipped with a light source;
- climate chambers or temperature regulated room or other adequate apparatus for temperature and lighting control;
- dissecting dish and dissecting instruments

Test organism

11. The species to be used in the test is *P. antipodarum* (Gray, 1853). Snails used for the validation exercises belong to haplotype *t* and morphotype "Warwick A" and should be used for testing. Städler et al. (6) and Warwick (7) describe distinct genetic and anatomical markers of haplotype *t* / morphotype "Warwick A".

12. Test animals should be laboratory-reared and taken from a healthy stock of female snails (i.e. showing no signs of stress such as high mortality, poor fecundity, infestation with parasites, etc.). The stock snails should be maintained in culture at the same conditions (light, temperature, medium and feeding) of the test (culturing methods for *P. antipodarum* are described in Annex 2). If snails are received from an external supplier, a pre-test acclimation period of at least 2 weeks should be applied to avoid stress for the animals. Field collected organisms should not be used because of the unclear (contamination) history.

Test medium

13. Reconstituted (synthetic) water should be used as sole test medium. The reconstituted water should be prepared with 3 g Tropic Marin® sea salt (or equivalent) and 1.8 g sodium hydrogen carbonate

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($NaHCO_3$) dissolved per 10 litre deionised water. It is further recommended that total organic carbon level in the medium (i.e. before addition of the food) be below 2 mg/l. The reconstituted water should be prepared in a container of inert material (e.g. glass or stainless steel) and of sufficient volume, e.g. a 50-litre aquarium, where the water is stored for further use, preferably at ambient temperature in the dark. The reconstituted water should be aerated for at least 24 hours before use. It should be used within a maximum storage period of 2 weeks.

14. The following parameters should be achieved and kept:

pH:	8.0 ± 0.5;
Oxygen saturation:	> 60% ASV (air saturation value);
Conductivity:	770 ± 100 µS/cm;
Total organic carbon:	<2 mg/L

15. Test vessels should contain 400 mL of reconstituted water and should be covered (with a perforated lid or gauze to allow air inflow). Glass beakers should be replaced weekly. Larger volumes may sometimes be necessary to meet requirements of the analytical procedure used for determination of the test chemical concentration, although pooling of replicates for chemical analysis is also allowable.

Test solution

16. Test solutions of the chosen concentrations are usually prepared by dilution of a stock solution. Stock solutions should be prepared by dissolving the test chemical in reconstituted water using mechanical means such as agitating, stirring or ultrasonication, or other appropriate methods. The use of solvents or dispersants should be avoided. For difficult test chemicals, the OECD Guidance Document on aquatic toxicity testing of difficult substances should be consulted (8). If a solvent is required in order to produce a suitably concentrated and homogeneous stock solution, its final concentration should be kept to a minimum and should not exceed 20 µL/L in the test vessels (= 0.002%). A solvent control should be added in addition to negative (dilution water only) control. The concentration of solvent should be equal in all test concentrations and in the solvent control.

17. The selection of an appropriate solvent depends on the physico-chemical properties of the test chemical and on the sensitivity of test, which should preferably be determined in a previous study. Only solvents or dispersants that have been shown to have no significant effects on reproduction in *P. antipodarum* should be used in the test. Regarding choice of solvent, the OECD Guidance Document on aquatic toxicity testing of difficult substances (8) should be consulted. During the validation exercises dimethyl sulfoxide (DMSO), triethylene glycol (TEG), acetone and glacial acetic acid were used as solvents. DMSO, TEG and acetone are characterised by a low toxicity (NOEC ≥ 12.5 mL/L which is at least by factor 625 higher than the maximum concentration of 20 µL/L in the test) to *P. antipodarum* and do not cause biofilm development in the test vessels (5). In contrast, the use of ethanol as a solvent may result in considerable development of fungi and bacteria even at concentrations as low as 0.003%.

PROCEDURE

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Conditions of exposure

Duration

18. The test duration is 28 days.

Loading

19. Snails used for the tests should be examined for their ability to reproduce. Therefore, the shell length, and the embryo numbers of 20 snails from the culture batch selected for the test should be determined prior to the start of the test (cf. Annex 3, Fig. 1A). The shell length should be between 3.5 and 4.5 mm. The mean number of embryos per snail to be used for the test should be between 5 and 20, which reflect the lower and upper limit of mean embryo numbers in long-range laboratory cultures of *P. antipodarum* (cf. Annex 2). Additionally, the coefficient of variation for the mean embryo number should not exceed 40%.

20. Six adult snails should be allocated randomly to each test vessel containing the exposure medium using tweezers. Treatments should be allocated to the test vessels and all subsequent handling of the test vessels should be done in a random fashion, e.g. by using a randomised block design, in order to minimise any bias due to the position on the testing area. Failure to do this may result in bias that could be construed as being a concentration 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 effects at the higher concentrations.

Feeding

21. Feeding should preferably be done daily, but at least 3 times per week with finely ground TetraPhyll® flakes or an equivalent food with the same nutrient composition (60 to 80 µg per animal per day). The use of other food sources or quantities should be reported and justified. For application of the food, a homogenised suspension with deionised or distilled water should be prepared. The appropriate amount of the suspension should be pipetted in each test vessel after stirring the suspension continuously to avoid sedimentation of food particles. The volume of suspension added to each test vessel should be minimised to avoid a dilution of the test concentrations. The suspension should be prepared immediately before use.

Light regime

22. The test photoperiod is 16L:8D hours throughout the test. Light intensity should be in the range 500 ± 100 lx at the surface of test medium. When testing photo-labile chemicals lower light intensities should be used. The light source should be positioned above the test vessels. Test vessels should be positioned on a dark surface. The snails show a negative photo-tactic behaviour, so bright, light-reflecting surfaces can trigger an avoidance-behaviour and should be avoided. Snails may creep out of the water and eventually escape from the test vessel. Deviations from the light regime should be reported and justified.

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Temperature

23. The temperature of the test media should be 16 ± 1.5°C throughout the test.

Aeration

24. Water should be aerated through glass pipettes (Pasteur pipettes) connected to an air tubing system. Adjustable valves should be used to ensure continuous and constant air flow. When testing volatile chemicals, consideration may be given not to aerate the test vessels. Highly volatile test chemicals should be tested in completely filled and closed vessels (with zero headspace) large enough to prevent oxygen becoming limiting or too low.

25. Dissolved oxygen content should remain >60% ASV, however the test vessels should be aerated gently to avoid stripping of test chemicals.

Test design

26. At least five concentrations with six replicates of six snails (36 snails/concentration) should be tested in a geometric series with a factor between adjacent nominal concentrations preferably not exceeding 3.2. Justification should be provided if fewer than five concentrations and/or a spacing factor larger than 3.2 (e.g., in the case of a shallow slope of the concentration-response curve from range-finding studies) are used. Prior knowledge on the toxicity of the test chemical (e.g. from range finding studies or other sources like read across, etc.) should help in selecting appropriate test concentrations. Chemicals should not be tested above their solubility limit in test medium. The design with 5 (or more) concentrations and 6 replicates is recommended as the best design to assess both the EC_X and the NOEC/LOEC. The test concentrations should bracket the expected value. If an EC_x is desired, whatever x is, ideally that value should be bracketed by the test concentrations.

Limit Test

27. If no effects are observed at the highest concentration in the range-finding test (e.g. at 10 mg/L or a concentration equal to the limit of solubility), or when the test chemical is highly likely to be of low/no toxicity based on lack of toxicity to other organisms and/or low/no uptake, the reproduction test may be performed as a limit test, using a test concentration of e.g. 10 mg/L and the control. Ten replicates with six snails each should be used for both the treatment and the control groups. A limit test will provide the opportunity to demonstrate that there is no statistically significant effect at the limit concentration, but if effects are recorded a full test will be required. Justifications should be provided if fewer than ten replicates are used.

Controls

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28. Control vessels without added test chemical should be included in the test with an appropriate number of replicates, six replicates for the dilution-water control and, if needed, six replicates for the solvent control containing the solvent carrier only. In case of a limit test, the number of control replicates should be extended to ten (see paragraph 27).

Test medium renewal

29. The frequency of medium renewal will depend on the stability of the test chemical, but should be at least three times per week. Glass beakers should be replaced weekly. If, from preliminary stability tests or from the physico-chemical properties of the test chemical, test concentrations are evaluated not to be stable (i.e. outside the range 80 - 120% of nominal or falling below 80% of the measured initial concentration) over the maximum renewal period, i.e. three days), considerations should be given to more frequent medium renewals. Alternatively, the test should be conducted under flow-through conditions when testing "difficult chemicals" (i.e. volatile, unstable, readily biodegradable and adsorbing chemicals). The test protocol has been validated under semi-static conditions. The option of flow-through conditions can be used but may require some adjustments of the test design and justification should be provided.

30. The following procedure is used for test medium renewal. Exposure medium is completely removed (gently) from the test vessels. A sieve can be used to collect the snails which might detach from the glass walls. Snails should be returned from the sieve to the test vessel immediately. Test vessels are refilled using reconstituted dilution water at the test temperature. The test chemical is immediately added to the renewed water, e.g. by using stock solutions. Freshly prepared exposed medium is homogenised by manual agitation. Snails are then placed back in the test vessel. Food is provided in the given range (see paragraph 21) to animals once the control and test water renewals have been completed.

Observations

31. The test vessels should be observed at least three times per week to achieve visual assessment of any abnormal behaviour (e.g. avoidance of water, avoidance of food or lethargy, i.e. immobile snails fully retracted into the shell or detached from test vessels with foot protruded out of the shell), preferably during medium renewal and following feeding, indicating stress for the animals. Any of these signs of stress should be recorded. If there are snails found on the lid or outside of control and test medium, they should be transferred back to the medium, immediately.

Mortality

32. An animal is recorded as dead when it is immobile, i.e. when it does not show any reaction after gently touching the foot or the operculum (in case of snails retracted into the shell) with a pair of tweezers. Dead snails should be removed from the test vessels and recorded preferably daily, or at least as frequently as test medium is renewed.

Reproduction

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33. The number of embryos in the brood pouch is analysed for all surviving snails at the end of the test. To this end, snails should preferably be quick-frozen (in liquid nitrogen or in a -80°C freezer) and stored at -20°C until analysis. Only if histopathologic examination is envisaged, snails can alternatively be narcotised for 45 to 90 minutes in a solution of 2.5% magnesium chloride hexahydrate ($MgCl_2$*6 H_2O) in deionised or distilled water before dissection.

34. Shell length of snails (from the tip of the shell to the lower edge of the aperture, see Annex 3, Fig. 1A) should be measured under a stereomicroscope with an ocular micrometer as a supporting parameter because shell length is positively correlated with female fecundity in *Potamopyrgus* (1).

35. The shell of the snails should be cracked carefully with a pair of pincers. Subsequently, the snails are placed into a dissecting dish containing a small volume of test medium or tap water. The soft body can be prepared by removing the shell with dissecting needles or pointed tweezers. After removal of the shell, *P. antipodarum* embryos can easily be seen through the epithelia (see Annex 3, Figs. 1B, 2). The brood pouch of the snails should be opened carefully with a dissecting needle. Subsequently, the embryos (both shelled and unshelled) should be removed and counted for determination of the reproductive success of each female (see Annex 3, Fig. 1C).

36. If snails did not produce embryos, animals should be sexed. Males are characterised by a penis in the neck (bottom of the mantle cavity behind the snout and the two ocular tentacles). The influence of the presence of males on the results of the reproduction test is not known since male snails do not occur in laboratory cultures of haplotype *t* (1). In case males may occur during a test, they should be discarded from the fecundity analysis.

37. Data are recorded in an appropriate data sheet (example in Annex 5). The mean and variability parameters such as standard deviation or standard error of the mean, for the shell length and the number of embryos are calculated.

Frequency of analytical determinations and measurements

Concentration of the test chemical

38. During the test, the concentrations of the test chemical are determined at regular intervals. Analytical methods required should be established, including an appropriate limit of quantification (LOQ) and sufficient knowledge on the test chemical stability in the test system.

39. In semi-static tests where the concentration of the test chemical is expected to remain within ± 20% of the nominal (i.e. within the range 80 - 120% - see paragraph 29), it is recommended that, as a minimum, the highest and lowest test concentrations are analysed with freshly prepared and the old test solution at the time of renewal. Analyses should be made on samples from the same test solution - when freshly prepared and at renewal. These determinations should be repeated at weekly intervals thereafter at least.

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40. For tests where the concentration of the test chemical is not expected to remain within ± 20% of the nominal, it is necessary to analyse all test concentrations, when freshly prepared and at renewal. However, for those tests where the measured initial concentration of the test chemical is not within ± 20% of nominal, but where sufficient evidence can be provided to show that the initial concentrations are repeatable and stable (i.e. within the range 80 - 120% of initial concentrations), chemical determinations could be reduced in weeks 2, 3 and 4 of the test to the highest and lowest test concentrations. In all cases, determination of test chemical concentrations need only be performed on one replicate vessel at each test concentration and changed alternating between replicates.

41. If a flow-through test is used, a similar sampling regime to that described for semi-static tests is appropriate (but measurement of 'old' media is not applicable in this case). However, increasing the number of sampling occasions during the first week (e.g. at least two sets of measurements) may help to demonstrate that the test concentrations remain stable. This also may be completed prior to the definitive test initiation. In these types of test, the flow-rate of diluent and test chemical should be checked daily.

42. If there is evidence that the concentration of the test chemical has been satisfactorily maintained within ± 20% of the nominal or measured initial concentration throughout the test, then results can be based on nominal or measured initial values, respectively. If the deviation from the nominal or measured initial concentration is greater than ± 20%, results should be expressed in terms of the time-weighted mean (see guidance for calculation in Annex 4).

Physico-chemical parameters

43. Water quality parameters such as pH, oxygen saturation, conductivity, temperature, ammonium and total nitrite content should be measured in one replicate per exposure group and control at least once a week in fresh and old media. Additional measurements of nitrate and water hardness should be done if abnormally high mortality is observed in the controls or in one replicate of the lowest concentration.

DATA AND REPORTING

Treatment of results

44. The purpose of this test is to determine the effect of the test chemical on the reproductive output. The survival of the parent animals and the embryo numbers in the brood pouch (without distinction of developmental stages) at the end of 28 days exposure should be reported using a spreadsheet (see Annex 5 for an example). For statistical evaluation of the embryo numbers, the mean number across replicates for each concentration should be calculated. The standard effect in the reproduction test is a decrease of the mean embryo number with increasing concentration of the test chemical. The reproduction test was validated exclusively with chemicals which caused a reduction of embryo numbers. Increase in reproduction may occur in the test and was observed with chemicals such as ethanol (4) and those with known or suspected estrogenic effects in vertebrates (9 - 12). However, the reproduction test is not suitable for proving an endocrine mediated mode of action solely on the basis of a decreased or increased embryo number.

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45. Before employing the statistical analysis, e.g. ANOVA procedures, comparison of treatments to the control by Dunnett's test, Williams' test, or stepdown Jonckheere-Terpstra test (see paragraph 49), it is recommended to consider transformation of data if needed for meeting the requirements of the particular statistical test (2). With non-parametric alternatives one can consider e.g. Bonferroni-U-test according to Holm or Jonckheere-Terpstra trend test. 95% confidence intervals are calculated for individual treatment means. Alternatively, a generalised linear mixed model (GLMM) with Poisson or negative binomial error structure can be used to for the statistical analysis of embryo numbers as count data.

46. The number of surviving adult snails in the untreated controls is a validity criterion, and should be documented and reported. Also all other detrimental effects, e.g. abnormal behaviour as specified under paragraph 31 and abnormal appearance of embryos (e.g. fully developed embryos without shell, embryos with deformed shells) should be reported in the final report as well.

EC_X

47. EC_X-values, including their associated lower and upper 95% confidence limits, are calculated using appropriate statistical models designed for count data, such as a logistic model with Poisson error structure. It should be noted that Weibull and the Spearman-Karber method are designed for quantal responses, such as survival, where a fixed number of subjects are at risk. This differs from reproduction where the number of embryos varies considerably from parent to parent. It will sometimes be acceptable to analyse counts as though they are continuous responses following a normal error structure, but this should be carefully evaluated on a case-by-case basis. To compute the EC_{10}, EC_{50} or any other EC_X, the complete data set should be subjected to regression analysis.

NOEC/LOEC

48. If a statistical analysis is intended to determine the NOEC/LOEC appropriate statistical methods should be used according to (2). In general, adverse effects of the test chemical compared to the control are investigated using one-tailed hypothesis testing at $p < 0.05$. This valuable document does not cover GLMM models with non-normal error structure, as that is an area that has opened up since its publication.

49. Normal distribution and variance homogeneity can be tested using an appropriate statistical test, e.g. the Shapiro-Wilk test and Levene test, respectively ($p < 0.05$). If a GLMM model is used, the overdispersion (extra-Poisson variability) should be checked and an alternative error structure, such as the negative binomial, should be used instead of the Poisson. One-way ANOVA and subsequent multi-comparison tests can be performed. Multiple comparisons (e.g. Dunnett's test) or step-down trend tests (e.g. Williams' test, or stepdown Jonckheere-Terpstra test) can be used to calculate whether there are significant differences ($p < 0.05$) between the controls and the various test chemical concentrations (selection of the recommended test according to (2)). Otherwise, non-parametric methods (e.g. Bonferroni-U-test according to Holm or Jonckheere-Terpstra trend test) could be used to determine the NOEC and the LOEC.

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Test report

50. The test report includes the following information:

Test chemical:

- Mono-constituent substance:
  physical appearance, water solubility, and additional relevant physico-chemical properties; chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc. (including the organic carbon content, if appropriate).
- Multi-constituent substance, UVCBs (substances of Unknown or Variable composition, Complex reaction products or Biological materials) and mixtures:
  characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physico-chemical properties of the constituents.
- analytical method for quantification of the test chemical where appropriate.

Test species:

- scientific name, supplier or source and culture conditions.

Test conditions:

- test procedure used (e.g. semi-static or flow-through, volume, loading in number of snails per litre);
- photoperiod and light intensity;
- test design (e.g. test concentration used, number of replicates, number of snails per replicate, etc.);
- method of test chemical pre-treatment and spiking/application;
- the nominal test concentrations, details about the sampling for chemical analysis and the analytical methods by which concentrations of the test chemicals were obtained;
- test media characteristics (i.e. pH, conductivity, temperature and oxygen ASV, ammonium, nitrite concentrations, total organic carbon content and any other measurements made);
- detailed information on feeding (e.g. type of food, source, amount given, frequency of feeding).

Results:

- results from any preliminary studies on the stability of the test chemical;
- the nominal test concentrations and the results of all analyses to determine the concentration of the test chemical in the test vessels; the recovery efficiency of the analytical method, the means of the measured values and the limit of detection should also be reported;
- water quality within the test vessels (i.e. pH, temperature, oxygen ASV, ammonium and nitrite concentration - see example data sheet in Annex 5);
- the full record of embryo numbers by replicate at the end of the test (see example data sheet in Annex 5);
- the number of deaths among the snails (see example data sheet in Annex 5);
- where appropriate, the LOEC for reproduction (embryo numbers), including a description of the statistical procedures used and an indication of what size of effect could be expected to be detected (a power analysis can be performed before the start of the experiment to provide this) and the

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NOEC for reproduction; where appropriate, the LOEC or NOEC for mortality of the animals should also be reported;

- where appropriate, the EC_X for reproduction and confidence intervals (e.g. 95%) and a graph of the fitted model used for its calculation, the slope of the concentration-response curve with its confidence interval;
- other observed biological effects or measurements: report any other biological effects which were observed or measured (e.g. abnormal behaviour of snails as specified under paragraph 31, abnormal appearance of embryos as specified under paragraph 46, effects on growth of parent snails) including any appropriate justification;
- an explanation for any deviation from the Test Guideline.

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ANNEX 1

DEFINITIONS

For the purposes of this guideline, the following definitions are used:

EC_X is the concentration of the test chemical dissolved in water that results in a x per cent reduction in reproduction of *Potamopyrgus antipodarum* within a stated exposure period.

Lowest Observed Effect Concentration (LOEC) is the lowest tested concentration at which the test chemical is observed to have a statistically significant effect on reproduction and mortality (at $p < 0.05$) when compared with the control, within a stated exposure period. However, all test concentrations above the LOEC should have a harmful effect equal to or greater than those observed at the LOEC. When these two conditions cannot be satisfied, a full explanation should be given for how the LOEC (and hence the NOEC) has been selected.

No Observed Effect Concentration (NOEC) is the test concentration immediately below the LOEC, which when compared with the control, has no statistically significant effect ($p < 0.05$), within a stated exposure period.

Limit of detection is the lowest concentration that can be detected but not quantified.

Limit of determination is the lowest concentration that can be measured quantitatively.

Mortality. An animal is recorded as dead when it is immobile, i.e. when it is does not show any reaction after gently touching the foot or the operculum (in case of snails retracted into the shell) with a pair of tweezers.

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ANNEX 2

RECOMMENDATIONS FOR CULTURE OF *POTAMOPYRGUS ANTIPODARUM*

INTRODUCTION

This Standard Operating Procedure (SOP) describes the laboratory rearing of *Potamopyrgus antipodarum*. The objective is to achieve reproducible results with each experiment conducted under the conditions described. Therefore, it is necessary that snails are kept under standardised conditions. Mortality should be on a low level not exceeding 20% and the mean number of embryos per snail should be between 5 and 20. To this end, a good food supply is required and a distinct population density should not be exceeded (see §11 of Annex 2).

TEST ORGANISM

Taxonomy

P. antipodarum (Gray, 1853), the freshwater mudsnail, belongs to the phylum Mollusca, class Gastropoda, order Neotaenioglossa and family Hydrobiidae.

Ecology

P. antipodarum originates from New Zealand, but has been introduced to other parts of the world. Typical habitats are running waters from small creeks to streams, lakes and estuaries, where its reproduction is often very intensive (1 - 4). The shell length of adult snails averages about 3.5 – 4.5 mm. *P. antipodarum* predominantly lives in freshwater, but it is also able to survive and reproduce in brackish water with a salinity up to 15‰ (5). Mudsnails prefer living in or on soft sediments of standing or slowly flowing water bodies as well as in estuarine areas on the coasts. The species feeds on detritus, algae and bacteria, which are rasped from the surface of plants, stones or the sediment.

Biology

In their ancestral distribution area, the populations have an almost balanced ratio of males to females with a sympatric coexistence of biparental and parthenogenetic populations. In other parts of the world populations consist almost entirely of female snails reproducing parthenogenetically. In this way, a single snail is capable of establishing an entire population. In Europe male snails are found rarely (6, 7) and never occurred in long-term laboratory culture. Snails used for the validation exercises belong to haplotype *t* and morphotype "Warwick A" according to Städler et al. (8).

Reproduction occurs throughout the year. *P. antipodarum* performs a very distinct kind of brood care. The eggs develop in the anterior part of the pallial oviduct section, which is transformed into a brood pouch. Older embryos are situated in the anterior and younger embryos in the posterior part of the brood

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pouch. The embryos are released through the female aperture when the egg shell tears open. This kind of reproduction is called ovovivipary (2).

EQUIPMENT, TEMPERATURE AND LIGHT REGIME

Temperature and light regime

The culturing of *P. antipodarum* should be carried out at a water temperature of 16 ± 1.5°C and a light-dark period of 16:8 hours. The light intensity should be in the range 500+/- 100 lx.

Aquaria and accessories

The following equipment is needed:

- Culturing aquaria (e.g. 15 litre; made of glass)
- Storage tank of appropriate volume for reconstituted water (e.g. 50-litre glass aquaria)
- Air pumps
- Flexible air tubes (Teflon-coated)
- Glass pipettes
- Measuring electrodes for conductivity, oxygen and pH
- Test kits for ammonium, nitrite and nitrate measurements in water
- Stereomicroscope
- Cold light source
- Dissecting dish & dissecting instruments

Chemicals, food and products for water conditioning

The following compounds and products are needed:

- Sodium hydrogen carbonate ($NaHCO_3$)
- Calcium source (e.g. cuttlebone or calcium carbonate)
- Magnesium chloride hexahydrate ($MgCl_2$ * 6 H_2O) if narcotisation is required for subsequent histopathologic examination (see paragraph 16),
- Tropic Marin® sea salt (Dr. Biener GmbH, Wartenberg, Germany) or an equivalent sea salt
- TetraPhyll® (Tetra GmbH, Melle, Germany) or an equivalent food with the same nutrient composition

OPERATING PROCEDURE

Culture medium: Reconstituted water

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For the culturing of snails reconstituted water is used. To produce reconstituted water, 3 g Tropic Marin® sea salt and 1.8 g sodium hydrogen carbonate ($NaHCO_3$) are dissolved per 10 litre deionised water. The reconstituted water is prepared e.g. in a 50-litre aquarium, where the water is stored for further use for up to 2 weeks. The reconstituted water should be aerated for at least 24 hours before use.

The following water parameters should be achieved and kept:

pH:	8.0 ± 0.5;
Oxygen saturation:	> 60% ASV (air saturation value);
Conductivity:	770 ± 100 µS/cm

Before the water is used in the culture aquaria, the compliance of these parameters should be checked.

Population density

The population density should not be higher than 100 snails per 1 litre.

Food and feeding

The snails are fed with finely ground TetraPhyll® flakes or an equivalent food with the same nutrient composition *ad libitum*, preferably daily but at least 3 times a week. The flakes are ground either with a porcelain mortar with pistil or with a coffee mill with a high-grade steel masticator.

Cleaning and care

Once a week temperature, pH-value, oxygen saturation, conductivity, ammonium and nitrite concentration of all aquaria in the breeding program should be measured. Additional measurements of nitrate should be done if necessary.

Once per week a partial renewal of the culture water is required. Weekly replacement of at least 50% of the water has been found appropriate. Water renewal is accompanied by removal of discarded food remains and detritus from the culture vessel. Care should be taken to ensure that juvenile snails are not removed from the aquaria.

Before renewing water for the culture aquaria, temperature, pH-value, oxygen saturation, and conductivity of the water in the storage aquarium should be measured. After a partial change of water a calcium source (e.g. a piece of cuttlebone) should be added to each aquarium. When measuring the parameters in the aquaria of the breeding program, all electrodes should be thoroughly cleaned before used in the next aquarium to prevent a potential transfer of diseases or pathogens.

Monthly registration of embryo numbers

Each month the reproduction of 20 adult snails (> 3.5 mm) should be registered, together with measurements of shell length. Procedure:

- Snails are shock-frozen (in liquid nitrogen or in a -80°C freezer) and stored at -20°C until analysis. Only if histopathologic examination is envisaged, snails can alternatively be narcotised for at least 45 minutes up to a maximum of 90 minutes in a solution of 2.5% magnesium chloride hexahydrate

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($MgCl_2$ * 6 H_2O) in deionised or distilled water.

- Shell length of the snails should be measured under a stereomicroscope with an ocular micrometer. Data are recorded in a spread sheet.
- The shell of the snails is broken open carefully with a pair of pincers. Subsequently, the snails are placed into a dissecting dish containing a small volume of culture water.
- The soft body is exposed by removing the shell with dissecting needles or pointed tweezers (see Annex 3, Fig. 1).
- The brood pouch of the snails is opened with a dissecting needle and all embryos are removed out of this pouch.
- Then all embryos should be counted. Data are recorded in a spread sheet. The mean and variability parameters such as the standard deviation or the standard error of the mean for the shell length and the number of embryos are calculated.

ALGAE GROWTH, DISEASES AND MORTALITY

If heavy algae growth occurs on the shell of the snails, algae should be removed manually if possible. If it is not possible to reduce the growth of the algae in this way, affected snails should be removed from the brood and the aquaria should be cleaned thoroughly.

If dead snails are found in an aquarium, they should be removed as soon as they are identified as such. If there is an aquarium with a high mortality (> 20%), the mudsnails in this aquarium should be observed for several days. If the mortality continues to be high, all snails should be removed from the breeding program and the aquarium with all equipment should be cleaned and disinfected thoroughly.

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ANNEX 3

PHOTOGRAPHS AND DIAGRAMS OF *POTAMOPYRGUS ANTIPODARUM*

Figure 1: *Potamopyrgus antipodarum* with shell (A) or with partly removed shell and uncovered brood pouch (white) (B) and the embryos extracted from its brood pouch (shelled ones on the right and unshelled ones on the left) (C).

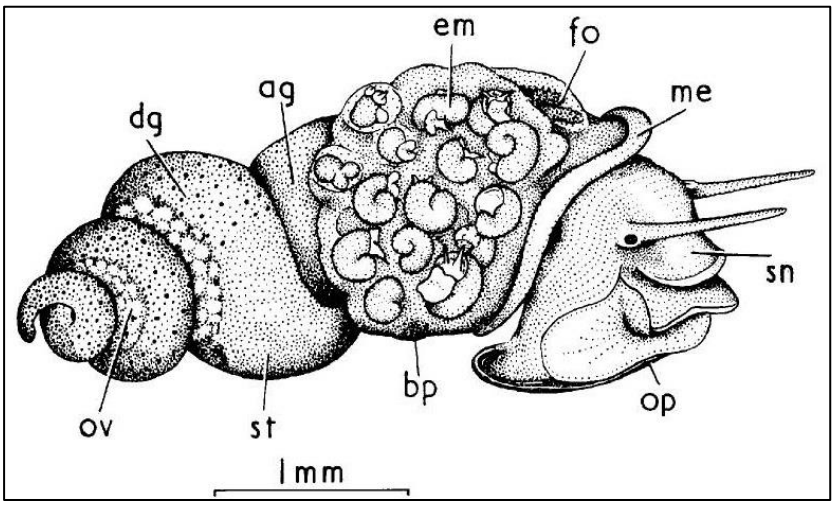

Figure 2:*Potamopyrgus antipodarum*, soft body after removal from the shell (modified after 2); ag = albumen gland, bp = brood pouch, dg = digestive gland, em = embryo, fo = female opening (vagina), me = mantle edge, op = operculum, ov = ovary, sn = snout, st = stomach.

A photograph of a male specimen of *Potamopyrgus antipodarum* is available in Fig. 5 of a document provided at http://el.erdc.usace.army.mil/ansrp/potamopyrgus_antipodarum.pdf.

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ANNEX 4

CALCULATION OF A TIME-WEIGHTED MEAN

Time-weighted mean

Given that the concentration of the test chemical can decline over the period between medium renewals, it is necessary to consider what concentration should be chosen as representative of the range of concentrations. The selection should be based on biological considerations as well as statistical ones. For example, if reproduction is thought to be affected mostly by the peak concentration experienced, then the maximum concentration should be used. However, if the accumulated or longer term effect of the toxic test chemical is considered to be more important, then an average concentration is more relevant. In this case, an appropriate average to use is the time-weighted mean concentration, since this takes account of the variation in instantaneous concentration over time.

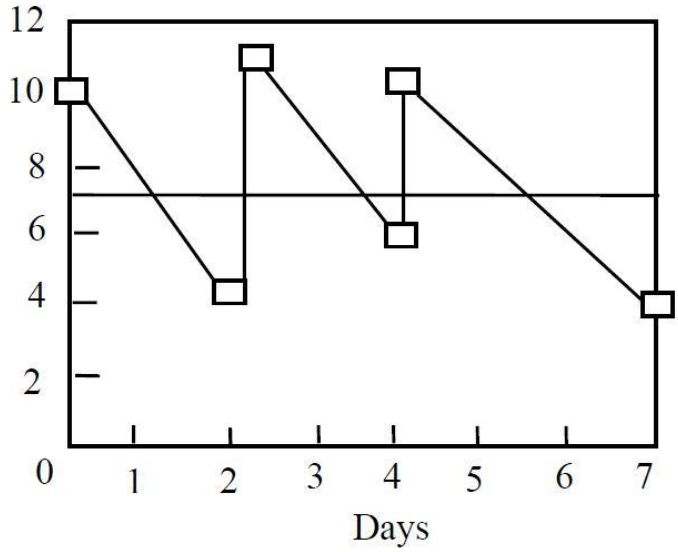

Figure 1:Example of time-weighted mean.

Figure 1 shows an example of a (simplified) test lasting seven days with medium renewal at Days 0, 2 and 4.

- The zig-zag line represents the concentration at any point in time. The fall in concentration is assumed to follow an exponential decay process.
- The 6 plotted points represent the observed concentrations measured at the start and end of each renewal period.
- The horizontal solid line indicates the position of the time-weighted mean.

The time-weighted mean is calculated so that the area under the time-weighted mean is equal to the area under the concentration curve. The calculation for the above example is illustrated in Table 1.

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Renewal No.	Days	Conc 0	Conc 1	Ln(Conc 0)	Ln(Conc 1)	Area
1	2	10.000	4.493	2.303	1.503	13.767
2	2	11.000	6.037	2.398	1.798	16.544
3	3	10.000	4.066	2.303	1.403	19.781
Total Days:	7				Total Area: TW Mean:	50.092 7.156

Days is the number of days in the renewal period
Conc 0 is the measured concentration at the start of each renewal period
Conc 1 is the measured concentration at the end of each renewal period
Ln(Conc 0) is the natural logarithm of Conc 0
Ln(Conc 1) is the natural logarithm of Conc 1

Area is the area under the exponential curve for each renewal period. It is calculated by:

$$\text{Area} = \frac{Conc\ 0 - Conc\ 1}{Ln(Conc\ 0) - Ln(Conc\ 1)} \text{ x Days}$$

The time-weighted mean *(TW Mean)* is the *Total Area* divided by the *Total Days*. Of course, for the reproduction test with *P. antipodarum* the table should be extended to cover 28 days.

It is clear that when observations are taken only at the start and end of each renewal period, it is not possible to confirm that the decay process is, in fact, exponential. A different curve would result in a different calculation for *Area*. However, an exponential decay process is not implausible and is probably the best curve to use in the absence of other information.

However, a word of caution is required if the chemical analysis fails to find any test chemical at the end of the renewal period. Unless it is possible to estimate how quickly the test chemical disappeared from the solution, it is impossible to obtain a realistic area under the curve, and hence it is impossible to obtain a reasonable time-weighted mean.

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ANNEX 5

EXAMPLE DATA TABLE FOR RECORDING MEDIUM RENEWAL, PHYSICAL & CHEMICAL MONITORING DATA AND FEEDING

Experiment No. Date started: Test chemical: Nominal conc:

Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	
Medium renewal (tick)																													
pH*																													new
																													old
O_2 [mg/L]*																													new
																													old
Temperature [°C]*																													new
																													old
Conductivity [µS/cm]																													new
																													old
Total organic carbon [mg/L]																													new
Food provided (tick)																													

EXAMPLE DATA TABLE FOR RECORDING POTAMOPYRGUS REPRODUCTION AND MORTALITY

Experiment No. Date started: Test chemical: Nominal conc:

	Replicate 1		Replicate 2		Replicate 3		Replicate 4		Replicate 5		Replicate 6	
	Shell length	Embryos	Shell length	Embryos	Shell length	Embryos	Shell length	Embryos	Shell length	Embryos	Shell length	Embryos
1												
2												
3												
3												
4												
5												
6												
Mean												
No. of dead adults												

* Indicate which vessel was used for the experiment box

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