Adopted:
29 July 2016

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Lymnaea stagnalis 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. In addition, hermaphrodite aquatic species are very poorly covered by the existing reproduction test guidelines. This guideline is therefore designed to assess effects of prolonged exposure to chemicals on the reproduction and survival of the hermaphrodite freshwater snail *Lymnaea stagnalis* (the Great Pond Snail).

2. Core test endpoints are survival and the cumulated number of egg-clutches produced per snail during a 28-day exposure. Moreover, individual growth of the reproducing snails (*e.g.*, increase in shell length) and the number of eggs produced per snail can be used as additional test endpoints.

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 the reproductive output of *Lymnaea stagnalis*. To this end, reproducing adults of *L. stagnalis* are exposed to a concentration range of the test chemical and monitored for 28 days for their survival and reproduction. Prior to the test, snails are sampled from a laboratory parasite-free culture, calibrated in size, and introduced into the test vessels for acclimation. Following exposure to the test chemical (day 0 of the test), survival is recorded daily, when feeding the snails, and dead snails are withdrawn from the test vessels. Fecundity is determined at least twice per week. Clutches are collected and counted. As additional information, the number of eggs per clutch may also be determined. Adult shell length may be measured at the beginning and at the end of the test.

5. Survival of the parent animals and the number of clutches produced per test vessel should be reported. Reproductive output should reflect the production of clutches per surviving parent organism.

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Other test chemical-related effects on parameters such as number of eggs per surviving parent organism or growth (*e.g.*, increase in shell length) can also be examined.

6. The toxic effect of the test chemical on the cumulated number of clutches produced per individual-day is expressed as EC_x by fitting an appropriate regression model to the data in order to estimate the concentration that would cause x% reduction in the reproductive output (2). Alternatively, the toxic effect of the test chemical can be expressed as the No Observed Effect Concentration and Lowest Observed Effect Concentration (NOEC/LOEC) values (3). Both EC_x and NOEC/LOEC can be determined from a single study.

INFORMATION ON THE TEST CHEMICAL

7. Information on the test chemical, which may be useful to establish the test conditions, includes the structural formula, purity, stability in light, stability under the conditions of the test, pK_a, P_{ow}, and results of a test for ready biodegradability (see OECD Test Guidelines 301 and 310; 4,5).

8. 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 should be available.

REFERENCE SUBSTANCES

9. Reference substances should be tested periodically as a means of reassuring sensitivity of test organism and consistency of experimental test conditions. Cadmium chloride is a common reference substance which has been successfully used in the international validation studies for this Test Guideline, with an $EC_{50,reproduction}$ range of 40 - 230 µg Cd/L^{-1} (6,7).

VALIDITY OF THE TEST

10. For a test to be valid, the following criteria should be met:

- the mean control mortality (accounting for all control replicates) should not exceed 20% at the end of the test;
- the mean cumulated individual fecundity in the controls at the end of the test should be at least four egg-clutches per individual-day (see § 49);
- 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 20 ± 1°C throughout the test in both control and exposure groups. Transitory deviation (for 1 to 2 days) from this mean value can occasionally occur but should not be more than ± 2°C.

DESCRIPTION OF THE METHOD

Apparatus

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11. Test vessels and other apparatus which are in contact with the test solutions should be made of glass or other chemically inert material.

12. Some or all of the following equipment are required:

- climate chamber or temperature regulated room or any adequate apparatus for temperature control;
- adequate apparatus for the control of the lighting regime and measurement of light intensity;
- oxygen-meter;
- pH-meter;
- conductivity-meter;
- equipment for the determination of water hardness;
- test kits or other equipment for ammonium, nitrite and nitrate measurements in water;
- 1 L glass vessels which can be covered with perforated lids, nets, or any device that prevents snails from escaping, but allows air inflow;
- air pumps, air tubes, Pasteur pipettes;
- adjustable valves for airflow control;
- (digital) calliper;
- razor blades and/or sharp-edged spoons;
- stereomicroscope equipped with a light source (for egg-counting);
- volumetric flasks and other laboratory glassware for the preparation of solutions;
- glass pipettes.

Test organism

13. The species to be used in the test is *Lymnaea stagnalis* (Linnaeus, 1758).

14. Adult *Lymnaea stagnalis* to be used in the test should come from an established parasite-free laboratory culture. They should have been maintained in culture conditions similar to those to be used in the test (the culture protocol for *Lymnaea stagnalis* is described in ANNEX 2). Especially, photoperiod and temperature should be similar in the culture and test conditions. Field collected organisms should not be used right away for the reproduction test.

15. Organisms to be tested should exhibit a shell length within the range 2.5 - 3.0 cm at the beginning of the test. Compliance with this length criterion should be checked by measuring the maximal shell length using *e.g.*, a (digital) calliper. Snails should be carefully manipulated to avoid breaking the shells; particular attention should be dedicated to preserving the apex of the shell while measuring/manipulating the snails.

Test medium

16. Tests can be implemented using tap water filtered with charcoal for dechlorination, reconstituted water, or any uncontaminated water which ensures compliance with water quality criteria listed below. Tap water should be used with care since water pipes could contain/release copper which might be highly toxic to test organisms and comparisons between results from different laboratories become difficult (8). It is recommended that the water used in the test be similar to the

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culture water (see ANNEX 2). Test water, including the water used for test medium renewal, should reach test temperature and dissolved oxygen concentration before use.

17. The following water parameter values should be achieved and kept:

- pH: in the range 6.5-8.5;
- oxygen concentration (in controls): > 6 mg L^{-1} at 20°C (> 60% of the air saturation value);
- conductivity: between 600 ± 200 µS cm^{-1};
- water hardness: in the range 140-250 mg L^{-1} as $CaCO_3$, preferably close to 250 mg L^{-1}.

Test solutions

18. 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 the test water using mechanical means such as agitating, stirring or ultrasonication, or other appropriate methods. If possible, the use of solvents should be avoided. For difficult test chemicals, the OECD Guidance Document on aquatic toxicity testing of difficult substances and mixtures should be consulted (79).

19. 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^{-1} (10). 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. Only solvents that have been shown to have no significant effects on adult response should be used in the test. The selection of an appropriate solvent depends on the physico-chemical properties of the test chemical and on the sensitivity of test organisms (10-12), which should preferably be determined in a previous study.

PROCEDURE

Conditions of exposure

Duration

20. The test duration is 28 days.

Loading

21. Two days before the beginning of the test, test vessels should be filled with 1 L of clean test water at test temperature. Adult *Lymnaea stagnalis* should be collected among free-floating snails and/or snails removed from the glass wall (using *e.g.*, a brush or a thin flat spoon that is carefully inserted between body and glass) of the culture. Apart from using gloved hands, snails can be manipulated without specific equipment. Animals injured before or during collection should not be included in the test. Appropriate shell length, which is between 2.5 and 3.0 cm, should be checked. Groups of five snails should be randomly introduced in test vessels containing clean water and fed *ad*

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libitum. The mean shell length of animals should not significantly differ (as determined by an appropriate statistical methodology) among test vessels.

22. During this two-day acclimation period, check to be certain that reproduction is occurring under test conditions before starting exposure to the test chemical. Otherwise, the acclimation period should be extended until reproduction has started (*i.e.*, the observation of at least one clutch) in at least 2/3 of the test vessels. In that case, test water should be renewed at least twice a week. The clutches that were laid during acclimation have to be removed from test vessels and discarded. At day 0, a randomized block design according to clutch production output should be used to allocate replicates to the various experimental levels to ensure balanced distribution of replicates. The test water should be renewed and test chemical added for the first time.

Feeding

23. Snails are fed with fresh lettuce after it has been washed in clean water. Preferably, lettuce should be of organic quality (as certified by the European *ECOCERT* or equivalent label). Round-head lettuce should be used if possible, or any other lettuce variety with smooth leaves. Hard parts of leaves are generally not eaten by the snails: they should be removed in order to avoid accumulation of leftovers in the test vessels and losses of test chemical.

24. Organisms are fed *ad libitum,* preferably every working day, but at least twice a week while renewing the test medium. The *ad libitum* feeding level in adults corresponds to *ca*. 3.5 g of fresh lettuce/individual/week. However, this quantity should be increased if snails consume the whole amount of food provided between two feeding dates. This quantity should be reduced if a decrease in the feeding rate of animals is observed due to the test chemical, in order to prevent the accumulation of leftovers, which may result in harmful ammonia and nitrite concentrations. Leftovers are withdrawn from the tests vessels at water renewal. Because feeding rate influences the reproductive output, a decrease in food intake should be reported.

Light regime

25. A constant light-dark period of 16/8 hours should be used. The light source (natural daylight spectrum) should be positioned above the test vessels so that the light intensity is in the range 250-500 lux at the surface of test water.

Temperature

26. The mean temperature of the test media should be 20 ± 1°C throughout the test. Transitory deviation, for 1 to 2 days, from this mean value can occasionally occur but should not be more than ± 2°C.

Aeration

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27. The water in the test vessels should be gently aerated through glass pipettes (Pasteur pipettes) connected to the air tubing system. Adjustable valves should be used to ensure continuous and constant air flow.

28. Dissolved oxygen content should remain >60% ASV, however the test vessels should be aerated gently to avoid stripping of test chemicals. Volatilization is reduced when the vessels are covered (see § 12).

Test design

29. The test protocol has been validated under semi-static conditions with water renewal twice a week. However, for test chemicals that dissipate quickly, water can be renewed more frequently in order to keep the exposure concentration above 80% of the nominal value (see § 36). *L. stagnalis* can also accommodate flow-through conditions (13). If the test protocol is implemented in such conditions, the system, including the flow rate, should be adjusted so that the validity criteria (see § 10) are met.

30. At least five concentrations with a minimum of six replicates of five snails (*i.e.*, in total, 30 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) is required in order to help in selecting appropriate test concentrations. If a NOEC is desired, the concentrations should bracket the expected value. If an EC_x is desired, whatever x is, ideally that concentration should be bracketed by test concentrations.

31. When designing the test, it should be considered whether the aim is to achieve a NOEC/LOEC, EC_x values, or other relevant endpoints from mechanistic models, as identified in (3). The demand for replicates is higher if the NOEC/LOEC is wanted, whereas the estimation of EC_x generally requires more concentrations. If necessary, the number of tested concentrations or the number of replicates per concentration can be increased to ensure adequate statistical power.

32. Introduction of the test chemical in the test vessels defines the beginning of the test (day 0). Introduction of the test chemical should be done before feeding the snails. Test solutions of the chosen concentrations are prepared by introducing the appropriate amount of stock solution in test vessels containing 1 L of clean test water at the test temperature. Water is then homogenised by gentle manual agitation. Alternatively, test solutions of the chosen concentrations are prepared by introducing the appropriate amount of stock solution in a tank containing at least 6 L of clean test water at the test temperature. Water is homogenised by agitation prior to dispatch in the test vessels.

Range finding test

33. The range-finding study should be conducted according to the best practices (14). The exposure conditions, including validity criteria, should be similar to those of the definitive test. However, the number of test concentrations can be reduced and/or the spacing factor between adjacent concentrations can be increased. In addition, the number of replicates per treatment can be reduced to 4 or the test duration can be shortened.

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Limit test

34. If no statistically significant effects are observed at the highest tested concentration in the range-finding test, 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^{-1} or the maximum water solubility of the test chemical, and a control. Ten replicates 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 statistically significant effects on the number of clutches per individual-day are recorded, a full test is required.

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Controls

35. At least six control vessels (*i.e.*, containing no test chemical) should be included in the test. If a solvent has been used for the application of the test chemical, six replicates of a solvent control using the same amount of solvent as in the treatments should also be used. In case of a limit test, the number of control replicates should be extended to ten (see § 34).

Test medium renewal

36. For semi-static exposure conditions, the water in each test vessel should be renewed completely at least twice a week in order to maintain exposure concentration above 80% of the nominal value and adequate physico-chemical parameters values between two successive water renewals. This renewal rate can be increased up to three times per week if a readily degradable test chemical is studied. Degradability rate can be estimated from preliminary stability tests or from the physico-chemical properties of the test chemical. Renewal days are chosen so as to ensure that the time interval between two successive renewals is kept constant (*e.g.,* renewal on Monday morning and on Thursday afternoon; interval between renewals is equal to 3.5 days).

37. The following procedure is used for water renewal. Exposure water is gently removed from the test vessels so that snails do not tumble in the test vessel, which might damage their shells. A sieve can be used to collect the snails which might detach from the glass walls and the food leftovers. Food leftovers are eliminated after having checked that no clutches remain on or in it. Test vessels are wiped off with a tissue to eliminate faeces, food leftovers and the biofilm. Alternatively, clean beakers can be used. Test vessels are then refilled using water at the test temperature. The test chemical is immediately added to the renewed water using stock solutions. Freshly prepared test medium is homogenised by manual agitation. Snails are then placed back in the test vessel. Food is provided *ad libitum* to animals once the water renewal and spiking have been completed.

Observations

38. Animals should be observed preferably daily while feeding them, but at least twice a week for visual assessment of any abnormal behaviour (*e.g.*, avoidance of water, avoidance of food, crowding of snails, lethargy, lack of retraction reflex or cannibalism). Any abnormal behaviour should be recorded.

Mortality

39. Dead snails can be easily identified because their foot sole abnormally lies out of their shell. They may also emit an unpleasant odour. In addition, the absence of reaction when the foot sole is stimulated with a blunt needle is a reliable indication of death.

40. The number of dead snails should be recorded daily when feeding the animals. Dead snails are removed from the test vessels and counted. Mortality should be taken into account in the calculation of the reproductive output (see § 49).

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Reproduction

41. The number of egg-clutches laid per replicate is recorded at least twice a week, while renewing the test medium. Clutches are carefully removed from the glass walls, using a razor blade or a sharp-edged metallic spoon and counted. As additional information, the number of eggs per clutch can also be counted. To this end, clutches are transferred to Petri dishes (or any other appropriate container, *e.g.*, 6-wells plates) for observation using a stereomicroscope, and the number of eggs per clutch is recorded. If abnormal eggs are produced (see ANNEX 3 for a definition of egg abnormalities), they may also be counted and recorded. Eggs are killed by rapid freezing of the egg-clutches, once their number (as well as the number of eggs per clutch and frequency of egg abnormalities, if desired) has been determined.

Growth

42. Adult shell length is measured at the end of the test, using a (digital) calliper, if growth is desired as an additional test endpoint. Average growths of the exposed and control groups are calculated from the increase in shell length between the initiation and end of the test.

Once their size has been determined and if no other endpoints (*e.g.*, histopathology) are investigated, exposed adults are killed preferably by rapid freezing at -80°C or cryopreservation.

Frequency of analytical determinations and measurements

Concentration of the test chemical

43. During the test, the concentrations of the test chemical are determined at regular time intervals. Results should be based on measured concentrations. However, if the concentration of the test chemical in solution has been satisfactorily maintained within ±20% of the nominal concentration throughout the test, then the results can either be based on nominal or measured values.

44. As a minimum, the highest and lowest test concentrations should be analysed in freshly spiked test medium at the start of the test (preferably within the first hour after application of the test chemical) and in the old solution at the time of renewal on one occasion during the first week of the test. These determinations should be made on pooled water from different replicates and repeated at weekly intervals thereafter.

45. 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. In this case, results should be expressed in terms of mean measured concentration or time-weighted average (TWA) concentration for test chemicals with fast dissipation kinetics (15; see guidance for calculation in ANNEX 4).

46. If the chemical analysis needs large samples which cannot be taken from test vessels without influencing the test system, analytical determinations should be performed on samples from additional test vessels treated in the same way (including the presence of test organisms) but not used for biological observations.

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Physico-chemical parameters

47. The pH, dissolved oxygen in the test water and temperature of the test vessels should be measured in one replicate of each treatment before and after every second renewal of the test medium using the appropriate equipment (see § 12). As a minimum, water hardness should be measured in the controls and one test vessel at the highest concentration at the start and the end of the test. Water hardness can influence the toxicity of ionic compounds. When working with such compounds, water hardness should be measured at the beginning, mid-term and end of the test. Ammonia, nitrite and nitrate concentrations should be measured when abnormal high mortality is observed in the controls or in one replicate of the lowest concentrations (see § 24). All the physico-chemical parameter values should be reported using *e.g.*, a spreadsheet (see ANNEX 5 for an example).

DATA AND REPORTING

Treatment of results

48. 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 number of clutches produced per test vessel should be reported using *e.g.*, a spreadsheet (see ANNEX 6 for an example). Other detrimental effects, *e.g.*, altered feeding or growth, abnormal behaviour and toxicological significant findings, should be reported in the final report as well.

49. Reproductive output should reflect the production of clutches per surviving parent organism over the test duration (28 days). For each replicate, the reproductive output is calculated by dividing the cumulative number of clutches collected over 28 days in a given test vessel by the number of individual-day (NID; see ANNEX 1) of this test vessel (2; see guidance for calculation in ANNEX 7).

50. Other test chemical-related effects such as production of eggs per surviving parent organism or average growth can also be examined. For each replicate, the production of eggs is calculated by dividing the cumulative number of eggs collected over 28 days in a given test vessel by the NID of this test vessel (2; see guidance for calculation in ANNEX 7).

51. If both water and solvent controls are included in the experiment and if there is no statistically significant difference between them, the preferred method is to pool the controls unless otherwise required by Regulatory Authorities, in which case only the solvent control is used to test for treatment effects (11,16,17).

EC_x

52. EC_x values, including their associated lower and upper credible/confidence limits, are estimated using any appropriate statistical method based on a regression analysis of the number of clutches (or eggs) per individual-day. Even if any statistical software can be used for regression analysis (3), the user-friendly web-platform MOSAIC_repro, freely available at http://pbil.univ-lyon1.fr/software/mosaic/reproduction/, is recommended because the procedures implemented within this software were developed during the validation process of the *L. stagnalis* Reproduction Test (see details in ANNEX 7).

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53. The proposed test design is well suited for the estimation of EC_{50} values and the determination of NOEC/LOEC values. It also enables estimation of EC_{10} or EC_{20} values, but the statistical reliability and the biological relevance of the estimated values should be carefully checked through the width of their associated 95% credible/confidence intervals. Test concentrations should be chosen with caution. Indeed, test concentrations bracketing the EC_{50} might not allow a proper estimation of EC_{10} or EC_{20} values due to the need of extrapolating below the lowest tested concentration. In the same way, test concentrations bracketing EC_{10} or EC_{20} values would not allow a proper estimation of EC_{50} due to the need of extrapolating above the highest tested concentration.

NOEC/LOEC

54. If a statistical analysis is intended to determine the NOEC/LOEC, appropriate statistical methods should be used according to OECD Document 54 on the Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application (3). In general, adverse effects of the test chemical compared to the control are investigated using one-tailed hypothesis testing at $p < 0.05$.

55. Step-down trend tests are recommended to determine whether there are significant differences ($p < 0.05$) between the control(s) and the various test chemical concentrations (3). Comparisons of the numbers of clutches per individual-day should be done either with parametric (*e.g.*, Williams' test) or non-parametric methods (*e.g.*, Jonckheere-Terpstra trend test) to determine the NOEC and the LOEC. Where a NOEC is computed, the ratio Reproduction/NID, where Reproduction is the number of clutches or the number of eggs per replicate, is analysed. R software can be used, as well as other R-based software such as StatCharrms available at www.epa.gov/med/Prods_Pubs/rscabs.htm, or commercial softwares such as ToxRat (www.toxrat.com) and Cetis (http://www.tidepool-scientific.com/Cetis/CetisStats.html) (see details in ANNEX 7).

Test report

56. The test report should include 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, UVBCs 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.

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Test conditions:

- test procedure used (*e.g.*, semi-static, volume, loading in number of snails per litre);
- photoperiod and light intensity;
- test design (*e.g.*, number of replicates, number of snails per replicate, etc.);
- details of test medium characteristics (including pH, conductivity, temperature, dissolved oxygen concentration, water hardness, ammonium concentration, and any other measurements made);
- detailed information on feeding (*e.g.*, type of food, source, frequency), including amount (in mg/snail/day or *ad libitum*);
- method of preparation of stock solutions and frequency of renewal (the solvent and its concentration should be given, when used).

Results:

- results from any preliminary studies on the stability of the test chemical;
- the nominal test concentrations and results of all chemical analyses to determine the concentration of the test chemical in the test vessels; the recovery efficiency of the analytical method and the limit of quantification should also be reported;
- water quality within the test vessels (*i.e.* pH, temperature, dissolved oxygen concentration, water hardness, and ammonium concentration) (see data spreadsheet example in ANNEX 5);
- the number of deaths among the snails and the day on which they occurred (see data sheet example in ANNEX 6);
- the full record of the production of egg-clutches per replicate during the test (see data spreadsheet example in ANNEX 6);
- plot of total number of egg-clutches produced per individual-day in each replicate *vs*. concentration of the test chemical;
- where appropriate the LOEC for reproduction, 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 NOEC for reproduction; where appropriate, the LOEC or NOEC for mortality of the snails should also be reported;
- where appropriate, the EC_x for reproduction with credible/confidence interval (*e.g.*, 90% or 95%), the slope of the concentration-response curve with its credible/confidence interval and a graph of the fitted model used for its calculation;
- other observed biological effects or measurements: report any other biological effects which were observed or measured (*e.g.*, number of eggs per egg-clutch, proportion of abnormal eggs in egg-clutches, snail growth, altered behaviour) including any appropriate justification;
- an explanation for any deviation from the Test Guideline.

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ANNEX 1

ABBREVIATIONS & DEFINITIONS

ABBREVIATIONS

ASV Air Saturation Value

EC_x *x*% effective concentration, that is the estimated concentration for which an effect of *x*% is expected.

LOEC The Lowest Observed Effect Concentration is the lowest tested concentration at which the test chemical is observed to have a statistically significant effect. However, all test concentrations above the LOEC should have a harmful effect equal to or greater than those observed at the LOEC:

NOEC The No Observed Effect Concentration is the tested concentration just below the LOEC.

NID Number of Individual-Day calculated by summing the observation periods during which each individual contributes to the reproduction process (see details in ANNEX 7).

TWA Time Weighted Average

DEFINITIONS

Fecundity: Actual reproductive rate of organisms, measured by the number of egg-clutches or eggs

Parent Animals: Reproducing adults of which the reproductive output is the object of study

Reproductive output: Production of clutches per surviving parent organism over the test duration (28 days)

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ANNEX 2

RECOMMENDATIONS FOR THE CULTURE OF *LYMNAEA STAGNALIS*

INTRODUCTION

1. This Standard Operating Procedure (SOP) describes the laboratory rearing of the freshwater snail *Lymnaea stagnalis* (the Great Pond Snail), in order to ensure optimal and reproducible biological quality of the cultured organisms to be used in further toxicity tests. Rearing should thus allow producing all year round a sufficient number of snails with optimal health and reproducible sensitivity to reference toxicants. Cultured snails must be parasite-free, exhibit a low mortality and be able to reproduce all year round. This can be achieved through an appropriate control of feeding, photoperiod and population density, as described in this SOP.

TEST ORGANISM

Taxonomy

2. *Lymnaea stagnalis* (Linnaeus, 1758) belongs to the phylum Mollusca, class Gastropoda, sub-class Euthyneura, order Basommatophora, family Lymnaeidae. This species is highly polymorphous, especially regarding the shape and colour of the shell (1).

Ecology

3. *Lymnaea stagnalis* is common in holarctic regions of Europe, northern Asia and North America (2). It inhabits stagnant or slow-running freshwaters (3), where it establishes in populations of generally low densities (4). Mostly herbivorous, its feeding regime varies depending on its size. Juveniles mostly feed on small/detrital plant material (5) whereas adults feed on both living and dead plants. Both juveniles and adults also graze on micro-algae, periphyton, bacteria, and hyphen, and occasionally feed on dead small invertebrates, including congeners.

Biology

4. Life-span is comprised between one and two years in the field, depending on environmental conditions (2). The average adult shell length varies in the range of 2-6 cm (measurement from the lower aperture edge to the apex; Fig. 1). *L. stagnalis* is a simultaneous hermaphrodite but the male reproductive organs mature prior to the female ones (protandric hermaphrodite). During mating, an individual can only take the male or the female part. *L. stagnalis*, as all Basommatophora, can self-fertilize and appears to show no or weak inbreeding depression (6,7). Yet, crossed fertilization is the most common reproductive pathway (8). After mating, snails can store sperm for one to three months (9). Reproduction occurs mostly at the beginning of summer (2), with egg-clutches being frequently deposited on aquatic plants. The number of eggs per clutch generally varies from 50 to 120 (10) and is a function of adult size. Hatching rate is generally above 70% in unpolluted waters. Embryonic development duration usually lasts between 10 and 30 days, depending on the number of eggs per clutch and on environmental conditions.

Laboratory specificities

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5. The expected life-span is also comprised between one and two years in the laboratory, and the snails usually exhibit a maximum shell length of 3 to 4 cm (measurement from the lower aperture edge to the apex; Fig. 1). Reproduction occurs all year round, with a seasonal decrease in fecundity in February-March. Renewal of the surrounding water is known to be a trigger for egg-laying (11). Egg-clutches are generally deposited on the glass surface of the aquaria. Embryos (from 20 to 160 per clutch, depending on the adult size) can easily be observed through the transparent clutch using a stereomicroscope. Four successive embryonic development stages can be distinguished (12). Hatching rate is usually above 90% after 28 days.

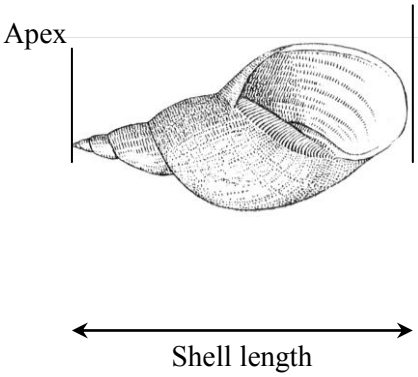


Figure 1. Measurement points to determine the shell length of *Lymnaea stagnalis*.

EQUIPMENT

Aquaria and accessories

6. The following equipment can be used:
- Climate chambers or temperature regulated room with temperature indicator;
- Culturing aquaria (5 L and 35 to 50 L; made of glass);
- Water filtering device (equipped with ceramic rings, coarse sand, cockleshell and/or coral fragments, or commercial filter wadding);
- Water pumps;
- Air pumps;
- Flexible air tubes (with glass pipettes);
- Measuring electrodes for temperature, conductivity, oxygen and pH;
- Test kits for ammonium, nitrite and nitrate measurements in water;
- Equipment to measure water hardness;
- Digital calliper;
- Sharp-edged metallic spoons or razor blades.

The filters (biological or commercial) and any plastic material used for culturing should ensure no leaching of plasticizers and other compounds. PVC materials should be avoided.

Chemicals and products for water conditioning

7. If tap water is used, a filtration on charcoal is required for dechlorination before use. Reconstituted water with appropriate hardness (ca. 250 mg L^{-1} as $CaCO_3$) can also be used (*e.g.*, ISO 6341 medium (13); preparation is described in Table I).

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Table I. Preparation of the ISO medium

Stock solutions (single substance)		To prepare the reconstituted water, add the following volumes of stock solutions to 1 litre water*
Subtance	Amount added to 1 litre water*	
Calcium chloride ($CaCl_2$, $2H_2O$)	11.76 g	25 ml
Magnesium sulfate ($MgSO_4$, $7H_2O$)	4.93 g	25 ml
Sodium bicarbonate ($NaHCO_3$)	2.59 g	25 ml
Potassium chloride (KCl)	0.23 g	25 ml

* Water of suitable purity, for example deionised, distilled or reverse osmosis with conductivity preferably not exceeding 10 µS cm^{-1}.

Biological material

8. Snails to be cultured can be sampled either from an existing culture or from the field. If existing cultures are used, at least ten clutches should be used per culture aquarium to be settled. If cultures are started from field populations, an unpolluted location with a sufficient snail density should be found. Note that snails originating from various field populations may exhibit varying reproductive performances. A minimum of 30 founders should be used. High parasite infestation rates have been reported for wild-caught *L. stagnalis* (14). Parasites (*i.e.*, mainly cercarians, trematodes, cestodes, protists and platyhelminths) may induce diseases in the infected snails. Therefore, the presence of parasites should be checked by dissecting at least 20 snails and observing (using a stereomicroscope) the digestive tract and full-length sagittal sections throughout the animal body and infected populations should not be used for culturing. If no parasite is found, founders should be acclimatized to culture conditions under quarantine during at least 30 days. The laboratory culture is built up with the second-generation clutches.

9. Clutches are incubated at 20°C in 1 to 5 L aquaria until hatching, which starts after ca.10 days after egg-laying. Newborn have the reflex of escaping from water to start breathing air: therefore, heavy newborn losses are expected during the first month (ca. 50%) because these young snails tend to dry out on the walls of aquaria. Using aquaria with a reduced depth and providing lettuce leaves on the surface of water, where newborn can hide and feed on, will reduce these losses. Once the escape behaviour no longer occurs (ca. three weeks after birth), juveniles are transferred into the 35 L culture aquaria by groups of 100-120 individuals per aquarium.

REARING CONDITIONS

Temperature and light regime

10. The culturing of *L. stagnalis* should be carried out at a constant water temperature of 20 ± 2°C and a constant light-dark period of 16/8 hours with a light intensity of 250-500 lux (natural daylight spectrum).

Culture medium

11. Dechlorinated charcoal filtered tap water or reconstituted water can be used. Any reconstituted medium that allows meeting the following water quality requirements mentioned in § 12 can be used. Examples of suitable culturing water can be found in ISO 6341:2012 (13; see Table 1) and OECD 2010 (15).

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12. The following water parameters should be achieved and kept:
- Temperature: 20 ± 2°C;
- pH should remain between 7 and 8.5;
- Oxygen concentration: > 6 mg L^{-1} at 20°C (>60% of the air saturation value);
- Conductivity: between 400 and 800 µS cm^{-1};
- Hardness: ca. 250 mg L^{-1} as $CaCO_3$.

Compliance with these parameters should be checked before the water is used in the culture aquaria.

Food and feeding

13. Snails should be fed lettuce (organic quality, *e.g.*, as certified by the European label *ECOCERT* or equivalent, and washed in clean water). Secondary food source may be added if needed to support growth, *e.g.*, commercial fish flakes. Adding a thin layer of coral sand in every aquarium facilitates digestion in snails and facilitates the maintenance of pH in the basic range.

14. Organisms are preferably fed every working day, but at least three times a week. The amount of food to be delivered depends on population density and development stage. The chosen value should ensure that animals have reached the appropriate development stage and size at the beginning of toxicity tests. Snails should be fed *ad libitum* at least during the two weeks preceeding toxicity tests.
If organisms are fed with lettuce, they should be provided with a minimum of 160 g of fresh food/100 ind./week. The *ad libitum* feeding rate in adults is expected to be ca. 320 g of fresh food/100 ind./week.
Alternatively, organisms can be fed with commercial feed such as TetraMin®, or equivalent proprietary food that shows no endocrine activity. For example, using TetraMin®, the *ad libitum* feeding rate in juvenile snails of 1.5 cm corresponds to 7 g of flakes/100 ind./week. The *ad libitum* feeding rate in adults is comprised between 70 and 100 g of flakes/100 ind./week (16). If accumulated at the bottom of the aquaria, unconsumed food particles have to be removed (at least 6 h after feeding) to reduce growth of bacteria and fungi. If the water has turned turbid due to leftover decomposition, it should be completely renewed.

OPERATING PROCEDURE

Population density

15. A density of 100-120 adults per 35 L aquarium is generally used. Population density can be modified to facilitate growing snails reaching the appropriate development stage and size for the implementation of toxicity tests. However, it should not be higher than five adults per litre.

Cleaning and care

16. Temperature, pH, oxygen concentration, conductivity and ammonium and nitrite concentration of all aquaria should be monitored. Additional measurements of ammonium and nitrate, as well as water hardness, should be done if necessary (*e.g.,* if the aquaria are newly installed or if any abnormality in the water and/or snail behaviour is noticed). Electrodes have to be thoroughly cleaned before they are used in the next aquarium to prevent a potential transfer of diseases or pathogens. Dead snails, if any, have to be removed at the same time.

17. Culture water should be renewed weekly if snails are fed with lettuce, or three times per week if snails are fed with fish flakes. One third of the aquarium volume is replaced by fresh water from the storage aquarium using *e.g.,* a siphon, which should be thoroughly cleaned before it is used in the next aquarium.

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Detritus (especially food leftovers) and clutches that are laid on glass walls by adults have to be removed at the same time. Clutches have to be carefully removed from the glass walls (using *e.g.,* razor blade), and can then be collected with the siphon.
Before replacing water for the culture aquaria, temperature, pH, oxygen concentration, and conductivity of the water in the storage aquarium should be measured. After renewal, a maximum of 1 ml calcium carbonate from a saturated solution can be added to each aquarium, if required (*e.g.,* if decalcified shells are observed in many animals), to support shell growth. During cleaning procedures, snails should be handled with care to limit/avoid stress.

18. Aquaria and filter pumps have to be cleaned thoroughly every 6 to 8 weeks. Depending on the cleanliness of the filters, the filter materials have to be cleaned or replaced up to a maximum of 2/3, in order to conserve useful bacteria such as the denitrifying bacteria.

Diseases and mortality

19. To our knowledge, no disease has ever been observed in parasite-free cultures of *L. stagnalis,* providing the above-cited conditions are met.

20. Aquaria should be checked daily (at the same time as feeding) for abnormal breeding conditions (*e.g.,* bacterial or fungal growth) or behaviour /health of animals (*e.g.*, escape behaviour, crowding, lack of grasping reflex or cannibalism). Handling may generate escape behaviour in snails: when disturbed or removed from aquaria, animals eject air and/or hemolymph. Crowding of snails and/or occasional cannibalism are commonly observed in mass-reared *L. stagnalis*. Those behaviours do not indicate bad health of the snails. The lack of grasping reflex and unresponsiveness to mechanical irritation indicate that the snail may die soon. Those symptoms generally affect only a few individuals, which should be removed from the culture if found. Yet, if such indications are frequently observed in an aquarium, or if avoidance of water is observed, the aquarium should be removed from the culture.

21. Dead snails should be removed from culture aquaria once per week. In case of abnormally high mortality (*i.e.,* sudden mortality affecting approximately 15% of stocks and occurring over a period of 15 days, 15;), the aquarium should be excluded from the culture. Snails are killed by *e.g.,* overdosing of narcotics (*i.e.,* 24 h exposure to a 2.5% $MgCl_2$ solution) before being eliminated. The aquarium and all equipment then have to be cleaned and disinfected thoroughly.

Control of the culture productivity

22. Reproductive success should be analysed regularly to monitor the annual reproductive cycle and assess the culture productivity, according to the following procedure:

- Twenty snails of homogenous size are sampled among free-floating snails and/or snails carefully removed from the glass walls;
- Shell length is measured using (digital) callipers. Snails are then distributed among replicates by groups of five individuals;
- At least five replicates consisting of glass vessels filled with 1 L of culture water should be used.
- Clutches produced in a three-day period are counted and removed from the vessel using *e.g.,* a razor blade or any sharp-edged spoon. The number of eggs per clutch is counted (using a stereomicroscope). Data are recorded *e.g.,* in a spreadsheet.
- The number of eggs produced per snail is calculated based on previous data, accounting for mortality in test vessels, if any.
- The mean, and variability parameters (such as standard deviation or standard error of the mean), are calculated for each of the endpoints.
- The number of eggs produced per snail and shell lengths are evaluated statistically. The

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number of clutches and the number of eggs per clutch can be studied as additional endpoints.

Maintenance of the cultured populations

23. Populations should be regularly renewed in order to avoid (i) the mixing of cohorts in a single aquarium and (ii) the culturing of old snails (*i.e.,* aged more than over eighteen months, which exhibit a lower fecundity (17). Clutches to be used for the renewal can be obtained from the adults to be replaced. Clutches from the various aquaria to be renewed can be pooled in 5 L hatching aquaria. New culture aquaria are then started according to the procedure described in § 8.

24. It is recommended to introduce new genetic resources in the culture (*e.g.,* once per year) using at least 30 snails sampled from another culture or collected in the field, breeding them in quarantine and adding second generation clutches to the mass-rearing, as described in § 8. In both cases, new clutches have to be mixed with clutches produced by adults from the culture in order to ensure a mixing of genetic characteristics.

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ANNEX 3

EGG ABNORMALITIES

Egg quality can be assessed by determining the frequency of 4 types of abnormalities (Figure 1B –E): polyembryonic egg (the presence of several embryos per egg; unfertilized egg (the absence of embryo in the egg, which only consists of the eggshell and albumen; atrophied albumen (damaged eggshell containing an abnormally low albumen quantity); and single embryo (presence of a nondeveloping embryo, without an eggshell and without albumen).

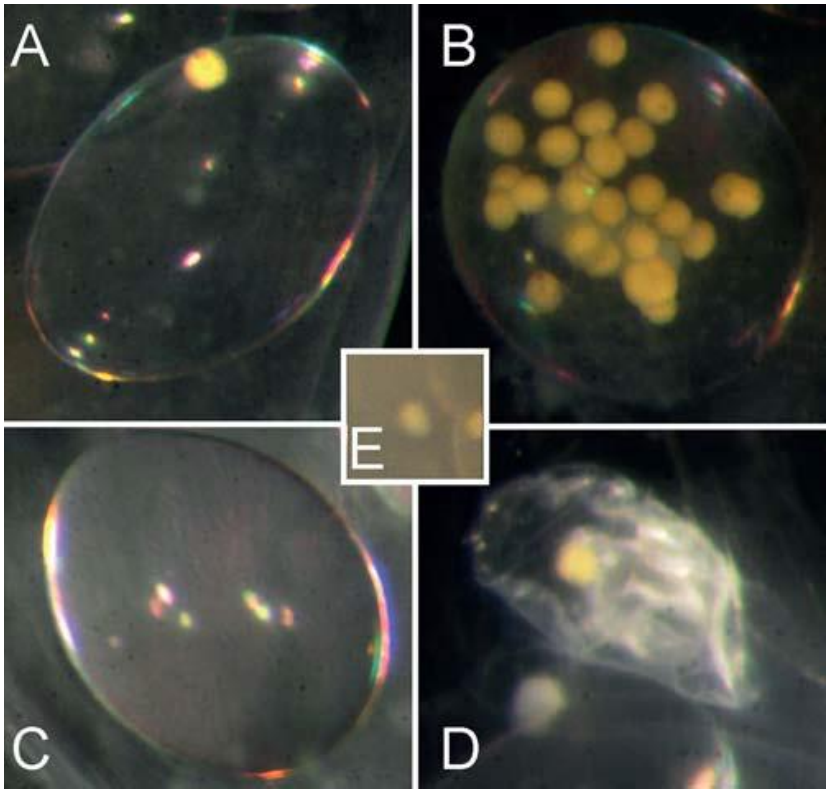


Figure 1. Egg abnormalities observed in *Lymnaea stagnalis*. (A) normal egg; (B) polyembryonic egg; (C) unfertilized egg; (D) egg with atrophied albumen; and (E) single embryonic cell. From Giusti *et al*., 2013.

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ANNEX 4

CALCULATION OF A TIME-WEIGHTED AVERAGE CONCENTRATION

Given that the concentration of the test chemical can decline over a measurement period ($\left[t_i\,;\,t_{i+1}\right]$, Fig. 1) after medium renewals (t_0, t_2, t_4, Fig. 1), it is necessary to consider what concentration should be chosen as representative of the concentration experienced by the exposed organisms. The selection should be based on biological considerations as well as statistical ones. For example, if reproduction is thought to be affected mostly by exposure to peak concentration, then the maximum concentration should be used. However, if the accumulated or longer term effect of the test chemical is considered to be more important, then an average concentration is more relevant. In this case, an appropriate average to use may be the time-weighted average (TWA) concentration which accounts for the variation in instantaneous concentration over time (plain lines, Fig. 1).

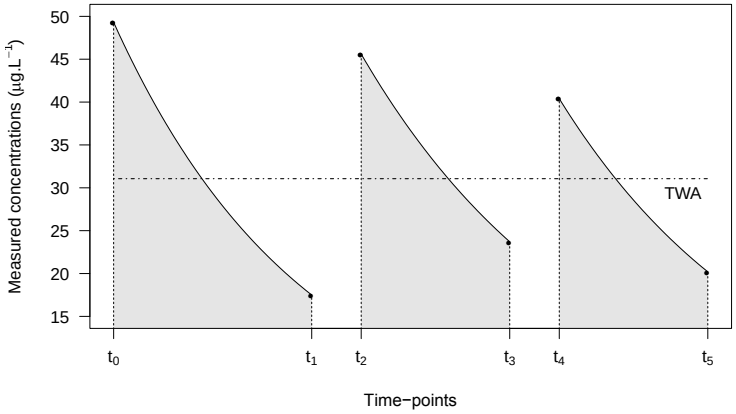


Figure 1. Example of graphical representation of the time-weighted average (TWA) concentration. Data points correspond to the measured concentrations over three time periods after medium renewals ($\left[t_0\,;\,t_1\right]$, $\left[t_2\,;\,t_3\right]$ and $\left[t_4\,;\,t_5\right]$). Plain lines correspond to the theoretical exponential decay of the concentration between two measurement time-points. The horizontal dashed line corresponds to the calculated time-weighted average (TWA) concentration value (see text for details).

The main assumption of the calculation of the TWA concentration is that the decrease in concentration between two measurement time-points (t_i and t_{i+1}) follows an exponential decay:

$$C\left(t\right) = c_i\, e^{-k_i\left(t-t_i\right)} \text{ for } t \in \left[t_i\,;\,t_{i+1}\right] \qquad \text{(eq. 1)}$$

where $c_i = C\left(t_i\right)$ is the measured concentration at time-point t_i.

Parameter k_i stands for the decay strength between t_i and t_{i+1}:

$$c_{i+1} = c_i\, e^{-k_i\left(t_{i+1}-t_i\right)} \Leftrightarrow k_i = \frac{\ln c_i - \ln c_{i+1}}{t_{i+1} - t_i} \qquad \text{(eq. 2)}$$

The time-weighted average concentration is then calculated so that the area under the dashed line is equal to the area under the concentration curve (sum of the grey areas, Fig. 1).

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Over one period $\left[t_i \, ; t_{i+1} \right]$, the area under the concentration curve is given by:

$$A_i = \int_{t_i}^{t_{i+1}} c_i \, e^{-k_i(\tau - t_i)} \, d\tau \Leftrightarrow A_i = \frac{c_i}{k_i}\left(1 - e^{-k_i(t_{i-1} - t_i)}\right) \qquad \text{(eq. 3)}$$

Replacing k_i by eq. 2 leads to:

$$A_i = \frac{\left(c_i - c_{i+1}\right)\left(t_{i-1} - t_i\right)}{\ln c_i - \ln c_{i+1}} \qquad \text{(eq. 4)}$$

Finally, the time-weighted average concentration is calculated as follows:

$$TWA = \frac{\sum_{i=1}^{n} A_i}{\sum_{i=1}^{n}\left(t_{i+1} - t_i\right)} \qquad \text{(eq. 5)}$$

where n corresponds to the number of medium renewal periods.

Table 1 gives some empirical data to illustrate the whole calculation process of TWA concentration.

Table 1. Empirical data (gray cells) to perform the calculation of the time-weighted average concentration. $t_{i+1} - t_i$ (in days) corresponds to the time period between two concentration measurements; c_i is the measured concentration at the beginning t_i of the period (medium renewal) and c_{i+1} is the measured concentration at the end t_{i+1} of the period; ln corresponds to the natural logarithm. A_i is the area under the decreasing exponential curve between t_i and t_{i+1} (eq. 4).

Renewal nbr	$t_{i+1} - t_i$ (days)	c_i (µg L^{-1})	c_{i+1} (µg L^{-1})	$\ln c_i$	$\ln c_{i+1}$	A_i
1	4	49.3	17.5	3.90	2.86	122.8
2	3	45.6	23.7	3.82	3.17	100.4
3	3	40.4	20.2	3.70	3.01	87.4
TOTAL	**10**					**310.6**

From the data in Table 1, the calculated TWA concentration is 31.06 µg L^{-1} (dashed line, Fig. 1).
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 actually exponential. A different theoretical curve would result in a different calculation for A_i (1). However, an exponential decay seems plausible and is probably the best compromise to use in the absence of other information.
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 disappears from the test medium, it is impossible to obtain a realistic area under the curve, and hence it is impossible to obtain a reasonable time-weighted average concentration.

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ANNEX 5

EXAMPLE OF A DATA TABLE FOR RECORDING MEDIUM RENEWAL, PHYSICO-CHEMICAL MONITORING DATA AND FEEDING

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 [mgL^{-1}]*																													new
																													old
Temperature [°C]*																													new
																													old
Conductivity [$\mu S\ cm^{-1}$]																													new
																													old
Food provided (tick)																													
Water hardness* [$mg\ L^{-1}$, as $CaCO_3$]																													

* Indicate which vessel(s) was (were) used

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ANNEX 6

EXAMPLE OF DATA TABLE FOR RECORDING REPRODUCTION OUTPUT AND SURVIVAL OF *LYMNAEA STAGNALIS*

A typical reproduction bioassay consists in the exposure of a group of individuals to a certain concentration of a test chemical, and reporting the number of offspring and the number of surviving parents at each time-point. The bioassay is usually replicated in order to assess the variability of the measured effects. Eventually, there are two biological measurements for each triplet replicate-dose-time.
As proposed within MOSAIC_repro (http://pbil.univ-lyon1.fr/software/mosaic/reproduction/), a convenient way to collect the data is as a tabular text file (see below). Each line of the table corresponds to a time-point for a given replicate and a given concentration of the test chemical for which the number of surviving parents (Nsurv) and the number of offspring are reported (Nrepro).
Please note that the **order of the columns should be respected** and that the first line of the file should contain the column headings. Finally, a single tabulation character should separate the columns.

Here is an example of the first lines of a data file:

Replicate	Conc.	Time (in day)	Nsurv	Nrepro
A	0	0	5	0
A	53	0	5	0
A	78	0	5	0
A	124	0	5	0
A	232	0	5	0
A	284	0	5	0
B	0	0	5	0
B	53	0	5	0
B	78	0	5	0
B	124	0	5	0
B	232	0	5	0
B	284	0	5	0
C	0	0	5	0
C	53	0	5	0
C	78	0	5	0
C	124	0	5	0
C	232	0	5	0
C	284	0	5	0
⋮	⋮	⋮	⋮	⋮
A	0	3	5	5
A	53	3	5	5
A	78	3	5	4
A	124	3	5	4
A	232	3	5	1
A	284	3	5	0
B	0	3	5	6
B	53	3	5	5
B	78	3	5	1
B	124	3	5	1
B	232	3	5	2
B	284	3	5	0
C	0	3	5	3
C	53	3	5	2
C	78	3	5	3
C	124	3	5	1
C	232	3	5	1
C	284	3	5	2
⋮	⋮	⋮	⋮	⋮

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ANNEX 7

METHODS FOR THE STATISTICAL ANALYSIS OF LYMNAEA STAGNALIS REPRODUCTION DATA

This ANNEX describes possible ways for statistical analyses of the data obtained in the *Lymnaea stagnalis* Reproduction Test. Data are analysed at the end of the test.

CALCULATION PRINCIPLE OF THE NUMBER OF INDIVIDUAL-DAY

A non-negligible mortality may be recorded at the end of the *Lymnaea stagnalis* Reproduction Test because individuals are monitored during a prolonged exposure duration. Nevertheless, individuals may have reproduced before dying and thus contributed to the observed reproduction outcome. Information on the reproduction of individuals which are dying during the test should therefore be taken into account to avoid any bias in the statistical analyses. This is particularly critical at high concentrations, when mortality may be very high.
In the *Lymnaea stagnalis* Reproduction Test, survival is regularly recorded at each time-point when clutches are counted. It is thus possible to calculate the period during which each individual is alive, corresponding to the period during which it may reproduce. As commonly done in epidemiology for incidence rate calculations, it is possible to calculate, for one replicate, the total sum of the observation periods of each individual before its death. When an organism is alive at time t but counted as dead at time $(t + 1)$, it is then assumed to be actually dead at $((t + 1) + t)/2$. The final sum for a replicate can then be expressed as a number of individual-days for this replicate. Hence, reproduction is evaluated in each replicate through the number of clutches per individual-day.

FIT PRINCIPLE OF A REGRESSION MODEL TO REPRODUCTION DATA ACCOUNTING FOR CONCOMITANT MORTALITY AND INTER-REPLICATE VARIABILITY

Let N_{ij} be the number of clutches for replicate j at the i^{th} concentration u_i, and NID_{ij} the number of individual-days at the i^{th} concentration for replicate j. As a first approximation, if the possible inter-replicate variability is neglected, a Poisson distribution can describe *Nij*:

$$N_{ij} \sim Poisson(f(u_i;\theta) \times NID_{ij})$$

$f(u;\theta)$ is the deterministic part of the model describing the mean tendency of the exposure-effect relation. MOSAIC_repro proposes the use of the three-parameter log-logistic model:

$$f(u_i;\theta) = \frac{d}{1+(u/EC_{50})^b} \text{ with } \theta = (EC_{50}, b, d)$$

where EC_{50} is the concentration inducing a halfway effect between upper limit d and 0, and b stands for curvature (shape) of the curve.
Depending on the shape of the exposure-effect relation, other deterministic parts would be possible: the 4 or 5-parameter logistic models, the Gompertz model, the 2- or 3-parameter exponential models, the Bruce-Versteeg model, or the Brain-Cousens model (1).

In order to explicitly account for the inter-replicate variability, the previous Poisson model may be extended with a gamma distribution:

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$$N_{ij} \sim Poisson\left(f_{ij} \times NID_{ij}\right) \text{ with } f_{ij} \sim gamma\left(\frac{f\left(u_i;\theta\right)}{\omega}, \frac{1}{\omega}\right)$$

Note that a gamma distribution of parameters α and β has mean $\frac{\alpha}{\beta}$ and variance $\frac{\alpha}{\beta^2}$. Here, parameters $f\left(u_i;\theta\right)$ and $\omega f\left(u_i;\theta\right)$ are respectively the mean and the variance parameters of the gamma distribution. Hence, parameter ω can be considered as an overdispersion parameter (the greater its value, the greater the inter-replicate variability).

Because non-standard stochastic parts (Poisson or gamma-Poisson) are required, classical frequentist statistical inference tools may appear difficult to use for parameter estimation. Known for its flexibility, the Bayesian inference framework may prove to be a convenient alternative. For that purpose, MOSAIC_repro proposes the combined use of freeware JAGS and R, in a transparent way through a web-interface.

The use of Bayesian inference requires the definition of appropriate priors based on expert knowledge on *Lymnaea stagnalis* reproduction process and the experimental design itself:

- $log_{10}(EC_{50}) \sim N(\mu, \sigma)$ with μ and σ defined from u_{min} and u_{max} the minimum (excluding the control) and the maximum tested concentrations, respectively, as follows:

  $$\mu = \frac{log_{10}(u_{min}) + log_{10}(u_{max})}{2} \text{ and } \sigma = \frac{log_{10}(u_{max}) - log_{10}(u_{min})}{4}$$

  thus assuming a normal distribution centred on the mean of $log_{10}(u_{min})$ and $log_{10}(u_{max})$, and a probability that $log_{10}(EC_{50})$ lies between $log_{10}(u_{min})$ and $log_{10}(u_{max})$ equals to 0.95.

- as *d* stands for to the reproduction output in controls, a normal prior $N\left(\mu_d, \sigma_d\right)$ based on the data themselves:

  $$\mu_d = \frac{1}{r_0} \sum_j \frac{N_{0j}}{NID_{0j}}$$

  $$\sigma_d = \sqrt{\frac{\sum_j \left(\frac{N_{0j}}{NID_{0j}} - \mu_d\right)^2}{r_0\left(r_0 - 1\right)}}$$

  where r_0 is the number of replicates in controls. Note that since they are used to define the prior distribution, data under control conditions are excluded from the fitting process.

- .
- $log_{10}(b) \sim U(-2, 2)$, a quasi non-informative prior for the shape parameter.
- $log_{10}(rate) \sim U(-2, 2)$, a quasi non-informative prior for the dispersion parameter of the gamma-Poisson distribution.

The major advantage of Bayesian inference lies in the posterior distribution it provides as an estimate for each parameter. A posterior distribution can also be obtained for any EC_x, thus providing a point estimate as well as a 95% credible interval extracted from 2.5, 50 and 97.5% quantiles, respectively.

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MOSAIC_repro provides final results as:

- Raw data in two plots: survival and cumulative reproduction data; a summary of the experimental design is also proposed to check that data have correctly been uploaded.
- The fitted curve superimposed to the reproduction data, expressed as the number of clutches per individual-day; the 95% credible band is also available.
- Parameter estimates of the Poisson (or gamma-Poisson) 3-parameter log-logistic model, as medians with 95% credible intervals (also named Bayesian confidence intervals);
- EC_x (x = 1, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80) median values with 95% credible intervals.

For a given dataset, the procedure implemented in MOSAIC_repro will fit both models (Poisson and gamma-Poisson) and use the Deviance Information Criterion (DIC) to choose the most appropriate. In situations where overdispersion (that is inter-replicate variability) can be neglected, using the Poisson model will provide more reliable estimates. Hence a Poisson model is preferred unless the gamma-Poisson model has a significantly lower DIC (in practice we require a difference of 10).

ALTERNATIVE ANALYSES OF MOLLUSC REPRODUCTIVE DATA

As has been made clear in the preceding discussion, it is important to adjust the analysis of individual eggs or egg clutches for parental mortality. Assuming gamma distributions to describe the number of independent days of parental survival in each replicate of each treatment group and a specific concentration-response shape, such as the 3-parameter log-logistic for the mean reproductive measure (clutches or eggs) is one valid method for doing that. As noted above, other shapes are needed for some datasets, especially where there is hormesis or low-concentration stimulation evident. Currently, MOSAIC_repro does not offer shapes other than the 3-parameter log-logistic model, but such shapes can be programmed, for example, in SAS, or added to MOSAIC in the future.

An alternative method for taking parental mortality into account is to analyse the ratio Reproduction/NID, computed for each replicate, where Reproduction is the number of clutches or the number of eggs per replicate. The models for the resulting concentration-response shapes can then be based on familiar normality-based regression methods (2). During the validation phase, it was found that this approach agreed well with the results from MOSAIC_repro, except where MOSAIC model was not or could not be fit, including, but not restricted to, when hormesis was evident in the data. The potential need for fitting other curves to the data requires use of some of the numerous statistical tools for assessing goodness-of-fit and model selection. This alternative approach is available in software packages such as ToxRat (http://www.toxrat.com), or programmable in SAS.

LITERATURE

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(2) OECD (2006). *Current approaches in the statistical analysis of ecotoxicity data: a guidance to application*. Environment, Health and Safety Publications. Series on Testing and Assessment No. 54. OECD, Paris.

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